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Drug Delivery

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DRUG DELIVERY RESEARCH ADVANCES

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PREFACE

Drug delivery is a term that refers to the delivery of a pharmaceutical compound to humans or animals. Most common methods of delivery include the preferred non-invasive oral (through the mouth), nasal, pneumonial (inhalation), and rectal routes. Many medications, however, can not be delivered using these routes because they might be susceptible to degradation or are not incorporated efficiently. For this reason many protein and peptide drugs have to be delivered by injection. For example, many immunizations are based on the delivery of protein drugs and are often done by injection.

Current efforts in the area of drug delivery include the development of targeted delivery in which the drug is only active in the target area of the body (for example, in cancerous tissues) and sustained release formulations in which the drug is released over a period of time in a controlled manner from a formulation.

This new book focuses on worldwide research on drug delivery and targeting at the molecular, cellular, and higher levels.

Chapter 1 discusses how ocular diseases such as age related macular degeration (AMD), diabetic retinopathy (DR), proliferative vitreal retinopathy (PVR), endopthlamitis and viral retinitis pose serious threat to vision if left untreated. The unique anatomy and physiology of eye offer many challenges to development of effective ocular drug delivery systems. Historically, drugs have been administered to the eye as simple drops instilled in the cul-de-sac. Recent advancements in the technology have led to the development of various polymeric ocular drug delivery systems such as biodegradable and nonbiodegradable implants, microspheres, nanoparticles, liposomes and gels. This book chapter aims to delineate various challenges, opportunities and recent advances in the development of ocular drug delivery systems.

Innovation in pharmacology can be achieved designing polymer nanoparticles to rationalize drug delivery by enhancing the oral bioavailability of peptides and proteins. New types of formulations were designed during the last couple of years promoting interactions with the mucus for bioadhesion, with the gastro-intestinal fluids for decreasing antiprotease activity and possibly with cells constituting the epithelium to enhance permeability. As they will be presented in Chapter 2, they result from the formation of core-shell poly(alkylcyanoacrylate) nanoparticles coated with chitosan and thiolated chitosan. The design and characterization of such core-shell polymer nanoparticles will be described in this chapter. Their evaluation as a tool to improve the bioavailability of peptides and proteins by the oral route will be discussed thanks to data obtained from different *in vitro* models and set ups.

The information presented in Chapter 3 will review the current findings on excipients with modulatory potential on intestinal transporters. With respect to excipients the article will mainly discuss Cremophor EL, Tocopheryl Polyethylenglycol Succinate (TPGS), Polysorbate 80, Pluronic P85, Polyethyleneglycol (PEG), Solutol HS15 and Labrasol. In addition, a comprehensive table of excipients with modulatory effects on drug transporters will be included. With respect to intestinal drug transporters the article will concentrate on interactions between excipients and two important members of the ABC-transporter family (P-glycoprotein and MRP) as well as two members of the solute carrier family (PEPT1 and the monocarboxylate transporter).

In Chapter 4, the authors review different techniques, including the flavoured additives (sweeteners, amino acids and lipids), physical methods (polymers coating, matrix granulation and encapsulation) and chemical methods (formation of inclusion complexes) as well as describe original emulsified dosage forms developed in their laboratory to mask the bitter taste of drugs incorporated into liquid oral dosage forms intended for oral paediatric applications. The e-tongue, an electronic device developed recently to evaluate the taste masking effect of dosage forms and used by developers in order to select the best combination of ingredients which provide “the best” testing formula is also described.

Chapter 5 reviews molecular transport between the nucleus and the cell cytoplasm which occurs through nuclear pore complexes (NPCs). Although access to the nucleus is a highly restricted process, a multitude of macromolecules have to enter and exit the nucleus, for the control of the basic cellular metabolism and to respond to changing environmental conditions. Except during mitosis, when the nuclear envelope disappears, the only way macromolecules can enter the nucleus is through the nuclear pore complex (NPC). The NPC is built of a diverse set of nucleoporins and associated nuclear and cytoplasmic filaments surrounding a central channel structure. This chapter will address the NPCs in detail and will introduce the mechanisms of entry through NPC such as NLS. Recent advances in applications of NLS is also presented.

Biomagnetism involves the measurement and analysis of weak magnetic fields detected from physiological activity or magnetic sources within the human body. The implementation of biomagnetic methods in pharmaceutical studies as a non-invasive monitoring of drug delivery is currently an alternative to nuclear medicine. Chapter 6 will present a short review of biomagnetic techniques, particularly AC Biosusceptometry (ACB) as an innovative method in pharmaceutical research.

In Chapter 7, live attenuated mutants of *Salmonella* are under evaluation as vectors for the delivery of heterologous antigens to humans via the oral route. To produce such recombinant *Salmonella* strains, live attenuated vaccine strains of *Salmonella* are genetically manipulated to express heterologous antigens. They are able to produce a limited infection *in vivo*, producing the heterologous antigen during replication. A range of technologies have been developed to allow the controlled and stable delivery of antigens. This has allowed the optimisation of delivery of various vaccine antigens, stimulating appropriate humoral and cellular immune responses. As a result, several live attenuated *Salmonella*-based vaccines are now in human clinical trials.

Regenerative therapy of bone tissue by cytokines is now in clinical application as discussed in Chapter 8. The most effective cytokines for bone tissue reproduction are the bone morphogenetic proteins (BMPs). Cytokine therapy using BMPs has been tested in clinical trials and is now being used for clinical treatment; however, because of obstacles,

such as high cost, it is not yet widely used. To optimize this new method of treatment, it is important to allow BMPs to act efficiently on the locus where bone growth is necessary. To achieve this, the authors first have to examine the effect of combination therapies to increase or activate cells reacting to BMPs. In addition, it is necessary to develop new drug delivery systems (DDS) for BMPs and determine the most suitable use of BMPs and DDS for the various clinical situations. The field is being advanced by the development of new DDS for BMPs using nanotechnology. If the technology of bone tissue regeneration using BMP progresses with these new developments, orthopedic treatment systems can be expected to change dramatically.

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Chapter 1

ADVANCEMENTS IN OCULAR DRUG DELIVERY

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ABSTRACT

Ocular diseases such as age related macular degeration (AMD), diabetic retinopathy (DR), proliferative vitreal retinopathy (PVR), endopthlamitis and viral retinitis pose serious threat to vision if left untreated. The unique anatomy and physiology of eye offer many challenges to development of effective ocular drug delivery systems. Historically, drugs have been administered to the eye as simple drops instilled in the cul-de-sac. Recent advancements in the technology have led to the development of various polymeric ocular drug delivery systems such as biodegradable and nonbiodegradable implants, microspheres, nanoparticles, liposomes and gels. Due to continuous success, transporter and receptor targeted prodrug modification strategy is rapidly gaining acceptance in various drug delivery avenues. A novel strategy of circumventing efflux proteins (P-glycoprotein and multidrug resistance proteins) using transporter targeted drug delivery may improve specific targeting to cancer cells. Recent advances in biotechnology have resulted in the development of gene, antibody and nucleic acid therapy with significant activity against AMD, DR, PVR and viral retinitis. However, the attainment of therapeutic drug levels at the target site in a safe and effective manner for the desired length of time remains a major challenge. The diversity of the approaches for ocular drug delivery is an indication of the fact that the current ocular drug delivery approaches may not be optimal and further research may require for the successful development of ideal ocular drug delivery systems. This book chapter aims to delineate various challenges, opportunities and recent advances in the development of ocular drug delivery systems.

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1. ANATOMY AND PHYSIOLOGY

The eye is a delicate organ held in position in the orbital cavity by various ligaments and muscles. A close examination of the physiology and anatomy of the eye is of great importance to understand the challenges associated with ocular drug delivery. The structure of the eye can be divided in two segments, namely anterior and posterior segments. Anterior segment comprises of two chambers, anterior (between the cornea and iris) and posterior (between iris and lens) chambers. Anterior segment tissues are cornea, pupil, aqueous humor, iris- ciliary body and lens while posterior segments tissues are sclera, choroid, retinal pigment epithelium (RPE), neural retina and vitreous humor. The conjunctiva is a protective layer which covers eyeball. It consists of a thin mucous membrane layer in the inside of the eyelids and anterior sclera (Figure 1).

Considerable amount of research work has been done to improve anterior segment drug delivery following systemic and topical administrations. However, limited research work has been done in the area of posterior segment drug delivery.

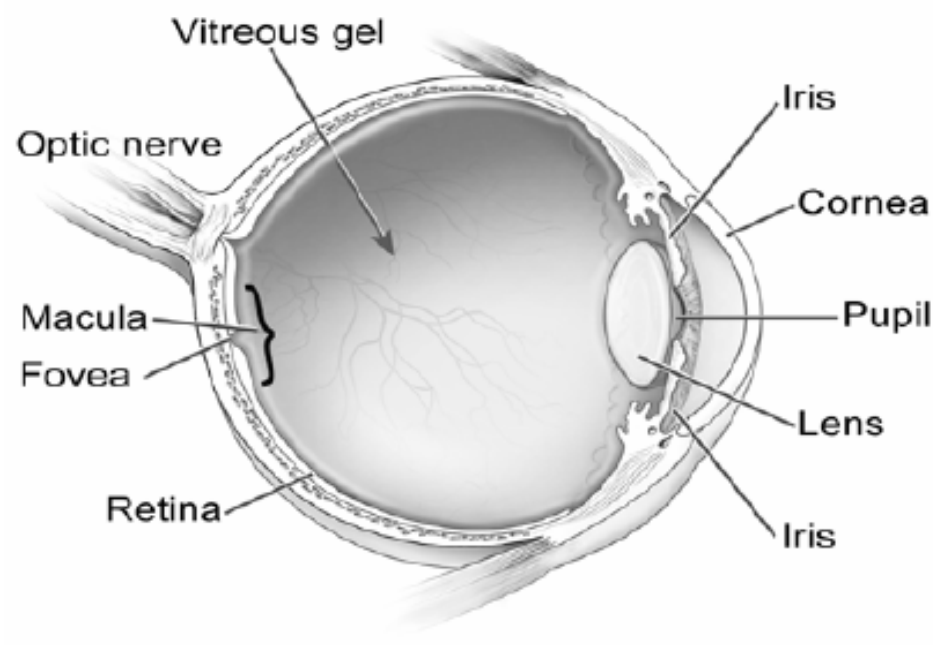

Figure 1. Anatomy of the eye. (www.nei.nih.gov/health/eyediagram/eyeimages3.asp).

With the advent of posterior segment diseases like age-related macular degeneration (AMD), proliferative retinopathy (PVR), cytomegalo virus (CMV) retinitis, bacterial and fungal endophthalmitis and diabetic vitreoretinopathies, achieving effective drug concentration in the retina, choroid and vitreous humor has gained high clinical attention.

2. CHALLENGES TO OCULAR DRUG DELIVERY

2.1. Anterior Segment Delivery Challenges

Cornea is a major barrier for traditional topical drug delivery in the treatment of anterior segment diseases such as glaucoma, keratitis and bacterial and viral infections. Cornea is a transparent, avascular and highly innervated tissue [1]. It mainly consists five layers. Corneal epithelium (5-6 layers of columnar cells), bowman's layer (a homogenous non-cellular layer), stroma (thickest layer of cornea composed of collagen fibers and 90% water), descemet's membrane (thick membrane separating stroma and endothelium) and endothelium (single cell layer with pinocytotic vesicles). The corneal epithelium layer possesses tight junction cells which limits the entry to small hydrophilic drug molecules following topical administration. Moreover, hydrophilic structure of stroma offers limited entry of lipophilic compounds [2, 3]. Presence of efflux proteins (P-glycoprotein and MRPs) on the corneal epithelium also restricts the entry of xenobiotics to the anterior segment tissues [4, 5]. Precorneal drainage, tears washout and limited contact time are major challenges to the anterior segment drug delivery following topical administration. To be clinically effective topical formulation has to possess a balance between the hydrophilicity and lipophilicity with higher contact time [6].

2.2. Posterior Segment Drug Delivery Challenges

Drug delivery to the ocular tissues, mainly posterior segment tissues, is a challenging and exciting field due to the unique physiology of the eye and presence of blood ocular barriers (BOB). Two major components of BOB are blood aqueous barrier (BAB) and blood retinal barrier (BRB) [7]. Endothelail cells of ciliary blood capillary forms BAB which prevents the entry of drug molecules from the blood to the anterior segment tissues. RPE and retinal endothelial blood vessels collectively form blood retinal barrier (BRB) and act in conjunction to prevent drug entry from the blood to the posterior segment tissues. Blood supply to the posterior segment tissues is achieved by choroidal and retinal blood vessels. Blood vessels in the choroid possess large fenestrations that allow easy diffusion of substances into and out of the choroidal stroma. However, RPE, a single monolayer structure separates the outer surface of the neural retina from the choroid [8]. The RPE cells are polarized in nature which faces the neural retina on apical side and choroid on basolateral side. The tight junctions between RPE cells form an outer blood retinal barrier (oBRB) which plays a vital role in supporting and maintaining the homeostasis of neural retina [9]. The endothelial cells of the retinal blood capillaries express tight junctions with poor or no fenestrations. Moreover, astrocytes are reported to present in close apposition with the retinal blood vessels similar to brain capillaries [10]. Thus, retinal endothelial cells form an inner blood retinal barrier (iBRB). Recently, efflux pumps such as P-glycoprotein and multi-drug resistance proteins (MRPs) have been identified on the RPE [11, 12] which may limit the penetration of xenobiotics and endogenous compounds from the systemic circulation to the posterior segment tissues and mainly retina.

3. ROUTES OF OCULAR DRUG ADMINISTRATION

1) Topical administration: It is the most commonly used route of drug administration for the treatment of anterior segment complications. Posterior segment drug delivery via topical route suffers from drug loss in the precorneal area and anterior segment, drug elimination from the anterior chamber by the canal of Schlemn or via absorption through iris-ciliary body [7]. Enzymatic metabolism in the anterior chamber limits the entry of intact drug into the posterior segment tissues. Limited success has been achieved with topical administration in the area of posterior segment drug delivery. Acheampong et al. [13] studied the distribution of brimonidine into anterior and posterior tissues of monkey, rabbit and rat eyes. Therapeutic level of brimonidine was achieved in the vitreous and retina upon topical instillation. Verapamil was also reported to enter into the posterior segment tissues following topical administration in rabbit eye [14]. However, topical administration is not a feasible approach for delivering therapeutic agents in the treatment of retinal diseases.

2) Intravitreal administration: With the help of recent advancement in the surgical procedures, intravitreal administration of therapeutic agents by direct injection into the mid-vitreous region and sustain and controlled released intravitreal implants have become a mainstay treatment option of posterior segment diseases. Various therapeutic agents, such as antiviral agents- ganciclovir (GCV), acyclovir (ACV), cidofovir and foscarnet and antibiotics-cephalexin, gentamicin and cefazolin have been evaluated to design the effective treatment of CMV retinitis and endophthalmitis [15-18]. Macha et al. [19] studied the vitreous disposition of GCV and its monoester lipophilic prodrugs following intravitreal injection. Longer retention time and higher vitreous concentration of GCV was obtained following this route of administration. Intravitreal injection circumvent the BRB and hence therapeutic concentration of drug may be achieved and maintain for a longer period of time. However, patient non-compliance, pain and discomfort are major obstacles to the clinical application. Inorder to overcome the risks related to direct intravitreal injection such as cataract, retinal detachment and vitreous haemorrhage [20], intravitreal implant was developed. Vitrasert® is a non-biodegradable GCV intraocular implant. Sustain therapeutic concentration of GCV into the vitreous humor for a period of 5-6 months can be achieved by this intravitreal implant. Removal of implant requires a skillful surgical procedure and possesses risks of retinal detachment and haemorrhage [21].

3) Scleral administration: Due to its large surface area, easy accessibility and relatively high permeability to macromolecules, the sclera recently has become a potential vector for posterior segment drug delivery [22, 23]. Scleral drug delivery has been attempted by different ways, such as scleral plugs and implants, sunconjunctival injection, subtenon injection etc. Sustained release transscleral device has also been studied to overcome complications related to the intravitreal injection. Sakurai et al. [21] studied the efficacy of scleral inserts composed of poly (DL-lactide-co-glycolide) and poly (DL-lactide) polymers. The inserts was reported to have sustained levels of GCV in vitreous and retinal tissues in HCMV retinitis treatment. However, scleral plugs and implants suffer from disadvantages such as patient discomfort and surgical risks. Subconjunctival administration of various therapeutic agents has recently gained a special attention due to the lower surgical risks and patients compliance. Kalsi et al. [24] reported that higher aqueous and vitreous levels of anticancer drug, dacarbazine, can be achieved compared to systemic administration. Various

studies by Weijtens et al. [25, 26] showed that higher vitreous concentration of dexamethasone was obtained following subconjunctival administration as compared to peribulbular injection. Closer and precise placement of drugs to the sclera by subconjunctival injection could be a possible reason to achieve higher vitreo-retinal concentration [27]. Subconjunctival administration of therapeutic agents could also result in higher aqueous and plasma concentration as compared to peribulbular administration. Transscleral administration of drugs offers a promising therapeutic approach for the treatment of various posterior segment diseases.

4) Systemic administration: Due to the presence of BRB, systemic administration has achieved a limited success to deliver drugs to the vitreo-retinal tissues. Only 1-2% of plasma drug concentration is achieved in the vitreous humor and therefore requires frequent administration to maintain therapeutic drug level. This route of administration may also result in non-specific binding of drug to other tissues and cause systemic cytotoxicity. Eventhough not an ideal strategy for ocular complication, intravenous administration of ganciclovir or foscarnet sodium has been used in the treatment of acute CMV reitinitis. Table 1 illustrates the advantages and disadvantages of various routes of administration. Figure 2 shows the diagram of various routes of ocular drug administration.

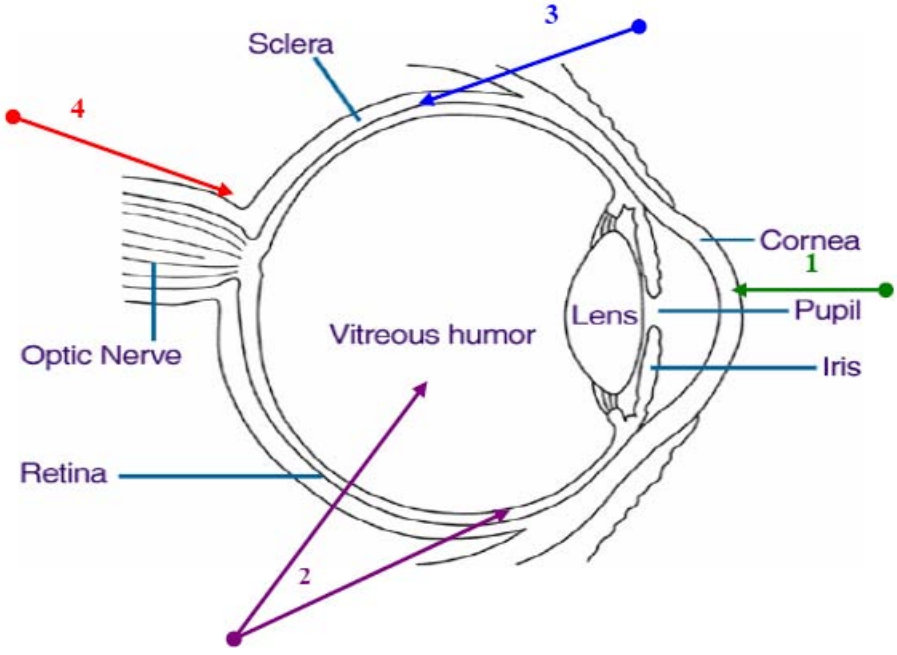

Figure 2. Routes of ocular drug administration. 1-topical; 2-intravitreal (injection/implants); 3-subconjunctival; 4-peribulbular administration.

Table 1. Comparision of various routes of ocular drug administration

Route of Drug Administration	Advantages	Disadvantages/Risks
Topical	Patient compliance Ease of administration	Subtherapeutic drug level Precorneal loss Conjunctival absorption
Systemic	Control the spread of infection to other tissues	Need of frequent administration Systemic toxicity Subtherapeutic ocular drug level
Intravitreal injection	Therapeutic drug level No or low systemic toxicity	Patient non-compliance Vision threatening surgical risks
Intravitreal implants	Sustain therapeutic level	Removal of implant Vision threatening surgical risks
Scleral plugs and implants	Lower surgical risks Sustain therapeutic level	Patient discomfort Repeated operation
Subconjuncitval injection	Relatively non-invasive Sustain therapeutic level	Systemic absorption Risk of ocular infection

4. OCULAR METABOLISM AND EFFLUX PUMPS

Efflux and metabolism of therapeutic agents are of great interest for the development of ocular drug delivery. Presence of efflux proteins and metabolic enzymes play an important role in determining the concentration of the drug delivered to the site of action.

4.1. Role of Metabolism in Ocular Drug Delivery

Metabolism is one of the defense mechanism of the body and a primary mode of drug elimination from the body. Eye, being a very delicate organ of the body, has well developed and designed metabolism mechanism to prevent the entry or accumulation of xenobiotics from external environment of systemic circulation [28, 29]. Drug metabolizing enzymes like esterases, peptidases, reductases and cytochrome P (CYP) family enzymes present in ocular tissues which serve as a metabolic barrier for ocular drug delivery. However, utilization of these enzymes by prodrug strategies has met considerable success.

Topical administration is the most common route of drug delivery in the treatment of anterior segment diseases like glaucoma, bacterial and viral infections (i.e. epithelial and stromal keratitis) [6]. Cornea, a transparent and avascular tissue, plays an important role as a major barrier for the topically administered drugs. Moreover, presence of various metabolizing enzymes in the cornea makes the topical drug delivery more difficult [30]. Abraham et al. [31] reported heme oxygenase activity in calf corneal epithelium. Important classes of CYP-450 enzymes (aryl hydrocarbon hydroxylase, 7-ethoxycoumarin-O-deethylase and benzphetamine demethylase) have been reported to present in the corneal epithelium and endothelium [32]. The CYP-450 enzymes detoxify the xenobiotics permeating the cornea through the tear film and mucus. The endothelial cells of blood capillaries in the iris-ciliary

body forms blood aqueous barrier which prevent the entry of xenobiotics into the anterior segment from the systemic circulation. Various researchers have reported high levels of CYP enzymes expression in the iris-ciliary. Expression of CYP enzymes in the iris-ciliary have been induced by administration of CYP inducers, β-napthoflavone and pentobarbital [33, 34]. Limited amount of reports are available for the metabolic activity of vitreous humor. The author's laboratory reported esterase and peptidase activities in the rabbit vitreous [35]. Various metabolic enzymes including phase I and II enzymes, esterases, peptidases, alcohol and ketone dehydrogenases have been reported on retina and RPE [36-38].

A wide variety of enzymes, present in the ocular tissues, involve in various stages of drug detoxification and metabolism. These enzymes also maintain ocular homeostasis by preventing environmental and systemic insults. These enzymes may be an attractive target for prodrugs delivery in the treatment of anterior and posterior segment diseases. Instantaneous conversion of the prodrugs into the active parent drug due to the presence of specific enzymes may improve the ocular drug delivery.

Dias et al. [35] reported high peptidases and esterases activity in the rabbit vitreous humor. Upon intravitreal administration, lipophilic prodrugs of nucleoside analogs acyclovir and ganciclovir were found to be converted to their respective parent drugs. A study from author's laboratory suggested that dipeptide prodrugs of ganciclovir were converted to their respective monopeptide prodrugs by peptidases prior to the final conversion to the parent compound by esterases [39]. The presence of esterases and peptidases in the vitreous humor and retina provide an opportunity for the development of prodrug for the treatment of posterior segment diseases like CMV retinitis, endophthalmitis, choroidal neovascularization [7]. Lipophilic prodrugs of ganciclovir has been extensively studied in author laboratory and found to be successful in CMV retinitis.

Helberg et al. [40] studied the role of metabolizing enzymes in human and rabbit ocular tissues (cornea, iris-ciliary body and sclera). Bimatoprost, an ethyl derivative of a prostaglandin analog, was hydrolyzed to carboxylic acid product, 17-phenyl-trinorPGF-2alpha in ocular tissues. Kawakami et al. [41] showed that O-palmitoyl tilisolol, an amphiphilic prodrug of tilisolol, ready convert into tilisolol in the cornea, aqueous humor, iris-ciliary following topical instillation. Enhanced transit time of prodrug and higher retention time of parent drug in the ocular tissues were observed.

Detailed understanding of the presence, distribution and activity of various enzymes in the ocular tissues is important to successfully implement the prodrug strategy. Knowledge of metabolic enzymes into ocular tissues enables us to predict the bioconversion rate at the site of action and elimination of metabolites from the target tissue.

4.2. Role of Efflux Pumps in Ocular Drug Delivery

Efflux pumps are important proteins mainly involve in the extrusion of toxic substances from within the cells into the external environment and thereby maintain the homeostasis. The presence of efflux pumps/proteins on ocular tissues plays an important role in absorption, distribution and elimination of therapeutic agents. P glycoprotein and multi-drug resistance proteins (MRP) are two efflux pumps being investigated by researchers. These efflux pumps are members of ATP binding cassette (ABC) family which use ATP hydrolysis to drive efflux. P-gp, a 150kDa protein, is the most widely studied human MDR1/ABC1 transporter.

P-gp has a wide range of substrate specificity including anticancer drugs, HIV protease inhibitors, steroids, antibiotics, immunosuppressive agents and antihypertensive agents. Despite of extensive research work, exact mechanism of action of P-gp is not perfectly understood. One of the various proposed mechanism is illustrated in Figure 3. P-gp proposed to act as a flipase [42], carrying its substrate from the inner leaflet of the lipid bilayer to the outer leaflet. Another model also suggested that P-gp can efflux drug from intracellular compartment as well as lipid bilayer [43]. MRPs are able to transport lipophilic anions, glucuronide/ glutathione/ sulfate conjugate compounds and cause alteration in drug distribution and confer resistance to therapeutic agents.

Greenwood et al. [12] showed expression of P-gp in brain and retinal endothelium in vitro using rat retinal endothelial cell line. Saha et al. [44] first reported the expression of P-gp in cultured rabbit conjunctival epithelial cells. Kawazu et al. [45] studied transport of cyclosporin A across cultured rabbit corneal epithelial cells. Cyclosporin A was found to be interacted with P-gp and therefore transport was inhibited. Yang et al. [46] also studied propranolol transport in cultured rabbit conjunctival epithelial cell layers. They reported that P-gp restrict the propranolol transport across this cell line. Dey et al. [5] first time reported molecular evidence and functional expression of P-gp (MDR1) in human and rabbit cornea and corneal epithelial cell lines. The same group also studied pharmacokinetics of erythromycin in rabbit cornea after single-dose infusion and evaluated the role of P-gp as a barrier to in vivo ocular drug absorption.

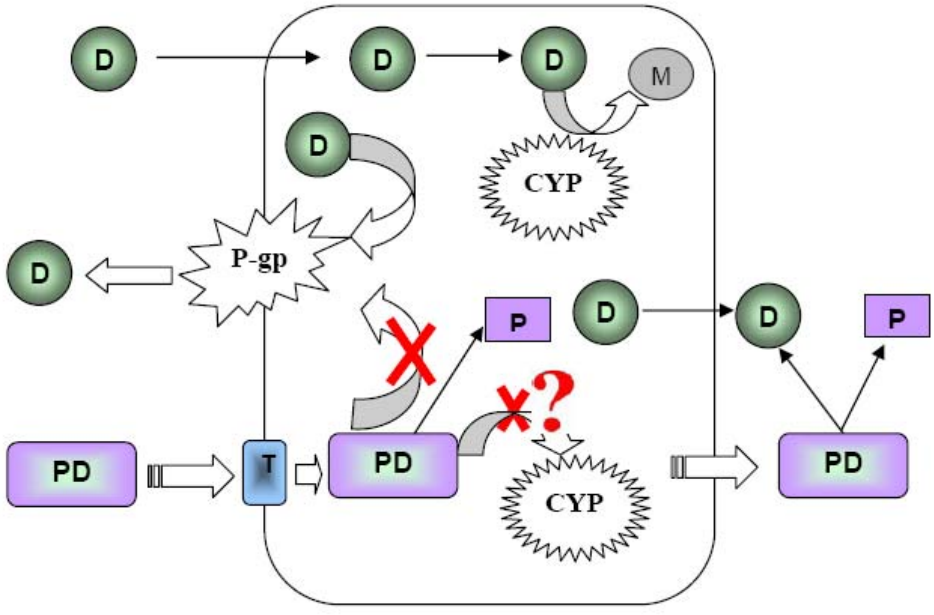

Figure 3. Schematic of P-gp cirmcuvention strategy. [?]- yet to be confirmed.

Kennedy et al. [47] reported P-gp expression in human retinal pigment epithelium. Aukunuru [11] studied the expression of MRP in human RPE cells. The expression of P-gp and MRP on ocular tissues is beneficial to remove toxins from the ocular epithelium linings but at the same time also restrict absorption and ocular bioavailability of therapeutic agents like cyclosporine A and erythromycin. In human RPE cells, expression of P-gp on both apical

and basolateral surfaces serve as a protective mechanism for the neural retina but restrict the drug delivery to the posterior segment tissues.

Various strategies have been evaluated to circumvent the physiological barriers created by P-gp and MRPs (Table 2). Inhibition of efflux pump activity could be a useful strategy to circumvent the efflux mediated lower bioavailability. Various P-gp and MDR modulators, such as chemical agents, polymers and monoclonal antibodies, have been studied. The use of chemosensitizers needs to be evaluated for the toxic concentration and drug-drug interactions. Alakhov et al. [48] and Batrakova et al. [49] studied the application of pluronic block copolymers to overcome multi drug resistant (MDR). They reported that pluronic block copolymers sensitized MDR cells and increased the therapeutic activity. Pluronic copolymers reported to have energy-depleting effects by reducing ATP levels in MDR cells. Use of pluronic copolymers in the sustain release formulation for the posterior segment disorders can be considerable clinical significant approach. Mano et al. [50] and Naito et al. [51] have reported that anti-Pgp monoclonal antibody, MRK-16, enhance the effectiveness of anti-MDR chemotherapeutics. The main advantage of monoclonal antibody approach is that direct targeting of P-gp avoids the side effects of chemosensitizers. Circumvention of efflux pumps using prodrug approach has recently gained attention. Katragadda et al. [52] studied the modulation of P-gp mediated efflux by prodrug derivatization using isolated rabbit cornea. Peptide prodrugs of quinidine reported to have a reduced or diminished affinity toward P-gp and suggested to be a possible viable approach to overcome P-gp mediated efflux.

Receptor targeted nanoparticles have been studied for the development of sustain release formulation in the treatment of posterior segment complications. Development of polymeric delivery may aid a new dimension in overcoming efflux pumps.

Table 2. Various strategies to overcome efflux pump effects

Strategies	Examples
Inhibition of efflux pump activity – chemotherapeutic agents – Polymers – Antibodies (Ab)	Ca-channel blockers, immunosuppressant Pluronic block copolymers MRK-16, P-gp monoclonal Ab
Circumvent efflux pumps – Prodrugs / monoclonal Ab – Polymeric drug delivery	Dipeptdie prodrugs of saquinavir Doxorubicin encapsulated nanoparticles
Inhibition of efflux pump expression – Transcriptional regulators	K2-5F, a designed MDR1 transcriptional repressor

Successful circumvention of P-gp by nanoparticle bound loperamide and doxorubicin in the brain delivery have been reported [53]. Advancement in the gene delivery may be utilized to reduce the P-gp expression on particular ocular tissues to enhance the ocular availability by avoiding efflux pump mediated biological barrier. However, immunological reactions and limited clinical data warrant further studies for the implementation of gene therapy.

5. TRANSPORTERS/RECEPTORS TARGETED OCULAR DRUG DELIVERY

The past decade has witnessed rapid developments in the field of carrier mediated transport of therapeutic agents across various biological barriers. Various nutrient transporters/ receptors such as peptide, amino acid, monocarboxylic acid, folate, organic anion and cation transporters have been identified in ocular tissues like cornea, lens, conjunctiva and retina (Table 2). These transporters are responsible for transferring exogenous and endogenous nutrients across the cell membranes and thereby regulate the exchange of these compounds. Utilization of the nutrient transporters present on the ocular barriers via prodrug derivatization is an exciting strategy recently being investigated in author's laboratory. Parent drug can be targeted to the membrane transporters/receptors by coupling wit a promoiety that is an endogenous substrate for the same transporter/receptor. Translocation of entire prodrug across cell membrane occurs due to the recognisation of promoiety by transporters/receptors. Once inside the cell, the prodrug releases drug by enzymatic hydrolysis (Figure 4).

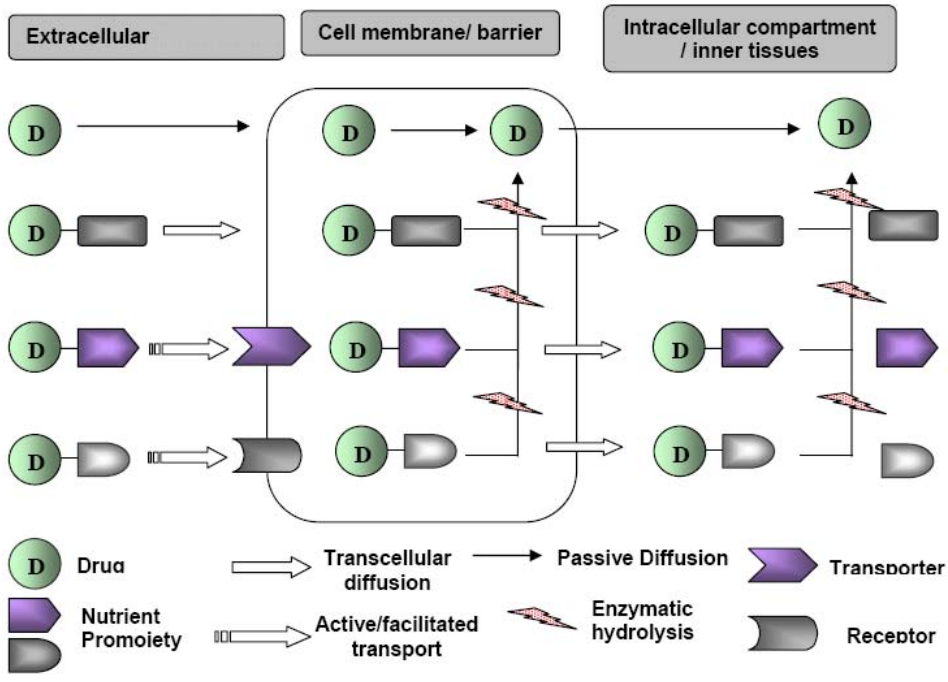

Figure 4. Schematic of transporter/receptor targeted drug delivery.

Moreover, prodrug design may improve undesirable biopharmaceutical properties such as aqueous solubility, chemical stability and specificity of therapeutic agents. Carrier mediated prodrug delivery is a novel strategy to improve specificity and therapeutic efficacy with lower cytotoxicity [6]. For ocular drug delivery, prodrugs can be targeted by mainly four delivery routes: topical, systemic, intravitreal and subconjunctival (Figure 5).

Various transporters and receptors have been identified on the ocular tissues and studied to improve ocular drug delivery (Table 3).

Table 3. Examples of transporters present on various ocular tissues

Tissue	Transporters	Subtypes
Cornea	Peptide	hPEPT1
	Amino acids	Phenylalanine, tyrosine, LAT1, LAT2
	Nucleoside	
	Glucose	GLUT1
Lens	Amino acids	System A, L, Gly, Ly+, β, ASC
	Glucose	GLUT1, GLUT3
	Ascorbic acid	SVCT2
	Glutathione	R-GSHT
Iris-ciliary body	Glucose	GLUT1, GLUT4
	Nucleoside	
Conjunctiva	Acid-base	NKCC, HE1
	Glucose	GLUT1
	Peptide	dipeptide
	Amino acids	$B^{o,+}$
Retina	Peptide	PEPT1?, PEPT2?, PHT1, PHT2
	Amino acids: (glycine, glutamine, arginine, taurine, proline, etc.)	
	Nucleoside	
	Glucose	GLUT1, GLUT3
	Vitamins (Ascorbic acid; folic acid; riboflavin; biotin)	SVCT2, RFT, FR-∞, SMVT
	Monocarboxylic acids	MCT1, MCT3

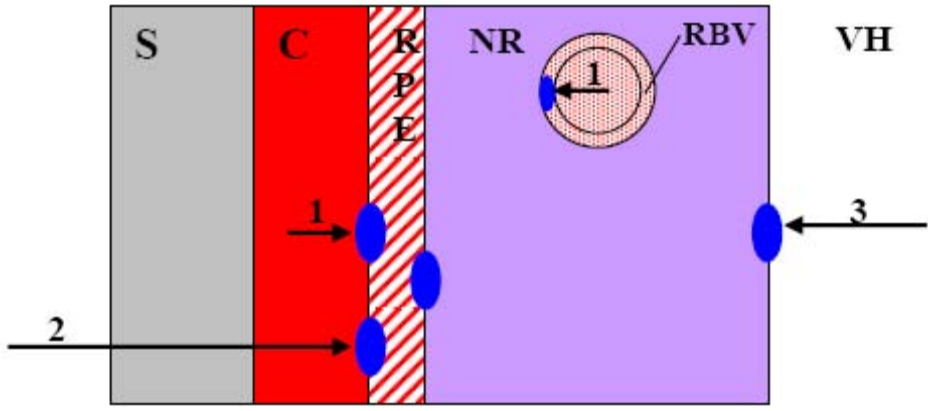

Figure 5. Schematic presentation of carrier mediated (transporters/receptors targeted) retinal drug delivery following systemic (1), scleral (2) and intravitreal (3) drug administration. S-sclera; c-choroid; RPE-retinal pigment epithelium; NR-neural retina; RBV-retinal blood vessel; VH-vitreous humor; 1- systemic administration; 2-episcleral (subconjunctival, bulbular) administration; 3-intravitreal administration.

5.1. Anterior Segment Delivery

Peptide transporters have been shown to transport peptides and peptidomimetic drugs across various epithelia. Anand et al. [54] have shown the functional evidence for the presence of oligopeptide transport system (OPT) on the rabbit cornea. Dipeptide prodrugs of ACV (US patent pending) were synthesized in authors' laboratory to target the OPT present on the cornea. These dipeptide prodrugs of ACV possess excellent stability, antiviral activity, higher permeability across cornea and most importantly affinity for OPT on the cornea and intestinal mucosa [55, 56]. Therefore the dipeptide prodrugs of ACV have a clinical potential in the treatment of HSV keratitis.

Amino acids are important nutrients for ocular tissue survival. Various types of amino acid transport systems (anionic, cationic and neutral) have been identified on the corneal epithelium. Besides natural amino acids, structurally similar compounds such as L-dopa, a drug for parkinsonism- melphalan, an anticancer agent- Phe mustard and anticonvulsant agent- gabapentin may be transported by different amino acid transporters. Thus amino acid transporters can be use to target and improve drug delivery.

Glucose transporter-1 (GLUT1) has been identified in the bovine corneal epithelium [57]. However, various studies suggested that inspite of high transport capacity GLUT may not be feasible approach due to their rigid structure requirements [58]. Various receptors including growth factor, insulin and bradykinin receptors have also been identified on corneal tissues [59]. These receptors are essential for cell division, differentiation and maintenance of cellular homeostasis. These receptors can be targeted for the treatment of various ocular diseases. Insulin receptors have been utilized to delver insulin via the ocular route.

Conjunctiva is a highly vascularised tissue and has the characteristics of expressing both secretory and absorptive transport mechanism. Various carrier mediated transport system have been identified on the conjunctiva. Glucose, potassium, chloride and sodium cross the conjunctiva by active transport mechanism [60]. Presence of a proton-coupled dipeptide [61]

and $B^{0,+}$ [62] transport system on the conjunctiva can be potential targets for ocular drug delivery following systemic and transscleral drug administration.

Molecular and function evidence for presence of nucleoside and glucose transporters and various receptors (muscarinic, adrenergic, growth factors) have been reported on the iris-ciliary body. A research study suggested that muscarinic receptors present on the iris-ciliary body may involve in the inhibitory effects of cholinomimetic drugs on ocular sympathomimetic neurotransmission [63]. Flesinoxan, a potent adrenergic receptor antagonist, found to reduce intraocular pressure in the rabbit eye [64]. Limited work has been done in the field of drug delivery by utilizing carrier mediated systems present on these tissues.

5.2. Posterior Segment Delivery

Various nutrient transporters and receptors such as peptide [65], glucose [66], amino acids [67], nucleoside [68], folate [69], biotin [70] and riboflavin [71] have been identified on the retinal tissues. Kansara et al. [69] have recently evaluated an ex vivo model for carrier mediated retinal drug delivery. Presence of peptide transporter on the retinal side facing the vitreous and on the RPE side facing the blood offers unique opportunity to target these transporters following vitreal or sunconjunctival administration of peptidomimetic prodrugs. Amino acid, dipeptide monoester and diester prodrugs of anti-CMV agent GCV have been synthesized and characterized in authors' laboratory [72]. These prodrugs possess chemical stability, aqueous solubility, antiviral efficacy and affinity towards oligopeptide transporters present on the cornea and retina. Dipeptide prodrugs of GCV found to have 7-8 fold higher permeability across cornea as compare to parent drug GCV. The amino acid esters of GCV have reported to be highly effective against herpes simplex viruses (HSV-1, HSV-2), varicella voster virus (VZV), and HCMV (unpublished data). Monocarboxylic acid transporters (MCTs) have been identified on the retina and conjunctiva [73]. MCT1 and MCT3 have been reported to be present on the apical and basolateral membrane of rat RPE, respectively [74]. Monocarboxylic drugs like salicylic acid and pravastatin are reported to be transported via MCTs in the small intestine [75]. Presence of MCTs on both side of the RPE can be targeted for improved retinal delivery following intravenous and transscleral administration. Various researchers have shown that folate receptors can be targeted for facilitating anticancer drug delivery and for liposome based gene delivery [76]. Moreover, tissue specific expression of folate receptors (kidney, placenta, choroids plexus, lungs and retina) provide a benefit for targeted delivery of the folate ester prodrugs to the retina.

6. CONTACT LENS AND COLLAGEN SHIELDS

Eye-drops are the conventional dosage forms that account for majority of currently accessible topical ophthalmic formulations. Despite the excellent acceptance by patients, one of the major problems encountered is rapid precorneal drug loss. Longer drug retention/contact time with cornea is one of strategies researchers have investigated to improve ocular drug bioavailability.

In 1971 Kaufman introduced the idea of using soft contact lenses to deliver drugs to the cornea [77]. Bandage soft contact lens made of hydrophilic polymer was used as a carrier vehicle to increase the retention time of therapeutic agents in the tear film and at the corneal surface. However, the soft contact lens has several limitations to be used for ocular drug delivery. The soft contact lens can be inserted and removed by ophthalmologist. Moreover, it may cause pathogenic ocular infection. Various studies also suggested that contact lens hydrated with drug is nearly devoid of drug within 1-2 hours on the cornea [78]. Therefore, soft contact lens failed to be a reliable approach for sustained ocular drug delivery.

Collagen, an essential protein of tissue, cartilage and bone, is considered safe and biocompatible protein for human medical applications. Collagen has been used in cardiovascular surgery, plastic surgery and ophthalmology. Collagen has been used for ocular lubrication as forms gel-like structure at physiological temperature. Collagen has used as a protective layer against trauma during ocular surgery. Incorporation of wound healing agents resulted in clinically beneficial application of collagen shield. The collagen shield was designed to be a disposable, short term therapeutic bandage lens for the cornea. It is available in different diameters, dissolution rates (i.e. rapid dissolution, 12, 24, 72 hrs) and water contents. It has been developed as a new continuous-delivery system for drugs that provide high and sustained levels of drugs to the cornea. Bloomfield et al.[79] showed that higher level of gentamicin was achieved in the tear film and ocular tissues using wafer shapped collagen inserts compared to drops, ointment or subconjunctival injection. Collagen shield has shown to be a suitable vehicle for sustained corneal drug delivery. In a study by Kaufman et al. [80] a new drug delivery system, collasomes, have been developed where collagen pieces or particles suspended in a viscous vehicle was instilled beneath the eyelid. The collasomes were found to be well tolerated, and because the collagen particles are suspended in carrier vehicles, safe and effective instillation in much the same fashion as drops or ointments was achieved.

Degree of cross-linking in collagen subunits is an important parameter to control the release of drug from the shield. Kuwano et al. [81] achieved higher and sustained aqueous humor and corneal drug concentrations with collagen shield and suggested that cross-linked collagen shields might be useful ocular drug delivery devices. However, collagen shield is not optically clear and therefore it reduces visual acuity and cause minor interference with vision. Collagen shields are designed to be inserted in a physician’s office and reported to produce some discomfort, and interfere with vision.

Owning the characteristics of providing ocular lubrication, protection, and sustain drug delivery, and better and faster wound healing properties, collagen shield could be promising approach for the development of novel ocular drug delivery systems.

7. DENDRIMERS

Dendrimers are three dimensional synthetic structure created by cross-linking or bridging of polymers. Due to their tree-like rigid and macromolecule structure, dendrimers are inflexible and insoluble in most liquids. Dendrimers can be assembled in a large variety of structures, sizes, surface chemistry and topology. The chemical structures, degree of cross-linking and steric properties of functional groups present on the internal and external surfaces

determine the physicochemical properties of dendrimers. They can be formed in various sizes (10 Å to 130 Å), masses (500-1500 daltons) and shapes (spherical, ellipsoid, cylindrical etc.) by polymerization technique.

Dendrimers has been investigated as a delivery vehicle of drugs and genes, imaging contrast agents, diagnostic kits, anticancer therapy, sensors and industrial catalysts. Dendrimers have a large capacity to hold a wide range of therapeutic molecules in their core or internal structure and provide protection against external environment until they are released in a time-dependant, controlled manner. Current gene-delivery vehicles such as viruses or liposomes suffer from limitations such as immunogenicity and carcinogenicity [82]. Being nontoxic, nonimmunogenic and nonviral, dendrimer-mediated gene delivery is considered a promising approach. The stable complex of positively charged dendrimers and negatively charged DNA were prepared by Kukowska-Latallo et al. [83].

Reddy et al. [84] studied the role of folate linked dendrimers in targeting solid tumors using cell culture model. Higher transfection efficiency was observed with folic acid-linked liposomal dendrimers compared to the control dendrimers. Higher expression of folate receptors have reported in retina during retinoblastoma. Utilization of this receptor for folate-linked dendrimers may be a viable approach for drug delivery during the treatment of retinoblastoma. Recently, Kansara et al. [69] reported the functional presence of folate receptor alpha on basolateral side of retinal pigment epithelium of rabbit retina using ex vivo model.

Implications of dendrimers for ocular drug and gene delivery have also been investigated by researchers. Hudde et al. [85] evaluated the efficacy of a plasmid containing reporter gene (β-galactosidase) delivery to the rabbit corneal epithelium using polyamidoamine (PAMAM) dendrimers in an ex vivo model. The staining of endothelium but not stroma and epithelium was observed without evidence of cellular toxicity from the dendrimers. Effective delivery of immunoconjugate, tumor necrosis factor receptor fusion protein (TNFR-Ig) via dendrimers was also achieved in rabbit cornea ex vivo model [85]. Utilizaion of PAMAM dendirmes for in vitro gene delivery (reporter gene, β-galactosidase and luciferase) in fetal primary human retinal pigment epithelial (RPE) cells was investigated by Urtti et al. [86]. Significant higher expression of luciferase was observed when delivered by dendrimers than other carriers (polyethylene amine and cationic lipids).

Due to capacity of carrying wide variety of drug molecules, providing protection from the surrounding environment, stability over a wide range of pH, conjugation ability, dendrimers seems to be a promising approach to deliver drugs and gene to the ocular tissues. Further in vivo experiments, however, are recommended before clinical application.

8. BIO-PHYSICAL APPROACH

8.1. Ocular Iontophoresis

Ocular iontophoresis is a non-invasive technique to deliver therapeutic agents to anterior and posterior segment tissues of the eye. Iontophoresis is based on the physical principle that ions with the same charge repel (electrorepulsion) and ions with opposite charge attract (electroosmosis) [87]. Iontophoresis causes increased transport of ionized substances into

ocular tissues (cornea, aqueous humor, iris, lens, vitreous humor, retina, choroids) with the help of an external electric current. Therapeutic agents of various classes (steroids, antifungal agents, antiviral agents, dyes, anesthetics, antibiotics and anticancer) have been studied for ocular drug delivery using iontophoresis technique.

Transcorneal iontophoresis has been proven to be a viable approach to deliver antiglaucoma agents. Kitazawa et al. [88] achieved higher intraocular tissues of 6-hydroxydopamine, a congener of norepinephrine, in rabbit model. The same group has also showed that iontophoresis technique can also be useful combination therapy (iontophoresed 6-hydroxydopamine and topical epinephrine) in the treatment of open angle glaucoma. However, Watanabe et al. found a moderate effectiveness of this technique in the treatment of primary open-angle glaucoma [89].

Transscleral iontophoresis has shown to be a better alternative of subconjunctival injection of 5-Flurofluracil (5-FU), an antiproliferative agent, after glaucoma surgery. Sustained therapeutic concentration ofionotphersed 5-FU in the conjunctiva and sclera was achieved [90]. This technique eliminates then need of subconjunctival injection and its unwanted complications such as risk of bleeding, infections, scarring and drug penetration to other ocular tissues. Application of ocular iontophoresis for delivering antiviral agents in the treatment of cytomegalovirus infection has been studied by researchers. A human cytomegalovirus (HCMV) infection is the most common cause of blindness in acquired immunodeficiency syndrome (AIDS) patients. Yoshizumi's group has demonstrated that repeated ocular iontophoresis of foscarnet could be an effective means of achieving enhanced, localized treatment of HCMV [91].

Ocular iontophoresis could be an alternative delivery system for the macromolecules with poor absorption characteristics.

8.2. Ultrasound Mediated Ocular Drug Delivery

Ultrasound is a 'physical' approach, in which sonoporation effect is created with the help of microbubbles, to potentiate pore formation in cell membrane. Advantages of ultrasound technique are non-invasiveness, deeper penetration into the tissue and can be controlled by various parameters such as frequency, duty cycles, duration of application and power density. Various mechanisms for biological action of ultrasound have been proposed including thermal energy, blood capillary permeability enhancement, sonoporation of cell membranes due to microconvection or internal cavitation [92]**.** Cavitation is defined as the formation and activity of bubbles and is believed to cause cell membrane ruptures as a results of shear stress and microstreaming [93, 94] around the bubbles in an ultrasound field. Reversible opening of tight junctions in the epithelium cell layer is one of the primary mechanisms of enhanced permeability of drugs following ultrasound application.

Mitragotri et al. [95, 96] showed that several fold higher transdermal delivery of various hydrophilic compounds can be achieved with the help of ultrasound without long-term damage to the skin. Zderic and collogues [97] support the therapeutic application of ultrasound in ocular drug delivery. Russian researchers have studied the ocular phonophoresis for transcorneal drug delivery in the treatment of corneal inflammation, wounds, and retinal dystrophy [98, 99]. Atleast 10 fold higher corneal permeability of hydrophilic compounds was reported with the application of ultrasound [100]. The ultrasound exposure is believed to

open the tight junctions in the epithelium of cornea. The ultrasound mediated pores in the surface of corneal epithelium may provide additional pathways for the corneal drug delivery. Mesiwala et al. [101] observed the reversible opening of tight junctions in the endothelial cells of blood-brain barrier.

Safety of the ultrasound application to the ocular tissues is a major concern. Histological changes of the cornea following ultrasound application have been reported. Saito et al. [102] suggested that plasma membrane disruption may result in corneal endothelium damage. Rutzen et al. [103] reported the production of corneal lesions using high intensity focused ultrasound. Minor structural alterations in the corneal epithelium have been reported with the ultrasound at a frequency of 880 kHz and intensities of 0.19-0.56 W/cm^2 for 5 minute exposure duration. However, careful investigation of the recovery of ocular tissues and barrier function after the ultrasound application, in vivo, is suggested. Ultrasound induced heating of cornea and other ocular tissue is a major obstacle to clinical applications of ultrasound. Food and drug administration outlined strict thermal safety requirements for diagnostic application of ultrasound in the eye with maximal allowed temperature rise of 1^oC. Therefore it is very essential to determine apoptosis of ocular tissues, degradation of stroma or production of cataract due to ultrasound.

In summary, ultrasound has the potential to provide a minimally invasive, efficient and controlled drug delivery to the ocular tissues. In vivo safety data must be carefully examined before ultrasound application to delicate organs such as eye.

9. SURGICAL APPROACHES

9.1. Tissue Implant/Transplant: Retinal Degenerative Diseases

Development of effective treatments for retinal degenerative disorders (diseases that affect the center of the retina) has been slow due to the complex etiology of disease and hence lack of appropriate animal models, which are essential to elucidate disease progression and to test the efficacy of many therapeutic agents. With the help of recent advancement in molecular technologies, the genetic and biochemical bases of many retinal diseases are well defined. Retinal implants, stem cell implants and gene delivery are some of the novel approaches in the treatment of vision threatening retinal diseases such as AMD, PVR and diabetic macular edema.

Transplants of the retinal pigment epithelium (RPE), neural retina, iris pigment epithelium (IPE) and photoreceptors have been evaluated by researchers to improve the vision by replacing the lost function of retinal cells. The RPE transplants approach shown to be clinically potential to integrate into the retina, to rescue photoreceptor cells and to improve vision. Little et al. [104] showed that grafts of human fetal RPE cells are capable of rescuing photoreceptor degeneration in animal (retinal degenerative rat) model.

Promising results of animal experiments lead researchers to the human RPE transplantation. A pilot study by a surgical team of ophthalmologist at John Hopkins Medical Institute involved patients with advanced RP and AMD for fetal human retinal transplants [105]. The results of suggested that the procedure was safe, graft rejection complications were less severe and light sensitivity was improved in all patients. However limited improvement

in the quality of vision was reported. However, human RPE transplant is still in its preliminary stage and further detailed investigation is required to be a feasible approach for treating retinal degenerative diseases. Cotransplantation of RPE-retina complex have shown to be more advantageous to promote retinal survival and repair by Sharma et al. [106]. Promising result of RPE-retina cografts was supported by recent morphological and biochemical studies [107].

The transplant of IPE cells have shown to be clinically potential because of the ease of availability, same embryological origin as the RPE cells, high trans-differentiation potential and functional autologus IPE transplants in patients with degenerative diseases. Rezai et al. and Abe et al. [108, 109] confirmed the capability of IPE cells to delay photoreceptor degeneration and suggested that this may be a useful alternative approach to retinal transplant. Thumann et al. [110] evaluated the efficacy of autologus IPE cells transplant in AMD patients where neovascular membrane was removed. The results from this study suggest that IPE transplant is a better choice than RPE transplant for conditions associated with loss of RPE cells after surgery. However, more clinical trials need to be carried out. Thus far, the results from animal and human retinal transplant studies provide promising vision improvement. However, various factors, such as choice of tissue, surgical procedure, immune response, proper host integration and transplantation and long term vision improvement should be considered to make this approach clinically feasible.

Genetic modification of transplant may be a viable strategy for gene delivery in the treatment of retinal diseases. Abe et al. observed high expression of bGFG in the retina and prolonged photoreceptor survival when genetically modified IPE (bGFG cDNA tansfection) was transplanted to rat retina [111]. Stem cells are precursor cells for various tissues in a human body. With the advancement of molecular technologies, such as cDNA subtraction hybridization and microarray-based transcription profiling, stem cells transplant is becoming a clinically attractive feature for tissue-replacement therapy. Stem cell transplant provides a unique opportunity for medical advancement in retinal diseases. However, this technique suffers from various limitations for clinical application. In vitro, homogenous culture of stem cells is very difficult to maintain since spontaneous differentiation results in multiple cell types and lose their multipotent characteristics.

9.2. Retinal Prosthesis

Retinal prosthesis is an exciting microelectronic technique currently being investigated by researchers to restore the vision loss of patient suffering from retinal degenerative diseases in which photoreceptor function is lost and retina becomes insensitive to light. This technique utilizes highly sophisticated, miniaturized electronic implants, which responds to light and generate signals that electronically stimulate the intact neuronal connection and thereby activate the visual system [112]. The aim of the implants is to mimic photoreceptor cell function in the visual transduction cascade.

Two types of retinal prosthesis implants, subretinal and epiretinal implants, were evaluated for their application for retinal degenerative diseases. The subretinal implant is surgically placed between RPE and photoreceptor cells while epiretinal implant is designed to rest on the inner surface of the retina. Chow et al. [113] showed that the array was stable functional position in the subretinal space and was sensitive to visible and infrared light.

However, non-permeable nature of implant blocked the supply of nutrients from choriocapillaries to the retina and resulted in degeneration of retinal cells. In order to overcome this limitation, epiretinal prosthesis was developed where implants utilizes the functional output potential of the ganglion cells and optic nerve to stimulate visual system. Hesse et al. [114] placed polyimide embedded platinum microelectrode arrays onto the inner surface of cat retina using cyanoacrylate adhesive. No signs of retinal detachment or ocular inflammation was observed after a prolong implantation time.

Inspite of promising preliminary results, retinal prosthesis approach suffers from serious surgical and biocompatibility problems. Long term retinal implantation may cause serious complications such as retinal detachment, ocular inflammation, blockage of nutrient to the retina, mechanical trauma to retinal tissues and immune response. So far, application of retinal prosthesis to restore the vision is not clinically proven and therefore construction of device that can overcome these limitations is a current focus in the field.

10. GENE THERAPY

Gene therapy, one of the most exciting fields, is being investigated by researchers for many life threatening diseases including ocular disorders like retinal pigmentosa (RP), age-related macular degeneration (AMD) and proliferative vitreoretinopathy (PVR) [115]. In gene therapy a specific gene is inserted into the cell to repair or replace the disease causing gene and to make “good”- normal functioning proteins. Recent advancement in biotechnology enables scientists to identify the disease causing genes. Due to the complex physiology and presence of blood-ocular barriers, systemic administration of genes does not provide therapeutic levels in ocular tissues. Currently, intravitreal and intracameral injections are the common delivery routes. Two types of vectors are being investigated for gene delivery: viral and non-viral vectors.

10.1. Viral Vectors

Since viruses have ability to enter the nucleus of the cells and direct the replication mechanism to viral replication, it is possible to use viral as a gene delivery vector in the treatment of diseases. The retrovirus, the adenovirus, adeno-associated virus and lentivirus are some of the most widely investigated viral vectors for ocular gene therapy.

Bradshaw et al. [116] and Murata et al. [117] studied the application of retrovirus for gene transferring in the treatment of corneal wound healing and retinal neovascularization have been studied by researchers. Retrovirus-mediated transferring of herpes simplex virus thymidine kinase (HSV-tk) resulted in efficient gene transferring and ganciclovir sensitive transduced cells. The retrovirus-mediated gene delivery was also studied for the treatment of PVR. Effective transduction and strong bystander effect after GCV treatment were reported in vitro and in vivo [118]. The use of retroviral vector therapy is constrained due to lack of infecting ability to nondividing cells and low viral load. Moreover, therapeutic gene larger than 10kb cannot be delivered using retrovirus.

Adenovirus are non-enveloped, double stranded DNA viruses that have ability to transfect wide range of host in postmitotic and proliferating cells. Adenoviruses are currently the interest of research for many retinal diseases. The genome of the adenoviruses is completely defined and modification of this genome results in production of large quantities without compromising their transfection efficiency. Adenoviruses have shown to be as an effective system for delivering a functional active therapeutic gene in the various ocular tissues like RPE, retinal ganglion cells, corneal epithelium and conjunctival epithelium [119, 120]. However, high immunogenicity, inflammatory responses and toxic reactions in the host need to be carefully evaluated for clinical applications.

Adeno-associated viruses (AAV), a DNA containing and non pathogenic viruses have ability to replicate in many cell lines with wide cell and tissue specificity [121]. They preferentially integrate in human genome in a site specific manner and therefore do not require host cell replication for integration [122]. Retina, RPE and optic nerves have been targeted using recombinant AAVs [123]. Application of AAVs as a potential vector in gene therapy is limited due to limited transgene capacity and need of helper virus for replication.

Herpes simplex viruses (HSVs), large DNA pathogens, have large gene transfer capacity and can infect wide range of retinal cells. Various ocular tissues (corena, subconjunctiva and anterior chamber) have also been studied as a potential target for gene delivery using HSVs [124]. Successful HSV-mediated transfer of lacZ gene in monkey eye, human trabecular meshwork and ciliary muscle cells, was reported by Rodhal et al. [125]. However, strong immune response from the host limits the use of HSVs for gene therapy and therefore further modification are required.

10.2. Non-Viral Vectors

Nonviral vector-mediated gene delivery has, recently, gained popularity. Nonviral vectors are synthetic and nonimmunogenic in nature. Due to their flexibility of synthesis and usage of well characterized chemical agents, physical interaction with the cells is reproducible. Moreover, the synthetic carriers are nontoxic in nature when degrade in the body. Inspite of above advantages, ocular application of nonviral vectors for delivery gene of interest is limited. Following internalization of nonviral vectors via phagocytic mechanism of human RPE, escape of DNA from the endosomes or nucleus entry of DNA could be the rate-limiting steps [126]. Possible complex degradation was reported by serum component adhering to the complexes [127].

1) Naked DNA

Direct transfer of naked DNA into the cells is simple, safe and nontoxic method of gene delivery. A therapeutic gene is often ligated to a specifically designed plasmid which is a circular DNA duplex, replicated extrachromosomally in bacteria, mostly, or sometimes in cells of other organisms. Highly negatively charged polymeric DNA can bind with a variety of agents by electrostatic and hydrophobic forces. Rapid degradation of naked DNA into the body fluid, poor transfection efficiency and need of surgical procedure to access internal tissues limit the clinical application of this approach.

Entry of naked DNA into the cells is a difficult task and requires mechanical or chemical forces. Different techniques (in vitro and in vivo) are available for preparation and

transfection of purified plasmid DNA. However, there is no standardized method available. The entry of naked DNA was improved by “gene gun” approach. In this approach, gold particle attached DNA is bombarded to the target cells with high velocity generated by high voltage electric arc. This improved technique helps in delivering naked DNA to ocular surface with minor damage or irritation to the tissues [128, 129].

2) Liposome

Utilization of liposomes as a nonviral vector is, currently, the most widely used strategy for gene delivery. This approach involves encapsulation of therapeutically active plasmid DNA into lipsomes (spherical particles composed of lipid bilayer). Based on the type of lipids, liposmes can be formulated in different sizes and charges (negative, positive and neutral). The surface characteristics of liposomes and types of linkers determine the tissue distribution, cellular uptake and intracellular trafficking of the delivered gene. Cationic liposomes are most commonly used liposomes for gene delivery due to its positively charged surface which readily interact with negatively charged DNA. Lipoplex (liposomes containing condensed DNA) enter the cell by endocytosis process and protect the DNA from the interaction with cell surface proteoglycans. However, endosomal escape and degradation inside the cell result in poor transfection efficiency and therefore limit the use of liposome-mediated gene delivery. Stealth loposomes (conventional liposomes covalently bound to polyethylene glycol) have been investigated to prevent steric hindrance and enzymatic degradation following systemic administration [130]. Such pegylated liposomes are shown to have higher biodistribution, lower reticuloenothelial system (RES) uptake and longer residence time into the systemic circulation [131].

A few studies have been reported the application of lipsome-mediated gene delivery to ocular tissues. Matsuo et al. [132] investigated the gene transfer efficiency of liposome eye drops to retinal ganiglionic cells of rats. They reported efficient gene transfer without any ocular inflammation. A recent study by Abul-Hassan et al. [133] reported efficient and nontoxic transfection of human primary retinal pigment epithelium cells with liposomes compare to other vehicles (calcium phosphate and dextran). Hangai et al. [134] evaluated a novel liposomes system (virus coated liposomes) for in vivo gene transfer and expression into adult mammalian retina. Surface modification of liposomes to achieve lignad-targeted delivery of gene has been currently research interest among many scientists. Immunoliposmes have shown to be a potential approach for targeting gene delivery.

3) Peptides

Recently, various membrane-active peptides such as pH-specific fusogenic, lytic peptides and bacterial proteins have been studied as a nonviral vector system to efficient gene transfer in ocular tissues. While cationic peptides form easy complex with negatively charged DNA, anionic peptides provide buffering into the endosomal environment and therefore prevent the endosomal degradation of DNA. Shewring et al. [135] studied the ability of peptides to deliver β-galactosidase reporter gene to the corneal endothelial cells of human, pig and rabbit. Due to the membrane- or vesicle-destabilizing property peptides from viral and natural or synthetic sequences of amphipathic peptide found to improve the gene transfection efficiency [136].

4) Polymers

Applications of polymers (polylysine and polyethylyneimine) as a nonviral vector for gene delivery have been studied. Entry of polymer-DNA complex (polyplex) occurs due to cell membrane destabilization. Polylysine-DNA complexes shown to have good transfection ability, however the polycation:DNA ration found to be toxic to normal cells and therefore limits the in vivo applications. Due to the phagocytic activity, RPE is considered as potential target for gene delivery. Chaum et al. [137] studied polyplex-mediated gene transfer into human retinal pigment epithelium cells in vitro and stable integration was reported. Application of polymers limits by concentration of polycations used for gene delivery. Most concentrations found to be toxic or associated with other adverse effects.

11. OLIGONUCLEOTIDES

All most all diseases are associated with dysfunctions of protein or gene. Oligonucleotide therapy involves blocking of the synthesis of disease causing proteins at genetic level by inhibiting transcription, translation or gene splicing events. At transcriptional level, oligonucleotides bind to the double stranded DNA and form the triples helix structure which prevents the transcription of specific mRNA. At translational level, antisence oligodeoxynucleotides interrupt the binding of complimentary DNA sequence. Splicing events which are necessary for RNA transcript can be disturbed by targeting oligonucleotides to the intron-exon junction of premature RNA.

Antisense Approach

Antisense drugs are synthetic short RNA or DNA sequence that is homologus to that contained in the target gene. Antisense oligodeoxynucleotides are synthesized in the opposite direction of the known complementary DNA sequence to bind with the specific target sequence and to interrupt the synthesis of the corresponding protein. Antisense approach has been employed as a powerful clinical tool in the treatment of ocular diseases.

Proliferative retinopathy (PVR) is an ocular disease associated with excessive proliferation of RPE cells. Capeans et al. [138] inhibited the human RPE cell proliferation by targeting antisense oligonucleotide to c-myc, an active protein in the mitogenic pathway. Fomivirsen (ISIS 2922), 21-phosphorothioate oligonucleotide, is the first antisense drug approved for the treatment of HCMV retinitis. Fomivirsen binds to complementary sequences of mRNA CMV retinitis transcripts and inhibits the viral replication in the eye [139]. Flores-Aguilar et al. [140] evaluated retinal toxicity and efficacy of fomivirsen. Higher intraocular pressure and mild inflammations were observed in the treated eye. However, these complications can be treated by topical steroid treatment. Robinson et al. [141] studied the application of oligodeoxynucleotide in the treatment of retinal neovascularization using murine model of PVR. Dose-dependant inhibition of VEGF synthesis and growth of new blood vessels were reported following intraocular administration of phosphorothionate antisense drug.

Antisense approach has encountered some serious problems. Cellular uptake, specific targeting, toxicity, determination of length of sequence with optimum activity and specificity and stability of antisense drugs are some of the important parameter in the design of effective antisense drug delivery. Antisense oligonucelotides are susceptible to nucleases present in the intra and extra cellular compartments [142]. Chemical modifications of nucleic acid backbone (phosphate, bases or sugar moieties) are very successful approach to improve the stability of antisense drugs. Matsukara et al. [143] studied the efficacy of phosphotothioate analogs of oligodeoxynucleotides against human immunodeficiency virus and reported that these analogs are less vulnerable to intracellular nuclease degradation. However, due to chiral structure phosphorothionate oligonucletotides are reported to lack efficiency and specificity and found to be toxic. Chemical modifications are also reported to reduce the affinity of the oligonucleotide towards targeted cells [144].

Recent research work is focused on the development of targeted delivery systems of oligonucleotide with the use of colloidal carriers. Intavenous administration of administration associated with higher cost and patient non-compliance issues. Polymer based delivery system is the current focus of the research. Liposomes and microparticles have been widely studied for delivering oligonucleotides. Polycations such as poly-L-lysine has been shown to have higher uptake rate of oligonucleotides [145]. However, use of polycations is limited due to non specific cellular binding and toxicity at lower concentrations. Application of liposomes as a delivery vehicle for nucleotides has shown to be biocompatible, biodegradable and effective [146]. Biodegradable micro- and nano- particles have also been studied for sustained oliogonucleotide delivery following parenteral administration. Various biodegradable polymers (albumin, gelatin and polysaccharides) and synthetic polymers (polycarprolactone, poly (lactide-so-glycolide), polyalkylcyanoacrylate, etc.) have been used for polymeric delivery of oligonucleotides [147, 148].

12. RIBOZYMES

Ribozymes, RNA enzymes, are single stranded RNA molecules act as molecular scissors to digest or edit the target RNA to prevent translation of corresponding proteins. Ribozymes have capability of forming three dimensional structures in order to induce site-specific cleavage, ligation and polymerization of nucleotides including DNA and RNA. The unique features of ribozymes can be utilized as a therapeutic tool in the field of ocular drug delivery.

With the recent advancement in the technology, unique characteristics of ribozymes, such as relatively small size, high specificity and catalytic activity, can be utilized as a genetic tool to inhibit viral replications. Ribozymes therapy has advantages over antisense drugs due to their physical, biochemical and biological properties. Ribozymes are much smaller in size than antisense nucleotides and therefore possess high selectivity. Ribozymes catalyzes self-cleavage as well as cleavage of external substances [149]. Ribozymes offer greater specificity and inhibit viral replication 2-10 folds compared to an antisense drug [150].

Limited amount of research work has been done in the treatment of ocular degenerative diseases utilizing ribozymes therapy. Autosomal dominated retinitis pigmentosa (ADRP), cause by mutated protein formation which leads to the apoptotic death of photoreceptor cells [151]. Mutation of rhodopsin gene (praline residue to histidine at 23 and serine to premature

termination at 334) cause ADRP. Lewin [152] at el. evaluated the application of ribozymes for the treatment of ADRP in transgenic rat model. Results showed that ribozymes successfully slowed down the apoptosis of rod cell and maintained cellular function for 8 months after a single injection. The constructed ribozymes had capability of specifically bind to the mutant but not wild forms of rhodopsin mRNA. Two types of ribozymes (hammerhead and hairpin) have shown most active against ADRP. Lewin et al. [153] also studied long term effect of ribozymes and reported that ribozymes appear to be a highly effective and potential long term therapy for ADRP. Ribozymes have also shown to be effective against ADRP when cloned (as DNA) in recombinant adeno-associated virus vectors.

13. NEUROTROPHIC FACTORS

Treatment of ocular diseases with soluble neurotrophic factors is a simple therapy which bypasses the need of viral or non-viral vectors. Intravitreal or subretinal injection of soluble neurotrophic factors have shown to be delay or prevent apoptotic process of retinal neurons in vision threatening ocular complications. Unlike vector-mediated delivery system, therapeutic efficacy of neurotrophic factors is not limited by immunological and cytotoxic reactions. However, clinical application of neurotrophic factors is limited due to the need for multiple treatments to complete reversal of the disease. One appropriate approach would be administering these agents using slow-release system into the eye. Pigment epithelium derived factor (PEDF), a 50 kDa protein, is an endogenous neurotrophic factor. PEDF has therapeutic potential for reducing ocular tumor progression and prevent degeneration, associated with the growing retinal tumor. Dawson et al. [154] studied a role of PEDF in ocular diseases, such as AMD where cell death and increased angiogenesis result to severe visual loss. A single intravitreal injection of PEDF has shown to inhibit the light damage effects on photoreceptors. Treatment of retinal degenerative diseases with neurotrophic factors has emerged as an attractive therapeutic tool. However, detailed studied are required to develop long-term sustained delivery of these factors to the ocular tissues.

14. POLYMERIC OCULAR DRUG DELIVERY

The treatment of vitreoretinal diseases has improved greatly in the advent of new therapies, biomaterials and sustained delivery devices. Sutained ocular drug delivery through nanopaticles, microspheres and liposomes has been attempted for improvement of various ocular diseases. Various sustained release devices such as scleral buckles, scleral implants, compressed tablets, subconjunctival implants and thermosensitive gels have gained attention.

Various polymers such as ethyl cellulose, chitosan, albumin, gelatin, poly(caprolactones) and most importantly poly (lactides) and their glycolide copolymers have been studied for preparation of sustained drug delivery [155]. Due to their biocompatibile and biodegradable nature, Poly (D, L-lactide-co-glycolide) (PLGA) polymers are the most studied polymer for drug delivery. A wide variety of these polymers are available in the wide range of molecular weight and lactide:glycolide ratio. Controlled delivery of drugs via PLGA polymers as implants, microspheres and nanoparticles has gained wide acceptance. Custom development

of a sustained release formulation becomes recent interest of pharmaceutical industry to obtain required duration of drug action in various pathological conditions. An ideal controlled release formulation should release the entrapped drugs in a continuous manner over desired time periods.

Release modifying agents such as ethylene-glycols, isopropyl myristate and surfactants have been studied to achieve constant release of a hydrophilic drugs like acyclovir and ganciclovir from PLGA microspheres [156, 157]. Addition of such agents in the ophthalmic formulation could result in potential tissue toxicity. Moreover, in order to modulate the desired drug release, blending of different types of PLGA polymers with different molecular weights and lactide/glycolide ratios have been studied by researchers including atuhtor's laboratory [158, 159].

Kompella's group has recently studied the size dependant disposition of subconjunctivally administered micro and nanoparticles [160, 161]. They have concluded that periocular administration of particulate systems of this size would likely be useful as sustained drug delivery systems. Same group has also evaluated the efficacy of celecoxib-PLGA mciroparticles in the diabetic mice following periocular injection and reported that celecoxib microparticles are useful sustained drug delivery systems for inhibiting diabetes-induced elevations in PGE2, VEGF, and blood-retinal barrier leakage [162].

Biodegradable and biocompatible thermogelling polymers are rapidly gaining acceptance as sustained drug delivery systems. Sustained release of therapeutic agents has been reported using triblock co-polymers prepared from polyethylene glycol (PEG) and PLGA. Aqueous solutions of these polymers at physiological temperatures have been reported to undergo phase transformation from the solution to gel state which makes them ideal polymers for drug administration. Amongst the various triblock co-polymers currently available, PLGA-PEG-PLGA is an attractive candidate for use in ocular drug delivery.

Mitra's group have reported that 23% w/w solution of the polymer gels at 32-34 ^{o}C and remain as free flowing solutions at room temperatures. Therefore, these polymers can be injected with ease and upon administration they rapidly get at the site of administration forming a drug depot. A novel approach of drug loaded microspheres suspended into PLGA-PEG-PLGA polymer has been recently studied [158]. The major advantages of this formulation prepared by dispersing drug loaded PLGA microsphreres into thermogelling PLGA-PEG-PLGA gel are:

- ease of administration
- biocompatibility and biodegradability
- modulation of drug release rates and durations by carefully manipulating the type of microspheres used in the dispersion
- minimal particle migration in vitreous
- frequent re-administrations possible without the need for removal of previous implant

A formulation containing GCV loaded PLGA microspheres dispersed in PLGA-PEG-PLGA gel has been prepared and investigated for its utility in drug delivery in author's laboratory [158]. A controlled release PLGA microsphere based formulation of GCV has been evaluated in vitro and in vivo for its release characteristics (Figure 6).

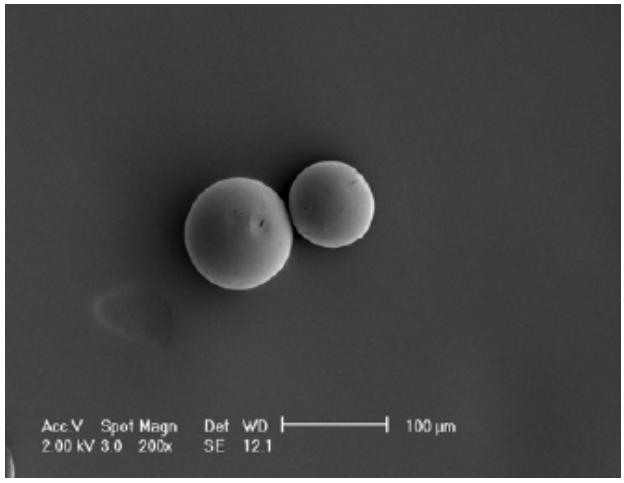

Figure 6. GCV loaded Resomer 502H microsphere.

Dispersion of GCV loaded PLGA microspheres in PLGA-PEG-PLGA gel could result in ability to administer exact amounts of drug and diminished particle migration in vitreous (Figure 7). Evaluation of the dose-toxicity response of the tri-block gel has been warranted before clinical application of biodegradable thermogel can be employed to tailor-make controlled release devices.

Inspite of extensive ongoing research, an ideal sustained release ocular drug delivery system has yet to be developed. The only systems currently in clinical use are the solid reservoirs containing ganciclovir in the treatment of HCMV retinitis. The development of thermogelling injection outside the eye is of great interest among pharmaceutical scientists. Recent advancement in polymeric ocular delivery seems to provide a great opportunity to develop biocompatible and biodegradable devices in the treatment various vitreoretinal pathologies.

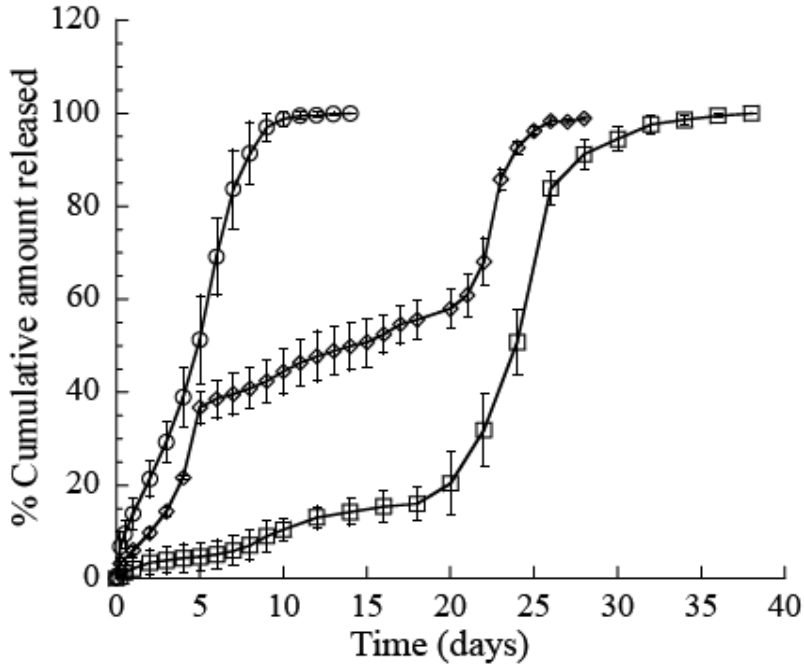

Figure 7. In vitro release of GCV from 10 mg of Resomer RG 502H microspheres (○), blend microspheres (□) and their 1:1 mixture (◊) dispersed in 200 µl of 23 % w/w aqueous solution of PLGA-PEG-PLGA polymer at 37 º C and 60 oscillations/min. (n=3).

FUTURE DIRECTION

Vision threatening ocular pathologies require immediate attention and long term treatments. Efficient and safe delivery of therapeutic agents to the ocular tissues, mainly posterior segment tissues, is a major challenge for ophthalmologists and pharmaceutics scientists due to the presence of various physiological barriers. Various approaches have been studied to achieve therapeutically effective concentrations of drugs into the ocular tissues. Recent technological advancement has changed the field of ocular drug delivery from conventional drops to biodegradable, sustained release ocular delivery systems. Application of iontophoresis and ultrasound has added a new dimension to the development of various ocular drug delivery devices. Recent advancement in the field of biotechnology has opened a new area of tissue-, cells- and disease- specific gene delivery. In the recent era of technology, combinatorial approach seems to be a focus of research in the development of safe and efficient ophthalmic drug delivery systems.

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Chapter 2

CORE-SHELL POLYMER NANOPARTICLE FORMULATIONS FOR THE ORAL ADMINISTRATION OF PEPTIDES AND PROTEINS

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ABSTRACT

Innovation in pharmacology can be achieved designing polymer nanoparticles to rationalize drug delivery by enhancing the oral bioavailability of peptides and proteins. New types of formulations were designed during the last couple of years promoting interactions with the mucus for bioadhesion, with the gastro-intestinal fluids for decreasing antiprotease activity and possibly with cells constituting the epithelium to enhance permeability. As they will be presented in the present chapter, they result from the formation of core-shell poly(alkylcyanoacrylate) nanoparticles coated with chitosan and thiolated chitosan. The design and characterization of such core-shell polymer nanoparticles will be described in this chapter. Their evaluation as a tool to improve the bioavailability of peptides and proteins by the oral route will be discussed thanks to data obtained from different *in vitro* models and set ups. The more advanced research will then be presented on the last part of this chapter opening the conclusion and the discussion for future developments.

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INTRODUCTION

Significant advances in biotechnology and biochemistry have led to the discovery of a large number of bioactive molecules and vaccines based on peptides and proteins (Jung et al., 2000, Morishita and Peppas, 2006). They have become the drugs of choice for the treatment of numerous diseases as a result of their selectivity and ability to provide effective and potent actions.

In comparison to other possible routes of administration, oral delivery of peptidic drugs has many advantages. It is non invasive and safe, it avoids complications arising from the need of sterile techniques including parenteral formulations. Moreover, it is convenient and easy dosage forms can be obtained at low preparation costs. Above all, it should encourage patient compliance (Pinto Reis et al., 2006). However, the suitable clinical exploitation of these pharmacologically active bio-engineered products including peptides by the oral route is seriously hampered by two main obstacles: their poor stability in the gastrointestinal tract (GIT), mainly due to enzymatic degradation (enzymatic barrier), and their limited transport across the intestinal epithelia (permeation barrier) (Zhou, 1994; Larionova et al., 1999; Madsen and Peppas, 1999; Ameye et al., 2000; Jung et al., 2000; Michael et al., 2000; Dorkoosh et al., 2002; Morishita and Peppas, 2006).

Most therapeutic peptides are still being administered by the parenteral route because of insufficient absorption from the gastrointestinal tract. It is clear that the development of suitable carrier systems enhancing drug delivery remains challenging for the pharmaceutical scientist but it is believed that it will make possible the oral delivery of peptides (Jung et al., 2000; Cox et al., 2001; Prego et al., 2005; Morishita and Peppas, 2006). The need to improve the administration of peptides by the oral route is not only focused on the increase of bioavailability of the pharmacologically active form of the therapeutic peptide such as insulin or calcitonin, but also includes the concept of mucosal vaccination which has become more prominent in the recent years. Oral dosage forms of vaccines would improve patient compliance and facilitate frequent boosting necessary to achieve an adequate protective immune response. Indeed, epithelia from mucosae are the entrance doors for many pathogens including viruses and bacteria hence mucosal immunity can be considered as an important protective barrier against infections (Jung et al., 2000; Le Buanec et al., 2001; Iqbal et al., 2003; Mansouri et al., 2004).

The aim of the present chapter is to highlight and discuss the advantages of recent innovations in pharmacology proposed to rationalize the oral delivery of therapeutic peptides and proteins. The first part will briefly summarize the different obstacles hampering so far peptide and protein therapies given by the oral route. The advantages of formulation strategies using nanoparticles will be discussed in the second part of the chapter. Recent results considering new types of formulations including core-shell poly(alkylcyanoacrylate) will be presented in the following 3 parts. First the synthesis of the nanoparticles will be described together with the physico-chemical characterization. The interactions of the chitosan-coated

poly(alkylcyanoacrylate) nanoparticles with the mucosae will then be considered. Finally, results showing how these nanoparticles can modulate the intestinal permeation will be presented. The more advanced researches on core-shell poly(alkylcyanoacrylate) nanoparticles presented on the last part of this chapter will open the conclusion and the discussion for future developments.

OBSTACLES TO THE ORAL ADMINISTRATION OF PEPTIDES AND PROTEINS

Obstacles to the oral administration of peptides and proteins include many parameters. Parameters inherent of the therapeutic molecules include the high molecular weight shown by peptides and proteins and their hydrophilic natures. These two properties greatly compromise their absorption through the gut epithelium. Additional problems come from the very unfavourable conditions encountered in the gut generally resulting in the degradation of the therapeutic peptides and proteins. Moreover, once in the body, peptides are genrally characterised by high elimination rate constants, hence requiring constant feeding with large amounts of the peptides to ensure sustained therapeutic concentration levels and even sometime accordingly to very specific time-delivery pattern. To better understand the efforts made developing suitable delivery formulations for therapeutic peptides and proteins, this part of the chapter summarizes the different physiological and biochemical obstacles those compounds are facing when they are administered orally.

Physiological Obstacles

In human, the small intestine is 6 to 7 m long and is roughly divided into three sections: duodenum, jejunum and ileum, which comprise 5%, 50% and 45% of the total length respectively. The intestinal epithelium normally functions as a selective barrier with a surface area of about 500 m^2 in human (Balimane and Chung, 2005). It allows the absorption of nutrients, electrolytes and water but restricts the passage of larger potentially toxic compounds from the lumen into the systemic circulation. This restriction prevents the translocation of entire proteins like toxins and of bacteria eventually responsible for systemic infections (Usami et al., 2003). Approximately 90% of the all absorption occurs in the small intestinal region of the gastrointestinal tract (Balimane and Chong, 2005; Lee et al., 2005). The epithelium is covered by protective mucus composed of glycoproteins (mucins) and covering the mucosa as a continuous adherent blanket. The thickness of the mucus layer varies regionally throughout the gastrointestinal tract, decreasing distally from 50 to 500 μm in the stomach to 15 to 150 μm in the colon. Over the Peyer's patches, the mucus layer is much thinner than everywhere else in the intestine (Norris and Sinko, 1997; Ponchel and Irache, 1998).

To reach the epithelium where absorption takes place, molecules have to cross first the mucus layer. Mucoadhesive formulations are designed to adhere to the mucus layer hence delaying the transit of the associated molecule and prolonging its residence time in contact with the sites of absorption. When the formulation is engulfed in the gel formed by the

mucins the established gradient of concentration in the drug between the delivery system and the epithelium is in favour to the absorption of the drug (Huang et al., 2000; Martinac et al., 2005; Morishita and Peppas, 2006).

In addition to the mucus layer, it is generally accepted that the main barrier for drug absorption is formed by the intestinal epithelium. Several routes can be followed to cross this epithelium (Mardones et al., 2004). Bio-engineered compounds including proteins and peptides are mostly large molecules which are highly hydrophilic in nature and do not partition to a large extent into cell membranes. They are consequently excluded from the transcellular transport pathway. The absorption of these compounds is for the most part limited to the alternative paracellular pathway (Tenhoor and Dressman, 1992), which occupies only a very small surface area of the intestine (around 1% of the total surface) compared to the transcellular route (Kotzé et al., 1998).

Epithelial cells of the intestinal mucosa are joined together at intercellular attachment zones by junctional complexes which represent 0.5 to 2 μm in length (Kriwet and Kissel 1996). The different elements of these complexes are known as *zonula occludens* or tight junctions, *zonula adherens* or intermediate junctions and *macula adherens* or spot desmosomes (Baker et al., 1991). The *zonula occudens* or tight junctions are located at the apical end of the lateral membrane of adjacent cells. They are continuous strand-like transmembrane proteins which interact with similar structures of adjacent cells. This interaction defines the paracellular permeability (Fasano and Nataro, 2004). They lead a pore with a range of 8 to 16 Å in diameter (Zhou, 1994) being the major restriction for the paracellular absorption of active molecules (Samanen et al., 1996).

Tight junctions are dynamic structures subjected to structural changes that dictate their functional status under a variety of developmental, physiological and pathological circumstances (Schulzke et al., 2005). To meet the many diverse physiological challenges to which the intestinal epithelial barrier is subjected, tight junctions must be capable of rapid and coordinated responses. This requires the presence of a complex regulatory system that orchestrates the state of assembly of the tight junction multi-protein network (Fasano and Nataro, 2004; Schulzke et al., 2005). Although these adaptive mechanisms and specific regulation of tight junctions are areas of active investigations, they still remain incompletely understood (Fasano and Nataro, 2004). It is well-known that the structure of the tight junctions is influenced by a variety of factors, e.g. protein kinase C, cytochalasin and hormones (Borchard et al., 1996; Cox et al., 2001; Usami et al., 2003). There are also evidences that tight junctions are linked to the cytoskeleton, so that intracellular processes might lead to change in their dynamic structure (Fasano and Nataro, 2004; Avadi et al., 2005). In addition, their intactness is linked to the presence of divalent cations, typically Ca^{2+} or Mg^{2+} (Borchard et al., 1996).

One approach to overcome the restriction of the paracellular transport pathway for active peptides and proteins delivery is the co-administration of *absorption-enhancing agents* regulating the integrity of the tight junctions by physicochemical interactions, either with the proteins of the tight junction or with environmental components such as extracellular calcium (Ameye et al., 2001; Roumi et al., 2001; Dorkoosh et al., 2002). The ideal enhancer will be non-toxic and act in a reversible way on the junctional complex maintaining it open just during the drug absorption. Currently, the search for safe and effective absorption enhancing agents is one of the major research fields in both academia and industry (Kotzé et al., 1998). Different absorption enhancers have been tested: calcium chelating agents (Boulenc et al.,

1995a; Thanou et al., 2001; Schulzke et al., 2005), surfactants (Boulenc et al., 1995b), fatty acids (Michael et al., 2000; Usami et al., 2003), etc. Several cationic and anionic polymers have been also studied as absorption enhancers. Although the action mechanism of those polymers has not been totally elucidated, the mechanism proposed for cationic polymers (e.g. chitosan, poly-lysine, etc) was related to their ability to interact with tight junction proteins promoting their re-organization and subsequent opening (Ewan et al., 1993; Ranaedi et al., 2002). For anionic polymers, such as polyacrylic acid, a calcium binding mechanisms was suggested (Borchard et al., 1996; Kriwet and Kissel 1996; Jung et al., 2000). Both types of polymers can be used in the strategy to elaborate a carrier system aiming the improvement of the paracellular absorption of active molecules.

Enzymatic Barrier

The physiologic role of gastrointestinal proteases is to degrade dietary proteins into di- and tripeptides and aminoacids being absorbed by the small intestine. Obviously, these proteases represent a risk for the oral administration of therapeutic peptides resulting in the total loss of their biological activity. Generally, the intestinal route presents the largest enzymatic barrier to the absorption of peptides because intestinal tissues contain relatively high amount of soluble proteins (Zhou, 1994). According to their location, intestinal proteases can be divided into three main groups: luminal, brush border membrane-bounded and intracellular (cytosolic and lysosomal) enzymes (Lueβen et al., 1996; Langguth et al., 1997).

Luminal enzymes. Once in the gastrointestinal tract, the first enzymes involved in protein degradation are the pepsins, secreted in the stomach lumen by the gastric pits. These proteases are mostly active at pH 2 to 3 and become inactive at a pH above 5 (Tenhoor and Dressman, 1992). Pepsins do not represent a real threat to orally administered pharmacologically active peptides because gastro-resistant coating materials can guarantee stability in the prevailing acidic conditions of the stomach. In contrast, the other important group of luminal enzymes including the pancreatic enzymes: trypsin, chymotrypsin, elastase and carboxypeptidases A and B represents a much serious risk for active peptides (Lueβen et al., 1996). They are highly active in the small intestine and are able to survive transit through the entire human digestive tract because their role is to transform proteins in assimilable molecules (e.i. di- or tri-peptides and even aminoacids).

Brush border membrane and intracellular proteases. Brush border membranes and intracellular proteases are present on all mucosal tissues. As pharmacologically active peptides will most likely choose the paracellular route rather than the permeation through the lipophilic cell membranes, the intracellular proteases have almost no influence in the active peptide stability. Conversely, membrane bound peptidases play the most prominent role in enzymatic degradation of active peptides (Quan et al., 1999; Ameye et al., 2000). Brush border membrane proteases are large glycoproteins facing toward from the membrane to hydrolyse peptides that come in contact with the cell surface. Examples of brush border proteases include aminopeptidases A and N, amino-oligopeptidase, dipeptidyl aminopeptidase IV, and angiotensin converting enzyme (Tenhoor and Dressman, 1992). Most of these enzymes, such as aminopeptidases, are metallopeptidases which need metal ions (Zn^{2+}, Co^{2+}, etc) as co-factors to be active (Sanderink et al., 1988; Taylor, 1993; Hooper, 1994).

Finally, it is also interesting to point out the metabolic activity of the microflora in the small and large intestine, especially in the colon. In the colon, it is composed of more than 500 species consisting of 10^{11} to 10^{12} bacteria per gram of gut content, and is capable of several metabolic reactions that can also influence peptide stability once they reach this part of the intestine (Pinto Reis et al., 2006).

Peptidic drugs can be absorbed through the gastrointestinal mucosa and enter the blood circulation in an intact form only if they are fully protected from enzymatic degradation down to their absorption site (Zhou, 1994; Larionova et al., 1999; Madsen and Peppas, 1999; Ameye et al., 2000). Because of the high variety of enzymes encountered, the many locations in the gut and the multiplicity of degradation sites in the peptide and protein structure it can be expected that there will be an upper limit to the percentage of an applied dose that will actually reach the absorption target site (Pinto Reis et al., 2006).

Some of the most important strategies tried to overcome the enzymatic barrier were: the design of drug delivery systems targeting a particular part of the gut where the luminal proteolytic activity is relatively low, typically the colon (Mackay et al., 1997), the structural modification peptides preventing proteolytic attack (Dass and Mahalakhmi, 1996; Jayawardene and Dass, 1999; Mizuma et al., 2000) or the use of protease inhibitors (Birk, 1985; Narayami, 2001). Although the use of protease inhibitors appeared to be an attractive strategy from a technological point of view, it raises serious problems of toxicity after simple co-administration of typical inhibitors, such as soybean trypsin inhibitor, aprotinin or Bowman-Birk inhibitor. Indeed, the toxic side effects of the inhibitors especially appeared during chronic therapy involving peptides because they can affect the digestion of dietary proteins (Lueβen et al., 1996; Morishita and Peppas, 2006). To prevent occurance of toxic effects due to the inhibitors, it was suggested to immobilize them in the pharmaceutical dosage form (Larionova et al., 1999; Madsen and Peppas, 1999). However, the inhibition of the proteases requires a direct interaction between the inhibitor and the enzyme. This is difficult to achieve when the inhibitor is immobilized on conventional dosage forms and the proteases are located in the mucus layer or at the apical membrane of the epithelial cells (Lueβen et al., 1996).

In the specific case of proteases requiring cations as co-factors (metallopeptidases) or to maintain their tridimensional structure (such as trypsin), one of the most effective inactivation approaches is to deplete the enzyme environment in this cation (Lueβen et al., 1996). This inhibition is interesting because it does not require a direct contact between the enzyme and the inhibitor (Lueβen et al., 1995). Indeed, it was demonstrated that chelating agents such as EDTA, EGTA, were efficient to inhibit different metallopeptidases. However, they must be immobilized in the pharmaceutical form to avoid toxic side effects (Bernkop-Schnürch and Krajicek, 1998; Bernkop-Schnürch and Kast, 2001). Some polymers showing intrinsic complexing properties, such as carbomers and polycarbophil, were able to inhibit trypsin activity thanks to a calcium depletion capability (Lueβen et al., 1995; 1996; Madsen and Peppas, 1999; Ameye et al., 2000; 2001).

Alternatively, another interesting approach is the use of *bioadhesive systems*. They are believed to be effective in enhancing the intestinal absorption of biologically active compounds vulnerable to proteolytic enzymes. Indeed, bioadhesive systems can minimize presystemic metabolism of peptide and protein drugs by the action of luminal peptidases during their transit throughout the intestine and during their transit between the delivery

system and the membrane of the enterocytes (Mao et al., 2001; Iqbal et al., 2003; Mansouri et al., 2004; Vila et al., 2004; Salman et al., 2005; Morishita and Peppas, 2006).

ADMINISTRATION OF PEPTIDES AND PROTEINS BY THE ORAL ROUTE USING NANOPARTICLES

From the previous section, it is obvious that peptides and proteins cannot overcome the gastrointestinal barriers by themselves. For instance, peptides of therapeutical interest such as TRH (Tyroïde Releasing Hormone) analogues, calcitonin or insulin are only poorly absorbed after oral administration. Their bioavailability remains very low being generally below 5%, 2% and 0.5% respectively (Smith et al., 1992; Tenhoor and Dressman, 1992).

Major efforts have been made to design effective and safe formulations for peptides and proteins using drug carriers. Several of the proposed strategies included the use of liposomes (Fukunaga et al., 1991; Takeuchi et al., 2005), emulsions (Matsuzawa et al., 1995; Silva Cunha et al., 1997), microcapsules (Morishita et al., 1992; Couvreur and Puisieux, 1993; Allémann et al., 1998) and nanoparticles (Couvreur et al., 1995; Couvreur et al., 2002; Panyam and Labhasetwar, 2003; Couvreur and Vauthier, 2006; Prego et al., 2006).

Nanoparticles may be defined as submicronic (< 1μm) colloidal systems. According to the process used for the preparation of nanoparticles, nanospheres or nanocapsules can be obtained. While nanospheres are matrix systems in which the drug is dispersed throughout the particles, nanocapsules are vesicular systems (“reservoir” systems) in which the drug is confined in an aqueous or oily cavity surrounded by a polymeric membrane (Alonso 2004; Couvreur et al., 2002). In many occasions, the literature has emphasized the importance of the size of the drug carrier and revealed the advantages of nanoparticles over microparticles (McClean et al., 1998; Zhi et al., 2005). It also emphasized the advantages of nanoparticles over liposomes because of their greater stability in biologic fluids as well as during storage (Soppimath et al., 2001).

Among the different kinds of nanoparticles that have been applied for drug delivery, polymeric nanoparticles were the most studied (Gupta et al., 2005). They present several characteristics that make them promising candidates to develop suitable carriers enhancing oral administration of bio-engineered drug products (Couvreur and Vauthier, 2006). The entrapment of active molecules within the polymeric nanoparticle would offer protection of the molecules against *in vivo* degradation after oral administration and control of the drug release. Moreover, due to their small size, they present an important specific surface area available for very define interactions with the absorptive epithelium and with tissues and cells (Zhou, 1994; Hillgren et al., 1995; Jung et al., 2000; Michael al., 2000; Dorkoosh et al., 2002; Mansouri et al., 2004; Morishita and Peppas, 2006; Pinto Reis et al., 2006; Prego et al., 2006).

The real utility of nanoparticles as carriers enhancing the oral delivery of drugs was demonstrated in many occasions in the scientific litterature. Briefly, nanoparticles have been used as oral drug carriers for several reasons: (I) improvement of the bioavailability for drugs with poor absorption characteristics, (II) prolongation of the residence time of drugs in the intestine, (III) high dispersion at the molecular level and consequently drastic decrease in the dissolution time for poorly soluble drugs, (IV) delivery of vaccine antigens to gut-associated

lymphoid tissue, (V) control of the release rate of drugs, (VI) reduction of the gastrointestinal tract irritation caused by drugs and (VII) improvement of the stability of drugs in harsh conditions of the gastrointestinal tract (Pinto Reis et al., 2006).

Fate of the Nanoparticles in the Intestinal Tract

One approach enhancing oral delivery of therapeutic peptides and proteins using colloidal systems is to exploit the uptake of particles by the gastrointestinal tract (Hillery et al., 1996a; Florence, 2005). Indeed, nanoparticles administered orally can be absorbed, albeit in small quantities. Absorption not only occurs through the M- cells of the Peyer's patches in the gut-associated lymphoid tissues (GALT) (Hussain et al., 2001; Florence, 2005; Morishita and Peppas, 2006) but also through the enterocytes which represent the majority of the cells of the gut epithelium (Davda and Labhasetwar, 2002, Pinto-Alphandary et al., 2003, Florence, 2005).

According to Florence (2005), some of the most important factors affecting uptake and translocation of nanoparticles by the gut epithelium after oral administration are: (I) the diameter: Decrease in diameter below 1 μm increases the uptake of particles. With a diameter above 3 μm, particles are preferentially taken up by the Peyer's patches and remain there. (II) The surface charge: charged particles can be taken up but in a lower extend than non-ionic hydrophobic nanoparticles. (III) The presence of surface ligands: ligands selective to epithelial cell receptors enhance the affinity of the carriers for the cells hence promoting uptake but not necessarily translocation. (IV) The elasticity of the nanoparticles: the elasticity appears potentially important to allow nanosystems to cross capillaries with diameter smaller than the nanoparticle diameter once they have crossed the epithelium, and (V) the colloidal stability: the absence of flocculation and aggregation is a key issue for the absorption of nanoparticles. It is related to the particle size effect mentioned above.

Although the very small size of nanoparticles is in favour increasing the interactions of these types of formulations with the mucus due to the large specific surface area, the effect of mucus in nanoparticle uptake phenomena is controversial. On the one hand, it can inhibit the uptake of particles because of their engufment in the viscous glycoprotein gel hampering their access to the absorption sites. In this sense, Behrens et al. (2002) demonstrated that the presence of mucus was a major barrier to the absorption of hydrophobic polystyrene nanoparticles and showed an even stronger effect with hydrogel nanoparticles. On the other hand, it can be argued that entrapment of nanoparticles in mucus delay the transit of the nanoparticles along the gut bringing then closer to absorption sites for a longer period of time (Florence, 2005). Nevertheless, whatever the previous considerations were, nanoparticle uptake at the level of the gastrointestinal tract has been suggested to be responsible for the increased oral bioavailability observed for several peptides such as LHRH (Hillery et al., 1996b) and cyclosporin (Ford et al., 1999).

Plain Nanoparticles

Maybe some of the most popular polymeric nanocarrier for the oral administration of peptides are poly(alkyl cyanoacrylates) (PACA) nanocapsules and nanospheres. They have

demonstrated to efficiently improve the oral bioavailability of peptides such as insulin (Damgé et al., 1988; Damgé et al., 1990; Lowe and Temple, 1994; Pinto-Alphandary et al., 2003), octeotride (Damgé et al., 1997), cyclosporine A (Allémann et al., 1998), calcitonin (Lowe and Temple, 1994) among other pharmacological peptides.

PACA nanoparticles can be prepared either by polymerisation of alkyl cyanoacrylate (ACA) monomers (Couvreur et al., 1979; Couvreur et al., 2002) or directly from PACA polymers and copolymers, by nanoprecipitation or emulsification solvent evaporation (Peracchia et al., 1997a; Couvreur et al., 2002). PACA are bioerodible polymers that are degraded in vivo mainly by esterases of the pancreatic juice in the intestinal tract or of serum in the blood. The degradation of PACA nanoparticles takes place in a couple of hours, depending on the alkyl side chain length, leading to degradation products (alkylalcohol and poly(cyanoacrylic acid)) that are soluble in water and easily eliminated (Vauthier et al., 2003b).

PACA nanoparticles can protect the active molecules and control their release by an erosion mechanism (Fawaz et al., 1997; Chattaraj et al., 1999; Dembri et al., 2001). There are evidences that PACA nanoparticles can be uptaken by M-cells (Michel et al., 1990), which might be favoured by the hydrophobic and anionic nature of PACA. The high esterase activity described along the M-cell apical side, might promote nanoparticle degradation and subsequent peptide release. PACA nanoparticles can also cross the epithelium by the paracellular pathway in some extent, releasing the peptide in the bloodstream after action of serum esterases. However, this mechanism is mainly limited to particles of small diameter (< 150μm) (Pinto-Alphandary et al., 2003). Finally, several authors have observed some tendency of PACA nanoparticles to develop intimate contact on large mucosal sites, evidencing the capture of PACA nanoparticles by the intestinal mucus (Ponchel et al., 1997; Dembri et al., 2001). Thus, the increase in bioavailability observed for active peptides administrated orally using PACA nanocarriers might be understood as a combination of several mechanisms: nanoparticle retention in the gastrointestinal mucus which creates a "reservoir" for peptide release and absorption, nanoparticle uptake by epithelium constituted by enterocytes and nanoparticle degradation at M-cell level (Damgé et al., 1990; Pinto-Alphandary et al., 2003).

Although it is not the aim of this chapter, it seems interesting to underline that PACA nanocarriers are also very promising tools in cancer therapy (for more detailed information audience is suggested to read Brigger et al., 2002; Vauthier et al., 2003a; Couvreur and Vauthier, 2006).

Other polymers of interest in the development of oral colloidal delivery systems are poly(lactic-co-glycolic acid) (PLGA) and poly(lactic acid) (PLA). They have gained some popularity mainly because of their noteworthy properties in terms of tissue compatibility and biodegradability (Anderson and Shive, 1997; Bilati et al., 2005). For example, Kim et al. (2002) demonstrated that a single administration of PLGA nanoparticles entrapping the peptide type II collagen was more efficient than repeated oral administrations of the peptide alone, in the induction of oral tolerance for the suppression of autoimmune rheumatoid arthritis. McClean et al. (1998) showed that about 10% of nanoparticles of PLA administered orally were adsorbed by the apical membrane of the gut in animals. No differences were found between the uptake patterns of tissues with and without Peyer's patches. The absorption mechanism was found to be size-dependent (nanoparticles better than microparticles) and predominately transcellular. However, the authors concluded that the

surface of PLA nanoparticles could be modified to improve their use as vaccine delivery systems in the gut.

Other nanoparticles that have raised much attention during the last decade are those made of chitosan. Several methods of preparation of chitosan nanoparticles such as coacervation/precipitation methods, spray-drying, and gelation have been studied for conventional drug encapsulation. However, the cross-linking methods have been the most used for peptides and drug encapsulation (Agnihotri et al., 2004). Although this method guarantee the preparation of well defined and size controlled chitosan nanoparticles, the conventional cross-linking agents, including glutaraldehyde, must be avoided preventing both damage in the loaded peptide structure and cellular toxicity (Janes et al., 2001; Wang et al., 2006). As an interesting alternative way to produce chitosan nanoparticles, the use of anionic counterions like tripolyphosphate, dextran sulfate or alginate as cross-linking agents was proposed. With the so-called ionic gelation process, chitosan nano- and microparticles loaded with peptides can be produced in gentle conditions. This method has gained much attention because it is simple, non-toxic and involves the mixture of two aqueous solutions at room temperature avoiding the use of organic solvents (Calvo et al., 1997; Janes et al., 2001; Sarmento et al., 2006; 2007). Chitosan constituting the nanoparticles retains its cationic nature required for bioadhesion at the mucosae surface and permeation enhancing properties (Boonsongrit et al., 2006). Pan et al. (2002) have elaborated insulin loaded chitosan nanoparticles by ionotropic gelation with tripolyphosphate. The ability of these nanoparticles to enhance the intestinal absorption of insulin made possible the lowering of blood glucose levels for an extended period of time after oral administration. Ma et al (2005) observed similar results showing strong interactions between rat intestinal epithelium and chitosan nanoparticles several hours after oral administration which also promoted an increase in the peptide bioavailability.

The group of Ferreira also used insulin as model peptide to develop dextran sulfate/chitosan and alginate/chitosan nanoparticles, demonstrating in both cases a high insulin association efficacy and preserving the peptide activity. Current studies of this group are focused on the in vivo evaluation of such a systems (Sarmento et al., 2006; 2007).

Chitosan is a very promising candidate for designing carrier systems for oral vaccine delivery (Van der Lubben et al., 2001a). Using confocal laser scanning microscopy, Van der Lubben et al. (2001b) demonstrated the uptake of ovalbumin loaded chitosan microparticles by Peyer's patches after intragastric feeding of mice.

Core-Shell Nanoparticles

According to Prego et al. (2006), the efficacy of polymeric nanocarriers to improve the absorption of labile macromolecules depends strongly on their composition. Polymers used should (I) provide the drug intact to the specific site of absorption, (II) preferentially prolong its residence time on the mucosa, and (III) reversibly and temporary increase the permeability of the mucosal epithelium to allow transport of macromolecular drugs to the blood circulation and/or to the lymphatic system. Additionally, a safety requirement should be that these excipients should not show adverse systemic effects, damage or exfoliate the epithelium (Thanou et al., 2001). It was suggested that a rational modification in the composition and structure of the nanoparticle surface, using safe materials, can increase the prospects of their

usefulness for mucosal protein delivery and transport (Vila et al., 2002). Several modifications of nanoparticle surface were already proposed to improve the biopharmaceutical characteristics of the oral administration of peptides and proteins. One of the most promising consist in coating the nanoparticle surface with *poly(ethylene glycol) (PEG).* Tobío et al. (2000) and Vila et al. (2002) observed an increment in the bioavailability of tetanus toxoid when poly(lactic acid) (PLA) loaded nanoparticles were coated with PEG. The PEG coating would compromise enzyme adsorption, thereby reducing the harsh conditions in which the particles are exposed until they reach the absorbing epithelium. Additionnaly, an increment of the bioadhesion of PLA nanoparticles was also suggested to occur in vivo after coating with PEG. Gref et al. (2001) analysed the benefit of PEG coating on PLA nanoparticles loaded with cyclosporin A. They noted that the presence of PEG not only improved the stability of nanoparticles in fluids medium and the peptide loading, but also performed a more adequate control of the release of the peptide *in vitro.*

The use of polysaccharides to coat nanoparticles was suggested to mimic the surface of cells, bacteria and viruses giving the nanoparticles new surface properties including the biological activity of the carbohydrate (Passirani et al. 1998a, Maruyama et al., 1999; Chauvierre et al., 2003c; Vauthier et al., 2007). In this view, the use of chitosan as coating material has been investigated. García-Fuentes et al. (2005a) prepared lipid nanoparticles coated with chitosan for the oral administration of calcitonin. The coating was performed by simple incubation of the lipid nanoparticle core in a chitosan solution thanks to the high affinity of chitosan for the lipid core. These nanoparticles showed a significant improvement of calcitonin oral bioavailability *in vivo*, which was attributed to the permeation enhancement properties already described for chitosan (García-Fuentes et al., 2005b). Prego et al. (2005) studied the permeation effect of chitosan coated nanosystems (lipid nanoparticles and oil nanodroplets) using a cell line developed as model of intestinal barrier representative of a monolayer of enterocytes (Caco-2 cell monolayer model). They observed that those chitosan-coated nanosystems caused a concentration-dependent opening of the tight junctions and particle internalisation in the cell monolayer. In subsequent *in vivo* studies performed in rats, it was suggested that this behaviour can explain the observed increased of intestinal absorption of calcitonin. In addition, the authors obtained more interesting results with the chitosan coated nanosystems than for nanoparticles elaborated exclusively with chitosan produced by ionotropic gelation (Prego et al., 2005).

With chitosan-coated submicron-sized liposomes, both the chitosan coating and the submicron size were key factors increasing enteral absorption of calcitonin as observed after oral administration to rats (Takeuchi et al., 2005). This mucoadhesive system is retained in the mucus partly owing the absorption by the intestinal epithelium.

Plain nanoparticles have almost no site-specificity in the gastrointestinal tract. To improve the specificity of nanosystems for the gastrointestinal mucosae, ligands binding to specific receptors shown by the gut were attached on the nanoparticle surface. This increased the binding specificity of the nanoparticles and decreased their elimination rate due to the mucus turnover. The most popular ligands used for this purpose included lectins, invasins and vitamine B12 (Nobs et al., 2004; Salman et al., 2005). L*ectins* raised special attention because they are involved in cell recognition mechanisms (Russell-Jones, 1999; Jung et al., 2000). Their specificity, multivalent featured, as well as their non-immunogenic properties render lectins appealing candidates for development of site-specific coated nanosystems as drug delivery carriers (Goldstein et al., 1980; Arangoa et al., 2000). For instance, Rodrigues et al.

(2003) developed core (polyester)-shell (polysaccharide) nanoparticles with lectins at the surface as a tool for site-specific oral administration of peptides. Arangoa et al (2000) prepared surface modified nanoparticles by binding lectins improving the specificity of the drug delivery device for the colonic mucosa.

The literature is riched in examples demonstrating the benefit of nanoparticulate formulations in the oral delivery of therapeutic peptides and proteins. They were shown to protect peptides and proteins from degradation in a very efficient way. Bioadhesive formulations were demonstrated to promote the bioavailability due to an increase of residence time near absoption sites. Morover, nanoparticles were found to serve as shuttle transporting active peptides and proteins through the intestinal epithelium. None of the systems proposed so far combined the different functionalities in a single nanoparticle. Thus, the purpose of our work, presented in the next parts of this chapter, was to develop a new nanoparticulate formulation combining the different functionnalities based on the use of core-shell poly(alkylcyanoacrylate) nanoparticle technologies.

ELABORATION OF CORE-SHELL POLY(ALKYL CYANOACRYLATE) (PACA) NANOPARTICLES

Considering the potential of plain PACA nanoparticles for the efficent oral administration of labile molecules, and the interest of nanoparticle surface modifications, efforts have been made to further improve the characteristics of this system by modification of their surface properties. From a technological view point, most of the approaches used in the development of stable surface modified nanoparticles have been based on the synthesis of amphiphilic copolymers (Peracchia et al., 1997b; Uchegbu et al., 1998; Brigger et al., 2001; Rodrigues et al., 2003; Archambault and Brash, 2004, Lemarchand et al., 2004; Park et al., 2006). Generally, this requires the synthesis of a copolymer between the hydrophilic coating and the hydrophobic core materials. The copolymer is then formulated as nanoparticles using methods of nanoprecipitation or solvent evaporation for instance (Peracchia et al., 1997b; Gref et al., 2001; Lemarchand et al., 2004). To obtain surface-modified PACA nanoparticles, both copolymers and nanoparticles are synthesized in the same time by emulsion polymerization thanks to different mechanisms of initiation of the polymerization of alkylcyanoacrylate monomers. Those monomers are so reactive that any nucleophilic groups can initiate a spontaneous anionic polymerization. This results in covalent linkage of the growing polymer chains on the component having initiated the polymerization. In this way, PEG-coated nanoparticles were synthesized by initiating the polymerization of isobutylcyanoacrylate with the terminal hydroxyl groups of PEG. The PEG-coated nanoparticles were designed as stealth® nanoparticles to avoid non-specific uptake by macrophages and to show long circulating properties in the blood after intravenous administration. PACA nanoparticles coated with polysaccharides can also be obtained thanks such a mechanism of polymerization initiation induced by the nucleophilic groups beared on polysaccharide chains. Taking advantage of this polymerization, dextran and chitosan coated nanoparticles were prepared (Couvreur 1979, Douglas 1986, Zhang et al., 2003). The polysaccharide chains standed at the nanoparticles surface adopt a side-on conformation because several anchorage chains of poly(alkylcyanoacrylate) can be grafted on a single chain

of polysaccharide due to the many nucleophilic polymerization initiator groups found on saccharide units.

Recently, Chauvierre et al. (2003a, 2003b, 2003c) developed a new method of polymerization for the elaboration of polysaccharide-coated poly(alkyl cyanoacrylate) nanoparticles. This method leading to poly(alkyl cyanoacrylate)-core polysaccharide shell nanoparticles can be coated with different polysaccharides, such as dextran, dextran sulphate, chitosan, heparin, and hyaluronic acid. The technique is based on a redox radical emulsion polymerisation of alkyl cyanoacrylate (ACA) monomers initiated on the carbohydrate structure by a radical polymerization. The copolymers which form during this polymerization showed a linear diblock structure which is quite different from the structure of the copolymer formed by the spontaneous anionic emulsion polymerization. Thus, a different conformation of polysaccharide chains on the nanoparticle surface can be obtained. While nanoparticles obtained by the anionic polymerization were characterized by a loop conformation of the polysaccharide chains arranged on the side-on position at the nanoparticle surface, radical polymerisation promoted brush like chain conformation thanks to the linear di-block structure of the copolymer formed (Bertholon et al., 2006a).

Polysaccharides grafted on the PACA nanoparticles did not only influence the physicochemical characteristics of the nanoparticles, such as surface charge or hydrodynamic diameter, but it also greatly affected their behaviour in physiological environments. For example, heparin gave antithrombic activity to particles (Chauvierre et al., 2006c) and dextran changed the response pattern of PACA particle interaction with blood opsonins typically responsible for the phagocytosis by macrophages after intravenous administration. Moreover, the interaction with proteins was demonstrated to be modulated by the polysaccharide molecular weight and the arrangement of the polysaccharide chains on the nanoparticle surface (Bertholon-Rajot et al., 2006a; 2006b).

Interest of Chitosan and Modified Chitosan in the Design of Surface Modified PACA Nanoparticles for the Oral Administration of Peptides and Proteins

Chitosan

Chitosan is a cationic high molecular weight heteropolysaccharide composed mainly of β-(1,4)-2-deoxy-2-amino-D-glucopyranose and secondary of β-(1,4)-2-deoxy-2-acetamido-D-glucopyranose. It is obtained from the natural polysaccharide chitin by partial deacetylation (Ding et al., 2003). Free amino groups of chitosan can undergo protonation hence making it soluble in aqueous acid solution (pH < 6.5) avoiding the use of hazardous organic solvents while handing (Agnihotri et al., 2004).

Because of a favourable biological profile including biodegradability, biocompatibility and absence of toxicity, chitosan has attracted attention in pharmaceutical and biomedical fields (Ravi Kumar, 2000; Agnihotri et al., 2004; Mao et al., 2004; Prego et al., 2005; 2006). Its cationic nature makes it an interesting candidate for DNA complexation involving electrostatic interactions and for proteins and vaccine encapsulation (Illum et al., 2001; Janes et al., 2001; Mao et al., 2001; Merdan et al., 2002; Li et al., 2003; Kim et al., 2004).

The inherent *bioadhesiveness* of chitosan has been denoted by many authors (Gaserod et al., 1998; Illum et al., 2001). Bioadhesive properties of chitosan involved electrostatic interactions with the negatively charged sialic acis groups on the mucus found at physiologic pH. It was demonstrated that chitosan can *enhance the intestinal absorption* of hydrophilic molecules. The mechanism behind this permeation enhancement effect seems to be based on the opening of the tight junction of the mucosal epithelium due to interactions of the positive charges of the polymer with tight junction-associated proteins therefore inducing a structural reorganization (Schipper et al., 1997; Jung et al., 2000; Leanes et al., 2004). Additionally, chitosan were found to bind divalent *cations* in neutral or weakly acid media, due to the free electronic doublet of nitrogen (Monteiro and Airoldi, 1999; Guibal et al., 2000; 2002; Lima and Airoldi, 2003). Thus chitosan may act as a cation scavenger interfering with the activity of cation-dependent proteases hence improving the chance for intact therapeutic peptides and proteins to be absorbed by the oral route. According to Lima and Airoldi (2003) the ion binding properties of chitosan is related to its high hydrophilicity owing to the large number of hydroxyl groups, the presence of a number of primary amino groups acting as potential base centers and the flexible polymeric chain structure which favours the adjustment of the cations dispersed in solution for complex formation.

All these characteristics make chitosan one of the most widely used biopolymers in development of nanoparticles for the oral administration of peptides (see the section: Plain Nanoparticles).

Modifiing Commercially Available Chitosan

Several types of modifications were considered to improve the properties of chitosan included in the formulation of drug delivery devices. Generally, the commercially available forms of chitosan are characterized by a high molecular weight (several hundred thousands g/mol) and by a certain degree of acetylation. In different works it was found that these characteristics are far from the optimal one. Thus several modifications of the commercial form of chitosan were undergone either to reduce the molecular weight of the initial compound or to introduce new chemical functions. As it will be explained bellow, there is a real benefice achieving such modifications. It generally allows to tune properties of the modified chitosan in such a way that the functionality of the polysaccharide is improved. For instance, the molecular weight of chitosan was influencing the stability of DNA/chitosan and peptides/chitosan complexes, the efficiency of cell uptake once in contact with the cellular membrane and the dissociation of active molecule from the complex after subsequent endocytosis. Reducing the size of chitosan to molecular weight ranging from 20,000 to 50,000 g/mol improved its transfection efficiency as demonstrated by Sato et al.(2001) and Lee et al. (2001). Vila et al. (2004) have demonstrated that tetanus toxin-loaded nanoparticles prepared with low molecular weight chitosan (28,000 g/mol and 38,000 g/mol) induced a stronger inmunoresponse towards the toxin at earlier times than the loaded nanoparticles elaborated with higher molecular weight chitosans.

Huang et al. (2004) developed an interesting method reducing the molecular weight of chitosan. This method is based on the oxidative degradation of the polysaccharide. It is performed by mixing an acidic solution of chitosan with different amounts of $NaNO_2$, because the molecular weight of the degraded chitosan will depend on the salt concentration. In our group, we have used this method to produce chitosans of different molecular weights from a commercial chitosan having a molecular weight of 400,000g/mol. The $NaNO_2$

concentrations were ranging from 7 to 1.5 mg/ml producing chitosan of molecular weight ranging from 10,000 to 100,000 g/mol respectively with a good production yield. All batches of low molecular weigh chitosan kept the same characteristics than the parent chitosan: general structure, degree of deacetylation, etc., (Bertholon-Rajot et al., 2006c; Bravo-Osuna et al., 2006; Bravo-Osuna et al., in press).

From an engineering point of view, the presence of hydroxyl and amino groups in the structure of chitosan make this polymer easy to modify introducing new chemical groups to modulate its characteristics and eventually to give it new properties (Kotzé et al., 1998; Zhang et al., 2003; Lemarchand et al., 2004; Sashiwa and Aiba, 2004). For example, several authors have modified chitosan by introducing diethyl or trymethyl groups in the structure. This increased the solubility of the polysaccharide at neutral pH (Thanou et al., 2001; Avadi et al., 2005) and gave the new polysaccharide potent intestinal permeation enhancement behaviour (van der Merwe et al., 2004a,b; Thanou et al., 2001; 2000). More recently, interesting peptide-chitosan polymers were created increasing the solubility of the chitosan derivatives over a wide range of pH (Batista et al., in press).

Another approach consists on copolymerising chitosan with other polymers. For instance, Prego et al. (2006) designed a new type of nanocapsules using chitosan chemically modified with PEG. The nanocapsules were obtained by the solvent displacement technique. Using the Caco-2 cell line model, it was observed that the pegylation of chitosan reduced the cytotoxicity of nanocapsules. In vivo studies showed that the capacity of the chitosan-PEG nanocapsules enhanced and prolonged the intestinal absorption of salmon calcitonin.

The group of Bernkop-Schnurch has introduced thiol groups in the structure of chitosan (Bernkop-Schnurch and Kast, 2001). The thiolated chitosans (chitosan-cysteine, chitosan-thioglycolic acid and chitosan-4-thiol-butyl-amidine) showed several interesting characteristics to be used for the development of pharmaceutical systems designed for the oral administration of drugs (Bernkop-Schnürch et al., 2004). Those products have improved mucoadhesive properties compared to chitosan because it can promote interactions with cysteine-rich domains of mucus glycoproteins resulting in the formation of disulphide bonds (Gum et al., 1992; Bernkop-Schnürch and Steininger, 2000; Bernkop-Schnürch and Kast, 2001; Kast and Bernkop-Schnürch, 2001; Marschutz and Bernkop-Schnürch, 2002; Roldo et al., 2004). Moreover, thiolated chitosans were found to develop additional mucosal permeation enhancing properties, which mechanism could be related with the gluthation (GSH) regeneration in the endothelial environment (Bernkop-Schnürch et al., 2003). Finally, as chitosan, thiolated chitosan can bind divalent cations including Zn^{2+} or Mg^{2+} suggesting that they will also have antiprotease activity (Bernkop-Schnürch and Kast, 2001; Bernkop-Schnürch et al., 2003; Bernkop-Schnürch et al., 2004; Roldo et al., 2004). The binding capacity was improved by the introduction of the thiol groups in the chitosan structure. Several possible binding mechanism were suggested: (I) the protonated form may bind divalent cations by ion pair formation thanks to an ion exchange mechanism; (II) the non protonated form of thiolated chitosan may bind cations via nitrogen and/or sulphur donor atoms by a coordination by ligand exchange mechanism; and (III) a more or like combination of the two previous mechanisms involving ion pair binding mechanism followed by slow ligand exchange. Among the different thiolated chitosan synthesized, those resulting from of the linkage of 2-iminothiolane (chitosan-4-thiol-butyl-amidine: chitosan-TBA) appeared the most promising in terms of stability. Moreover, the bioadhesive, permeation enhancement and

antiprotease activity were also maximized with these compounds (Bernkop-Schnürch et al., 2003; Roldo et al., 2004).

In our group, we have prepared chitosan-TBA of different molecular weights. Both the total sulphur content and the free thiol groups were determined by elemental analysis and iodine titration respectively (Bravo-Osuna et al., submitted (1)). Interestingly, an inversed relationship between the molecular weight of chitosan and the percentage of amino groups involved in the reaction with 2-iminothiolane was observed suggesting a lower accessibility of those groups when the molecular weight of chitosan increased. In all cases, total sulphur content was higher than the amount of thiol groups suggesting the presence of intra- and/or inter disulfide bonds in the polymeric structure. This effect was increased with chitosan of high molecular weight reducing the solubility of the macromolecule (Bravo-Osuna et al., 2006; Bravo-Osuna et al., in press).

Thiolated chitosan have demonstrated their usefulness improving the oral bioavailability of several peptides. For instance, tablets and minitablets targeted towards the stomach prepared with thiolated chitosan were evaluated in vivo. Using such formulations, a moderate increment of bioavailability of encapsulated peptides (antide, calcitonin) was observed (Guggi et al., 2003; Bernkop-Schnürch et al., 2005). The use of these polymers in the elaboration of microparticles has also raised interest for the oral delivery of peptides and proteins. Krauland et al. (2006) developed thiolated chitosan microparticles loaded with insulin. They were administered by the nasal route showing moderate increment of peptide bioavailability. Very recently, formulations from the nanotechnologies were also investigated. Thiolated chitosan was conjugated with DNA forming particles in the nanometer-range. Transfection studies performed in Caco-2 cell culture evidenced a high transfection efficiency using these complexes making these systems promising new vectors for gene delivery (Schmitz et al., 2006).

According to these interesting results, the use of thiolated chitosan for the preparation of nanocarriers appears to be a very attractive tool and merit to be associated with nanotechnologies to formulate new carriers for the oral delivery of peptides and proteins.

Chitosan-Coated PACA Nanoparticles

As other surface modified PACA naoparticles, chitosan-coated PACA nanoparticles were obtained by emulsion polymerization of the alkylcyanoacrylate monomers initiated by the anionic or the redox radical polymerization mechanisms (Zhang et al., 2003; Chauvierre et al., 2003c; Bertholon et al., 2006b, Bravo Osuna et al., in press), Chitosan-coated PACA particles obtained by redox radical emulsion polymerisation using commercial polysaccharide (400,000 g/mol), showed a diameter within the micrometer-range (hydrodynamic diameter of 59 μm). Chauvierre et al. (2003c). To reduce the size of the particle, Bertholon-Rajot et al. (2006c) and Bravo-Osuna et al. (2006; in press) suggested the use of depolymerised chitosan with molecular weight ranging between 10,000 and 200,000 g/mol. The nanoparticles obtained showed mean hydrodynamic diameters with values varying from 200 to 600 nm when increasing the molecular weight of chitosan. Slightly different diameters were shown by nanoparticles prepared by the anionic polymerization, suggesting a different copolymer arrangement of the copolymer within the nanoparticle structure. In all cases the presence of the cationic polysaccharide, chitosan, on the nanoparticles surface was demonstrated by ζ

potential measurement, obtaining positive values. This has to be compared with the negative values characterizing PACA nanoparticles coated with dextran, a neutral polysaccharide. The stability of nanoparticles was evaluated in a wide range of pH (3-9) demonstrating that the covalent attachment of chitosan to the PIBCA core ensured no aggregation over the range of physiological pH encountered in the gastrointestinal tract. Thus the potential bioadhesive nature of the nanoparticles due to positively charged surfaces was also completed over the range of pH of the gastrointestinal tract.

In Figure 1, SEM and TEM micrographs showed the PIBCA nanoparticles coated with chitosan of molecular weights of 20,000 and 100,000 g/mol. Both microscopic methods showed spherical and individualised nanoparticles. A layer of polysaccharidic material standing at the nanoparticle surface was clearly highlighted by TEM for radical formulations, especially when the nanoparticles were prepared with high molecular weight chitosan (100,000 g/mol) (Bravo-Osuna et al., in press).

The versatility of the method of elaboration of core-shell nanoparticles based on anionic and redox radical polymerisation was demonstrated by modifying both core and surface compositions (Bravo-Osuna et al., 2006; Bravo-Osuna et al., in press).

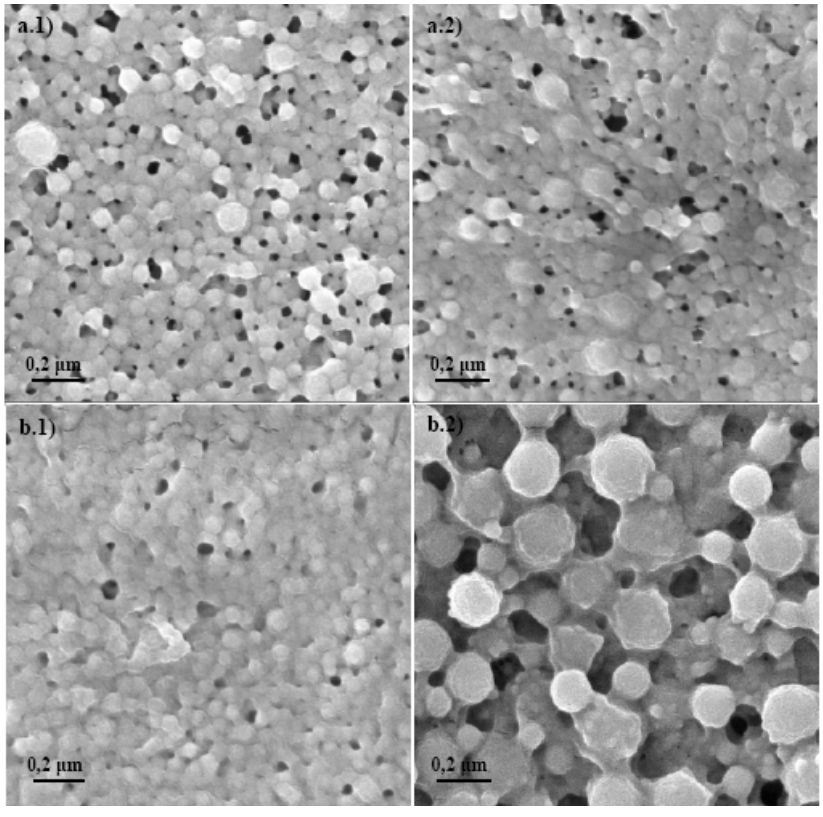

Figure 1. Scanning electron microphotographs of coated PIBCA nanoparticles elaborated with chitosan of 20,000 (a), and 100,000 (b) g/mol following anionic (1) or radical (2) polymerisation. Modified from Bravo-Osuna et al., in press.

The introduction of a second acrylic monomer in the formulation was investigated in order to modify the core properties, which can be useful for adjusting the core properties for

drug encapsulation and control of the release kinetics of the drug. This can be also very usefull as a simple method for labelling the nanoparticles required for further biopharmaceutical evaluation. In this sense, the inclusion of moderate percentages of methylmethacrylate in the alkylcyanoacrylate monomer (5-10%) did not essentially modify the morphology of the nanoparticles obtained by both anionic and radical polymerization. In all cases, monodisperse suspensions of nanoparticles were obtained. The labelling of the nanoparticles by this method involved the copolymerization of isobutylcyanoacrylate with a fluorescent labelled acrylic monomer. This successful method allowed the covalent linkage of the rhodamine containing marker in the nanoparticle core during the polymerisation process (Bravo-Osuna et al., in press).

Nanoparticle surface modification was also evaluated by replacing chitosan by thiolated chitosan as shell component. As was previously pointed out, inclusion of thiolated polymers in the formulation should add several interesting characteristics to the nanocarrier systems that would improve their efficacy for the administration of biotechnology products by mucosal routes. To this aim, nanoparticles were elaborated using mixtures of chitosan and chitosan-TBA of different molecular weights in different proportions. Due to the lack of solubility of the high molecular weight chitosan-TBA (Bravo-Osuna et al., 2006), nanoparticles with a wide range of chitosan-TBA content was only obtained with the lower molecular weight analysed (Bravo-Osuna et al., in press). The presence of thiol groups on the nanoparticle surface was verified by the iodine titration method. This method allows the determination of thiol groups at acid pH promoting oxidation of thiol groups in presence of iodine (Bravo-Osuna et al., submitted (1)). The density of those “active” or “reactive” thiol groups at the nanoparticle surface increased with the percentage of chitosan-TBA used in the nanoparticle preparation. However, the incorporation of high percentages of chitosan-TBA in the formulation enhanced the formation of disulphide bonds, leading to a higher cross-linked structure of the shell of the nanoparticles and to an increase of the viscosity of the gel layer formed by these molecules at the nanoparticle surface (Bravo-Osuna et al., in press). For each series of nanoparticles, the hydrodynamic mean diameter of the nanoparticles increased with the percentage of chitosan-TBA, while the ζ potential values decreased.

INTERACTION OF CHITOSAN-COATED PACA NANOPARTICLES WITH THE INTESTINAL MUCOSA

Bioadhesion

When micro- or nanoparticles are administered orally in the form of a suspension, they diffuse into the liquid medium and they rapidly encounter the mucosal surface. The particles can be immobilized at the intestinal surface by an adhesion mechanism so-named “bioadhesion”. More specifically, when adhesion is restricted to the mucus layer lining the mucosal surface, then the term “mucoadhesion” is employed. From a qualitative point of view, the following framework is generally accepted: After dilution in the gastrointestinal fluids, the mucoadhesiveness of a colloidal suspension depends basically (I) on the kinetics of the particles to diffuse into the suspension medium and (II) on the ability of the particles to

interact with mucin glycoproteins or other mucus components being immobilized within the mucus layer (Durrer et al., 1994a; 1994b; Ponchel and Irache, 1998).

In recent works, we have evaluated the bioadhesiveness of several chitosan-coated PIBCA nanoparticles (Bertholon-Rajot et al., 2006c, Bravo-Osuna et al., submitted (2) submitted (3)) following a method developed by Durrer et al. (1994a; 1994b) and presented in Figure 2. Bertholon-Rajot et al. (2006c) evaluated different chitosan-coated PIBCA nanoparticles elaborated by anionic and radical polymerisation with chitosan of molecular weight ranging between 30,000 and 200,000 g/mol. They demonstrated that polysaccharides grafted at the nanoparticles surface in the brush conformation (radical polymerisation) appeared more favourable to promote interactions of nanoparticles with glycoproteins of mucus in comparison with more compact loop conformation of polysaccharide chains obtained by anionic polymerisation. Bravo-Osuna et al. (submitted (2) (3)) analyzed the bioadhesion behaviour of chitosan core-shell PIBCA nanoparticles elaborated by radical polymerisation using chitosan and thiolated chitosan of molecular weights between 20,000 and 100,000 g/mol. They observed that the presence of a cross-linked structure, due to the formation of intra- and interchains disulfide bonds, partially reduced the interaction with mucins.

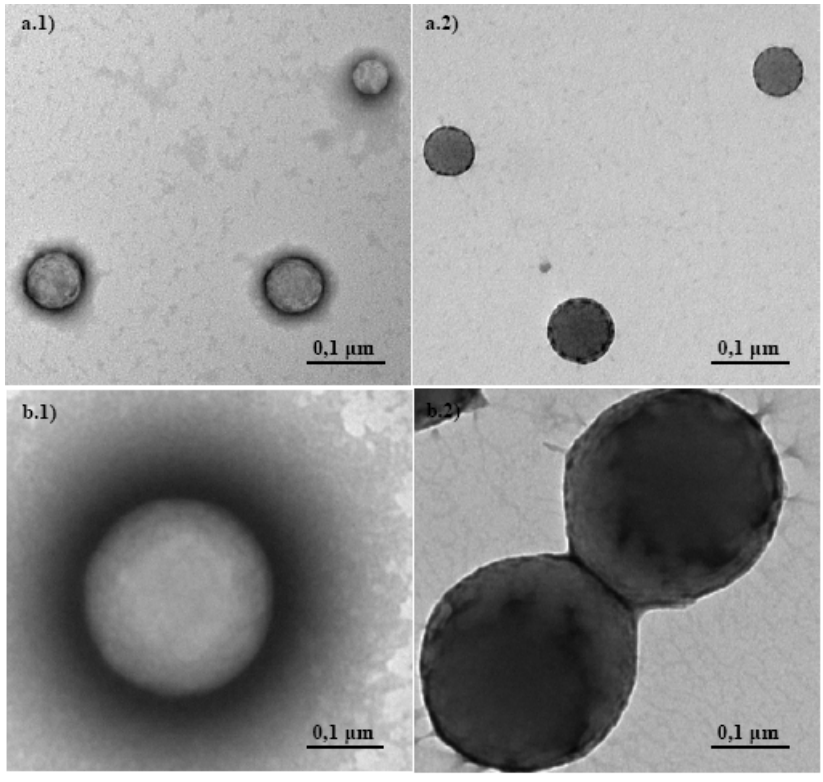

Figure 2. Transmission electron microphotographs of coated PIBCA nanoparticles elaborated with chitosan of 20,000 (a), and 100,000 (b) g/mol following anionic (1) or radical (2) polymerisation. Modified from Bravo-Osuna et al., in press.

However, the presence of high amounts of free (reduced) thiol groups in the nanoparticle surface importantly increased the bioadhesion of the nanoparticles because covalent bonds

with cysteine residues of glycoproteins of the mucus can be formed. In all cases, the presence of chitosan on the PIBCA nanoparticle surface importantly increased the bioadhesiveness of the system in comparison to PIBCA nanoparticles coated with dextran. This was in agreement with the bioadhesive nature of the chitosan used as coating material. The diffusibity of nanoparticles through the mucus hydrogel seemed to be mainly governed by their hydrodynamic diameter, so high bioadhesive capacity was observed for the nanoparticles of small hydrodynamic diameter (200-300 nm). These results were consistent with those of Durrer et al. (1994a; 1994b), who suggested that small particles can be considered as adsorbates penetrating into a porous adsorbent formed by the mucus layer.

Figure 3 presents the bioadhesion mechanism proposed for the chitosan-coated PIBCA nanoparticles (Bravo-Osuna et al., submitted (2)). In general, the deposition of model colloids on well-defined solid surface involves two steps: *transport* and *attachment*. In this work, the *transport* of the particles analysed was mainly governed by their particle size and the presence of hydrophilic chitosan on their surface.

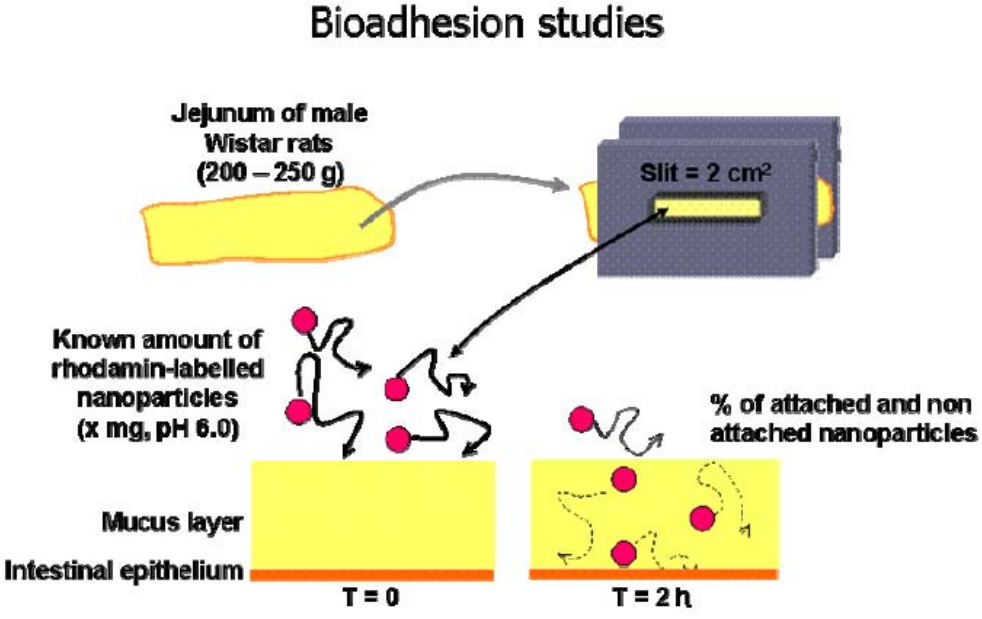

Figure 3. Scheme of in vitro bioadhesion studies according to the protocol developed by Durrer et al., (1994a; 1994b).

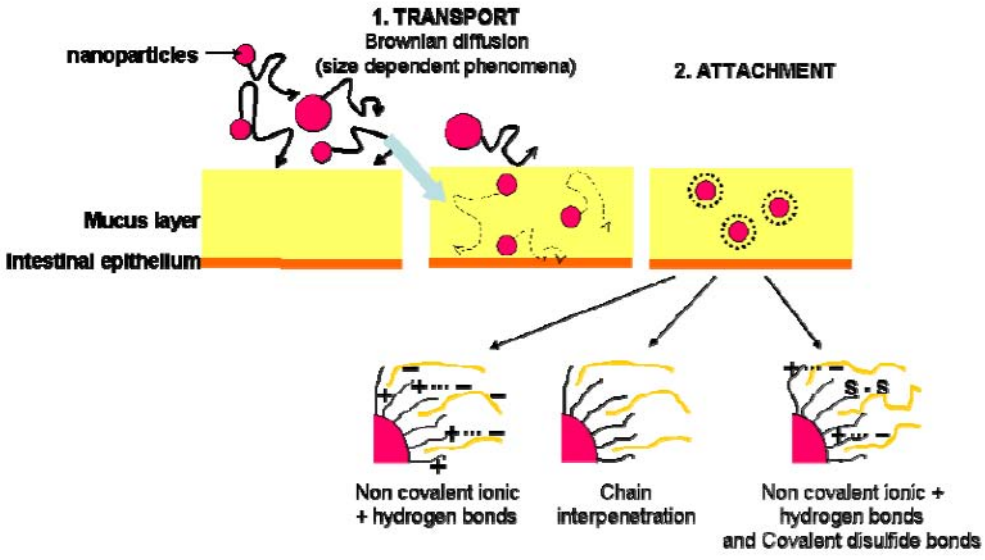

Figure 4. Suggested mechanisms describing the mucoadhesion of chitosan and thiolated chitosan-coated PIBCA nanoparticles. Mucin chains : yellow lines, chitosan and thiolated chitosan at the nanoparticle surface: black lines.

Different mechanisms can occur simultaneously in the *attachment* of the mucoadhesion process. Their intensity depends on the nanoparticle surface characteristics (Durrer et al., 1994a; Huang et al., 2000) (I) Non-covalent bonds can be created within the mucus-polymer interface (electronic and adsorption theory). For the systems analysed in this work, those non-covalent unions were improved by the presence of cationic chitosan. (II) The polymer and protein chains can interpenetrate (diffusion theory) and entangle together. This phenomenon depended on the chain conformation and on the degree of cross-linking in the gel layer formed by the polysaccharide on the nanoparticle surface. (III) Further non-covalent (physical) and covalent (chemical) bonds (electronic and adsorption theory) can be formed (Cilurzo et al., 2003; Dodou et al., 2005). In the case of thiolated nanoparticles, it can be expected that the presence of high amounts of reduced thiol groups on the nanoparticle surface should ensured the formation of covalent disulphide bonds with the mucus cysteine groups promoting the bioadhesion of the nanoparticles by covalent linkage.

Ion Binding Capacity

In the gastrointestinal tract, cations play an important role maintaining permeation properties of the epithelium and enzymatic activities. Thus potential cation binding capacity of colloidal systems might be an interesting tool for nanocarriers designed for the oral administration of pharmacologically active peptides because they may act as modulators of the enzymatic activity and permeation of the epithelium.

Thus, the cation binding capacity of chitosan and thiolated chitosan core-shell PIBCA nanoparticles was investigated (Bravo-Osuna et al. submitted (4), submitted (5)). Two cations of physiological interest calcium and zinc were selected as model ions. While calcium ions are necessary to maintain tight junction integrity and the tridimentional structure of several

proteases such as trypsin, zinc ions are part of the active site of gastrointestinal metallopeptidases. With both cations, the binding capacity of chitosan appeared 2 to 3 folds higher when the polysaccharide was bound on the nanoparticle surface than when it was in solution. In agreement with data published by other authors, this demonstrated that physical changes in chitosan presentation (beads or films manufacturing) can improve the cation binding capacity of this polysaccharide (Chassary et al., 2004; Guibal 2004). According to Rhazi et al. (2002), this phenomenon can be explained by an increase of the accessibility of the free amine groups of chitosan when it is exposed at the nanoparticle surface. This accessibility to active sites was also limited by the formation of a cross-linked structure observed when thiolated chitosan was included in intermediate percentage. The negative effect of cross-linking structures on the binding capacity of polymer was clearly demonstrated by other authors (Madsen and Peppas, 1999; Becker et al., 2000; Dzul Erosa et al., 2001; Jeon and Holl, 2003; Guibal, 2004). However, the formulations with high percentage of free thiol groups on the nanoparticle surface allowed higher binding ability, overcoming the negative effect of the cross-linking structure assumed for the gel layer standing at the surface of these nanoparticles.

Antiprotease Activity

The inhibitory effect of chitosan and thiolated chitosan core-shell PIBCA nanoparticles was tested against two model metallopeptidases of the gastrointestinal tract: Carboxypeptidase A (CP A) and Leucine aminopeptidase M (LAP M) (Bravo-Osuna et al., submitted (5)).

Carboxypeptidase A is a prototypic zinc-containing proteolytic enzyme, representing a large family of physiologically important zinc proteases such as Angiotensin-Converting Enzyme (ACE) and matrix metallopeptidases. This enzyme catalyses the hydrolysis of the C-terminal amide bonds of peptide substrate (Chung and Kim, 2001; Park and Kim, 2004). As a representative zinc protease, the enzyme has received much attention as a model enzyme for devising design protocols of inhibitors that are effective against zinc proteases of medical interest (Lee and Kim, 2000; Li and Solomon, 2001; Cho et al., 2002). Removal of zinc leads to the formation of metal-free carboxypeptidase which is enzymatically inactive (Vallee et al., 1960; Li and Solomon, 2001; Phoon and Burton, 2005).

Leucine aminopeptidase M (EC.3.4.11.2.) is probably the aminopeptidase that has been studied the most extensively. It is located on the brush border of intestinal epithelial cells of the gastrointestinal tract, where it cleaves degraded small peptides in the final stages of protein digestion. Hence, the enzyme performs an important nutritional protein processing role (Sanderink et al., 1988). It is, for example, the main cause for degradation of insulin after administration by the oral route (Zhou, 1994). This membrane-bound enzyme is also a zinc-dependent metallopeptidase (Himmelhoch, 1969; Hooper, 1994) that can be inactivated by depletion in the cation (Bernkop-Schnurch and Krajicek, 1998).

The zinc binding capacity of chitosan and thiolated chitosan in solution was not high enough to develop effective ion depletion from the active site of CP A and LAP M. However, the same amount of the biopolymers presented as the coating material of nanoparticles showed inhibition capability against the two metallopeptidases. The inhibition factor values were higher than one, determined in the first five minutes of the study. The inhibition

capacity decreased from nanoparticles elaborated with chitosan/chitosan-TBA proportions of 100/0 to 25/75, which is in total agreement with the zinc binding capacity previously observed. However, the high binding capacity observed for nanoparticles elaborated with 100% of chitosan-TBA did not correspond to the higher inhibitory effect expected. For this formulation, the affinity between polymer and ions was not high enough to induce sufficient ion depletion. Not only the zinc binding capacity of the biopolymer but also the biopolymer presentation on the nanoparticle surface clearly played a key role in the inhibitory effects observed on the peptidase activities.

MODULATION OF THE INTESTINAL ABSORPTION OF ACTIVE MOLECULES IN PRESENCE OF CHITOSAN-COATED PACA NANOPARTICLES

In vitro permeability studies are relevant approaches to evaluate the absorption enhancing effect of colloidal drug carrier systems (Hillgren et al., 1995; Lang et al., 1998; Watanabe et al., 2004). They offer many advantages over *in vivo* studies being performed more rapidly, involving fewer animals and simpler analytical procedures. They are also easier to standardised (Wadell et al., 1999). However, there is one restriction that must be considered concerning the viability and integrity of the tissue (Wadell et al., 1999). The feature of Ussing-like chambers is that they allow monitoring of the electrical parameters of the membrane during the course of the experiment assessing viability of tissues over the time course of the experiment (Hillgren et al., 1995; Samanen et al., 1996). In addition, the electrical parameters can be also used to determine the opening of tight junctions (Wadell et al., 1999) as complement of the monitoring of permeability of paracellular transport markers such as mannitol (Lang et al., 1998; Wadell et al., 1999; Boulenc et al., 1995a; Tanrattana et al., 2004).

The permeation enhancement properties of chitosan and thiolated chitosan coated PIBCA nanoparticles was evaluated using Ussing-type chambers and monitoring of the opening of tight junctions by both electrical and mannitol permeability measurements (Bravo-Osuna et al. submitted (3)). In a first set of experiments, the different formulations of core-shell nanoparticles were introduced in the donor compartment of the Ussing chamber allowing the direct contact of nanoparticles with the mucosal side. Surprisingly, the non-thiolated chitosan-coated nanoparticulate formulations did not show any enhancement of the paracellular pathway permeability during the study, considering the well-established enhancing permeation properties reported for chitosan in previous reports (Illum et al., 1994; Lueβen et al., 1997; Kotzé et al., 1998; Dodane et al., 1999). However, in agreement with other works, when the experiments were carried out with chitosan solutions at the same concentration than those used in the nanoparticles, an increment in the paracellular permeability was observed. Other authors have also reported a different behaviour between chitosan solutions and chitosan nanoparticles using the Caco-2 cell monolayer model (Prego et al., 2005). Similarly, they observed that chitosan solutions promoted a significant opening of the tight junctions, while the same amount of polymer formulated in nanoparticles did not lead to any significant permeation. Thus, our results confirmed that a different presentation of chitosan, in the form of a soluble solution or as solid nanoparticles, led to different permeation effects which can be

explained considering the high interactions between particles and mucus observed in bioadhesion studies. Indeed, high interactions can decrease the possibility of direct contact between nanoparticles and epithelial cells requested to promote structural changes in the tight junction proteins responsible for the increase of the intestinal permeation.

Interestingly, thiolated formulations which were found to be far less bioadhesives demonstrated a higher tendency to induce paracellular permeation enhancement. This effect can be related to the bioadhesion mechanism of nanoparticles. Indeed, once in contact with the mucus layer, nanoparticles begin to diffuse in the three-dimensional gel. The higher they attached the mucus chains, the lower they can interact with the tight junction system to open it. The high mucus-nanoparticle interactions might also be the cause of the lack of correlation observed between the calcium binding capacity demonstrated for these nanoparticles in vitro and their permeation behaviour observed in the presence of the biological tissues.

A second set of experiments was performed in which the adhesion of nanoparticles on the mucosa was avoided by interposing a semi-permeable membrane between the tested formulations and the intestinal tissue. In this case, no permeation enhancement was obtained with any of the formulation tested. This undoubtedly confirmed that the absorption enhancing effect of the nanoparticles was intimately related to their bioadhesion on the mucosa.

From all the results presented in this study, the following sequence of events can be proposed to explain the permeation enhancing effect of chitosan or of thiolated-chitosan coated-nanoparticles: (I) The presence of chitosan (modified or non-modified) on the surface of nanoparticles promoted their bioadhesion with the mucus layer by electrostatic interactions. (II) The cross-linked structure due to the presence of disulphide bonds in the thiolated chitosan coating, might limit the development of strong interactions of the nanoparticles with the mucus components hence enhancing the interactions of the nanoparticles with endothelial cells. (III) At the level of the endothelial cells, the combination of both the remaining cationic nature of the particle surface and the presence of thiol groups, could promote the opening of the tight junctions (Bravo-Osuna et al. submitted (3)).

CONCLUSIONS AND NEW TRENDS

Nanotechnology and more specifically nanoparticles is a very interesting approach for the oral administration of peptides and proteins. They can protect peptides from the harsh gastrointestinal environment and present a high surface area able to develop intimate contact with biological tissues. In addition, polymers used in their elaboration can give them useful additional characteristics to improve the bioavailability of active peptides and proteins. One of the most promising materials used with this objective are chitosan and some of their derivatives. They can modulate the gastrointestinal protease activity and can improve the peptide permeability across the intestinal epithelium.

Recent works focused on the elaboration of PACA nanoparticles allowed the development of an interesting method of preparation of PACA nanocarriers with modified surface based on emulsion polymerisation. The high versatility observed for this technique in the tuning of core-shell PACA nanoparticles opens a huge door for the development of such kind of nanocarriers with specific characteristics. The combination of this nanotechnology with the use of chitosan and their derivatives led to the creation of new nanoparticles having

different advantageous functions required to promote the oral bioavailability of therapeutic peptides and proteins. There is no doubt that research will continue with approaches aiming to develop multifunctionnal drug delivery systems at the nanoscale size. In this view, the nanoparticles reported here are interesting platforms for multifunctional systems developments because they can be further modified by the introduction of new functions at the nanoparticle surface thanks to the easy chemistry that can be perfomed on chitosan and thiolated chitosan used as coating material.

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Chapter 3

EXCIPIENTS AS MODULATORS OF DRUG-CARRIER MEDIATED ABSORPTION IN THE INTESTINE

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ABSTRACT

Over the past years the awareness of the importance of intestinal drug transporters for the bioavailability of certain drugs has grown steadily. More and more drugs are being identified as substrates or inhibitors of one or another transporter. Aside from drugs, however, numerous excipients have been found to modulate the functioning of drug transporters, too. Thus, they might also interfere with the absorption of drugs on a physiological level either enhancing or decreasing their absorption.

For developing rational drug formulations it is important to take into account such effects when conceiving a new formulation or redesigning existing formulations. In the first case, choosing the right excipient might result in higher bioavailability or even allow for absorption in the first place. In the second case unwanted changes of the performance of the redesigned dosage form might be prevented. The knowledge of such excipients is also important from a drug regulatory point of view in order to evaluate post-approval changes in the formulation.

This article will review the current findings on excipients with modulatory potential on intestinal transporters. With respect to excipients the article will mainly discuss Cremophor EL, Tocopheryl Polyethylenglycol Succinate (TPGS), Polysorbate 80, Pluronic P85, Polyethyleneglycol (PEG), Solutol HS15 and Labrasol. In addition, a comprehensive table of excipients with modulatory effects on drug transporters will be included. With respect to intestinal drug transporters the article will concentrate on interactions between excipients and two important members of the ABC-transporter family (P-glycoprotein and MRP) as well as two members of the solute carrier family (PEPT1 and the monocarboxylate transporter).

The article shall serve both as an introduction and reference for those active in the field of drug formulation or drug regulatory affairs.

1. INTRODUCTION

Absorption of drugs via the intestinal epithelium can be brought about by either passive or carrier-mediated transport. The dominant force of passive transport is diffusion which enables transport by the paracellular or transcellular pathway along the concentration gradient of a compound. A special case of passive transport is the so called facilitated transport which is mediated by carrier systems in the cell membrane and allows accelerated transfer of a compound (e.g. fructose) across the membrane along its concentration gradient without consumption of energy. Active transport requires the presence of a transport protein and consumption of energy which can be provided either directly by ATP-hydrolysis or indirectly by an ion gradient (e.g. H^+, K^+, Na^+) which has been generated by the use of energy or eventually ATP-hydrolysis. Transport proteins relying directly on ATP-hydrolysis are referred to as pumps and their action is also called primary active transport. Transporters working by ion gradients are also referred to as carriers and can be further classified into cotransporters and countertransporters. They use energy provided by the downhill transport of one substrate to propel the uphill transport of another substrate. In the case of cotransport both substrates flow in the same direction, in the case of countertransport the flux of the two substrates is in opposite directions.

In the past diffusion, has been regarded as the prevalent mechanism of absorption. But in recent years more and more transport proteins have been discovered and recognized to affect absorption or secretion of physiological substrates, drugs and their metabolites in liver, kidney, blood-brain barrier, placental barrier, tumor tissue and the intestine. These include P-glycoprotein [1], multi-drug-resistant protein [2], peptide transporter [3, 4], organic cation transporter [5] and the monocarboxylate transporter [6, 7].

Meanwhile drug-carrier mediated absorption and secretion across intestinal cell monolayers like Caco-2 and isolated or perfused rat tissue has been shown to be a relevant factor for a variety of drugs. These include celiprolol [8], digoxin [9], ranitidine and cimetidine [10], saquinavir [11] and fluorquinolone antibiotics [12] as well as enalapril [13] and valacyclovir [14]. Furthermore, secretion of intravenously administered digoxin and its inhibition by quinidine, a P-gp inhibitor, has been observed in the human intestine [15] which points out the in vivo relevance of drug-carrier mediated transport.

Drug carriers and pumps are proteins in nature and their action is influenced by various factors. On the transcriptional level higher expression could change activity, posttranscriptionally stabilization or destabilization of mRNA could play a role, translation itself, posttranslational events and trafficking of the protein to membranes can influence the actual amount of protein present in the membrane at any one time. Once in the membrane the protein activity can be altered by competitive, noncompetitive, or uncompetitive inhibitors or allosteric effectors. The functioning of the ABC-transporters, like P-gp and the MRPs, depends on ATP and the activity of the secondary-active transporters, like PEPT or MCT of the solute carrier family, is influenced by the ion-gradient of the propelling moiety. For example, a variety of inhibitors are already known for P-gp, besides the above mentioned

quinidine, verapamil, vinblastine and GF120918 may be mentioned. Pretreatment of Caco-2 cells with verapamil or vinblastine [16] or in situ of rats with vinblastine and rifampin [17] can also increase P-gp expression and protein levels. Furthermore, propranolol and verapamil were shown to cause a rapid induction of P-glycoprotein in colonic cells in vitro [18].

Polymorphisms are known for some of the drug carriers and pumps e.g. MDR-1 [19] and might account for inter-individual differences in drug absorption.

Interfering with the expression or activity of drug carriers and pumps might thus provide an opportunity to increase absorption of such drugs, that are substrates of these transport proteins. E.g. in the case where there is a secretory transporter inhibition or down-regulation could lead to increased absorption and in the case where there is an absorptive transporter, enhancing its activity and expression could lead to higher absorption.

This is not just hypothetical, since e.g. rifampin enhanced intestinal secretion of talinolol by induction of P-glycoprotein in humans [20]. Of course, one would need to find less toxic compounds or compounds devoid of systemic action in order to make use of these properties in the treatment of otherwise healthy patients.

Not only drugs can influence drug carriers and drug pumps. There are now quite a few publications available which describe excipients as modulators of drug-carrier activity in vitro and in vivo. For the most part, these excipients have been used for years in pharmaceutical formulations and thus when administered orally their toxicity is considered to be very low, their systemic availability poor and no relevant systemic effects are known. So they might be potentially valuable for modulation of intestinal drug carriers in those cases where bioavailability is limited by drug-carrier mediated efflux or absorption.

This review shall give an overview of excipients which have already been tested for their modulatory actions on intestinal drug carriers and pumps. It includes a list with several of those excipients but we do not claim completeness. Further discussion will concentrate on some selected excipients, since it is not possible to cover all of them in this review.

2. DRUG TRANSPORTERS IN THE INTESTINE

A variety of intestinal drug transporters has been described and we refer to reviews dealing exclusively with this topic [21]. Here, we will focus on some transporters that have been dealt with in the literature with regard to interaction with excipients. These transporters fall into two families: the solute carrier family (SLC), which uses ion gradients as driving force or functions by the principle of facilitative transport, and the ABC (ATP binding cassette) family, which uses energy from ATP-hydrolysis. Steffanssen et al. [22] give a fairly comprehensive overview of the absorptive solute drug carriers found in the intestine and Chan et al. [1] provide insight into ABC-tranporters and their regulation in intestine and liver.

For the SLC family we will consider the peptide transporter and the monocarboxylate transporter and for the ABC familiy P-glycoprotein (MDR-1) and multi drug resistance protein (MRP).

2a. P-glycoprotein (P-gp)

P-gp is a MDR-1 gene product and belongs to the ABC-transporter family. It is localized in the apical cellular membrane and exerts a secretory function. In the human intestine P-gp expression increases from proximal to distal (small intestine to colon) [23]. Several authors suggest a coordinate response of P-gp and Cyp P450 to exposure of cell to xenobiotics [24, 25]. Its substrate specificity is rather broad and includes drugs from a range of different therapeutic groups e.g. anticancer agents such as taxol [26] or doxorubicin [27], antibiotics e.g. grepafloxacin [28], HIV-protease inhibitors [29-31] and immunosuppressive agents as cyclosporin A [32] , drugs used in cardiovascular disease as talinolol [33] and digoxin [34]. Additionally, verapamil is not only a substrate but also acts as a potent inhibitor of P-gp [11]. Table 1 illustrates the large number of compounds interacting with P-gp in one or another way.

Talinolol and digoxin are often chosen as substrates for transport studies because of their metabolic stability, such that metabolic influences in absorption studies can be excluded. Furthermore, the fluorescent dye rhodamine 123 is a P-gp substrate and has often been used in transport studies for its relatively easy detection by fluorescence.

2b. Multi-Drug-Resistance Protein (MRP)

MRPs belong to the ABC-transporter family. Only MRP2, MRP3 and perhaps MRP1 are known to be relevant for intestinal transport. They are all secretory tranporters. While MRP2 is located in the apical membrane MRP1 and MRP3 are routed to the basolaterol membrane [1, 2]. The basolateral location of MRP1 and MRP3 prevents direct exposure to excipients on the luminal side, whereas MRP2 could possibly interact directly with high excipient concentrations. Substrates of MRP2 and MRP3 are shown in Table 1. Some of the substrates of MRPs show or affinity to P-gp (see Table 1); thus using them in transport studies might yield ambiguous results.

MRP1-3 might play a role in the secretion of anionic compounds generated during metabolism by conjugation with glutathione or sulphate as can be seen by their substrates (Table 1) dehydroepiandrosterone sulphate (MRP1), taurodeoxycholate-3-sulphate (MRP2 and MRP3) and leukotriene C4 (MRP1-3).

Table 1. Substrates, inhibitors and inducers of MDR-1, MRP1, MRP2, MRP3 and BCRP

Substrate	**MDR-1**	**MRP1**	**MRP2**	**MRP3**	**BCRP**
2,4-Dinitrophenyl-S-glutathione		S [35]	S [36]	S [37]	S [38]
Adefovir			S [39]		
Aldosterone	S [40]				
Amiodarone	I [41]				
Amitryptiline	S [42]				
Substrate	MDR-1	MRP1	MRP2	MRP3	BCRP
Amprenavir	S [43] ; U [44]				

Table 1. (Continued)

Substrate	MDR-1	MRP1	MRP2	MRP3	BCRP
Atorvastatin	S/I [45, 46]				
Bisantrene					S [47]
Bromocriptine	I [48]				
Carvedilol	I [49]				
Celiprolol	S [8]				
Chloropromazine	I [50]				
Cidofovir			S [39]		
Cimetidine	S [51]				
Cisplatin	N [52]	N [53]	S [54]		
Clarithromycin	I [55]				
Colchicine	S [11]				
Cortisol	S [40]				
Cyclosporin	S [32, 56]				
Daunorubicin	S [27]	S [57]		N [58]	S [59]
Dehydroepiandrosterone sulphate		S [60]	N [60]	N[60]	
Dexamethasone	S [40] U [61-63]				
Diethylstilbestrol					I [64]
Digoxin	S [34, 65]		N [66]		
Diltiazem	I [67]				
Docetaxel	S [68]				
Domperidon	S [69]				
Doxorubicin	S [27] ; U [70]		S[54]	N [58]	S [59]
Epirubicin					S [71]
Erythromycin	S/I [72]				
Estradiol-17-β-glucuronide	S [73]	S [74, 75]	S [75]	S [37]	
Etoposide	S [76]		S [54]	S [58]	S [76]
Fexofenadine	S [77]				
Flavopiridol					S [78]
Fluoxetine	I [79]				
Furosemid			I [80]		
Glibenclamid		I [81]	I [81]		
Grepafloxacin	S [12, 28, 82]	S [28]	S [12]		
Indinavir	S [29] ; U [83]	N [84]	S [84]	N [84]	N [84]
Itraconazole	S/I [85]				
Ketoconazole	N/I [86]				
Lamivudin					S [87]
Lansoprazol	S/I [88]				
Leukotriene C4		S [35]	S [36]	S [37]	
Levofloxacin	S [82]				
Loperamide	S [69]				
Losartan	S [89]				

Table 1. (Continued)

Substrate	MDR-1	MRP1	MRP2	MRP3	BCRP
Methadone	I [90]				
Methotrexat	S [91]	S [92]	S [93, 94]	S [92, 95]	S [96, 97]
Mitoxantrone	S [98]	S [99]			S [47]
Morphine	S [90] , U [100]				
Nelfinavir	S [29] ; U [83]				I [101]
Octreotide	S [102]		S [102]		
Omeprazole	S/I [88]				I [103]
Ondansetron	S [69]				
Paclitaxel	S [26]	N [53]		N [58]	
Pantoprazol	S/I [88]				S/I [103]
Pentazocine	I [90]				
Phenytoin	S [69]				
Prazosin					S [47]
Probenecid			I [80]		
Quinidine	S/I [104]				
Ranitidine	S [10]				
Reserpine	I [105, 106]; U [106]				
Retinoic acid	U [107]				
Rhodamine 123	S [108]	S [109]			S [59]
Rifampin	S [110]; U [106]		S [111]		
Ritonavir	S [29]; U [83]		S [84]		I [101]
Saquinavir	S [29, 31] ; U[83]		S [84]		I [101]
Sertraline	I [112]				
Simvastatin	I [113]				
Spironolactone	I [114]				
St John´s wort	U [115]				
Tacrolimus	S [32]; I [116]				
Talinolol	S [33]				
Taurodeoxycholate-3-sulphate			S [117]	S [118]	
Teniposide				S [95]	S [76]
Terfenadine	S [86]				
Tetracycline	S [119]				
Topotecan	S [120]				S [47]
Verapamil	S/I [11]				
Vinblastine	S [68]		S [121]		
Vincristine	S [122]	S [123]	S [54]; U [124]	S [58] ; U [124]	

Note: S = substrate ; I = inhibitor ; U = induction (Up-regulation), N = no substrate.

2c. Peptide Transporter (PEPT)

A couple of peptide transporters have been described by now including PEPT1, PEPT2 and peptide/histidine transporter PHT (reviewed by Steffansen et al. [22]). Not much is known about PHT and PEPT2 is not expressed in the intestine. So it will only be dealt with PEPT1.

PEPT1 belongs to the SLC15A gene family and is systematically referred to as SLC15A1 gene product. It is a secondary active H^+-co-transporter and is expressed in human intestinal epithelial cells as well as in Caco-2 cells [3, 125]. The transporter is found in the apical membrane and acts as an absorptive transporter. Typical substrates of PEPT1 are di- and tripeptides. Additionally, further substrates have been identified e.g. the ACE-Inhibitor enalapril [13], some β-lactam antibiotics e.g. cyclacillin and ceftibuten from the group of the cephalosporines [126]. Furthermore, for peptidyl-prodrugs higher oral bioavailabilities have been shown in comparison to the simple parent drugs e.g. L-valine aciclovir [14] or biphosphonate-peptide drugs [127] and they are thought to be substrates of the peptide transport system.

2d. Monocarboxylate Transporter (MCT)

Eight members (MCT1-MCT8) of the family of monocarboxylate transporters have been identified until today. Most is known about MCT-1, which is involved in the uptake of weak organic acids such as benzoic acid, lactate, pravastatin or salicylate. Additionally, atorvastatin has been recognized as a MCT substrate by Wu et al. [45]. MCT1 is encoded by the gene SLC16A1 and has been characterize in Caco-2 cells and rat small intestine.

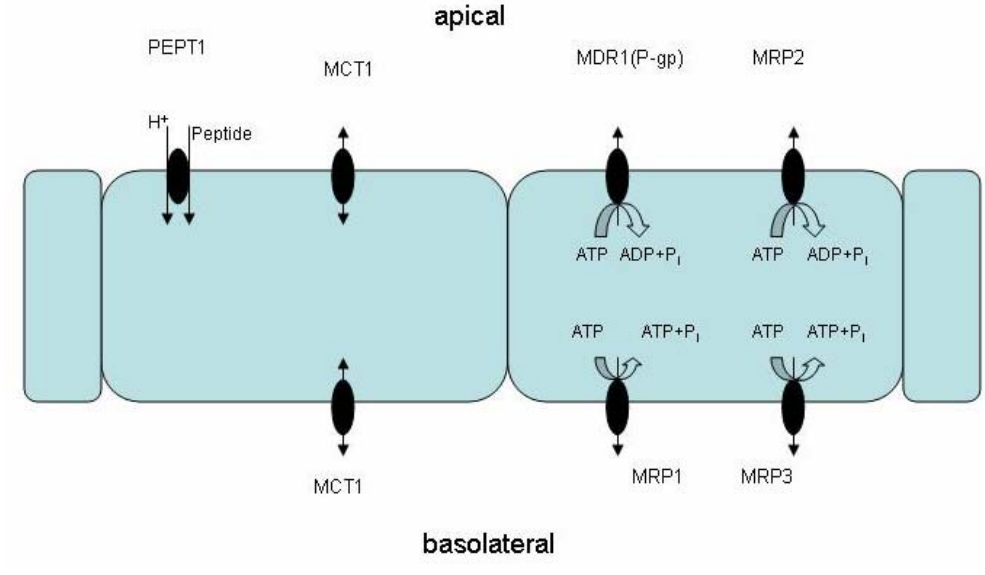


Figure 1. Transporters in intestinal epithelium, detailed explanation in the text.

It has been detected predominantly in the basolateral membrane of rat enterocytes [128] but also in apical membrane vesicles from pigs and humans [129, 130].

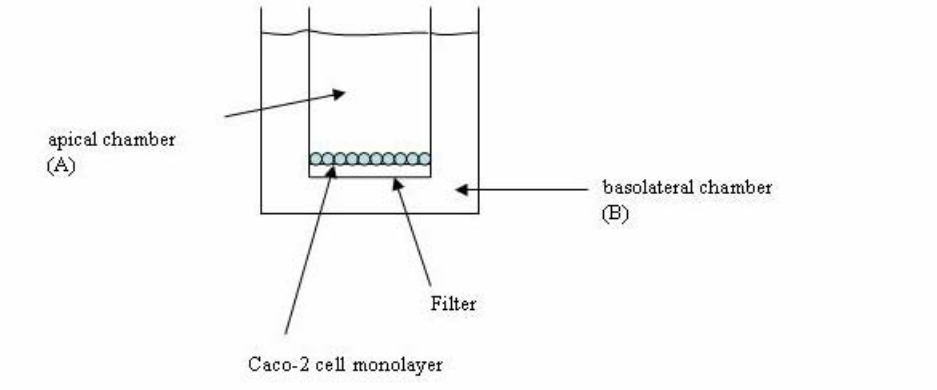
3. MODULATION OF DRUG-CARRIER ACTIVITY BY EXCIPIENTS

3a. Overview

Modulatory potential of excipients on drug carriers has first been discovered by reversal of drug resistance, which is at least in part due to drug efflux pumps, of multi-drug resistant cancer cells. One of the first reports by Woodcock et al. [131] about excipient inhibition of drug efflux was in cancer cells using Cremophor EL. They pointed out its potential usefulness as an additive in cancer therapy to overcome drug resistance of cancer cells.

Nerurkar and Borchardt [132] expanded that idea by outlining the potential usefulness of excipients for increasing absorption of drugs which are substrates for drug efflux pumps in the intestine.

Until today several excipients have been screened for their potential to modulate drug carrier mediated transport in cancer cells, intestine or other epithelial tissue. Studies have been conducted using in vitro, in situ and in vivo models such as cell culture (Caco-2, NIH3, MDCK), BBMV (brush border membrane vesicles), isolated rat tissue mounted in diffusion chambers or perfusion models. Only few clinical trials have been undertaken.



Substrate and excipient can be given either to the apical or basolateral chamber or to both and face the apical or basolateral membrane of the cells. Active transport will be different from A→B than from B→A if secretory or absorptive transport takes place. If only diffusion takes place, transport should be the same for A→B and B→A.

Figure 2. Transport studies – principle.

As a tendency rather nonionic than ionic surfactants have been examined with respect to inhibition of P-gp as can be seen in Tables 2 and 3, probably because of lower toxicity. Furthermore, ionic surfactants such as sodium dodecyl sulfate often enhanced paracellular transport possibly by changing permeability of tight junctions, thus indicating another mechanism than modulation of drug carriers [133, 134].

Table 2. Excipients that showed modulatory effects on drug-carrier-mediated transport for the substrates, models and concentrations indicated

Excipient	Concentration	Test model	Substrate	Parameter	Reference
Acconon E	0,05-0,5% [w/v]	Everted rat gut sac	Digoxin	1	[135]
Cremophor EL	0,05-0,5% [w/v]	Everted rat gut sac	Digoxin	1	[135]
	ca. 0,05-2,5% [w/v] (0,02-1mM)	Caco-2	Rhodamine 123	2	[136]
	0,1% [w/v]	Caco-2	^{3}H-Taxol	2	[137]
	0,10%	Rat intestinal membrane	Rhodamine123	2	[138]
Imwitor 742	0,1-0,5% [w/v]	Everted rat gut sac	Digoxin	1	[135]
	0,5% [w/v]	Everted rat gut sac	Celiprolol	1	[135]
Labrasol	0,05-0,5% [w/v]	Everted rat gut sac	Digoxin	1	[135]
Miglyol	0,05-0,5% [w/v]	Everted rat gut sac	Digoxin	1	[135]
MO-310 (Tetraglycerol monooleate)	0,03% [w/v]	BBMVesicles	Ceftibuten	1	[139]
MS-310 (Tetraglycerol monostearate)	0,03% [w/v]				
MS-500 (Hexaglycerol monostearate)	0,03% [w/v]				
n-dodecyl-β-D-malto-pyranoside	0,10%	Rat intestinal membrane	Rhodamine123	2	[138]
PEG 300	20% [m/v]	Caco-2	^{3}H-Taxol	2	[137, 140]
	20% [m/v]	MDR1-MDCK	^{3}H-Taxol	2	
PEG 400	1-10% [w/v]	Rat jejunal tissue	Digoxin	3	[141]
Pluronic L61	0.04% [w/v]	LLC-MDR1	^{3}H-Vinblastine	2	[57]
Pluronic L43/L61/L62/ L64/ L81/ L92/ L101	below corresponding CMC	KBv cells	Rhodamine 123	1	[142]

Table 2. (Continued)

Excipient	Concentration	Test model	Substrate	Parameter	Reference
Pluronic P85	0,1% [w/v]	Rat jejunal tissue	Digoxin	3	[141]
	0,01%-1%	Caco-2	Fluorescein	2	[143]
	0,1-1%	Caco-2	Doxorubicin	2	
	0,01% ; 1%	Caco-2	Etoposide	2	
	0,01-1%	Caco-2	^{3}H-Taxol	2	
	0,01-1%	Caco-2	Azidodeoxythymidine	2	
	0,01-1%	Caco-2	Loperamide	2	
	0,01%	BBMEC cells	Fluorescein	2	
	0,1%-1%	BBMEC cells	Doxorubicin	2	
	0,01%-1%	BBMEC cells	^{3}H-Taxol	2	
	0,1-1%	BBMEC cells	Azidodeoxythymidine	2	
Pluronic P103/P104/P123	below corresponding CMC	KBv cells	Rhodamine 123	1	[142]
Polysorbate 20	0,5% [w/v]	Everted rat gut sac	Digoxin	1	[135]
	0,03% [w/v]	BBMVesicles	Ceftibuten	1	[139]
Polysorbate 40	0,03% [w/v]	BBMVesicles	Ceftibuten	1	
Polysorbate 80	1,8mg/ml	Caco-2	Ranitidine	2b	[134]
	1,8mg/ml	Caco-2	Furosemide	2	
	1,8mg/ml	Caco-2	Cimetidine	2	
	1,8mg/ml	Caco-2	Hydrochlorothiazide	2	
	0,5%[w/v]	Everted rat gut sac	Digoxin	1	[135]
	ca. 0,015-0,6% [w/v] (0,025 -1mM)	Caco-2	Rhodamine 123	2	[136]
	0,05% [w/v]	Caco-2	^{3}H-Taxol	2	[137, 140]
	0,10%	Rat intestinal membrane	Rhodamine123	2	[138]
Sodium lauryl sulfate	0,04mg/ml	Caco-2	Ranitidine	2	[134]
	0,04mg/ml	Caco-2	Furosemide	2a	
	0,04mg/ml	Caco-2	Cimetidine	2a	
	0,04mg/ml	Caco-2	Hydrochlorothiazide	2	
Excipient	Concentration	Test model	Substrate	Parameter	Reference
	0,04mg/ml	Caco-2	Acyclovir	2	

Table 2. (Continued)

Excipient	Concentration	Test model	Substrate	Parameter	Reference
Softigen 767	0,5% [w/v]	Everted rat gut sac	Celiprolol	1	[135]
	0,5% [w/v]	Everted rat gut sac	Digoxin	1	[135]
Solutol HS 15	0,05-0,5% [w/v]	Everted rat gut sac	Digoxin	1	[135]
Sucrose stearate palmitate (7:3) monoester	0,03% [w/v]	BBMVesicles	Ceftibuten	1	[139]
SS-500 (Hexaglycerol sesquistearate)	0,03% [w/v]	BBMVesicles	Ceftibuten	1	[139]
TPGS 1000	0,05-0,5% [w/v]	Everted rat gut sac	Digoxin	1	[135]
	0,5% [w/v]	Everted rat gut sac	Celiprolol	1	[135]
	0,01 %-0,02%	Caco-2	Talinolol	2	[144]
	0,04%	Human	Talinolol	4	
TGPS 800	ca. 0,01-0,4% [w/v] (0,025-0,75mM)	Caco-2	Rhodamine 123	2	[136]
TS-500 (Hexaglycerol tristearate)	0,03% [w/v]	BBMVesicles	Ceftibuten	1	[139]
TS-750 (Decaglycerol tristearate)	0,03% [w/v]	BBMVesicles	Ceftibuten	1	

Note: Parameter 1 = increased uptake; 2 = increased transport; 3 = inhibition of efflux; 4 = increased AUC.

Table 3. Excipients without positive effects on carrier –mediated transport for the substrates, models and concentrations indicated

Excipient	Concentration	Test model	Substrate	Reference
Acconon E	0,05-0,5% [w/v]	Everted gut sac	Celiprolol	[135]
Anhydrous cherry flavor	0,006% [v/v]	Caco-2	Acyclovir, furosemide, hydrochlorothiazide	[134]
Cremophor EL	0,05-0,5% [w/v]	Everted gut sac	Celiprolol	[135]
	0,1% [w/v]	MDR1-MDCK	[^{3}H]taxol	[137]
	0-1mM	Caco-2	Gly-Sar	[136]
	0,10%	Rat intestinal tissue	Gly-Sar	[138]
	0,10%	Rat intestinal tissue	3-O-methylD-glucose	
Docusate sodium	0,02mg/ml	Caco-2	Ayclovir, atenolol, hydrochlorothiazide, ranitidine	[134]
EDTA	0,06mg/ml	Caco-2	Ayclovir, atenolol, cimetidine, ranitidine	
F-140 (sucrose stearate palmitate(7:3)polyester)	0,03%	BBMVesicles	Ceftibuten	[139]
F-160 (sucrose stearate palmitate(7:3)polyester)	0,03%	BBMVesicles	Ceftibuten	
Gelucire 44/14	0,05-0,5% [w/v]	Everted rat gut sac	Digoxin	[135]
Hexadecyltrimethyl ammonium bromide	0,10%	Rat intestinal tissue	Rhodamine123	[138]
HPMC	0,12 mg/ml	Caco-2	Acyclovir, atenolol, ranitidine	[134]
Imwitor 370	0,05-0,5% [w/v]	Everted rat gut sac	Digoxin	[135]
Labrafil M1944CS	0,05-0,5% [w/v]	Everted rat gut sac	Digoxin	
Lactose	2mg/ml	Caco-2	Acyclovir, atenolol, ranitidine	[134]
ML-310 (Tetraglycerol monolaureate)	0,03%	BBMVesicles	Ceftibuten	[139]
ML-500 (Hexaglycerol monolaureate)	0,03%	BBMVesicles	Ceftibuten	
ML-750 (Decaglycerol monolaureate)	0,03%	BBMVesicles	Ceftibuten	
MO-500 (Hexglycerol monooleate)	0,03%	BBMVesicles	Ceftibuten	
MO-750 (Decaglycerol monooleate)	0,03%	BBMVesicles	Ceftibuten	
N-octylglycoside	0,034mM	Caco-2	Benzoci acid, gly-sar, rhodamine 123	[136]
PEG 400	1,5% [v/v]	Caco-2	Acyclovir, atenolol, ranitidine	[134]
PEG 400	0,05-0,5% [w/v]	Everted rat gut sac	Digoxin	[135]
Excipient	Concentration	Test model	Substrate	Reference
Plurol oleic	0,05-0,5% [w/v]	Everted rat gut sac	Digoxin	

Table 3. Excipients without positive effects on carrier –mediated transport for the substrates, models and concentrations indicated

Excipient	Concentration	Test model	Substrate	Reference
Poloxamer 188	0,80%	Caco-2 cells/ human	Talinolol	[145]
Polysorbate (Tween 21)	0,03%	BBMVesicles	Ceftibuten	[139]
Polysorbate (Tween 60)	0,03%	BBMVesicles	Ceftibuten	
Polysorbate (Tween 80)	0,03%	BBMVesicles	Ceftibuten	
Polysorbate (Tween 81)	0,03%	BBMVesicles	Ceftibuten	
Propylenglycol	0,05-0,5% [w/v]	Everted rat gut sac	Digoxin	[135]
Propylenglycol	1.5 % [v/v]	Caco2	Cimetidine, furosemide	[134]
Span 20	0,03%	BBMVesicles	Ceftibuten	[139]
Span 30	0,03%	BBMVesicles	Ceftibuten	
Span 80	0,03%	BBMVesicles	Ceftibuten	
Span 85	0,03%	BBMVesicles	Ceftibuten	
Sucroester 15	0,05-0,5% [w/v]	Everted rat gut sac	Digoxin	[135]
Sucroester 7	0,05-0,5% [w/v]	Everted rat gut sac	Digoxin	
TPGS-1000	0,01% [w/v]	Rat intestinal tissue	Digoxin	[141]
TPGS-800	0-0,75mM	Caco-2 cells	Gly-Sar	[136]
TPGS-800	0-0,75mM	Caco-2 cells	benzoic acid	

Not all of the excipients given in the list will be covered in this review. Instead we have selected some interesting examples which will be discussed in more detail.

3b. Cremophor EL

Cremophor EL is used in several oral pharmaceutical formulations containing paclitaxel, cyclosporine A or ritonavir. It mediates solubilization of theses drugs and thus enhances their bioavailability. However, recently more and more evidence appeared that bioavailability enhancement might also be due to inhibition of P-glycoprotein-mediated secretion for such drugs that are P-gp substrates. Evidence came from in vitro and in vivo transport studies. Most of the studies found positive effects on absorption or transport, however some studies did not which triggered controversial discussion.

Shono et al. [138] investigated the use of Cremophor EL as an inhibitor of drug-carrier mediated uptake of rhodamine 123 in a diffusion chamber with rat jejunal intestinal tissue. They used a concentration range from 0.005-1% [w/v] Cremophor EL and found increased transport in the absorptive and decreased transport in the secretory direction at concentrations as low as 0.05% [w/v]. At higher concentrations the effect diminished. Equilibrium studies with dialysis membranes with rhodamine 123 and Cremophor EL showed that the amount of micelle-bound dye increased remarkably above Cremophor EL concentrations of 0.05% [w/v]. This seems a reasonable explanation for the waning effects at higher excipient concentration, since the free drug concentration and thus the amount of rhodamine 123 available for transport is reduced and so would be absorption. The CMC of Cremophor EL was given as 0.0095% [m/V] but had not been adapted to the chamber condition and a higher CMC in the presence of cell tissue might well be possible because of partitioning of Cremophor EL monomers into membranes.

Rege et al. [136] found increased permeability of rhodamine across Caco-2 monolayers in the apical-to-basolateral direction and a substantial decrease of permeability in basolaterol-to-apical direction in the presence of Cremophor EL (0.02-1 mM or ca. 0.005-0.25% estimated by a molecular weight of ~2500 Da), However, they did not find a decline of Cremophor EL effect at higher concentrations (> 0.25%) as was described by Shono. Bogman et al. [145] determined the CMC of Cremophor EL in HBSS buffer in the presence of cells as 0.03% [w/v] . The differences might be due to the different models, buffers or rhodamine 123 concentrations used (rat jejunal tissue, Krebs Ringer Bicarbonate Solution pH 7.4 vs. Caco-2 monolayers and HBSS/HEPES buffer pH 6.8).

The phenomenon of decreasing apparent permeability at very high excipient concentrations has already been described by Nerurkar et al. [146]. They carried out transport studies in Caco-2 cell monolayers with a peptide drug which showed P-gp like mediated transport when Cremophor EL was present in the apical chamber and demonstrated that permeability reaches a minimum for basolateral-to-apical transport in the proximity of the CMC of Cremophor EL and that for apical-to-basolateral transport a maximum is reached which starts to decrease again at higher surfactant concentrations. The concentration at which this occurs will be dependent on the substrate´s affinity to the surfactant micelles. The higher its affinity the faster should be the onset of permeability decrease above the CMC. Furthermore, whether or not the substrate´s permeability will be higher in the presence of surfactant also depends on its likeliness to associate with micelles. Therefore, depending on the substrate, micelle formation might not always exert a negative effect on transport.

The study of Nerurkar et al. further suggested that it is primarily the surfactant monomers which increase drug permeability, since increases above the CMC did not yield in higher permeability. The monomer concentration remains about the same above the CMC (and then

is about equal to the CMC) and therefore no further enhancement would be expected if the monomers are the species contributing to the permeability enhancement.

Using rhodamine 123 as substrate primarily aims at analysing P-gp mediated transport since it is a known P-gp substrate. However, rhodamine 123 is also known as MRP [109] and BCRP [59] substrate. So, if those carriers were also present in the cell lines or rat tissue used, the overall effect on rhodamine transport might be the consequence of their common action.

MRP1 and MRP3 are secretory transporters and located at the basolateral membrane. Shono et al. added Cremophor EL to the basolateral side of cell tissue and no transport inhibition was observed suggesting that MRP1 and MRP3 either do not play a role in the transport of rhodamine in rat tissue or that Cremophor EL does not alter their transport activity. MRP2 is a secretory transporter present in the apical membrane, as well as BCRP. Currently, it is not clear to which extent these carriers are involved in drug secretion in the intestine.

Other substrates have been used in order to investigate the effects of Cremophor EL on peptide transporters and the monocarboxylate transporter in Caco-2 cell monolayers. Rege et al. [136] used gly-sar (substrate of PEPT1) and benzoic acid (substrate of MCT) but did not find altered transport of these substrates in the presence of 0.01-1 mM Cremophor EL.

This might indicate that the effect of Cremophor EL is transporter specific.

Cornaire et al. [135] used the P-gp substrates digoxin and celiprolol to investigate Cremophor EL effects on absorption in rats in vivo (1 mg/kg) and by ussing chamber technique with rat intestine in vitro (0.05-0.5% [w/v]). Cremophor EL enhanced uptake of digoxin (10µM) in vitro but not of celiprolol. Celiprolol (100µM) was used at concentrations ten times higher than digoxin (10µM) therefore it is possible that active secretion at this concentration is neglible compared to passive transport and not rate-limiting for absorption. The concentration dependence of carrier-mediated absorption reflects in the Michaelis-Menten-equation and has been reported for talinolol by Wagner et al. [147] and for vinblastine [148].

However, celiprolol absorption could be effectively increased by verapamil, a competitive P-gp inhibitor. Furthermore, TPGS was shown to enhance both absorption of celiprolol and digoxin in the same study, though for absorption enhancement of celiprolol higher TPGS concentrations than for digoxin were needed. Therefore, different inhibitory mechanisms might also be involved and sensitivity of transport inhibition may also depend on the substrate itself. Another explanation could be the participation of other drug-carriers like MRP or BCRP together with specific inhibition of only P-gp or MRP/BCRP, respectively. Digoxin has been shown to be a substrate of P-gp and it has been shown that it is not a substrate for MRP2. Nevertheless, Lowes et al. remarked that besides P-gp another transporter might be involved in digoxin secretion [66]. For celiprolol it is not known whether it has or has no affinity to MRP2 or other transporters. Supposed it had, selective inhibition of P-gp might not suffice to stop secretion of digoxin efflux because of the efflux mediated by another transporter.

Neither of the authors (Cornaire, Nerurkar, Rege, Shono) observed relevant decreases of cell viability either measured by LDH release or trypan blue assay or increases in paracellular transport as measured by mannitol transport. So the effects of Cremophor EL are not due to cell death or changes in paracellular transport.

In in vivo trials [135] in rats with Cremophor EL and digoxin the AUC was reduced and t_{max} shifted to later times indicating diminished permeability of digoxin in the presence of the

non-ionic surfactant. This is contrary to what would be expected from in vitro experiments. In this experiment 1 mg/kg Cremophor EL together with digoxin was administered by gavage in a mixture of water/propyleneglycol/ethanol. The use of cosolvents can change the CMC of surfactants, normally decreasing it and thus also lowering monomer concentration which might decrease the inhibitory effect. If the surfactant concentration was too high micellar incorporation of digoxin might have reduced the free drug concentration and thus drug absorption. However, the actual concentration at the epithelial boundary layer as well as the CMC of Cremophor EL under in vivo conditions are currently unknown and therefore one can only speculate about possible mechanisms.

In a clinical trial Cremophor EL was administered up to 5000 mg [149]. Cremophor EL in single daily doses of 100, 1000 and 5000 mg together with 600 mg saquinavir (Invirase ®), which is also a P-gp substrate, was given to fasted volunteers. Doses of 100 mg and 1000 mg slightly increased $AUC_{0-\infty}$ by 1.4 fold compared to placebo. 5000 mg led to 5-fold increases. The effect of 5000 mg Cremophor EL on saquinavir pharmacokinetics was said to be similar to an opulent breakfast. In this regard, metabolic effects, solubilization by bile salts and altered intestinal and hepatic blood flow may be contributing. Furthermore, AUC_{0-4h} was 2-3 times enhanced by 100 mg or 1000 mg and 13 times enhanced by 5000 mg Cremophor EL. This shows an increased uptake in the early phase of absorption up to four hours after drug administration. The purpose of the study was to evaluate dose-dependant effects of Cremophor EL on saquinavir absorption and not to elucidate the possible mechanism. Besides P-gp inhibition CYP3A4 inhibition, i.e. decreased metabolism, favourable dissolution or solubility, might also play a role. Furthermore, the viscosity of gastric or intestinal fluid might be changed and thus the ability of the drug to diffuse freely.

Besides Cremophor EL Cremophor RH40, a structurally related compound, has also been assessed for its potential to enhance absorption of the P-gp substrate digoxin in humans. A 19-22% increase in AUC_{0-3h} was observed [150].

Cremophor EL is already used as an absorption enhancer in marketed products. To which extent enhancement is mediated by improvement of solubilization, P-gp inhibition or other physiological effects such as metabolism or changing gastric fluid viscosity remains to be determined.

As to whether high concentrations of surfactants (above CMC) rather hamper than enhance absorption depends on the substrate.

Bardelmeijer and Malingré [151, 152] as well as Chiu [153] reported that Cremophor EL might actually lower the intrinsic permeability of paclitaxel and cyclosporine A, respectively, in humans because of its association with Cremophor EL micelles whereas Nerurkar et al. [146] found a decrease in permeability for their peptide drug only at very high surfactant concentrations (100-1000 times higher than CMC) in studies conducted with Caco-2 cell monolayers.

In vitro experiments strongly suggest a role of P-gp inhibition (or other carriers) as shown e.g. with the metabolically stable substrate digoxin in Caco-2 cell monolayers. Most publications report an approximately twofold increase in permeability [135, 136, 138]. Flux is not only dependent on permeability but also on the concentration that remains on the luminal side. Thus effects might attenuate faster in vivo because unlike in in-vitro experiments surfactant and drug pass along the intestine and surfactant concentration continuously decreases over time [154] . However, in vitro only little amounts of drug are transported so that drug and surfactant concentration remain virtually constant in the donor chamber. On the

other hand in vivo mRNA levels of P-gp have been shown to be higher in duodenal enterocytes than in Caco-2 cells [155]. However, in the jejunum expression levels of P-gp have been shown to be equal or somewhat lower than in Caco-2 cells [156] and the jejunum is regarded as the more important absorption site. It is not possible to directly correlate in vitro and in vivo experiments and therefore the relevance of modulatory effects on drug-carriers and pumps can only be shown by in vivo experiments.

As a conclusion, Cremophor EL employed at appropriate concentrations with a suitable drug might be a useful absorption enhancer of drug-carrier substrates but it should be kept in mind that enhancing solubility by micellization can counteract an effect mediated by P-gp inhibition depending on the drug´s affinity to the micelles. If it is low however, then the P-gp inhibition produced by surfactant (monomers) might further increase permeability and yield a synergistic effect together with micellar solubilization.

Whether P-gp inhibition by Cremophor EL might play a substantial role in absorption of P-gp substrates in humans remains to be shown. In vitro evidence, however, suggests such a role.

3c. Tocopheryl-Polyethyleneglycol-1000-Succinate (TPGS)

TPGS is used as water-soluble form of vitamin E to treat vitamine E deficiency. Furthermore, it has been shown to enhance absorption of drugs e.g. amprenavir [157], cyclosporine A [158] and vitamine D in chronic cholestatic liver disease of infancy and childhood [159]. TPGS is listed as an excipient in the USP24 and is used as an embedding agent for the formulation of soft-gelatine capsules.

Dintaman and Silverman [160] observed that TPGS added to both sides of an ussing chamber simultaneously may inhibit basolateral-to-apical transport of rhodamine 123 (13 µM) and paclitaxel (0.1 µM) in Caco-2 and HCT-8 cells in concentrations from 0.001 to 0.005%, i.e. well below its CMC of 0.02% [w/w]. Furthermore, they showed that TPGS was able to reverse multi-drug resistance in NIH 3T3 G185 cells towards the P-gp substrate doxorubicin to the level of drug-sensitive NIH 3T3 cells whereas no such effect was observed using the non P-gp substrate 5-fluorouracil.

Cornaire et al. [135] observed a 2.5-fold increase mucosal to serosal digoxin transport using the everted rat gut model at a TPGS concentration of 0.05% [w/v]. Transport was still enhanced at concentrations of 0.1% (2.4-fold) and 0.5% (1.9-fold) but to a smaller extent than at 0.05%. The digoxin concentration used was 10 µM.

In a study by Johnson et al. [141] no reduction in serosal to mucosal digoxin flux across isolated rat jejunum was found at concentrations of 0.01% TPGS and 1 mM digoxin, which is twofold higher than that used in Caco-2 cell transport studies by Dintaman and Silverman, but would still be in accordance with the results of Cornaire et al.

However, the different experimental set-ups (e.g. different subtrates, substrate concentrations and transport models) do not allow a direct comparison Chiu et al. [153] found a reduction of basolaterol-to-apical and apical-to-basolateral transport of cyclosporin A (0,4 µg/ml, corresponding to approximately 3.3 µM) across Caco-2 cell monolayers at TPGS concentrations of 0.02% and 0.2% [w/v]. In their method TPGS was used together with cyclosporin A either in the donor or acceptor chamber. Therefore these results indicated rather a sequestration of cyclosporin A into TPGS micelles to be the underlying mechanism

for decreased transport as opposed to transporter inhibition as this might have lowered the free drug concentration. If TPGS is not present on the apical cell side no P-gp inhibition should take place since P-gp is expressed predominantly on the apical side and movement of TPGS to the apical membrane via the epithelium is restricted by tight junctions whereas transcellular transport should be negligible. Therefore, no reduction should have been expected. Nevertheless, one cannot absolutely deny concurrent inhibition of TPGS on P-gp in these experiments, since one has to consider the fact that in this study TPGS concentrations were equal or higher to the CMC of TPGS and thus association with micelles might have dominated over any other possibly observable inhibition of P-gp. Nevertheless, it indicates that TPGS similar to Cremophor EL might be more useful below or equal to its CMC for increasing bioavailability by inhibition of P-gp for certain substrates.

In vivo rat studies by Cornaire et al. [135] using TPGS at a concentration of 1 mg/kg did not enhance the $AUC_{0\text{-}480min}$ of digoxin, though the $AUC_{0\text{-}40min}$ was significantly higher. This shows that TPGS has a potential to modify the absorption of a drug and indicates that TPGS might be more effective in enhancing the rate of absorption rather than the extent.

None of the authors found significant cell toxicity or increases in paracellular transport with TPGS demonstrating its non-toxicity on rat tissue and cell cultures.

Varma and Panchagnula [161] showed that TPGS at concentrations of 0.1 mg/ml was superior to 1 mg/ml in enhancing the permeability of paclitaxel in in situ rat experiments, although at 1 mg/ml TPGS permeability was still significantly higher than in the control experiment. Moreover, they demonstrated that solubility of paclitaxel substantially increased above the CMC and that therefore improved solubility of paclitaxel could not be the reason for enhanced transport at the lower TPGS concentration.

An in vivo study in rats using the substrate colchicine in a 10% [v/v] TPGS solution showed a more than twofold increase in colchicine´s AUC. Colchicine has good inherent solubility leading the authors of the study to conclude that the solubilizing effect alone could not account for the increase in AUC [162].

TPGS effects on talinolol absorption have recently been studied with regard to P-gp-inhibition in humans [144]. The study was designed to exclude absorption phenomena mediated by improved solubility, dissolution, wetting or other generally surfactant-associated effects by using a non-P-gp inhibiting excipient, poloxamer 188, as a control and administration of solutions via nasogastral tubes. Possible metabolic effects on bioavailability were excluded by the use of talinolol as substrate as it can be considered as metabolically stable. The study provided good evidence that oral TPGS is able to enhance the bioavailability of talinolol and that this effect is due to inhibition of P-glycoprotein. The $AUC_{0\text{-}\infty}$ of talinolol was reported to augment by 39% compared to talinolol administered in the control vehicle.

Collnot et al. [163] studied the effect of polyethyleneglycol chain length of TPGS on absorptive transport of rhodamine 123 with the Caco2-cell model. The chain length of TPGS was varied from 200-6000 and it was shown that TPGS-1000 has the optimal chain length with respect to enhancement of rhodamine 123 transport.

Brouwers et al. [154] measured the TPGS concentration in duodenum and jejunum after administration of TPGS-containing amprenavir soft gelatine capsules. TPGS concentration reached levels as high as four mM in the jejunum and as high as eight mM in the duodenum. This is in the range of 0.01 to 0.1% , concentrations which have been shown to be effective in inhibiting P-gp in vitro as well.

In conclusion, there is broad evidence that TPGS can enhance bioavailability of P-gp substrates such as digoxin, talinolol and paclitaxel by both enhancing their solubility and inhibiting their efflux in the intestine. Evidence comes from in vitro, in situ and clinical trials. The efficacy of TPGS has been shown for different substrates. Thus TPGS might be a useful excipient in particular for increasing bioavailability of P-gp substrates.

3d. Polysorbate 80

Polysorbate 80 (Tween 80) is a non-ionic surfactant used as an emulsifier in O/W emulsions and as solubilizing agent. Furthermore it is used in mixture with other excipients in some formulations with self emulsifying properties containing e.g. cyclosporin A (Gengraf®) and sirolimus (Rapamune®) [164].

Nerurkar et al. [146] performed mechanistical studies on the effect of Polysorbate 80 on P-gp-mediated transport of a peptide drug in Caco-2 cell monolayers. The maximum decrease in basolateral-to-apical permeability was attained already below the CMC of Polysorbate 80 (given as 50 µM) indicating again the monomer and not micelle as the active moiety. Apical-to-basolateral permeability was enhanced by increasing Polysorbate concentrations up to 30 µM. The effect remained constant up to a concentration of about 25 times higher than the Polysorbate CMC. Then, like for Cremophor EL, permeability decreased suggesting associaton of relevant amounts of the peptide drug with surfactant micelles.

In a study by Rege et al. [134] it was demonstrated that Polysorbate 80 at concentrations of 1.8 mg/ml increased the overall permeability of furosemide, ranitidine and cimetidine in Caco-2 cell monolayer. Furthermore they showed that Tween 80 increased apical-to-basolateral permeability and decreased basolateral-to-apical permeability of rhodamine 123 in Caco-2 cell monolayers at concentrations ranging from 0.01 mM to 1 mM in a dose-dependent manner [136]. Membrane fluidization (DPH anisoptropy) was observed at a concentration as low as 0.025 mM. Polysorbate 80 also decreased apical-to-basolateral permeability of gly-sar, suggesting inhibition of the absorptive peptide transporter.

In Caco-2 cell monolayers ^{3}H-Taxol transport was increased at a concentration of 0.05% [w/v] without increasing mannitol transport measured as a marker of passive paracellular transport. The onset of transfer enhancement at 0.05% [w/v] correlated with the onset of membrane fluidization as observed by fluorescence anisotropy of DPH whereas no change occurred in trimethylammonuiumphenyl-DPH (TMA-DPH) anisotropy [137].

Furthermore Polysorbate 80 increased the digoxin transfer across isolated rat jejunal tissue by 1.8-fold at a concentration of 0.5% [w/v] [135, 165] without enhancing LDH release measured as a marker of cell toxicity.

Zhang et al. [166] examined the effects of Polysorbate 80 on digoxin absorption in rats. Digoxin was given orally in solutions containing 1% and 10% Polysorbate 80. Moreover digoxin was given intravenously while Tween 80 was administered orally to estimate the effect of oral Tween 80 on extraintestinal P-gp in liver and kidney. For 1% Polysorbate 80 the $AUC_{0\text{-}10h}$ increased by 30%, for 10% Polysorbate 80 by 61%. C_{max} climbed to about 160% for both. No influence of orally administered Polysorbate 80 on the AUC of intravenous digoxin was found. Therefore the increased AUC is most probably due to inhibition of intestinal P-gp by Polysorbate 80.

Seeballuck et al. [167] addressed a different aspect of Polysorbate 80. They demonstrated that it can enhance chylomicron secretion in Caco-2 cell monolayers and thus might be able to enhance lymphatic transport of lipophilic drugs. This would result in a synergistic effect for lipophilic P-gp substrates.

Oral formulation of paclitaxel with Polysorbate 80 results in higher bioavailability than with Cremophor EL as shown by Malingré et al. [152]. Less paclitaxel was found in faeces when formulated with Polysorbate 80 than with Cremophor EL. Moreover, the amount of paclitaxel in the faeces was correlated to the Cremophor EL concentration in the Cremophor EL formulation.

Though modulation of drug carriers by Polysorbate 80 has not yet been directly shown in vivo the results of in vitro studies strongly suggest it. Together with its potential as a solubilizing agent and perhaps its capability of enhancing lymphatic transport it seems a valuable excipient for increasing bioavailability of low-solube, lipophilic drugs with affinity to P-gp and P-gp like transporters. However, Polysorbate 80 may inhibit PEPT1 and therefore possibly decrease the uptake of substrates of absorptive carriers.

3e. Pluronic P85 (and other Pluronics)

Pluronics (also called poloxamers) are ABA block copolymers which are built from polyoxypropylene and polyoxyethylene. The hydrophobic polyoxypropylene chain (B) takes a central position and is flanked by two polyoxyethylene chains (A) on either side. Pluronics can be used for different purposes e.g. as O/W- type emulsifiers, to increase viscosity or for surfactant gels. A review about the use of pluronics as novel polymer therapeutics for drug and gene delivery is given by Kabanov et al. [168].

Batrakova et al. [169] studied the effects of Pluronic P85 on drug permeability in BBMEC (Bovine brain microvessel endothelial cells) and Caco-2 cells using rhodamine 123 as substrate. They found that at a concentration of 22 µM (CMC ~ 67µM) Pluronic P85 effectively eliminated polarized transport of rhodamine 123 across Caco-2 cell monolayers, i.e. flux in basolateral-to-apical direction and apical-to-basolateral direction practically equals each other, whereas without P85 apical-to-basolateral flux was 7.8-times higher than basolateral-to-apical flux. The effect was only seen when P85 was included on the apical cell side. This implies the involvement of an apical efflux transporter like P-gp, whose activity was inhibited by Pluronic P85.

Like the non-ionic surfactants discussed above, here again the monomers (or unimers) and not micelles are the active moiety, since inhibition was observed well below the CMC.

Working with a P85 concentration of 11 mM (i.e. above the CMC) on the apical side decreased apical-to-basolateral transport as compared to buffer plus rhodamine 123 only (control). Basolateral-to-apical flux was decreased likewise when P85 was added to the basolateral side. With no inhibition of drug transporters on the apical side, one would have expected increased transport because of accelerated efflux. At a P85 concentration of 11 mM about 50% of rhodamine 123 was associated with micelles indicating that decreased transport was due to decreased concentration of free rhodamine 123. Earlier, Batrakova et al. had shown that P85 at concentrations slightly above the CMC can still mediate P-gp inhibition by free unimers. However, when concentrations were further increased uptake of rhodamine 123

can be inhibited as compared to control (assay buffer + rhodamine 123 only). Similar phenomena were observed for Pluronic L81 and F68 [170].

The authors also described endocytosis of whole micelles into cells, but suggested that micelles were then routed back to the apical membrane and recycled out of the cell so that they do not positively contribute to uptake or transport of rhodamine 123 in Caco-2 cells [169]. Furthermore, it was shown in Caco-2 (and BBMEC) cell monolayers that not only rhodamine 123 transport can be enhanced by Pluronic P85 but also transport of fluorescein and drugs including doxorubicin, taxol, etoposide, azidodeoxythymidine, loperamide and valproic acid [143, 171]. Fluorescein is regarded as MRP substrate [172, 173] though its affinity to other drug carriers is not well defined. Nevertheless, it might be possible that Pluronic P85, as well as the other surfactants, can modulate activity of more than one drug carrier or pump.

The effects of Pluronic P85 on MRP1 and MRP2 overexpressing MDCK cells were studied and increased accumulation of vinblastine and doxorubicin was found suggesting that P85 is capable of inhibiting MRP1 and MRP2 [174]. On the other hand Evers et al. [57] studied effects of Pluronic L61, a compound structurally related to P85, on vinblastine transport in MDCK2-MRP transfected cells and concluded that neither vinblastine transport by MRP1 nor MRP2 were significantly inhibited by Pluronic L61. However, it is not known how important MRPs are for the intestinal absorption of drugs and not many conclusions can be derived from experiments in MRP overexpressing cells since they do not reflect MRP expression and activity in the human intestine.

Pluronic P85 (0.001%-1% [w/w]) has been shown to inhibit P-gp ATPase activity (also MRP1 and MRP2 ATPase activity but to a smaller extent) with concomitant increase of membrane fluidity in BBMEC cells. At high concentrations (> 0.01%) P85 also depletes intracellular ATP stores to a certain level [175-177]. Both actions, P-gp ATPase inhibition and ATP-depletion contribute to efflux inhibition of rhodamine 123.

Rhodamine 123 uptake was increased twofold by P85 concentrations from 0.001%-0.01% in BBMEC cells. At higher concentrations uptake was lowered again because of micelle formation. Thus the onset of enhanced uptake falls together with inhibition of P-gp, whereas at higher concentrations ATP-depletion adds up to the effect and at concentrations higher than the CMC (~ 0.03%) uptake enhancement levels off again because of the incorporation of drug into micelles resulting in lower free drug concentrations.

Transport studies have been performed with ^{3}H-P85 in LLC-MDR1 and MDCK-MRP2 and no directionality of transport was found indicating that ^{3}H-P85 is not a substrate of P-gp and MRP2 [177] and it was therefore concluded that inhibition rather operates by conformational changes of the transporter induced by membrane fluidization or by sterical hindrance of the drug binding site by P85.

Batrakova et al. [178] also reported inhibiton of MCT1, probably by inhibition of H^{+}-ATPase in BBMEC cells. However, BBMEC cells are model cells for blood brain endothelial cells and might show other physiology, expression levels and transporter activity than intestinal epithelial cells. The same study also investigated toxicity of Pluronic P85 administered intravenously but did not find histological or immunohistochemical difference as compared to control.

Batrakova et al. [142] studied several pluronics with varying content of polyoxyethylene and polyoxypropylene. It was concluded that with increasing polypropylenoxide content and higher hydrophobicity, compounds had higher potency in efflux inhibition. However, higher

hydrophobicity also went along with lower CMCs, i.e. lower concentrations of Pluronic monomers. Since therefore the concentration of the free monomers decreases, so does efflux inhibition. Therefore at a certain point, further increasing compound hydrophobicity will counteract efflux inhibition. Pluronic P85 turned out to be a compound with near optimal properties with respect to polypropyleneoxide content, hydrophobicity and CMC. No efflux inhibition was found for Pluronics with high HLB (above 20), i.e. with high polyoxyethylene content.

In conclusion, several of the more hydrophobic Pluronics can inhibit P-gp and probably other ATP-dependant carriers as MRP1 and MRP2, whereas hydrophilic Pluronics (HLB higher than 20) do not seem to have such properties. Investigations have mainly focussed on Pluronic P85, because it turned out to be one of the most potent pluronics with respect to modulation of carrier-mediated transport.

Pluronic P85 might also inhibit MCT1, but no studies are available for intestinal cells. Enhanced transport of several substrates including doxorubicin, taxol and etoposide was demonstrated for Pluronic P85. Its toxicity seems to be low in mice even if administered intravenously. As for the surfactants the monomers are the active moiety. The mechanism of inhibition includes P-gp ATPase inhibition and at higher concentration ATP-depletion. Data is available mainly from in vitro studies. Until now, in vivo experiments in humans dealing with drug-carrier mediated transport have not been conducted to our knowledge.

3f. PEG 400 and PEG 300

Polyethyleneglycols are polymers made by polymerization of ethyleneoxide. They are good solvents for several drugs though some incompatibilities e.g. with phenols are known. Wagner et al. [147] reported on the effects of PEG 400 and other surfactants on radioligand binding of ^{3}H-talinolol in a Caco-2 cell suspension. The IC50 of PEG400 was determined as 56.25 mM while IC50 values of the surfactants Cremophor EL and Tween 80 were as low as 0.41 mM and 0.23 mM respectively. This means that PEG 400 was less effective in displacing talinolol than Cremophor EL and Tween 80. Absorptive permeability of talinolol in Caco-2 cells was increased by 3.5 times while basolateral-to-apical permeability did not change significantly.

Johnson et al. [141] studied the potential of PEG 400 to enhance P-gp mediated transport of digoxin and metabolism of verapamil. They found that PEG 400 (5% w/v) inhibited basolateral-to-apical digoxin transport no matter if included on the apical or basolateral side or both. They suggested that changes in osmotic pressure at the apical membrane due to PEG 400 might result in decreased P-gp activity. Since PEG 400 also decreased basolateral-to-apical transport when added exclusively to the basolateral side and partitioning effects alone could not account for this effect they suggested that PEG 400 might also have an indirect effect on intracellular ATP levels as seen for Pluronic copolymers.

Furthermore, they demonstrated a decrease in verapamil metabolism when PEG400 was present indicating that PEG 400 might also act by inhibiting metabolism. Hugger et al. [179] studied the effect of PEG 300 on ^{3}H-taxol and ^{3}H-doxorubicin transport across Caco-2 cell monolayers. PEG 300 was evaluated in concentrations from 2.5% to 20%. Because of the high concentration of PEG 300 used it was added basolaterally and apically sumltaneously in order to exclude any effects due to differences between basolateral and apical osmolarity. The

b-to-a/a-to-b ratio was decreased for all concentrations and at the highest concentration of 20% PEG polarized transport was practically eliminated. No effect of PEG 300 was observed on testosterone and mannitol flux indicating that passive transcellular and paracellular transport is not enhanced. Therefore it was concluded that PEG 300 could inhibit P-gp and/or MRP mediated transport in Caco-2 cells. As a probable mechanism changes in membrane fluidity were proposed , since PEG300 at 20% significantly decreased fluorescence anisotropy of TMA-DPH (though not of DPH) which means that it decreases the fluidity of the polar head group region of the bilayer. This is different from the surfactants in that they rather decreased DPH anisotropy which monitors fluidity of the hydrophobic core part of the bilayer.

Interestingly , Hugger et al. [137] also showed that PEG300 inhibited transport in MDR1-MDCK cells while Cremophor EL and Tween 80 did not. Furthermore, Tween 80 decreased DPH anisotropy in Caco-2 cells while no such effect was seen in MDR1-MDCK cells. This supports the notion that inhibition of transporters also depends on their proximate environment since the membrane composition in these cells might differ. Compared to the other surfactants PEGs seem to exert their effects at comparatively high concentrations (5-20%) while they had no effect at low concentrations (0.05-1.5%) (Table 3). To our knowledge no in vivo studies investigating the effects of low molecular weight PEG on drug carrier activity in vivo have been published yet.

3g. Solutol HS 15

Solutol HS 15 contains mainly polyethylene glycol-15-hydroxystearate. It is used as a solubilizer for vitamin A, D, E and K and for lipophilic active pharmaceutical ingredients such as nifedipine, piroxicam, miconazole and alfadolone [180]. Solutol HS 15 was studied by Dudeja et al. [181] in the MDR (multi drug resistant) cell line KB 8-5-11 (human epidermoid carcinoma cell line). They showed that at a Solutol HS 15 concentration of 10 µg/ml (0.001%) multi-drug resistance of the KB 8-5-11 cells was reversed to a great extent as measured by rhodamine 123 uptake. Due to the fact that Solutol HS 15 is composed of several molecular entities a wide range is specified for the CMC of this product (0.005 – 0,02%).Thus, the concentration used in this study was above the CMC. Membrane fluidity studies with different probes including DPH and TMA-DPH were performed and it was concluded that Solutol HS 15 decreased membrane fluidity as measured by DPH and TMA-DPH.

Solutol HS 15 at concentrations ranging from 0.05% - 0.5% was effective in increasing digoxin transfer across rat everted intestinal tissue by about twofold [135]. LDH release by the tissue used as a marker of cellular toxicity was higher than control but less than 200% of control which was the limit regarded as not yet toxic by the investigators.

Bittner et al. [162] also showed that AUC of colchicine following oral administration was enhanced 4-fold by coadministration of Solutol HS 15 as compared to control. Since solubility of colchicine, a highly soluble drug, could not be enhanced by Solutol HS 15, other factors like P-gp inhibition or inhibition of CYP3A4 metabolism need to account for the improvement in colchicine bioavailability.

In conclusion, Solutol HS 15 was shown to enhance digoxin and rhodamine transport in vitro and colchicine bioavailability in rats in vivo, probably by P-gp inhibition or inhibition of metabolism and not by increasing drug solubility.

3h. Labrasol

Labrasol (caprylocaproyl macrogolglycerides EP/caprylocaproyl polyoxylglycerides USP/NF) is composed of a well-defined mixture of mono-, di- and triglycerides and mono- and di-fatty acid esters of polyethylene glycol. It is used as solubilizing agent, bioavailability enhancer of poorly soluble drugs, as surfactant in microemulsions and as permeation enhancer in topical preparations [182].

Effects of Labrasol on cephalexin a PEPT1 substrate were studied in vitro and in situ with rat intestine by Koga et al. [183]. It was found that permeability was enhanced by 10% when Labrasol (0.5%) was co-administerd under ATP-depleted conditions. Only about 50% of the enhancement was accounted for by active transport i.e. only 5% of the overall transport enhancement was due to modulation of active transport. Labrasol did not change the transepithelial electrical resistance of the tissue, though the short circuit current ($I_{sc)}$, a measure of flow of electrogenic ions, was reduced. The authors concluded that the membrane barrier was maintained. Gelucire 44/14 (PEG-32 glyceryl laurate) did not affect transport at the same concentrations. The result would suggest an enhancement of absorptive transport since cephalexin is known as PEPT1 substrate. However, if it were a substrate of MRP or P-gp too, the result could as well be due to efflux inhibition.

Cornaire et al. [135] found a 5-fold increase of digoxin transport through everted rat intestinal tissue by 0.5% Labrasol, which indeed would suggest inhibition of P-gp. In these experiments, Labrasol also liberated LDH in significant amounts, which might indicate a potential toxic effect on the tissue, so that decreased cell viability might have been the reason for increased permeability.

Labrasol contained in a self microemulsifying drug delivery system was shown to increase paracellular transport across Caco-2 cell monolayers as measured by mannitol transport [184].

The sensitivity of cell viability and tight junctions towards Labrasol might be higher in Caco-2 cells than in living tissue because the latter shows inherent heterogeneity and superior protective mechanisms. A toxic effect on a monoculture cell line must not necessarily entail a toxic effect under in vivo conditions. To which extend LDH release can be correlated with cellular toxicity remains unresolved.

Hu et al. [185] studied bioavailability of gentamicin in a Labrasol containing and a non-Labrasol formulation. Though no polarized transport was observed for gentamicin alone, verapamil significantly enhanced apical-to-basolateral transport and decreased basolateral-to-apical transport of gentamicin in rat intestinal tissue. Gentamicin shows high solubility, is not metabolised and it is not well absorbed. In vivo bioavailability in rats was significantly enhanced when gentamicin was administered directly to the colon together with Labrasol. An implication of active transport systems has not been shown, but could also not be excluded.

For the moment data is too scarce to judge whether Labrasol can significantly enhance drug transport by modulation of drug carrier activity. It might as well enhance passive

transcellular or paracellular transport. Nevertheless, it has been shown that to be effective in enhancing bioavailability of some low-bioavailability drugs [185].

3i. Others (Softigen 767, Imwitor 742, methoxypolyethylene glycol-block-polycaprolactone diblock copolymer Eudragit L100-55)

Softigen 767 (PEG-6 Caprylic/Capric Glycerides) and Imwitor 742 (caprylic/capric glycerides) were studied by Cornaire et al. [135]. Both increased digoxin and celiprolol absorption across rat gut tissue and AUCs for digoxin following oral administration in rats. Softigen 767 additionally increased AUC of celiprolol in the rat. Both increased LDH release of rat intestinal tissue less than twofold, which was set as an absolute toxicity boundary by the authors. I.e. any compound increasing LDH release above 200% was regarded as exerting substantial toxicity, those below as possibly but not necessarily toxic. The authors further state that Softigen 767 also increased mannitol transport suggesting that increased paracellular transport might contribute to the overall effect but that this route is not a common pathway for rather lipophilic substrates as digoxin and celiprolol.

A special aspect observed with these compounds in the in vivo experiments was that not only AUC increased but that the pharmacokinetic profile changed in that a second C_{max} appeared i.e. that the curves showed two peaks. The additional peak (as compared to control) appeared in the early stage of absorption. It was suggested that the compounds might have been more effective in the upper GI tract where P-gp expression is lower than in the lower parts of the GI tract [23]. Inversely, the concentration of the surfactants would be highest in the upper GI tract and due to dilution it would decrease in the lower GI tract.

Zastré et al. [186, 187] studied the effects of a methoxypolyethylene glycol-block-polycaprolactone diblock copolymer on rhodamine 123 uptake. Unlike shown for other surfactants rhodamine 123 accumulation in Caco-2 cells continued to increase even at concentrations 10 times higher than the CMC of the diblock copolymer. Partitioning into cell membranes might be a possible explanation. However, the CMC of Cremophor EL is 0.0095% [w/v] in water but 0.03 % [w/v] in the presence of cells and buffer [145] which only is about a threefold difference. The diblock copolymer was as effective in decreasing basolateral-to-apical flux as verapamil and enhanced apical-to-basolateral transport of rhodamine 123 at concentrations of 0.25 % while verapamil (50 µM) did not. Transport was also enhanced under ATP-depleted conditions so that probably both passive diffusion of rhodamine across the membrane and inhibition of active secretion by P-gp contributed to the effect.

The absorption of the antibiotics cefixim and cefadroxil, PEPT1 substrates, could be enhanced in an in situ study in rats by increasing the H^+-gradient in the rat ileum using a proton-releasing polymer (Eudragit L100-55) [188]. This complies with the idea of an H^+-dependent absorptive transporter. It is an example of actually enhancing absorptive transport in contrast to inhibiting efflux.

4. POSSIBLE MECHANISM FOR INHIBITION OF DRUG-CARRIERS BY EXCIPIENTS

P-gp as well as MRP are saturable and ATP-dependent enzymes. Therefore their activity can be affected by substrate concentration or ATP depletion. Saturable transport means that at high substrate concentrations V_{max} is reached and no further increase in active transport or secretion. V_{max} can be reduced by uncompetitive inhibition or noncompetitive inhibition. A change in membrane fluidity could trigger conformational changes of the transport protein into inactive, less active or even more active states which would be reflected in changes of V_{max} or K_m. Surfactants might interact with hydrophobic parts of the transmembrane domains of the transporter or might hinder access to putative binding sites sterically.

Transport of substrates by P-gp is coupled to ATP-hydrolysis. In fact P-gp contains two nucleotide binding sites with ATPase activity [189]. Any changes in binding affinity of ATP or availability of ATP (ATP depletion), any change in stability of intermediate states of ATP and the binding sites and changes in the release of ADP or P_i can affect the overall activity of P-gp. Such changes can be brought about by alterations of the three dimensional structure of the transporter by binding of a substrate or modulator or by changing the proximate environment of the protein i.e. membrane perturbations.

Depletion of intracellular ATP can inhibit P-gp mediated transport as was discussed for Pluronic P85. A proposed mechanism for ATP-depletion involves reduced mitochondria function [168] e.g. by uncoupling of oxidative phosphorylation [190] or inhibition of the NADH dehydrogenase complex [191].

Similarly, for secondary active transporters of the solute carrier type, as PEPT1, attenuation of the pH- gradient can lead to decreased activity.

Regev et al. [192] hypothesized that P-glycoprotein might be more sensitive to changes in membrane fluidization than other proteins. Most of the surfactants were able to alter membrane fluidity in one way or the other (e.g. TPGS decreased membrane fluidity, Polysorbate 80 increased membrane fluidity) at concentrations which had effects on transport, too. The question arises as to whether changes in membrane fluidity alone can cause inhibition of transporters. Rege et al. [136] studied the effects of benzyl alcohol, a membrane fluidizer, and cholesterol, a membrane rigidizer on rhodamine 123 transport. Benzyl alcohol was shown to increase membrane fluidity but not transport of rhodamine 123 across Caco-2 cell monolayers and cholesterol pretreatment decreased membrane fluidity but did not significantly affect the rhodamine 123 transport. Therefore, alterations in membrane fluidity alone might not be sufficient to elicit changes in transport activity or might just reflect partitioning of surfactants into the membrane.

P-gp has been shown to be a substrate of protein kinase A and C which might change transporter conformation and activity by phosphorylation of certain residues. However, P-gp mutants, where the putative phosphorylation sites have been eliminated, showed normal transport function and generated drug resistance in formerly sensitive cells [193]. Furthermore, Rege et al. [136] showed that the PKC inhibitors staurosporine and chelerythrine chloride were not able to increase rhodamine 123 transport across Caco-2 cell monolayers. Therefore, it was regarded as unlikely that PKA or PKC play an important role in regulation of P-gp activity.

5. CONCLUSION

Excipients have been regarded as pharmacological inert substances formerly. However, the above cited works disclosed interactions between surfactants and polymers with members of at least four different transport protein types (P-gp, MRP, MCT and PEPT1). Though the mechanisms of inhibition have not been fully unravelled yet, it has been shown that transport activity of the mentioned transporters in the intestine can be altered, especially by surfactants, on a functional level.

Nevertheless, more clinical studies would be necessary to determine the in vivo relevance of these findings.

The review includes a row of excipients that seem to be safe with regard to acute and probably long-term toxicity, since these excipients have been used for years in pharmaceutical formulations and have been admitted to use by the responsible governmental bodies.

However, concomitant intake of two or more drug formulations, one containing the modulatory excipient the other a transporter substrate, might alter pharmacokinetics of the drug and may lead to toxicity for drugs with low therapeutic index.

Interestingly, most of the drugs formulated with surfactants (e.g. HIV-Protease inhibitors, Cyclosporine, Paclitaxel) are themselves transporter substrates or inhibitors (especially P-gp substrates), so that one would not normally co-administer them with other P-gp substrates in the first place. It might however be more than a coincidence that the formulations containing the surfactants with modulatory potential turned out most effective in formulation studies with P-gp substrates.

The cited works illustrate the importance of characterizing excipients not only by their physical but also by their biological properties. This would not only contribute to their safe use but could also lead to new ideas in drug delivery.

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Chapter 4

TASTE MASKING OF UNPLEASANT ORAL DRUGS

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ABSTRACT

Since several decade, in the field of the development of oral dosage forms, a lot of attention was focused on the design, the optimization of the processes of manufacture, the choose of the ingredients to improve the bioavailability of the drugs and to ensure in some particular cases a specific release of these ones into the gastrointestinal tract. Besides these progresses, innovations have been also realized in order to provide palatable dosage forms of many drugs – especially bitter and astringent drugs –, thus aiding compliance. To allow this, for solid dosage forms, formulators developed various techniques to mask the bitter taste including the flavoured additives (sweeteners, amino acids and lipids), physical methods (polymers coating, matrix granulation and encapsulation) and chemical methods (formation of inclusion complexes). In this chapter, we review these different techniques and also describe original emulsified dosage forms developed in our laboratory to mask the bitter taste of drugs incorporated into liquid oral dosage forms intended for oral paediatric applications. The e-tongue, an electronic device developed recently to evaluate the taste masking effect of dosage forms and used by developers in order to select the best combination of ingredients which provide "the best" testing formula is also described.

Keywords: *taste masking, drug delivery systems, dosage forms, bitter, and astringent.*

INTRODUCTION

Humans can perceive and distinguish between five components of taste, namely: sourness, saltiness, sweetness, bitterness, and umami (in Japanese).

The bitterness of human pharmaceutical medicines plays a critical role in patient compliance, as the oral administration of bitter drugs is often hampered by their unpleasant taste, leading to noncompliance and thus decreasing therapeutic efficacy, especially in children and the elderly. The quantitative evaluation of the bitterness of medicines is, therefore, an important factor in drug formulation design.

Excessive bitterness of the active pharmaceutical ingredients in oral liquid or suspension formulation, sublingual or buccal formulation is a major taste problem facing pharmaceutical scientists. In the early development stage, bitterness of formulations can have an impact on clinical study design when a double-blinded trial is needed. Later, the bitterness of formulations can influence pharmaceutical selection by physicians and patients and thus affect acceptance and compliance. To inhibit or block the bitterness, both physical and chemical methods have been employed.

Depending on the character of the active substance and the desired dosage form, the methods commonly involved for achieving taste masking include various chemical and physical methods that prevent the drug substance from interaction with taste buds.

Although various physical methods, such as film coating, have been used in attempts to decrease the perception of bitterness of medicines, some drugs are still administered as a syrup or solution, using additives such as sucrose to reduce the bitterness of the formulation. Unfortunately, this method often fails to suppress the bitterness sufficiently.

Use of capsules, polymer coatings, microencapsulation, complexation, taste-masking excipients, inclusion complex with cyclodextrin, use of ion exchange resins, solubility limiting methods, liposomes, multiple emulsions, use of anesthesic agents, etc have been reported.

1. WHAT IS TASTE?

Taste, smell, touch, sight and hearing are the five senses which all humans and animals use to interpret the world around them. Specifically, taste is the sense for determining the flavour of food and other substances. All over the tongue, there are little bumps called taste buds (Figure 1).

Generally speaking, taste is comprised of five basic qualities (Figure 1): sourness produced by hydrogen ions such as hydrochloric acid, acetic acid, and citric acid; saltiness produced mainly by sodium chloride; sweetness produced by sugars; and bitterness produce by quinine, caffeine and magnesium chloride. The last one is umami, which is the Japanese term for “deliciousness”, and is produced by monosodium glutamate contained in seaweeds, disodium inositate in meat and fish and disodium guanylate in mushrooms (Pfaffmann, 1959; Kawamura and Kare, 1987).

Biologically, the sensations of taste in humans occur when molecules trigger signals in the mouth that are sent to the brain, where a specific taste sensation is registered.

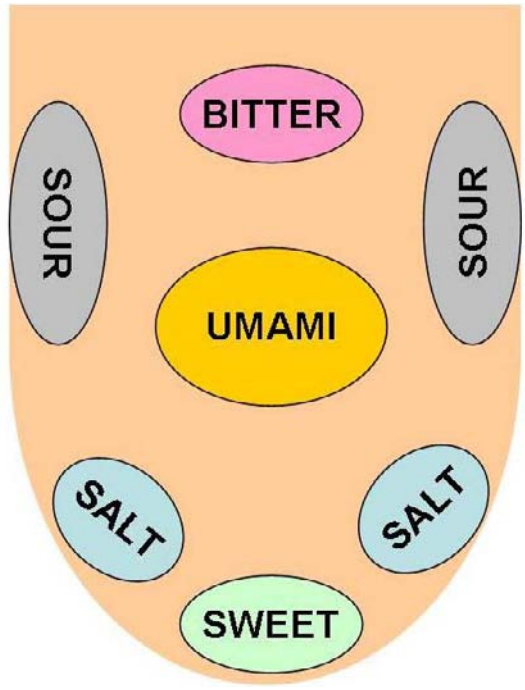

Figure 1. Map of human tongue.

The taste transduction is mediated by specialized neuroepithelial cells, referred to as taste receptor cells, organized into groups of 40-100 cells, which form taste buds. Taste buds are ovoid structures, the vast majority of which are embedded within the epithelium of the tongue. The number of taste buds declines with age. Different taste modalities appear to function by different mechanisms. For example, a salty taste appears to be mediated by sodium ion flux through apical sodium channels (Keast et al., 2001), while a sour taste seems to be mediated via a hydrogen ion blockade of potassium or sodium channels (Kinnamon and Roper, 1988). Sweet and bitter tastes are transduced by G protein-coupled receptors (Kinnamon and Cummings, 1992). To date, more than 80 putative bitter receptors have been identified (Matsunami et al., 2000). Nevertheless, the taste transduction mechanisms are complex and not fully yet elucidated. Moreover, the classical “taste map” is an over simplification. Sensitivity to all tastes is distributed across the whole tongue and indeed to other regions of the mouth where there are taste buds (piglottis, soft palate), but some areas are indeed more responsive to certain tastes than others.

1.1. Taste Perception

The biological definition of taste (gustation) is a chemical reaction derived from sensory responses from the four main taste perceptions: salt, sour, bitter, and sweet. Two other perceptions (umami and trigeminal) should be included when considering taste. Clusters of small organs called taste buds are located in the mouth, mainly on the surface of the tongue. Taste buds (named so because under the microscope they look similar to plant buds) lie in small projections called papillae and contain taste receptors that bind to food molecules down

by saliva. These receptors send messages along nerves to the brain, which interprets the flavour as sweet, sour, salty, or bitter.

Taste drives appetite and protects humans from poisons. So, the taste of sugar is well appreciated because people have an absolute requirement for carbohydrates (sugars etc.). Humans get cravings for salt because they must have sodium chloride (common salt) in their diet. Bitter and sour cause aversive, avoidance reactions because most poisons are bitter and off food goes sour (acidic). Why do medicines all taste bitter? Because they are, in fact, poisons and if the administration of these ones is too much, they will harm the patient. People have an absolute need for protein, and amino acids are the building blocks for proteins, so the taste quality umami which is the meaty, savoury taste drives their appetite for amino acids.

Smell (olfaction) contributes also significantly to the taste of something. This is due to aromas being released into the nasal passages as food is being chewed. The brain interprets the combined signals from both the nasal passages and the taste buds into one taste/flavour response. The human tongue is packed with nerves, and many of these detect subtle differences in the consistency of the food being masticated. Whether the food is creamy, rough, granular, or sticky, the tongue is a master in differentiating those characteristics. A characteristic that is agreeable with one taste/flavour may be unfavourable with a different combination. People perceive flavours in different ways, and this is influenced primarily by the types of foods eaten. People of different ethnicity will have greater or lesser reactions to spicy or sweet foods. Informal taste tests with different flavours have shown that age is also significant in taste preferences. Younger people tend to favour more exciting and dynamic flavours, such as tropical punch, tutti-fruity, and mixed berry. Older generations typically prefer more traditional flavours, such as orange or mint. The indication can also play a part in flavour selection. For example, treatments for indigestion often incorporate a mint flavour, and this has become the flavour that patients expect for this type of medication. Consequently, from one generation of patients to another one generation, changing the flavour to something new may act against the product, even if the effectiveness is the same.

Taste disorders, in which either the sense of taste or smell is impaired, can be the result of allergies and viral or bacterial infections that produce swollen mucus membranes (behind the nose). They also may be due to a brain injury or disease that permanently damages the neural pathway through which taste and smell is transmitted. Lumps or ulcers on the tongue can be also a problem for people of all ages. It is caused by a papillae disorder. In addition, exposure to environmental toxins such as lead, mercury, tobacco, alcohol and insecticides can damage taste buds and sensory cells in the nose or brain.

1.2. Sensorial Psychophysiology

Although there is considerable speculation that early flavour experiences influence later food and flavour preferences in humans, much of the published research, which is not extensive, fails to provide strong evidence for such effects. Moreover, previous studies showed that similarities in food preferences between children and their parents or siblings are often small or non-existent. Studies lead in this field demonstrated that there is an effect of early experience on later preference, involving flavours, not specific foods since experimental animal model studies demonstrate that early experiences with odours, a major component of flavour, in specific nursing-like contexts, results in long-term preferences. Research programs

designed to investigate the long-term effects of early feeding experiences allowed to conclude that there is for example a substantial flavour variation inherent in three classes of commercially available infant formulas: traditional milk-based formulas, formulas based on soy proteins, and those based on hydrolyzed proteins. From these research programs, it emerged that although the flavour of each brand has its own characteristic profile, milk based formulas are often described as having low levels of sweetness and “sour and cereal type” whereas soy-based formulas are described as tasting sweeter, more sour and bitter and having a relatively strong “hay/bean” odour. On the contrary, for adults, the formulas appeared to be extremely unpalatable, having offensive taste and off odour of the hydrolyzed formulas due primarily to its sourness and bitterness, perhaps because many amino acids have a taste sour or bitter, and also due to their unique volatile profiles.

2. Classification of Drugs for Taste Making

Many drugs, both in development (> 40%) and already available in the market, have the problem of being only poorly soluble (< 0,1% w/v) in aqueous media. This can lead to poor bioavailability and frequently results in variable dissolution rates. For these reasons, when it is possible, the formulators prefer to manufacture the dosage forms by using soluble (solubility > 3% w/v) drugs in order to avoid the problems of low bioavailability of drugs. Between these two categories, there are the large majority of drugs which are partially-soluble (0.5-3%) in aqueous media and into the digestive tract media (pH comprised from 1.5 to 7.0), particularly for the ionisable drug depending of the dissolution media. When the drug is poorly water-soluble, the formulators try to use adequate formulations or dosage forms in order to improve this solubility. For these reasons, ingenious devices can be implemented such as use of co-solvents, modification of the pH of the solution, micellar solubilization, complexation, adding of hydrotropic compounds, chemical modification of the drug, co-precipitate, co melting, mixtures with oily or fat excipients, etc…If the bioavailability of the drug could be solved by using some of these techniques, it increases the problem of the taste masking since a soluble drug can lead to a problem of taste making which is not necessary encountered with the same poorly soluble molecular structure. Indeed, the poor solubility of a drug can prevent this one to interact with the receptors located on the mouth and therefore to not give the unpleasant taste of the drug.

3. Dosage Forms for Taste Masking

If the taste of an active pharmaceutical ingredient is not too unpleasant, simply adding a flavour may mask its taste. However, if the drug is especially bitter or foul tasting, as is the case for many antibiotics, analgesics and central nervous system (CNS) drugs, coating of the active ingredient particles or forming other controlled-dissolution dosage forms may be required.

Taste exhibits almost complete adaptation to a stimulus – perception of a substance fades to almost nothing in seconds. For example, taste can be suppressed by local anaesthetics applied to the tongue. Amiloride, a blocker of epithelial Na channels, reduces salt taste in

humans and adenosine monophosphate (AMP) may block the bitterness of several bitter tasting agents.

3.1. Solid Dosage Forms

3.1.1. Granules, Powders

The use of granules for reconstituting as liquids (e.g. sachets, sprinkle capsules and powders) is very often employed for paediatric and geriatric patients. Taste masking of granules is a very important factor in product development and varied technologies and methodologies for this exist.

A granular composition for taste masking comprising of drug core of a non steroidal anti-inflammatory drug (NSAID) and methacrylate ester copolymers as coating agents for taste masking was prepared by Hayward *et al.* (1998). The method comprises of coating the drug cores with separate layers of aqueous dispersions of the copolymers. These granules could be used to obtain the chewable tablets, which had good palatability and bioavailability.

The mannitol and lactose having different weight ratios (1:1.5 – 1:5) are used as coating materials for masking bitter taste of solid drug preparations (Kishimoto *et al.* (1997)).

Another composition comprising of a drug with unpleasant taste of polymer solution and D-crystals of monoglycerides was described by Yajima et al (1997). Eudragit E (100 g) was dissolved in melted stearic acid monoglyceride (600 g) and then Erythromycin (300 g) were added to the mixture to obtain a powder, which was again mixed with sorbitol, magnesium oxide and starch to give taste masked granules of Erythromycin (Yajima *et al.* (1997)).

A novel dosage form was invented by Danielson et al (1999). These one comprised granules containing the histamine receptor antagonist which are provided with taste masking coating comprising a water insoluble, water permeable methacrylate ester copolymer in which the coating is applied to the granules in an amount which provides a taste masking effect for a relatively short period during which the composition is being chewed by a patient but which allows substantially immediate release of the histamine receptor antagonist after the composition has been chewed and ingested (Danielson *et al.* (1999)).

The use of polymer having at least one free carboxyl group and polyvinylpyrrolidone was also described as a mean and another interesing method for manufacturing palatable drug granules (Kumar (2002)).

3.1.2. Fast Disintegrating Drug Delivery

The key of the success of orally disintegrating tablets (ODT) is their good taste. If the product does not taste good, patients and physicians will find another ODT or other product that does taste good. ODT technology is relatively new to the pharmaceutical industry and has had a significant impact on patients of all ages. ODT solid dosage forms dissolve or disintegrate in the oral cavity in a relatively short time (within a matter of seconds when placed upon the tongue) and do not need to be swallowed with water. By using these dosage forms, it’s easier to take the medicines, especially for children and the elderly, who have traditionally difficulties by swallowing more conventional dosage forms. In fact, the introduction of fast dissolving dosage forms solved some of the problems encountered in administration of drugs to the paediatric and elderly patient, which constitutes a large

proportion of the world's population. The single most significant issue with ODT is the bitterness of the drug that can be exposed as the tablet breaks apart. Skilful taste masking is needed to hide this bitterness, and combining this with the right flavour/sweetness levels will result in a superior product. Medicines have always been unique as the only product that consumers took on blind faith. However, consumers have become more knowledgeable and demanding due to many reasons, including direct-to-consumer advertising, medical websites, and the increasing number of medicines available over the counter (OTC). Consequently, consumers now have a greater power of choice than ever before, and as with any other orally consumed product, taste is the deciding factor. In today's competitive world, there are increasing numbers of ODT products targeting the same indications. This means that a pharmaceutical laboratory's product needs to stand out over its competitor's.

Some drugs have relatively no taste, and simply adding a suitable flavour can "hide" any slightly unpleasant sensations. However, most drugs do require taste masking if they are to be incorporated into an ODT formulation. There are many different ways to produce an ODT, including compressed tablets, compression moulded tablets, freeze-dried (lyophilized) wafers, spray drying, coacervation, microencapsulation into pH sensitive acrylic polymers, and mass extrusion. Some of these technologies have led to products that are approved by the Food and Drug Administration (FDA).

The time for an ODT to disintegrate in the oral cavity also varies by product and the method of manufacturing. Compressed tablets will typically take slightly longer to disintegrate than freeze-dried wafers due to a different bonding mechanism and differences in porosity between the two dosage forms. Compression-moulded tablets would be expected to have disintegration times that are similar to compressed tablets. The method used to determine the disintegration time is critical, and the disintegration method stated both in the United States Pharmacopeia (USP30-NF25) and in European Pharmacopeia (EP) 5.0 for conventional "hard" tablets may not be appropriate. The lack of an appropriate disintegration test for ODT products, results in USP or EP method being the only official method available. However, this test is quite aggressive for ODT products and results in disintegration times as short as a few seconds. Several pharmaceutical companies have developed their own internal, more discriminating method to measure disintegration times of these dosage forms. *In vivo* disintegration times will vary tremendously depending on how the patient processes the dosage form. A patient that actively moves the product around the oral cavity with the tongue will experience a faster disintegration time than one who allows the product to disintegrate without any additional encouragement. It is important to note that even though this type of dosage form has inherent patient-to-patient disintegration time variability, an approved ODT must still meet the typical requirements for bioequivalence, independent of disintegration time.

The method of manufacture can influence the quality of the finished product, and this is especially true for lyophilized wafers. Insoluble drugs pose a significant challenge as they can settle out of suspension prior to being poured into the pockets and freeze dried, resulting in content uniformity issues. This dosage form also cannot be placed into the more convenient and cost-effective bottle packaging, unlike the more robust compressed tablets. Drugs have varying levels of bitterness and dosage; therefore, some are more challenging to taste mask than others. It may be acceptable to have a small amount of drug taste present in the final product, but it will depend on the relative merits of the product as to how well the product is received by patients. How the product is packaged will also make a difference to the patient.

If the ODT product has the advantages of being taken anywhere, at anytime, the access to the drug product is clearly important. Most of the ODT products currently on the market are packed into some form of blister package, and depending on how much protection the product needs both from the environment and access by children, some blistered products are easier to access than others. The significant advantage of not needing water to swallow the product can be negated by the need for scissors to open the blister. Being able to package the product into bottled will reduce the manufacturing costs, and may improve ease of access, but this will only be an option for those ODT technologies that are suitable for the rigors of bottle packaging.

3.1.3. Chewable Tablets

The dosage forms which can be produced simply and that the patients can swallow easily maybe important and valuable for the masking of the drug taste. One of the simple way amongst others to achieve such dosage forms is to add appropriate masking agents to powder, liquid or chewable dosage forms. Amongst these ones, a few decades ago, chewing gum was rarely considered when pharmaceutical industry searched for an applicable delivery vehicle for an active pharmaceutical substance. Yet, the 1980s and 1990s saw gum become the most successful nicotine delivery form; unawareness changes to cautious scepticism, and today the merits of chewing gum drug delivery technologies are generally appreciated.

As example to illustrate this, acetaminophen, an antipyretic, has a bitter taste, but is often applied to infants and children due to its safety. Suppository and syrup dosage forms are often used to take the drug more comfortably. Commercial syrups of acetaminophen exhibit fairly strong sweetness and do not suppress the bitterness very much. Chewable dosage form may be useful for improving such problems. Chewable dosage forms to mask bitter taste of acetaminophen were investigated in efforts to obtain oral dosage forms which could be taken easily. Therefore, for acetaminophen, the dosage form easy to swallow with inhibited bitter taste was necessary to achieve good compliance and simple administration way. To improve these matters, acetaminophen-containing chewable tablets using Witepsol® H-15 or cacao butter as a matrix base and some correctors as bitter masking agents were developed (Suzuki *et al.*, 2003). These chewable tablets can be prepared simply, and were considered to be available to patients having trouble in swallowing because they could be chewed. However, these tablets did not necessarily show comfortable oral feeling, which appeared to be mainly due to the stickiness of the hard fat. Therefore, further examination of the formulations was required to improve oral feeling in addition to bitterness suppression. To optimize these formulations, various kinds of hard fats and many sweetening agents were examined to obtain acetaminophen chewable tablets with suppressed bitter taste and improved oral feeling. The dose of acetaminophen per oral administration was 300-500 mg for adults, but it was adjusted to 10mg/kg for infants. As to hard fats, Witocan® was examined in addition to Witepsol®, Witocan® being utilized as special hard fats in the chocolate and confectionery industry. Currently available sweetening agents such as sucrose, xylitol, saccharin, saccharin sodium, aspartame and sucralose, were used as sweetening agents. These compounds show different sweet taste intensities and oral feeling. Xylitol has sweet taste intensity similar to sucrose but gives a brisk feeling orally. Saccharin and saccharin sodium are 500 times as sweet as sucrose, and aspartame and sucralose showed 200 and 600 times as the sweet taste intensity as sucrose, respectively. Sucrose is often utilized as a sweetening agent against drugs with unpleasant tastes or stimuli (Yin *et al.*, 1996). In addition to the above sweetening agents,

commercial bitter-masking powder mixture made from lecithin (Benecoat® BMI-40) (Katsuragi *et al.* 1997) and cocoa powder (Koyama and Kurihara, 1972; Pickenhagen *et al.*, 1975; Aremu *et al.* 1995) were utilized as correctors. Cocoa has been shown possibly useful for suppression of bitter taste of drugs (Popova, 1969; Takano, 2002). Benecoat® BMI-40 is known as a corrector against bitter taste. The corrector system of 1 or 5% (w/w) sucrose plus 5% (w/w) Benecoat BMI-40, or 1% (w/w) sucrose plus 1% (w/w) cocoa powder, or 5% (w/w) sucrose alone gave the best suppression of the bitter taste intensities of acetaminophen-containing Witepsol® H-15 chewable tablets (Suzuki *et al.*, 2003), the obtained tablets being evaluated based on suppression of bitter taste, sweet taste intensity, oral feeling and drug release.

3.1.4. Jellies

Some of the main problems in formulation for infants and children are unsavoury or bitter taste, formulations which are very powdery, and multiple dosages. Powdery medicines in particular often taste unpleasant, even in taste-masked formulations. Recently, a number of jelly products have been developed that aim to improve compliance by aiding swallowing. When the drug is incorporated in the jelly product, swallowing is made easier due to its moderate adhesion and liquidity while drug absorption by the tongue is prevented, and bitterness perception is thereby decreased. The physicochemical characteristics of jellies, such as viscosity, strength, loss of water content have a positive effect on swallowing and on bitterness suppression.

Jellies have been first envisaged in order to suppress the bitterness of dry commercially available syrups containing the macrolides clarithromycin (CAM) or azithromycin (AZM). The bitterness intensities of mixtures of the dry syrups and acidic jellies were significantly greater than those of water suspensions of the dry syrups in human gustatory sensation tests. On the other hand, the mixture with a chocolate jelly, which has a neutral pH, was less bitter than water suspensions of the dry syrups. The bitterness intensities predicted by the taste sensor output values correlated well with the observed bitterness intensities in human gustatory sensation tests. When the concentrations of CAM and AZM in solutions extracted from physical mixtures of dry syrup and jelly were determined by high performance liquid chromatography (HPLC), concentrations in the solutions extracted from mixtures with acidic jellies were higher than those from mixtures with a neutral jelly (almost 90 times higher for CAM, and almost 7 to 10 times higher for AZM). Thus, bitterness suppression was correlated with the pH of the jelly. Finally a drug dissolution test for dry syrup with and without jelly was performed using the paddle method. There was no significance difference in dissolution profile. It was concluded that the appropriate choice of jelly with the right pH is essential for taste masking. Suitable jellies might be used to improve patient compliance, especially in children. The taste sensor may be used to predict the bitterness-suppressing effect of the jelly.

3.2. Liquid Formulations

A lot of drugs have an unpleasant taste and in particular when they are administered under a liquid dosage form which is well appropriated for the administration of drugs to kids or old people who can encounter some swallowing problems with solid dosage forms like tablets, capsules, … For this reason, pharmaceutical liquid and fluid dosage forms like syrups,

potions, emulsions and suspensions are preferred. Nevertheless, the administration of such drugs having an unpleasant taste by using these dosage forms proves to be difficult and lead to a decrease of the compliance of such dosage forms in particular for the categories of people mentioned above.

3.2.1. Solutions, Liquid Suspensions, Syrups

The majority of paediatric preparations are formulated as syrups and suspensions although, the aforementioned methodologies have also been used for improving liquid taste and few patents in this area are worth mentioning.

The bitter taste of vitamin B1 derivatives such as dicethimine, was masked by formulating oral liquids with menthol, polyoxyethylene and /or polyoxypropylene (Nakona *et al.* (1999)).

To manufacture oral liquids containing diclofenac and its salts, Osugi et al. (1999) suggested to use a mixture of glycine, glycyrrhizin acid or salt thereof, in order to mask the bitter taste and to prevent the irritation of the throat upon oral administration.

The use of prolamine (class of simple proteins having a high proline content and found in the seeds of wheat, rye, maize, and barley …) for the application as single coating in weight ratio 5% to 100% relative to active substance being coated result in the production of a liquid suspension which effectively masked the taste of orally administered drugs which are extremely bitter. Prolamine coating does not restrict the immediate bioavailability of the active substance and is effective in masking the taste of antibiotics, vitamins, dietary fibres, analgesic, enzymes and hormones.

Pharmaceutical composition comprising polyhydric alcohol based carrier was reported by Swaminathan *et al.* (1997) to mask the bitter taste of a drug like cimetidine formulated into a liquid dosage form containing talin, peppermint oil and glycerol.

A liquid suspension of microcapsules for taste masking was invented by (Morella *et al.* (2001). This one allowed to taste mask a bitter drug as a function of a polymer coating and the pH of suspended medium at which pharmaceutically active ingredients remain substantially insoluble.

The another liquid composition comprising a pharmaceutically active medicament coated with a taste masking effective amount of polymer blend of dimethylaminoethylmethacrylate and neutral methacrylic acid ester and a cellulose ester in an aqueous vehicle was invented by Yu *et al.* (2002). The liquid composition utilizes a reverse enteric coating, which is soluble in acid pH of the stomach generally about 1 to 4 but relatively insoluble at the non-acidic pH of the mouth. The coating provides the rapid release and absorption of the drug, which is generally desirable in the case of liquid dosage forms.

When pharmaceutical compositions comprised of ingredients with an unpleasant taste are formulated, it is important not only to mask the taste by an appropriate pharmaceutical technology but also to evaluate the degree of improvement in taste. The surest method to evaluate taste is sensory testing with human volunteers. However, since the sensory test is difficult to perform, a simple alternative method is required. *In vitro* release tests, such as the paddle method in the Japanese Pharmacopoeia, measurement of the amount of release in a centrifugal tube with shaking or in an injector with continuous inversion and other tests, are often substituted for sensory tests as surrogate methods of evaluation. Clarithromycin (CAM), a macrolide antibiotic, has a strong bitter taste. The effect of CAM concentration on the bitterness was studied by Koyama et al. (1990) who reported the threshold of bitterness of

CAM solution is 14 mg/l, the concentration at which half of human volunteers recognize bitterness. To mask the bitterness for the commercial CAM paediatric dosage form (dry syrup), several pharmaceutical technologies are used (Yajima, 1996). Manufacturing conditions also affect the degree of bitterness of CAM dry syrup (Yajima, 1999). Since that dosage form consists of matrix comprised of CAM, the elevation of temperature during the drying process enhances the bitterness. In the first stage, the paddle method was used to evaluate the release rate of each batch manufactured under various conditions. No correlation was found between the amount of release and the degree of bitterness, however. In this dosage form, as the amount of release of CAM from dry syrup increases, the bitter taste increases. Thus, perfectly taste-masked batches do not always exhibit a low release pattern. For this reason, a new method for the evaluation of bitterness of CAM dry syrup using a mini-column technique was examined and optimum measurement conditions were defined. The threshold of bitterness of CAM from dry syrup was also obtained using this method. In the case of CAM paediatric dosage form, it is not enough to put a good coating on the drug particle. For suspension formulations, there are problems which must be solved and each requires quite different considerations. Firstly, the suspension must have an acceptable taste after storage, usually from two weeks to six months. Secondly, only a small amount of the drug must be released from particles which are trapped in the mouth. Thirdly, the drug must be released within the desired period of time in the proper part of the digestive tract after swallowing. Failure to solve any one of these problems will produce an unacceptable suspension.

3.2.2. Emulsions, Microemulsions

The emulsions and microemulsions deliver one or more actives in the same dosage form at predetermined rate(s) to enhance disease management and patient compliance.

Taste masking of therapeutic agents is also inherent with the liquid emulsion and microemulsion drug delivery technology, and the ease of oral administration making the system ideal for the geriatric and paediatric markets.

An emulsion is a dispersion of two or more immiscible liquids stabilised by a surfactant or emulsifier coating the droplets and preventing coalescence by reducing interfacial tension or creating a physical repulsion between the droplets. Two common types of emulsions are found in oral drug delivery systems. Water-in-oil emulsions (W/O) and oil-in-water (O/W) or lipid emulsions.

Microemulsions are clear, stable, isotropic mixtures of oil, water and surfactant, frequently in combination with a co-surfactant. These systems are currently of interest to the pharmaceutical scientist because of their considerable potential to act as drug delivery vehicles by incorporating a wide range of drug molecules and for their stability.

Microemulsions are defined as thermodynamically stable dispersions of two non miscible liquids and stabilized by surfactants. They can be obtained at room temperature when all the constituents are mixed together, which is particularly interesting for the thermolabile drugs. Generally, the microemulsions appear limpid since the size of the internal phase is comprised between 100 and 200 nm. The microemulsions are constituted of four major constituents, namely (i) an aqueous phase, (ii) an oily phase, (iii) a surfactant and (iiii) a co-surfactant.

In our laboratory, we designed the liquid water-in-oil (W/O) microemulsions intended for the oral administration of ranitidine hydrochloride. The unpleasant taste of the drug can be masked by incorporating this active compound in the internal phase of a W/O

microemulsion. The direct contact of the drug dissolved in the aqueous phase with the taste buds and the receptors whose are present on the tongue are prevented by the lipid external phase which is able to cover these ones. In order to design these liquid water-in-oil microemulsions, studies have been focused on the stability and on the taste of the excipients used to formulate this new dosage form.

To optimize this choice the different excipients has to be done in order to obtain a stable and limpid W/O microemulsion having a pleasant taste.

The Oily Phase

Different oily phases (Montane® 85, 65 and 20, Labrafil® M 1944CS and Montanox® 85) having various hydrophilic/lipophilic balances have been studied. Several W/O microemulsions have been formulated and had the following compositions : 10 ml of aqueous phase buffered at pH 6.9 containing 1000 mg of ranitidine hydrochloride, the preservative agents and the sweetener, 32.27 ml of an oily phase containing also the antioxidizing agents, 37.05 ml of Montanox® 80 as the surfactant and 16.67 ml of propylene glycol as the co-surfactant.

The Surfactants

Several surfactants (Montanox® 65, 80, 40 and 20) having different hydrophilic/lipophilic balances were studied. Several W/O microemulsions have been formulated and had the following compositions : 10 ml of aqueous phase buffered at pH 6.9 containing 1000 mg of ranitidine hydrochloride, the preservative agents and the sweetener, 32.27 ml of Labrafil® M 1944CS containing also the antioxidizing agents, 37.05 ml of the surfactant and 16.67 ml of propylene glycol as the cosurfactant.

The Co-Surfactants

The determination of the domains of the W/O microemulsions were established by the drawing of a ternary diagram for which the ratio Surfactant/Co-surfactant has been determined (figure 3-11). Three ratios (1, 10 and 50) and three co-surfactants (glycerol, propylene glycol, Transcutol® P) have been studied.

The Sweeteners

Different sweeteners (saccharose, sodium saccharinate, saccharine, neohesperidine dihydrochalcone (NHDC) and sodium cyclamate) having various sweetening powers have been tested.

The Determination of the Direction of the Microemulsions

The determination of the direction of the microemulsions was realized by the measure of the conduction of the electrical current, by using hydrophilic colouring like methylene blue or lipophilic colouring like soudan red and, by the method of dilution. The water-in-oil microemulsions were manufactured by making use of different steps as shown in figure 2.

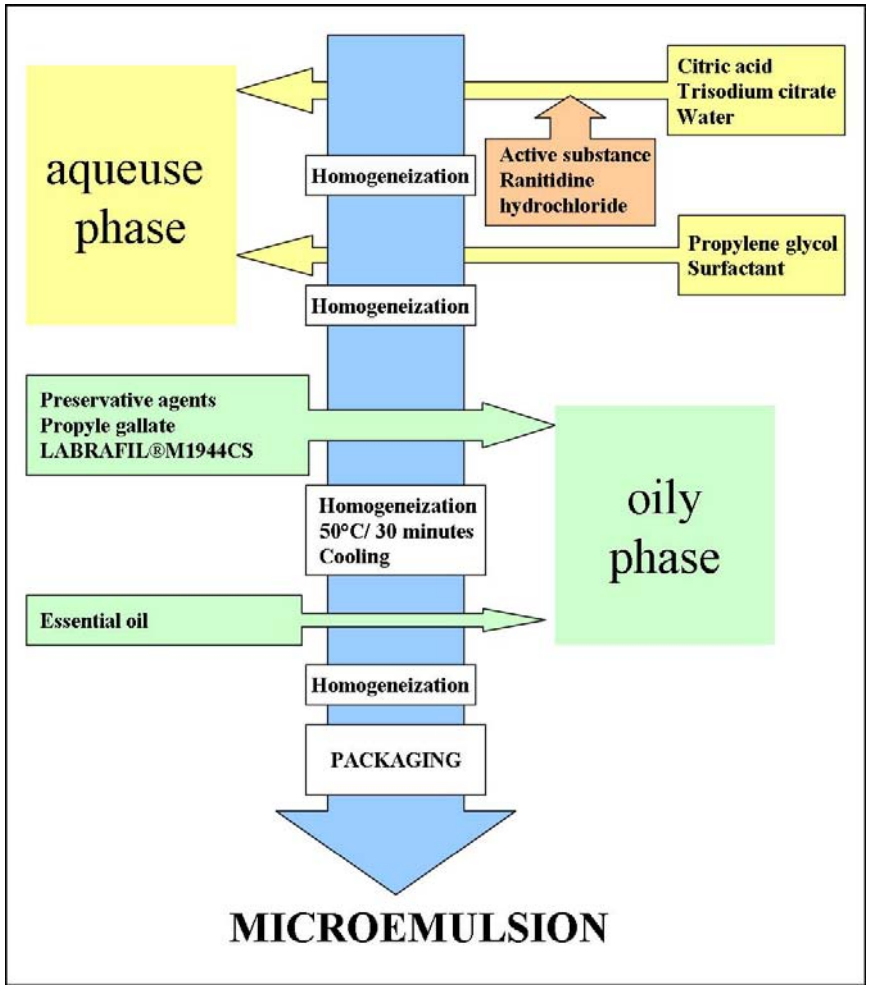

Figure 2. Manufacture scheme of W/O microemulsion containing ranitidine hydrochloride.

The process of the manufacture of the W/O microemulsions takes into account the physicochemical parameters of the different excipients (figure 2). The quality checks of the dosage form comprise the determinations of the viscosity of the microemulsions, of the size of the droplets constituting the internal phase of the dispersed system, the uniformity of the amount delivered and the quantitation of the drug and the preservative agents (figure 2).

The Oily Phase

The obtained results depending of the different oily phases are presented in table 1.

From these trials, it was concluded that the ideal constituents of the oily phase must have an hydrophilic/lipophilic balance comprised between 4 and 11 in order to obtain an optimal stability. Concerning the use of the more appropriate surfactants, the obtained results are presented in table 2.

Table 1. Results of stabilities of the different tested W/O microemulsions by using different oily phases, depending of different hydrophilic/lipophilic balances (HLB)

	Oily phase	Results
1st trial	Montane® 85 (HLB=1.8)	unstable
2 nd trial	Montane® 65 (HLB=2.1)	unstable
3 rd trial	Labrafil® M 1944CS (HLB=4)	stable
4 th trial	Plurol oleique CC (HLB=6)	stable
5 th trial	Montane® 20 (HLB=8.6)	stable
6 th trial	Montanox® 85 (HLB=11)	stable

Table 2. Results of stabilities of the different tested W/O microemulsions by using different surfactants having different HLB

	Surfactants	Results
1st trial	Montanox® 65 (HLB=10.5)	stable
2 nd trial	Montanox® 80 (HLB=15)	stable
3 rd trial	Montanox® 40 (HLB=15.6)	stable
4 th trial	Montanox® 20 (HLB=16.7)	unstable

From these trials, it was concluded that the ideal surfactants must have a hydrophilic/lipophilic balance comprised between 10.5 and 15.6 in order to obtain an optimal stability. The importance of the area of the domains of W/O microemulsions was different by using the three different co-surfactants mentioned below and is the following (figures 3-13):

Glycerol < Propylene Glycol < Transcutol® P.

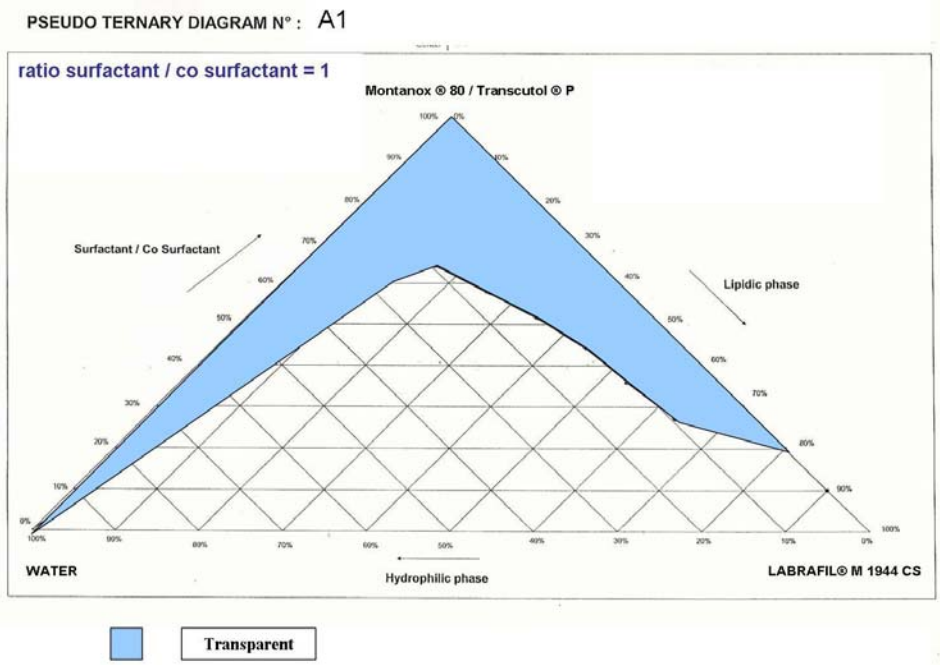

Figure 3. Pseudo ternary diagram of microemulsion containing Water/ Labrafil® M 1944CS/ Montanox® 80/ Transcutol® P with the ratio of S/CoS = 1.

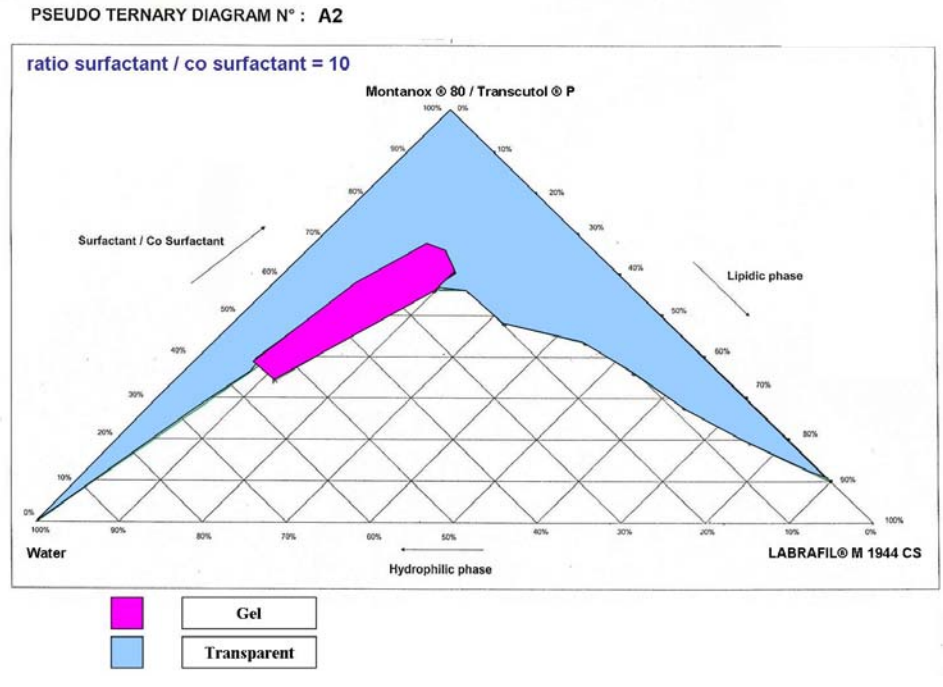

Figure 4. Pseudo ternary diagram of microemulsion containing Water/ Labrafil® M 1944CS/ Montanox® 80/ Transcutol® P with the ratio of S/CoS = 10.

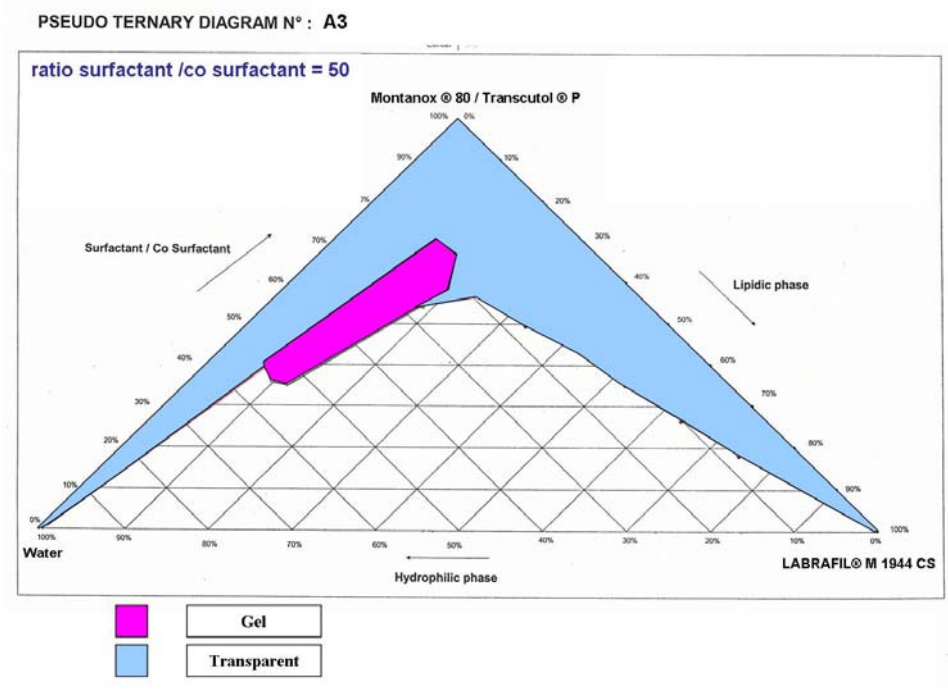

Figure 5. Pseudo ternary diagram of microemulsion containing Water/ Labrafil® M 1944CS/ Montanox® 80/ Transcutol® P with the ratio of S/CoS = 50.

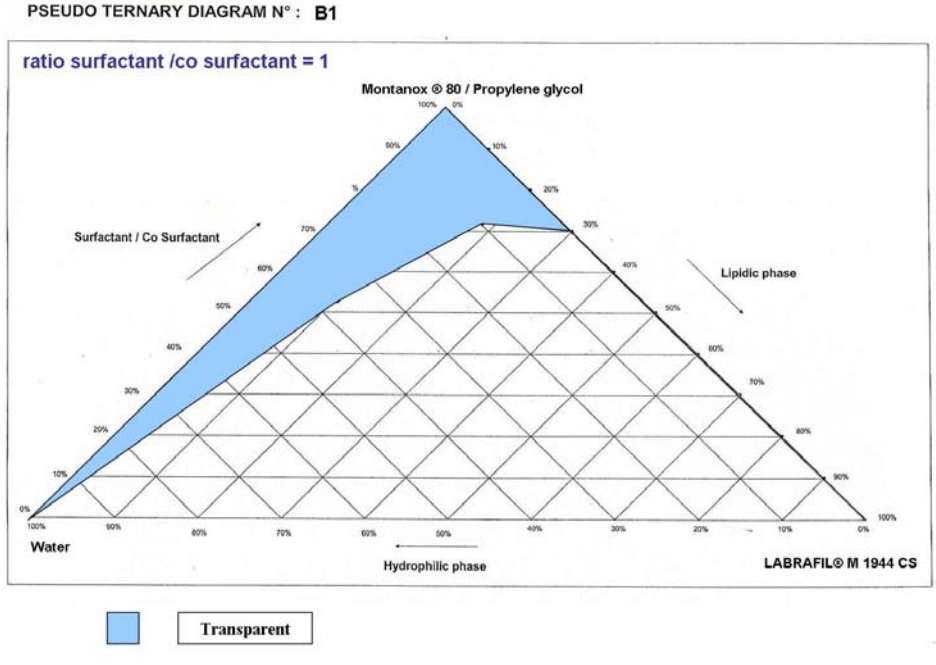

Figure 6. Pseudo ternary diagram of microemulsion containing Water/ Labrafil® M 1944CS/ Montanox® 80/ Propylene glycol with the ratio of S/CoS = 1.

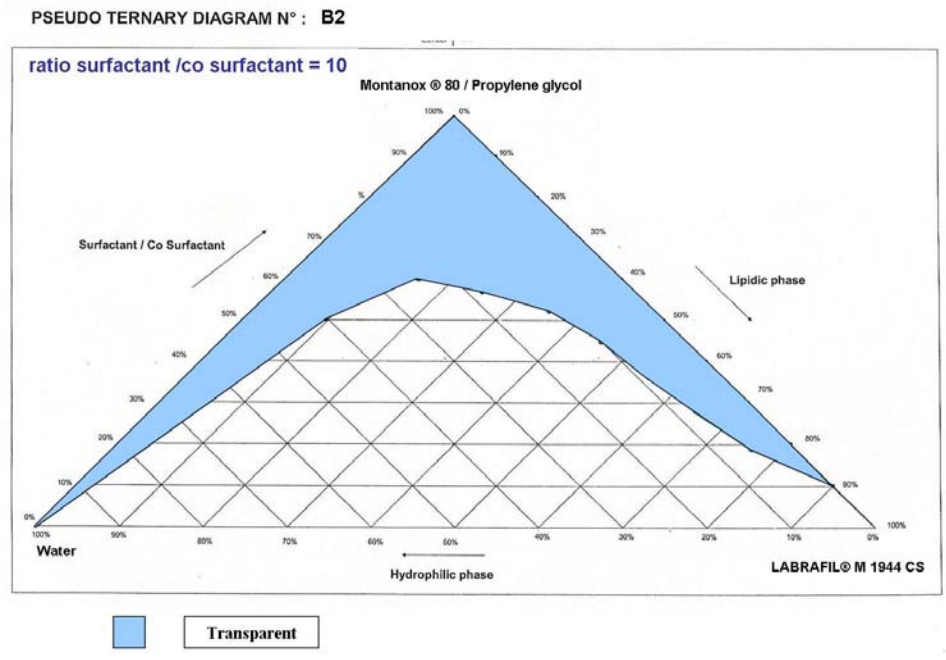

Figure 7. Pseudo ternary diagram of microemulsion containing Water/ Labrafil® M 1944CS/ Montanox® 80/ Propylene glycol with the ratio of S/CoS = 10.

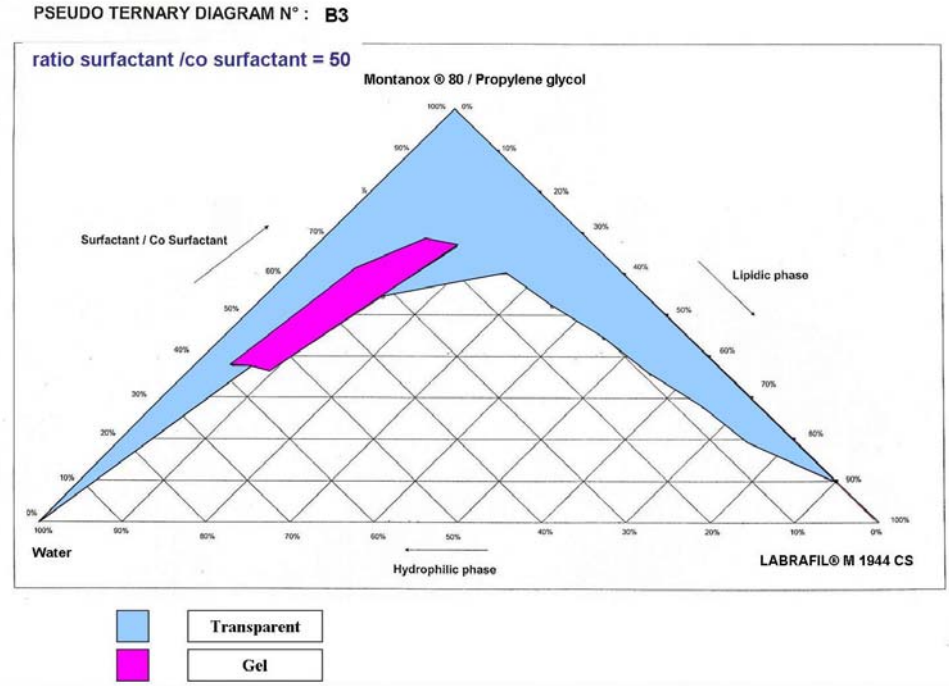

Figure 8. Pseudo ternary diagram of microemulsion containing Water/ Labrafil® M 1944CS/ Montanox® 80/ Propylene glycol with the ratio of S/CoS = 50.

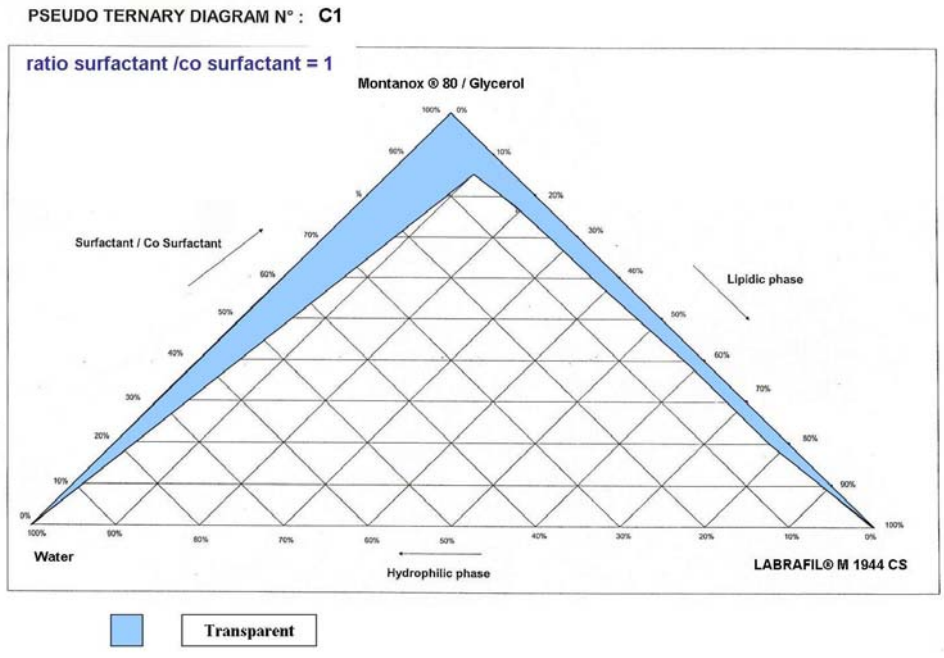

Figure 9. Pseudo ternary diagram of microemulsion containing Water/ Labrafil® M 1944CS/ Montanox® 80/ Glycerol with the ratio of S/CoS = 1.

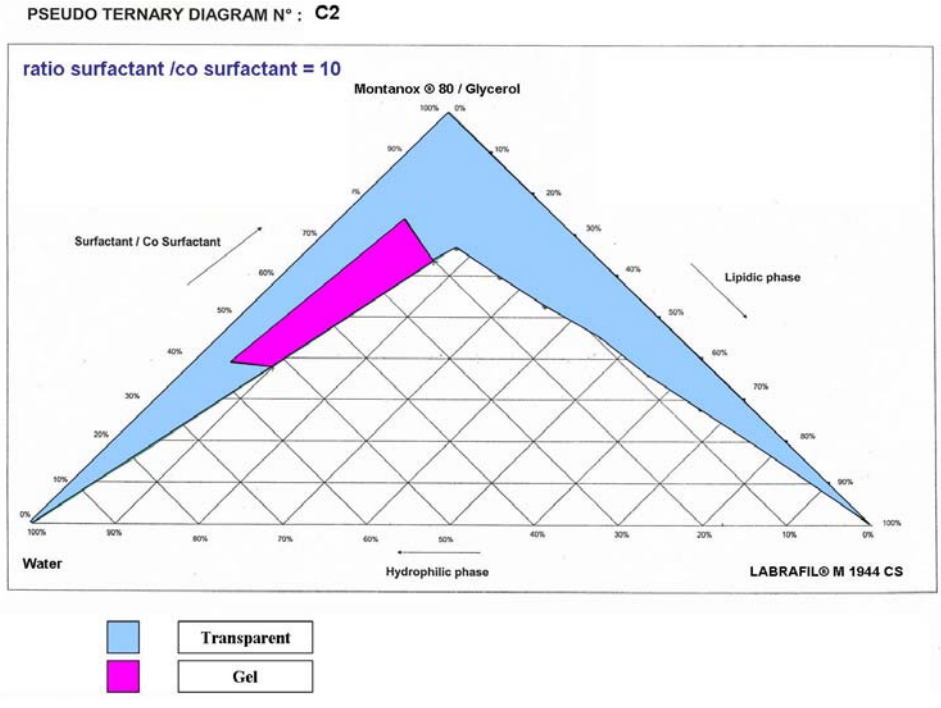

Figure 10. Pseudo ternary diagram of microemulsion containing Water/ Labrafil® M 1944CS/ Montanox® 80/ Glycerol with the ratio of S/CoS = 10.

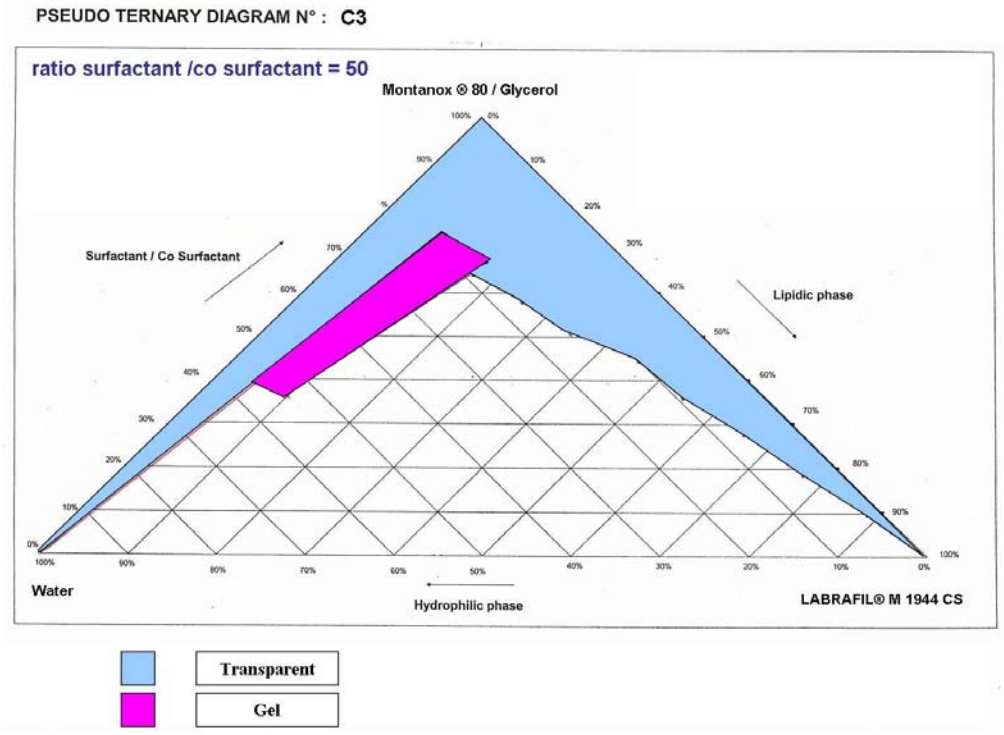

Figure 11. Pseudo ternary diagram of microemulsion containing Water/ Labrafil® M 1944CS/ Montanox® 80/ Glycerol with the ratio of S/CoS = 50.

The influence of the choice of the sweeteners is presented in table 3. From these trials, it was concluded that only sodium cyclamate can be used in order to design a stable and pleasant microemulsion dosage form.

Table 3. Results of stabilities of the different tested W/O microemulsions by using different sweeteners and their taste

	Sweetening power/saccharose	Physical stability of dosage form	Taste
Saccharose	Low	unstable	seamark
Sodium saccharinate	300 times more	unstable	pleasant
Saccharine	500 times more	Stable but degradation of the drug	pleasant
NHDC	1500-1800 times more	unstable	pleasant
Sodium cyclamate	50 times more	stable	pleasant

Table 4. Different ratios of the surfactants and the co-surfactants used to construct the ternary diagrams of the W/O microemulsions

Ternary diagram	Surfactant/co-surfactant	Ratio S/Co-S
A1	Montanox® 80 / Transcutol® P	1
A2	Montanox® 80 / Transcutol® P	10
A3	Montanox® 80 / Transcutol® P	50
B1	Montanox® 80 / Propylene glycol	1
B2	Montanox® 80 / Propylene glycol	10
B3	Montanox® 80 / Propylene glycol	50
C1	Montanox® 80 / Glycerol	1
C2	Montanox® 80 / Glycerol	10
C3	Montanox® 80 / Glycerol	50

The studies of stability of the W/O microemulsions were realized at three different temperatures (8°C, 25°C and 40°C) and allow to conclude that the W/O microemulsions were stable at 8°C and 25 °C and unstable at 40 °C.

The sizes of the internal phases of the different tested W/O microemulsions were determined by using a Malvern Mastersizer 2000 and allow to conclude that the mean size of the internal phase of the W/O microemulsions constituted of Montanox® 80, Labrafil® M 1944CS, Transcutol® P were of 276± 8 nm.

From these studies, it was concluded that a stable and pleasant W/O microemulsions containing ranitidine hydrochloride, can be manufactured to mask the bitter taste of this active compound.

The best formulation was constituted of: 1000 mg of ranitidine hydrochloride, 10.295 g of citric acid/trisodium citrate buffer, 17.10 g of propylene glycol, 30.39 g of Labrafil® M 1944 CS, 40.00 g of Montanox® 80, 0.400 g of sodium cyclamate, 3.20 g of essential oil of lemon.

4. STRATEGIES FOR TASTE MASKING

4.1. Use of Flavour Enhancers

There is a wide structural diversity in chemicals that elicit sweet taste, e.g. glucose, fructose (carbohydrates), sorbitol (sugar alcohol), saccharin, acesulfame-K (*N*-sulfonamides), cyclamate (sulfamate), aspartame (dipeptide), D-phenylalanine (amino acid), thaumatin, monellin (proteins), stevioside (diterpenoid glycoside), and lead and beryllium salts (ions).

To obtain flavouring and perfuming agents, natural or synthetic sources can be used. The family of natural products include: fruit juices, aromatic oils such as peppermint and lemon oils, herbs, spices and distilled fractions of these ones. They exist as concentrated extracts, alcoholic or aqueous solutions, syrups or spirit. Another family of effective taste masking agents includes: alkaline earth oxide, alkaline earth hydroxide, an alkaline hydroxide and other compositions like phosphorylated amino acid such as phosphotyrosine, phosphoserine, and phosphothreonine and mixtures thereof.

Concerning the essential oils, anethole can effectively mask a bitter taste as well as the aftertaste. For formulations which are intented to be chewed or dissolve in mouth prior to ingestion in solution the unpalatable drug can by masked by using clove oil and calcium carbonate.

4.2. Coating Methods

Suitable polymers or lipids used as coating agents, offer an excellent method of concealing the drug from the taste buds. The coated composition may be incorporated into much number of pharmaceutical formulations, including chewable tablets, effervescent tablets, powders, and liquid dispersions.

Microcapsules containing cefuroxime axetil were prepared with various cellulosic polymers having a pH dependent solubility with the final aim to mask its taste while assuring its release in the intestinal cavity (Cuna *et al.* (1997)). The drug release studies and the stability assay of the encapsulated moiety demonstrated that microspheres manufactured with cellulosic polymers represent a useful approach to achieve the proposed objectives.

The low melting point substances can be used for masking bitter taste of the drug (Kato *et al.* (1996)). Beef tallow (a low melting point substance) was mixed with micropulverized active ingredients (e.g. antiulcer methyl benactyzium bromide) and the mixture was nozzle sprayed to form coated spheres having homogeneous particle size.

A special study was conducted by Maccari *et al.* (1990) to assess the bioavailability of a flucoxacillin preparation microencapsulated for taste abatement with 17% ethyl cellulose made up as a granular product for extemporaneous resuspension compared to commercially available Flucoxacillin preparations. Both dosage forms were bioequivalent proving that Flucoxacillin microencapsulated for taste abatement is as available from the dosage form as the raw unprocessed antibiotics.

A novel method of taste masking using a spray congealing technique was developped by Yajima et al. (1996). The spray congealing technique, which uses a spray dryer, is an effective method of taste masking because this method is cost effective and requires no solvent and it can produce a more dense film than other methods without moving materials for drying. Also, since this method is easy to industrialize, many attempts using this technique have been undertaken. They reported spherical matrices containing Clarithromycin (a macrolide antibiotic), amino alkyl-methacrylate, and glyceryl monostearate as the ingredients, the objective being the prevention of drug release in the mouth while ensuring a rapid release in the gastrointestinal tract (GIT).

Microparticles of Imdeloxazine (a bitter tasting drug) were prepared and coated with a mixture comprising of hydrogenated oil and surfactants in a fluidized bed using side spray

method (Hiroya Sugao *et al.* (1998)). Drug release from the coated particles was significantly delayed which was overcome by heat treatment.

In order to mask the bitter taste of drugs, a novel microencapsulation process combined with wet spherical agglomeration technique by using modified phase separation method , was described by Udea *et al.* (1993) whereas Yekta Ozer and Atilla Hincal (1993), microencapsulated Beclamide by a simple coacervation method using gelatin.

To ensure that the coating remains intact when in the mouth, but then releases in the appropriate region of the gastrointestinal tract, formulators experiment with an array of polymers and lipids. The materials that are usually used are polymers that are pH-dependent in terms of release. One of the most widely used is Eudragit®, which is an acrylate derivative. Also, cellulose derivatives can be used as well. Examples of pH-dependent polymers used for coating bitter actives are methylmethacrylate, ethyl cellulose and ethylene propylene oxide. When exposed to an acidic medium, they dissolve. So, once the product reaches the stomach, it will release the active drug. Some lipids, such as hydrogenated vegetable oil, are also effective coating materials because of their hydrophobic nature. On the market, for example, Ceftin® (cefuroxime) is manufactured by using lipid coating such as stearic acid for taste masking. Also a melt coating with lipids can be used rather than a straight coating. The technological difference is that the material is melt rather than dissolved in a solvent that should be evaporated. Choose of a lipid with the right melting point is the key parameter. Because of the fact that the active drug cannot be exposed to very high temperatures, lipids or mixtures of lipids having a relatively low melting point has to be selected. However, these ones cannot to be too low since they could melt in the bottle or in the box. So, to avoid this, usually lipids having a melting point of around 60°C are chosen. Also, in the case of lipids, the release is mostly intestinal because lipids won't dissolve in the stomach as fast. Furthermore, several variables come into play when determining what type of coating material has to be used. There are a lot of important things to consider – the particle size, the solubility, of the active substance, and the melting point. All of that will determine what process and what coating material will be selected. The most critical factors to consider are chemical compatibility, the effects of processing, and bioavailability. Chemical compatibility is the first parameter which has to be taken into account, and the others are the processing requirements as for example, high temperatures that the drug can't tolerate. Most excipients used for coating are relatively inert, so they are not highly reactive. But precautions have to be taken to be careful about incompatibility, and the processing methodology has to be adjusted to be as benign as possible toward the active. Also, care has to be brought of how the drug will be released. Indeed, each coating material has its advantages and disadvantages, depending on the application. The polymers are more diverse, so the options are more numerous with them. The polymer coatings are more advanced, there are many more options than for the uses of lipids, and the manufacturing can be realized at lower processing temperatures.

The plasticity of most polymers is particularly important for formulators to consider when they are developing compressed (as opposed to lyophilized) dosage forms. With microencapsulation using fluid-bed systems as the easiest technology to make microspheres, the coating of the active particles can be damaged if the compaction of these microspheres has been considered to make tablets. So, coating fracture is one of the factors that should be considered. With careful manipulation of the formulation and the tabletting process, it is possible to solve a lot of problems of that sort.

On the other hand, a lipid coating has a better mouth-feel because it doesn't have the plasticity that the polymer coating materials have. Meltable materials such as hydrogenated vegetable oils, vegetable waxes or saturated fatty acids such as stearic acid can be utilised as either distinct coatings over the drug particles or as matrix materials containing dispersed drug particles. The matrix particles are the simplest and least expensive particles for taste-making drugs of low to moderate solubility where the dosage is fairly low. The use of stearic acid or stearyl alcohol can allow the incorporation of moderate amounts of ethyl cellulose for improving barrier properties while still providing flow properties necessary for processing.

Granulation or co-granulation using a spray dryer, a high-shear granulator, or other co-processing systems is another approach to taste masking, albeit a less effective one, especially for very bitter actives. The substance active is co-granulated with a certain excipient system, which forms sort of a coating with the active substance. But it's not the most efficient technique, because it still could leave the substance active on the surface and the patient could taste it. This technology is used sometimes with fairly mild substance active. In such cases, formulators typically use lipids to coat the active substance. Basically, the lipids are melted and the drugs are added, and then granulate together. Co-precipitation with polymers is a possibility, but it's not the preferred coating method because it involves dissolving the polymer and the active together in a solvent, and then evaporating it. Also, it would be risky in terms of changing the physical properties of the active.

Fluid-bed apparatus can be used to apply barrier coatings, either as organic solutions or in aquous suspension. The active ingredient particles, which should generally be above 100 microns in diameter for this process, are fluidised in an upward flow of air. Other equipment fluidises the bed of particles by physically putting them in motion by a spinning rotor. Coating solutions or suspensions are atomised into the fluidised bed of particles. Since the coating is applied slowly, in many small bursts as the particles move through the spary, this technique can provide the most uniform coating on irregularly shaped particles.

Spinning-disk processes can provide the least expensive approach to taste-masking small particles of drug. The product can be a matrix particle formed from a meltable material containing five to forty per cent of drug. The suspension is simply atomised and chilled to form the protected particles. If possible, a narrow size distribution should be formed to reduce premature dissolution from the fine particles. If the solubility of the active ingredient is reasonably low, this may be a good approach for taste-masking.

For more difficult taste-masking cases, where a definite protective coating is needed, roundish granules of the drug are formed and suspended in the melted of dissolved coating. When this suspension is metered to a specially designed rotary atomiser at a preferred speed, single coated particles are formed, along with finer particles of excess coating which are then separated. This process can easily coat the finest particles needed for taste masking.

4.3. Complexation with Ion Exchange Resins

Pharmaceutically-approved ion-exchange resins that can react with ionised active ingredient molecules to reduce objectionable taste by forming a less soluble drug-resin complex are available. The complex should be unstable in stomach acid to provide release of the active ingredient. Such complexes can be used in suspension or tablet formulations.

Besides other techniques described above, ion exchange resination is another effective approach to taste-masking bitter active substance. By combining the active with a compatible resin, formulators create a chemical complex called a drug resinate. In this approach, the entrapping of the drug is based on bonds that don't really change the chemistry of the drug said. It just creates relatively weak bonds that break based on pH or other triggers.

Not all active substances are suitable candidates for ion exchange resination, however. The primary requirement for using ion exchange resins is that the drug needs to be ionisable. If the drug is ionisable, then there is a reasonable chance that ion exchange resins will do the trick. One of the very useful things about ion exchange resins is that when they form these complexes with the drug, they are almost always reversible. It just takes copeting ions, such as chloride found in the gastrointestinal tract to displace the drug back off the resin and right into the solution where it can then get absorbed.

As with microencapsulation, choosing an appropriate co-processing material is key. The real trick for taste masking is to find the right resin that gives a slow enough release so that the patient doesn't taste it, but a fast enough release that once it hits the stomach it can lead to the kind release profile. So, there is really two aspects to it. There is not only the taste masking, but there is also the problem of the release profile. These aspects really govern the choice of resins that one tends to use. The manufacturing process for ion exchange resination is relatively straightforward. The process for making the complex is really very simple for many drugs, because the loading of the drug onto the resin is thermodynamically favourable. In most cases, the drug is dissolved in water and is mixed with the ion exchange resin. Because the resin is completely insoluble, the mixing can be variable, maybe one hour, maybe six hours, depending on the physicochemical characteristics of the drug. Then, the resin has to be filtered off, washed with water, dried it in a vacuum tray dryer or in a tumble dryer in order to obtain the resinate. Formulators also may spray-dry the slurry instead of filtering it. Unlike microencapsulation, which coats the exterior of active substance particles, ion exchange resination causes the drug to penetrate and saturate the resin substrate, essentially creating a homogeneous particle. It is an ionic bond, because the ion exchange resins are either acids or bases. So, if the drug has a basic behaviour, this one can be loaded onto an acidic ion exchange resin – what is called a cation exchange resin. This technology is especially useful for developing compressed orally disintegrating tablets (ODTs). Even if the particle is broken during tabletting, it wouldn't make any difference. It would be like breaking a crystal.

The adsorption of bitter drugs onto synthetic ion exchange resins to achieve taste coverage has been well documented. The high potency adsorbates of methapyrilene, dextromethorphan, ephedrine, pseudoephedrine, were prepared by Borodkin et al. (1971), by column procedures using a polymethacrylic acid ion exchange resin. Taste evaluation of the adsorbates showed a significant reduction in the bitterness of the drugs. Coating the adsorbate particle with 4:1 ethylcellulose – hydroxypropylmethylcellulose mixture reduced the bitterness further. Taste coverage was maintained after incorporation of the coated adsorbate into chewable tablets. Strong acid cation resins (sulfonated styrenedivinylbenzene copolymer product) can be used for masking the taste of basic drugs (Roy, 1994). Polystyrene matrix cation exchange resins have been used to mask the bitter taste of chlorpheniramine maleate, ephedrine hydrochloride, and diphenhydramine hydrochloride (Manke, 1991). Extreme bitterness of quinolones has been achived by ion exchange resin such as methacrylic acid polymer crosslinked with divinylbenzene (Gao, 2003).

4.4. Inclusion Complex Formation with Cyclodextrins

Amongst the different techniques described in this chapter to taste mask, cyclodextrin is the most widely used complexing agent for inclusion complex formation which is capable of masking the bitter taste of the drug either by decreasing its solubility on digestion or decreasing the amount of drug particles exposed to taste buds there by reducing its perception of bitter taste. Due to its low water solubility (18.5 g/L at 25°C), β-cyclodextrin is the preferred compound in this family, for this application. For example, bitter taste of ibuprofen and gymnima sylvestre has been effectively masked by cyclodextrin (Motola1991 and Ueno 1992).

Cyclodextrin complexation is similar to ion exchange resination in terms of relying on temporary molecular bonds to mask bitter actives. Cyclodextrin acts like a bucket. The active substance gets into the bucket, but it doesn't chemically react. It just stays bonded because of strong affinity. It is not a new chemical entity. Cyclodextrins are an unusual set of molecules in that the cyclodextrin molecule itself is a ring. The outer surface of the ring is relatively hydrophilic, whereas the inner surface of that ring is actually hydrophobic. So, if the dosage form contains hydrophobic drug, the drug molecule can sit inside the hole, or inside the ring, of the cyclodextrin. In this case, what the patient get is what it is called a molecular inclusion complex. And of course it no longer tastes like the drug, because what's on the surface is cyclodextrin, which is derived from sugar. Cyclodextrin complexation can be highly effective for taste masking, but as other systems, this technology has its drawbacks. One of these ones is the expenses of this approach since cyclodextrins are not the cheapest of materials. Plus, if it so happens that the drug molecule that doesn't fit inside the cyclodextrin ring, then there is nothing that the formulator can do about it. Either it does or it doesn't. In addition, making a cyclodextrin complex is a much more involved process than microencapsulation or ion exchange resination. Indeed, the formulator has to find the right amount of water present to force the drug to partition into the cyclodextrin. The formulator has to force the drug to get to such a high concentration that the drug ends up going into the cyclodextrin. In fact, the release mechanism for cyclodextrins is water. Therefore, if too much water is added, the drug comes back out again.

The bitter taste of drugs, food components, and any other substances which get in the mouth as dissolved in an aqueous solution, or in the saliva, can be strongly reduced or fully eliminated, if the bitter component forms an inclusion complex with an appropriate cyclodextrin (CD). The value of the complex association constant (determined by the structure of the bitter "guest" molecule and the size and eventual substitution of the "host" CD molecule), the temperature and the host/guest ratio determine the extent of complexation of the guest molecule (percentage of complexation) at the equilibrium. The complex association constant (K_{ass} for most drug/CD complexes at 36°C buccal cavity temperature is between 102 and 104 M^{-1}. If the unit dose (of a sublingual or chwing tablet, chewing gum) with a bitter drug (molecular weight of about 150, forming a 1:1 complex with β-CD) is approximately 10 mg, then the β-CD can be taken in a 5- or even 10-fold molar excess. Under such conditions, more than 99% of the bitter drug is complexed, and because the β-CD is present in a large excess, dissolved very quickly in the saliva and results in a saturated CD solution. Therefore, the complexation of the bitter drug is completed very rapidly. Only dissolved substances have taste and only CD complexable drug molecules can become debittered by CDs. Bitter, astringent components of foods (e.g. soya), beverages (e.g. naringin

in citrus fruit juice, or chlorogenic acid and polyphenols in coffee) cigarette smoke (nicotine) also can be complexed and their taste reduced or fully eliminated.

4.5. Other Techniques

These techniques include incorporation of drugs in vesicles like liposomes, chemical modification (Adjei et al, 1992 and Popescu, 1991) and solubility-limiting methods.

For the drugs whose taste profiles are dependent on aqueous solubility the solubility limiting method can be applied.

In order to reduce aqueous solubility and taste, chemical modification such as derivatization or lipophilic counter ion selection may be an effective method, as example for Erythromycin monohydrate, a bitter tasting drug with a solubility of 2 mg/ml which is chemically converted into erythromycin ethyl succinate, the aqueous solubility being reduced to <50mcg/ml. This form is tasteless and can be administered as a chewable tablet. The use of liposomes, to incorpore drugs into vesicles, is although an ideal technique, yet a challenge to formulate without altering the regulatory status of the product (*in vitro* dissolution kinetics, physical or chemical stability or bioavailability) (Adjei et al, 1992).

In order to inhibit the perception of the bitter taste of a formulation, it can be very helpful to use an anesthetizing agent like sodium phenolate, which numb the taste buds sufficiently within 4-5 seconds (Fuisz, 1991).

The multiple emulsions represent another novel technique for taste masking. The drug must be dissolved in the inner aqueous phase of w/o/w emulsion under condition of good shelf stability. The formulation is designed to release the drug through the oil phase in the presence of gastric fluid (Rosoff, 1988). Another technique has been described to allow masking the bitter taste of a drug by combining multiple emulsions with non ionic surfactants to form composites by hydrophobic interactions (Masahiro,1999).

5. EVALUATION AND OPTIMIZATION OF TASTE MASKING EFFECT

Taste is comprised of five basic qualities of sourness, saltiness, sweetness, bitterness, and umami. Amongst these taste qualities, bitterness is usually an undesirable taste, because it is a sign of a harmful substance, while sweetness and umami are signs of nourishment such as saccharide and amino acids. Umami is the fifth independent taste produced by monosodium glutamate (MSG) contained mainly in seaweed and disodium inosinate (IMP) in meat and fish. It is important especially for pharmaceutical and food sciences to express the extent of bitterness, e.g., in the case of developing syrups. To remedy this problem, formulators combine the drug with other ingredients in an effort to mask the bitterness of the drug and provide the patient with a pleasant product experience.

One an active substance is taste-masked, formulation scientists have to determine what additional functional excipients are required for the final dosage form. These include sweeteners, flavouring agents, and super-disintegrants. After all, the active substance may no longer taste bitter, but the final dosage form needs to taste pleasant and have a good mouth-

feel. Also, orally disintegrating tablets (ODTs), chewable tablets, and fast-dissolve films must disintegrate, or even dissolve, rapidly in the mouth.

Sweeteners and flavours are very important in any formulation that would come into contact with the tongue. The whole point is balance – the balance of the basic taste, and the balance of the flavour with that basic taste. When the formulator is working on an active component, he has to establish the whole preformulation framework, looking at the physicochemical characteristics of the active. Also he has to consider the chemical compatibility issues, and then to determine what sweeteners he can use. Sweeteners not only make oral dosage forms palatable but they are able to optimize product performance. Sometimes, sweeteners can be added for taste reasons, and sometimes for stability and functional reasons, such as by using polyols to prevent crystallisation of syrup made of sucrose. But if he is looking just for a balanced taste, he could use a blend of the nutritive sweeteners and the polyols. Also, a strong non-nutritive sweetener such as aspartame or sucralose can be selected. When incorporating flavouring agents into a formulation, pharmaceutical decision-makers tend to favour market expectations over what the base formulation requires. Usually, people think that if the market requires a strawberry flavour, the formulator has just to buy the flavour, to add it to the formulation, and it will come up as strawberry. But often that is not the case. Certain active ingredients have an off-taste, somewhat of a metallic taste, that may work well with certain flavours but not with others. Fortunately, formulators typically add flavouring agents late in the development process, making it easy to change out one flavour for another if there is a disparity between market expectations and base formulation requirements. The flavours are used in very low levels. For a formulator, switching from one flavour to another doesn't really pose any processing challenges. It is just a matter of preference, and it can be changed later on in the project, taking into account stability concerns, of course. The challenges with mouth-feel come from grittiness, so preferably the choice will be fond of a smaller particle size that would be less detectable in the mouth and less likely to be chewed. Particle size is particularly crucial for microencapsulated actives because if they are coated, chewing would break the coating and there would be bitterness out of that. Many sweeteners, especially polyols like sorbitol and xylitol, enhance mouth-feel in addition to improving taste, stability, or functionality. Xylitol is widely used in ODTs because of the cooling sensation and creamy mouth-feel it gives. Formulators generally rely on fast-dissolving excipients to ensure that ODTs and chewable tablets break apart rapidly in the mouth. Fast-dissolving ingredients are basically a blend of superdisintegrants, which would also be used in regular tablets to optimize disintegration.

The formulation departments of pharmaceutical companies bear the responsibility for the selection of ingredients and development of formulations that provide best taste performance for products taken orally. Several challenges exist that further complicate this process for the pharmaceutical formulators. Even if the level of unpleasant taste from oral solid dosage forms can be evaluated by *in vitro* methods, for liquid or semi-solid dosage forms it is not thinkable.

Evaluation of the taste masking effect from coated microspheres can be done by determining the rate of release of the drug from the microspheres. Similarly, for evaluating the taste masking effect by ion exchange resin, the drug release rate can serve as an index of the degree of masking achieved. Other methods include evaluation by a trained flavour profile panel and time intensity method in which a sample equivalent to a normal dose was held in mouth for ten seconds. Bitterness level are recorded immediately and assigned values between nought to three.

To accomplish the task of designing good tasting liquid or semi-solid products, formulators rely heavily on human testing or sensory panels. The use of sensory panels is a complicated procedure due not only to the subjectivity of panel members but also to the potential toxicity of drugs. Problems in recruiting taste panellists, motivation and panel maintenance are significantly more difficult when working with products that have an unpleasant taste attribute (bitter, sour, and numbing). Non-FDA approved compounds cannot be human tasted delaying when the formulators can begin development of taste formulations for new medicines. These hurdles coupled with the requirement that products being tested by sensory panels must be formulated or manufactured under GMP guidelines in a GMP approved facility; the time and cost to develop test batches can be prohibitive. Unfortunately, despite the challenges and expense of developing formulations with acceptable taste and aroma, product acceptance has become an important marketing factor for the pharmaceutical industry for both Over-the-Counter (OTC) and prescription drugs. The need for palatable products is particularly important when formulating oral delivery products for children and elderly patients.

To provide a tool for the formulators who address these difficulties, Soutakagi *et al.* (1998) invented a multichannel taste sensor whose transducer is composed of several kinds of lipid/polymer membrane with different characteristics, which can detect taste in manner similar to human gustatory sensation. Taste information is transformed into a pattern composed of electrical signals of membrane potential of the receptor part. It was reported that suppression of bitterness of Quinine and a drug substance by sucrose could be quantified by using multichannel taste sensor. The present method can be expected to provide new automated method to measure the strength of drug substance in place of sensory evaluation. The software tools provided with the system include an advanced chemometric software package for data measurement and analysis of results. The tools provide the ability to compare the formulation candidates and perform a classification process. The results can be quantitatively compared based on analytical parameters such as concentration, taste intensity scale, etc.). Other analytical assessment can be performed to define the qualitative aspect of the formulations such as identification, conformity, palatability, etc. The formulation comparison can be related to other samples in a comparative mode or to a “taste” or “flavour” database, which can be defined by the system operator, based on a selected training set of samples.

Therefore, the E-tongue quickly evaluates the bitterness masking efficiency of the different formulation candidates to provide the formulator with the ability to select the best combination of ingredients, which provide the “best” tasting formula. These analytical methods provide a fast, objective and simple assessment of oral formulations such as chewable tablets, liquid, rapid dissolve tablets and films, oral dispersive lozenges, sublingual delivery methods, and nasal delivery products which is highly correlated with the organoleptic taste panel methods. The E-nose and E-tongue provide the formulation scientist the ability to rapidly qualify the acceptability of a formulation taste and flavour and permit the definition of acceptable or “best” tasting products. By objectively measuring the effect of ingredient substitution and formulation selection, it is possible to identify the “best” tasting new formulations in accordance with sensory measurements without the need for human tasting of the experimental formulations. The reported studies conducted by pilot e-Tongues with short life sensors, limited significantly its application. Recently, a taste analyzing system manufactured by Alpha MOS has become commercially available. The taste sensors consist

of silicon transistors with an organic coating that governs sensitivity and selectivity of each individual sensor. The life of the sensors could last as long as 1 year.

6. APPLICATION FIELDS

6.1. Infants, Children

In pharmacology and clinical paediatrics, the focus is on active drug substance (or active pharmaceutical ingredient (API)) when determining dosage, clinical effects and adverse drug reactions. However, the formulation is fundamentally important since it determines, in practice, whether the dose can be successfully delivered to the paediatric patient. Moreover, it is important to consider the formulation excipients and the potential for any adverse effects in this potentially vulnerable age group.

The development of formulations which are appropriate for children (age-adapted dosage forms) can present significant challenges to the pharmaceutical scientist. Childhood is a period of maturation requiring knowledge of developmental pharmacology to establish dose but the ability of the child to manage different dosage forms and devices also changes. Paediatric formulations must allow accurate administration of the dose to children of widely varying age and weight. Whilst the oral route will be preferred for long term use and the intravenous route for the acutely ill, many of the dosage forms designed for adults, such as oro-dispersible tablets, buccal gels and transdermal patches, would also benefit children if they contained an appropriate paediatric dose. The age at which children can swallow conventional tablets is of great importance for their safety. Liquid medicines are usually recommended for infants and younger children so the ability to mask unpleasant taste with sweeteners and flavours is crucial. Compared to the conception of dosage forms intended for adults, the ones intended for children necessitate more research and clinical feedback because a formulation with poor acceptability may affect compliance, prescribing practice and ultimately commercial viability.

Unlike adults, where oral solid dosage forms such as tablets or capsules will be acceptable to the majority of patients, potential paediatric patients may include neonates, newborn, toddlers, young children and adolescents, and as such, will have widely varying needs. The development of multiple dosage forms for different ages will rarely be commercially viable and liquid formulations, which can be given to a broad age group, present particular pharmaceutical challenges. For example, taste masking a bitter-tasting drug is a major formulation hurdle which can be very costly and may not be totally achievable. Some considerations for the development of acceptable paediatric drug formulations are discussed below.

There are various reasons for the formulation of drugs into appropriate dosage forms; one of the most important relates to accurate measurement of the dose. Many active drugs are very potent and only require milligram or microgram amounts to be administered. For children, the amount of drug required for the dose varies with age and weight. Active drugs must be diluted in a vehicle which allows accurate and convenient dose measurement. Active drugs must also be protected during their shelf life from degradation, for example by oxygen and humidity and, when administered orally, may require protection from degradation by

gastric acid. It may be necessary to conceal taste and smell and to produce liquid preparations of insoluble or unstable drugs.

Concerning the ages and the abilities, there is a change in the magnitude of dose required during childhood and adolescence which may be fifty-fold. There are also significant changes in the ability to handle different dosage forms with small volume liquid medicines being appropriate for oral use in the younger age groups; liquid medicines and fast dissolving "melt" formulations suitable for most ages and tablets and capsules being more convenient to the lifestyle of adolescents. Paediatric practice requires a range of dosage forms that are acceptable at different ages and abilities and a range of strengths or concentrations allowing administration of the correct age-related dose.

The first challenge with oral liquid paediatric formulations concerns the dose and the volume of liquid medicines which may be limited by the solubility of drug substances requiring the addition of co-solvent and surfactant excipients. Physical, chemical and microbiological stability must be assured with buffering agents, antioxidants and preservatives. Of crucial importance is the ability to mask unpleasant taste with sweeteners and flavours. If this is not achievable, more sophisticated formulation approaches such as encapsulation of drug particles, may be required. The more complex formulation approaches bring higher technical challenges and consequently, research and development will be more lengthy and costly. Formulation homogeneity is required and dosing devices such as droppers or syringes may be required for accurate dosing. There are limitations on choice and concentration of excipients for paediatric patients.

To formulate industrial paediatric formulations, there are many gaps and many challenges to face if suitable preparations are to be available for all age ranges. These include:

- Acceptable dose volumes and sizes
- Safety, e.g. risk of aspiration or choking for solid dosage forms
- Excipient acceptability
- Taste (and how best to assess during development).

6.2. Geriatric Patients

Traditional tablets and capsules administered with glass water may be inconvenient or impractical for some patients. For these reasons, recent technological developments have presented viable dosage alternatives for patients who may have difficulty in swallowing tablets or liquids. Indeed, some patients, particularly paediatric and geriatric patients, have difficulty in swallowing or in chewing solid dosage forms and are unwilling to take these solid preparations due to fear of choking (Slowson, 1985). For example, a very elderly patient may not be able to swallow a daily dose of antidepressant, antiparkinsonian, antipsychotic, or a H2-blocker by people undergoing radiation therapy for breast cancer and who may be too nauseous, .etc. Fast dissolving/disintegrating tablets are a perfect fit for all of these patients (Seager, 1998) and have rapidly gained acceptance as an important new way of administering drugs. These are multiple fast-dissolving drug delivery systems on the market worldwide, most of which have been launched in the past 3 to 4 years.

These new dosage forms allow the disintegration and/or dissolution rapidly in the saliva without the need of water. Some tablets are designed to dissolve in saliva remarkably fast,

within a few seconds, and are true fast-dissolving tablets. Others contain agents to enhance the rate of tablet disintegration in the oral cavity, and are more appropriately termed fast-disintegrating tablets, as they may take up to a minute to completely disintegrate. When put on tongue, this tablet disintegrates instantaneously, releasing the drug, which dissolves or disperses in the saliva. Some drugs are absorbed from the mouth, pharynx and oesophagus as the saliva passes down into the stomach. In such cases, bioavailability of drug is significantly greater than those observed from conventional tablet dosage form (Chang, 2000).

6.3. Domestic Animals

As described above, pharmaceutical drugs often have certain desirable properties but an undesirable smell and/or taste. These properties have a great impact on patient compliance and marketing of the pharmaceutical product. This is especially true for domestic animals (cats, dogs) having an extremely well-developed sense of taste and smell.

In the veterinary field, only a few classes of medicines are concerned by this topic such as anti-inflammatory compounds, antibiotics, anthelmintic agents, etc … since other ways (skin, transdermal, injectable) to administer drugs can be envisaged. In the contrary of dosage forms intended for humans, in the veterinary field, the formulators have to take into account the price on one hand of the raw materials (drugs, excipients) and on the other hand of the process developed to manufacture dosage forms. To mask the bitter taste of drugs, similar techniques than those developed for human applications can be used such as barrier methods (embedding, coating, and encapsulation), use of flavours (beef, pork, fish, chicken, etc.) and sweeteners and complexation and absorption approaches. Thus, taste masked powders are formed by methods known such as spray drying, granulation and coacervation.

7. CONCLUSION

The bitterness of veterinary and human pharmaceutical medicines plays a crucial role in patient compliance, as the oral administration of bitter drugs is often hampered by their unpleasant taste, leading to non-compliance and thus decreasing therapeutic efficacy, especially in children and the elderly. The quantitative evaluation of the bitterness of medicines is therefore, an important factor in drug formulation design. To mask the high bitterness intensity of drugs, several innovative approaches have been taken to mask their bitterness in pharmaceutical formulations and then allowing improving the compliance by the patients or the domestic animals. With success, recently, to compare *in vitro* different taste-masked formulations, a taste sensor, called “electronic tongue or E-tongue” with global selectivity, was developed and has been applied to evaluation of the taste of new dosage forms.

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Chapter 5

MECHANISMS OF DRUG ENTRY INTO NUCLEUS

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ABSTRACT

Molecular transport between the nucleus and the cell cytoplasm occurs through nuclear pore complexes (NPCs). Although access to the nucleus is a highly restricted process, a multitude of macromolecules have to enter and exit the nucleus, for the control of the basic cellular metabolism and to respond to changing environmental conditions. Except during mitosis, when the nuclear envelope disappears, the only way macromolecules can enter the nucleus is through the nuclear pore complex (NPC). The NPC is built of a diverse set of nucleoporins and associated nuclear and cytoplasmic filaments surrounding a central channel structure. A protein destined for the nucleus and/or cytoplasm contains a specific sequence which can be recognized directly by importin/exportin or through an adaptor protein. Two major types of signals called nuclear localization signals (NLS) have been identified for the nuclear import of proteins: SV40 type and bipartite type. The former was first found in the large T antigen of the SV40 virus. The bipartite type was first identified in *Xenopus* nucleoplasmin. Such a detailed understanding of the processes of signal-mediated nuclear protein import/export and its regulation enabled the application and optimization of approaches to target molecules of interest. One of their practical and potential uses in delivering molecules of interest to the nucleus in a clinical context includes gene transfer, where NLS tagged plasmid DNA encoding a gene of interest is delivered to the nucleus. Other applications of NLS are delivery of toxins which are targeted to the nucleus of tumor cells in cancer therapy. This chapter will address the NPCs in detail and will introduce the mechanisms of entry through NPC such as NLS. Recent advances in applications of NLS will be presented.

1. INTRODUCTION

A. Transport Into and Out of Nucleus in the Eukaryotic Cell

Possession of a nucleus (karyon) by a eukaryotic cell has far-reaching implications for cellular function, since the genetic information, the DNA, is separated from the site of protein synthesis, the cytoplasm. Separation is achieved by a double membrane structure, the nuclear envelope (NE), which is both contiguous with the endoplasmic reticulum (ER) and largely ER-like in terms of lipid and enzyme composition (Figure 1). Since gene transcription and translation take place in separate subcellular compartments, specific transport events must occur for the cell to function: firstly, mRNA must make its way from the nucleus into the cytoplasm in order to be translated into protein; and secondly, the proteins that are required in the nucleus need to be specifically transported from their site of synthesis in the cytoplasm into the nucleus. Nucleocytoplasmic transport in both directions is precisely controlled, whereby the regulation of the subcellular distribution of proteins which regulate gene transcription, RNA splicing or other nuclear events is integral to many cellular processes. In a signal transduction context, any growth factor or hormonal signal that affects gene expression must by definition be communicated to the nucleus, this almost without exception involving a protein translocation event from the cytoplasm to the nucleus. The nuclear localization of many transcription factors (TFs), morphogens, and oncogene products very precisely accompanies changes in the differentiation or metabolic state of eukaryotic cells, indicating that nuclear protein import is a key control point in regulating gene expression and signal transduction [1].

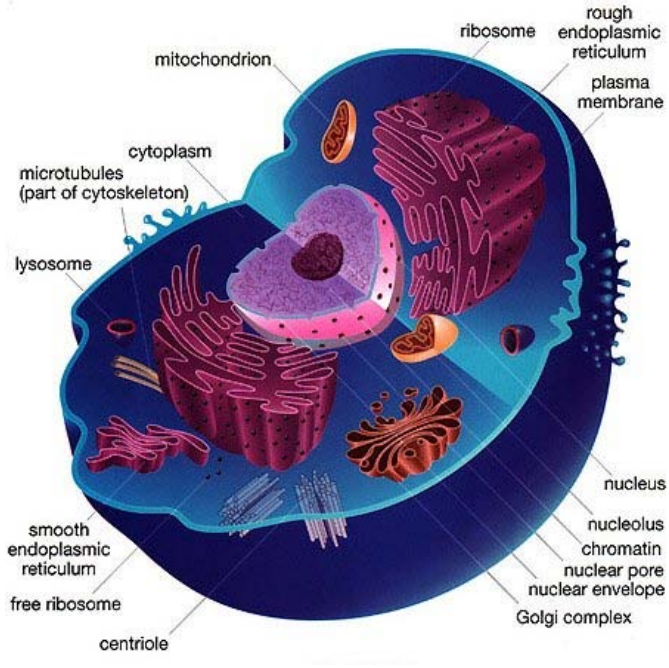

Figure 1 (Continued on next page)

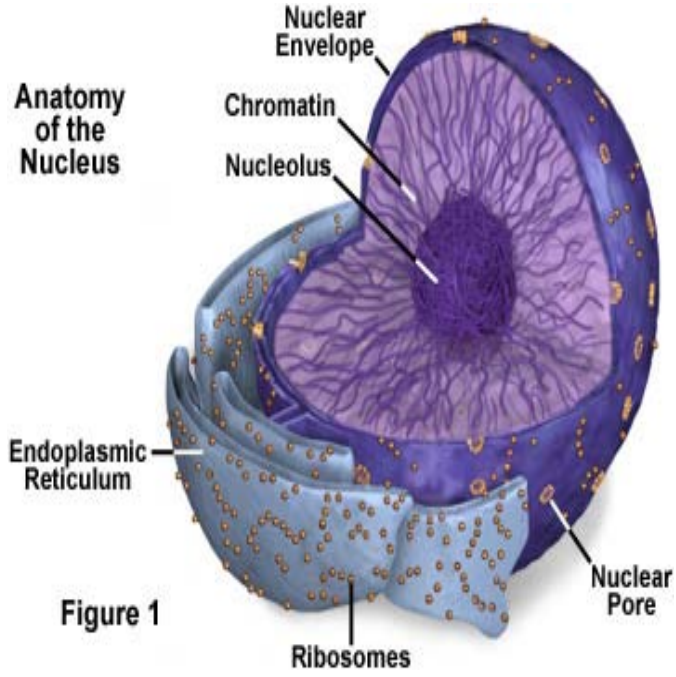

Figure 1.

B. The Nuclear Pore Complex; Central Avenue of Nucleocytoplasmic Transport

All passive and active transport into and out of the nucleus occurs through the nuclear pore complexes (NPCs) [3–5] present in the nuclear envelope. The structure and function of the NPC appears to be conserved in all eukaryotes indicating its central role in eukaryote cell function, with its unique properties determining the nature of transport through it. It is composed of at least 20 distinct protein components in varying stoichiometries and has a near organelle-like mass of about 105 kDa. Between 10^2 and 5×10^7 NPCs are present per nucleus depending on the metabolic or differentiation state of the cell. This corresponds to a range of about 3 NPCs/μm^2 in the nuclear envelope of an inactive oocyte, to about 15–20/μm^2 in that of normal, metabolically active, differentiated somatic cells. As its name suggests, the NPC has a pore-like, molecular sieve function, whereby molecules smaller than 40–45 kDa can diffuse freely into and out of the nucleus. Proteins larger than 45 kDa require specific targeting signals in order to pass trough the NPC. The structure of the NPC has been extensively investigated by electron microscopy (EM), and a consensus model of its central framework has emerged (Figure 2). Accordingly, the vertebrate NPC exhibits an 8-fold symmetric (i.e., perpendicular to the plane of the NE) tripartite architecture with a total mass of ~125 MDa. Its ~55 MDa central framework is a ring-like assembly built of eight multi-domain spokes consisting of two roughly identical halves each so that its asymmetric unit (i.e., one half-spoke) represents one 16th of its mass or roughly the size of a ribosome. This

central framework is sandwiched between a ~32 MDa cytoplasmic ring and a ~21 MDa nuclear ring.

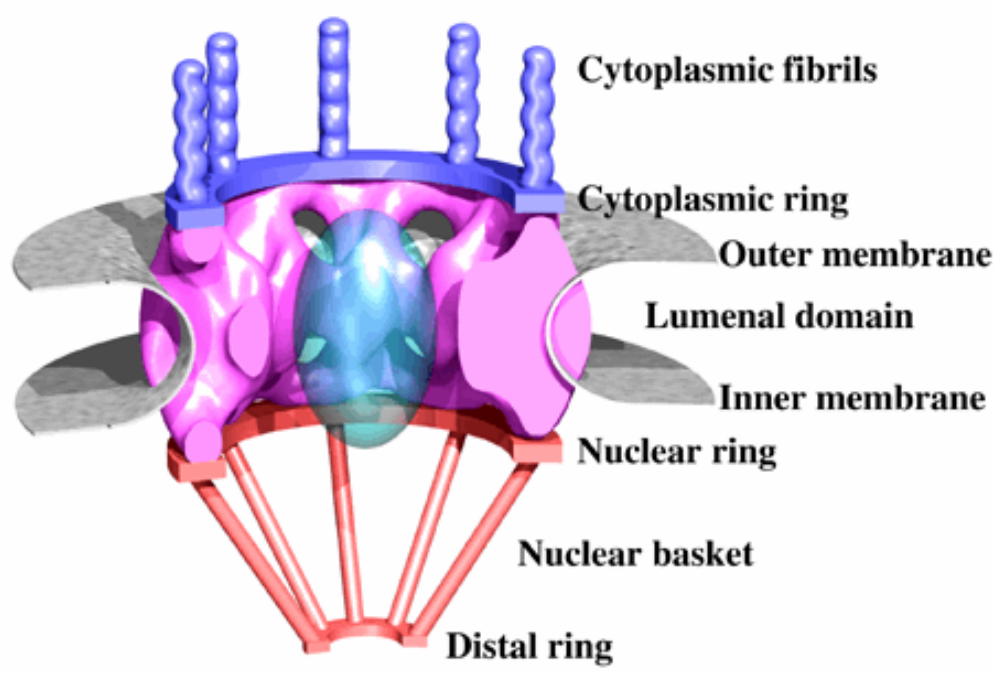

Figure 2.

From the cytoplasmic ring eight short, kinky fibrils emanate, whereas the nuclear ring anchors a basket (or fishtrap), assembled from eight thin, ~50 nm long filaments joined distally by a 30- to 50-nm-diameter ring. The ring-like, ~822-symmetric central framework embraces the central pore of the NPC which acts as a gated channel (Figure 2). In the case of nuclear entry, a nuclear localization sequence (NLS) [6–9] (see Sec. 2-A) is generally required by large proteins in order to be targeted specifically to the nucleus. NLS-dependent nuclear protein import is ATP- and temperature-dependent [10, 11] and can be inhibited by the lectin wheat germ agglutinin (WGA) [12, 13] which binds to NPC proteins, or by antibodies specific to NPC components [14–17]. The transport properties of the NPC appear to be regulated by mitogenic signals [18] probably either through the phosphorylation of NPC components [19] or by effects on Ca^{2+} concentrations within the ER and nuclear envelope lumen [20]. A number of protein components constituting the NPC have been characterized at the molecular level (see Ref. [21]). These include the nuclear envelope-anchored concanavalin A (ConA)- binding high-mannose Gp210 protein, most of whose mass is located within the nuclear envelope lumen adjacent to the NPC and whose function appears to anchor the NPC in the nuclear envelope [16, 22]. The 121 kDa WGA binding protein POM12123 and yeast 152 kDa ConA-binding protein POM15224 also appear to be localized in the pore membrane region, and probably play a similar role. An anchoring or NPC attachment role appears to be played by nup180, which is located at the cytoplasmic ring of the NPC and may constitute a link between it and the cytoskeleton [25]. Five to ten percent of the mass of the NPC is made up of a class of peripheral, nonmembrane anchored NPC protein

components called nucleoporins. They possess O-linked glycosidic N-acetylglucosamine moieties, which appear to be the basis of WGA inhibition of nuclear protein transport (see above). NPCs depleted of WGA-binding proteins are inactive in terms of nuclear protein import; [26] as mentioned above, antibodies to nucleoporins block nuclear protein import [14–16, 27, 28]. Nucleoporins identified by molecular cloning appear to form at least two classes (see Ref. [21]) based on the presence of FG (single letter amino acid code) repeat units; either FXFG (where X is any amino acid) or GLFG repeats. Specific oligomerization of nucleoporins, including those binding to Gp62, [3, 29] Nsp1p, and Nup1p, [30] has been documented, while a number of nucleoporins have been shown to complex with Gp210. Particular nucleoporins can be localized to specific locations within the NPC; e.g., the zinc finger-containing nup153 appears to be localized exclusively on the nucleoplasmic side of the NPC, possibly associated with the nucleoplasmic basket structures of the NPC (see Ref. [3]), while p62 appears to be associated with both the cytoplasmic and nucleoplasmic sides of the central channel of the NPC (see Ref. [6]). Significantly, nucleoporins nup98 and nup214 have been identified as fusion partners in chromosomal translocations associated with myeloid leukemias (see Refs. [1, 6, 31]), while the recently characterized 265 kDa nucleoporin Tpr has been found in oncogenic fusions with the protooncogenic kinases Met, Trk, and Raf.[32]. These observations stress the central role of the NPC and its components in vitally important cellular processes such as transformation and oncogenesis. The work of several groups, including that of Blobel and coworkers [31, 33] has provided insight into the role of the nucleoporins in docking transport substrate complexes, whereby the mammalian nucleoporins nup214 (on the cytoplasmic side of the NPC), and nup153 and nup98 (on the nucleoplasmic side of the NPC), appear to be directly involved [34–37] Radu *et al*.[34] showed that the FG repeat-containing amino-terminus of nup98 is necessary for docking transport substrates at the NPC, while in yeast, the central repeat-containing domain of the nucleoporin Nup159 (localized on the cytoplasmic side of the NPC) is similarly necessary for the nucleoporin's transport-substrate docking role [38]. It is thus postulated that the repeats, as well as those in many other nucleoporins, are the sites of association and dissociation of transport substrates as they move through the NPC [31, 33, 34]. Certain nucleoporins are able to bind some of the cytosolic factors mediating association with and transport through the NPC. RanBP2 (nup358) [28, 39] and Nup2p, [40] for example, are able to interact with the monomeric GTP-binding protein Ran/TC4, which plays a key role in translocation through the NPC in both directions, [28, 39, 40] while Gp62 binds NTF2 which interacts with Ran and is important in signal-dependent nuclear protein import [41]. RanBP2, as well as nup116, are able to bind factors such as importin β which mediates the docking of signal-containing transport substrates at the NPC (see Sec. 2-A) [36]. The current consensus of opinion with respect to the NPC is that nucleoporins represent multiple binding or assembly sites at which substrates and transport factors are brought into close vicinity, and thereby synergize to ferry substrates from one side of the pore to the other.

2. TARGETING SIGNALS

A. Nuclear Localization Sequences (NLSs)

NLSs are the short peptide sequences which are necessary and sufficient for nuclear localization of their respective proteins [6–9]. They have been largely identified based on two criteria: (1) mutation or deletion of the NLS leads to cytoplasmic localization of the protein in question, and/or (2) the NLS is active in nuclear targeting of a normally cytoplasmically localized carrier protein, either as a peptide covalently coupled to the carrier, or when encoded in the sequence of a fusion protein.

The basis of NLS-dependence of nuclear protein import is recognition/ligand-receptor-like interaction with specific NLS-binding proteins (see below), rather than through DNA or histone binding [42–46]. That conventional NLSs are entry rather than retention signals has been demonstrated by the fact that NLS-deficient carrier proteins (lacking specific signals for nuclear export) microinjected into the nucleus remain nuclear [45, 47] while nuclear accumulated NLS-containing proteins are highly laterally mobile [48] indicating that binding in the nucleus to chromatin or other structures such as the nucleoskeleton, is not the mechanism by which NLSs mediate nuclear localization [4, 5, 49] (see Refs. [3, 47]). NLSs are fundamentally different from other peptide signal or targeting sequences in that they are not cleaved during transport. This is because, in contrast to ER and other targeting signals, NLSs may be required to function several times, and through a number of cell divisions, which in the case of most eukaryotes involve dissolution of the nuclear envelope [3–5]. Breeuwer and Goldfarb [50] showed that the addition of an NLS to a small protein normally able to enter the nucleus by passive diffusion renders its nuclear entry temperature- and ATP-dependent, characteristics of active, NLS-dependent transport. This indicates that possession of an NLS confers quite specific regulatory properties upon the protein harboring it. There appear to be several types of NLSs; conventional or “directional” NLSs are those that mediate exclusively nuclear protein import, and are thus distinct from other categories of NLS (e.g., the M9 sequence of hnRNP A1—see Sec. 2-C below) that can also mediate nuclear export and hence are better termed “shuttle signals.” The process of NLS-mediated transport into the nucleus involves two basic steps, [10, 11] the first of which involves energy-independent recognition of the targeting signal of the transport substrate and subsequent docking at the NPC, while the second is energy-dependent and involves translocation through the pore and into the nucleus.

The first step is mediated by the “NLS receptor” the heterodimeric complex of the specific NLS-binding protein importin α (also known as importin 58/Srp1/Rch1/Kap60/karyopherin α), and the importin α- and nucleoporin binding NPC docking protein importin β (importin 97/Kap95/karyopherin β1), [35–37, 51–54] which is also required for high affinity NLS binding on the part of importin α. [54–57]. As alluded to above, importin β’s affinity for nucleoporins such as RanBP2 and nup11636, [41] enables initial binding of the importin-NLS-containing protein complex to the distal end of the cytoplasmic fibrils of the NPC, after which it is transferred to the cytoplasmic side of the central channel which may involve bending of the fibrils [58]. The subsequent step of energy-dependent translocation through the NPC of the importin/NLS-containing protein complex to the nucleoplasmic side of the NPC appears to require the GTPase Ran in its GDP-bound

form, [55, 59–61] and modifying factors such as NTF2, RanBP1 (a soluble Ran binding protein), and RanGAP1 (Ran GTPase activating protein 1) [62–66]. NTF2 and RanBP1 may function largely to stabilize the importin/transport substrate complex, thus preventing its dissociation before arrival at the nucleoplasmic side of the NPC [67, 68]. Once the complex has reached the nucleoplasm (about a 100–120 nm translocation), Ran-GTP, at a high concentration in the nucleus through the action of the guanine nucleotide exchanger RCC1, effects dissociation of the complex such that the NLS-containing protein and importin α are released into the nucleus while importin β remains at the NPC [36, 51]. Directionality of the translocation is purported to be provided through compartmentalization of the major effectors of the Ran GDP/GTP state, RanGAP1 and RCC1, which are predominantly cytoplasmic and nuclear, respectively (Figure 3) [66–68].

Importin α recycling/export from the nucleus is mediated by the specific transporter CAS, [69] while importin β, most likely complexed to RanGTP, is recycled back to the cytoplasm through an unknown mechanism; importin α probably then displaces Ran from importin β in the cytoplasm to regenerate the importin heterodimer for further cycles of transport. Nonhydrolyzable GTP analogs can block NLS-dependent nuclear protein import [34–37, 41, 57] through blocking Ran activity.

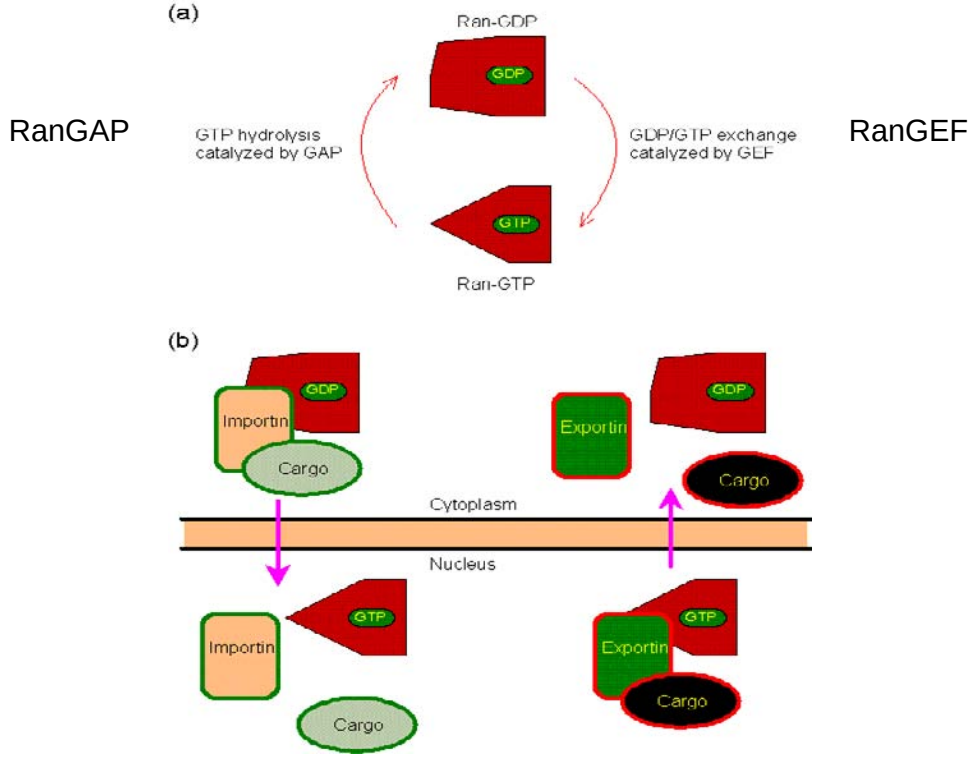

Figure 3.

That GTP-dependent transport requires Ca^{2+} in the lumen of the nuclear envelope has been shown by the fact that Ca^{2+}-ionophores such as A23187 inhibit transport [20] A Ca^{2+}-stimulated NLS-dependent nuclear import pathway has recently been reported where, under conditions of depletion of intracellular Ca^{2+} stores and elevated free Ca^{2+}, calmodulin can

support nuclear import which is not inhibitable by GTP analogs and does not require cytosolic factors [70]. This pathway is believed to have a physiologically important role under circumstances where, in response to stimulation of signal transduction or the onset of mitosis, Ca^{2+}-transients in and around the nucleus may perturb GTP-dependent nuclear import; the calmodulin-dependent pathway enables nuclear transport to continue and the cell to function under these special conditions [70].

1. NLSs Recognized by α/β-Importin

Conventional NLSs fall into at least three classes, two of which are predominantly basic in nature. They are those resembling the NLS of the SV40 large tumor antigen (T-ag-PKKKRK^{132}), [6–8] comprising a short stretch of basic amino acids, as indicated in Table I; and bipartite NLSs, which consist of two stretches of basic amino acids separated by a spacer of 10–12 amino acids [71] typified by the NLS of the *Xenopus* phosphoprotein nucleoplasmin (KRPAATKKAGQAKKKK [170]—see Table IB). As is immediately obvious from Table I and the subsequent tables within this chapter, a great number of NLS and other targeting signal-containing proteins are either viral proteins, or oncogene or cellular protooncogene-related products, underlining the central role of targeting signals in the function of viral protein and oncogene products. Understanding the properties and regulation of these targeting signals may thus enable the development of important new antiviral and anticancer therapies. The NLS of the protooncogene *c*-myc (PAAKRVKLD^{328}), although ostensibly falling into the T-ag class of NLS, has several unique properties; the PAA sequence appears to be essential, whilst the C-terminal LD sequence strongly enhances NLS activity [132]. Perhaps significantly, PAA sequences can be found in several other NLSs, including within the spacer of the nucleoplasmin bipartite NLS, implying a role more general than that exclusively relating to the *c*-myc NLS. Indeed, basic sequences not sufficient for nuclear import can be rendered active in nuclear targeting through N-terminal inclusion of the PAA sequence132 The third and less well-characterized class of NLS is made up of NLSs resembling those of the yeast homeodomain containing protein MATα2 (NKIPIKDLLNPQ^{13} and VRILESWFAKNI1) [59, 9] where charged/polar residues are interspersed with nonpolar residues (see Table I). All three classes of NLS are held to be recognized specifically by the αβ/-importin heterodimer during the first step of nuclear transport, as has been shown directly for the importins from several species (e.g., Refs. [57, 133–135]).

2. NLSs Recognized by β-Importin

It has been recently demonstrated that certain apparently conventional NLSs appear to be recognized specifically not by the α/β-importin heterodimer, but by β-importin alone [136–139] (see Table II). These include the T-cell protein tyrosine phosphatase (TCPTP), [136] human immunodeficiency virus (HIV-1) Rev protein, [138] and the yeast transcriptional activator GAL4 [137]. Whether these particular NLSs are recognized because they mimic the importin β-binding ("IBB") domain of α-importin (see Table II) or through an alternative mechanism is unclear at this stage (see Ref. [137]).

Table I. Selected Examples of Conventional NLSs Resembling That of T-ag

Protein[a]	**NLS**[b]	**Carrier Proteins Targeted** [c]
SV40 T-ag[6–8,48]	PKKKRKV[132]	PK/BSA/IgG/βG/CSA
Polyoma T-ag[72,73]	VSRKRPRP[196]	PK/CSA
Lamin LI[73]	VRTTKGKRKRIDV[420]	CSA
Lamin B[274]	RSSRGKRRRIE[419]	
Cofilin[75]	PEEVKKRKKAV[36]	BSA
h c-myc[73,76]	PAAKRVKLD[328]	PK/HSA/CSA
	RQRRNELKRSF[374]	PK
Ad7 E1a[73,77]	KRPRP[289]	IgG/GK/CSA
SV40 VP2/3[78]	PNKKKRK[323]	POL/PK/BSA/βG/IgG
h p53[73,79]	PQPKKKPL[323]	PK/βG
NF-kB p50[80,81]	QRKRQK[372]	
NF-kB p65[82]	EEKRKR[286]	
Chicken v-rel[83]	KSKKQK[295]	βG
Dorsal[84]	RRKRQR[340]	
v-jun[85]	KSRKRKL[253]	IgG
Yeast histone 2B[86]	GKKRSKAK[36]	βG
mMx[187]	REKKKFLKRR[6,15]	
SRF[88]	RRGLKR[100]	IgG/βG
Prothymosin a[89]	TKKQKT[107]	βG
MyoD[90]	CKRKTTNADRRK[112d]	IgG/βG
h PDGF A[91e]	RESGKKRKRKRLKPT[207]	PK/CAT/DHFR
hnRNP B[192]	KTLETVPLERKKREK[17]	CAT
NF-AT[93]	CNKRKYSLN[271f]	
	GKRKK[6,85f]	
PI-2[94]	REKKRQFEMKRKLH[147]	
cGMP-PK[95]	KILKKRHI[4,11]	βG
IL-1a[96]	KVLKKRRL[86]	
dHSF[97]	KRQQLKENNKLRR[412]	βG
Basonuclin[98]	PKKKSRKSS[5,41]	
h DNA ligase I[99]	PKRRTARKQLPKRT[132]	CAT
Ribosomal protein	KTRKHRG[12]	βG
L29[100]	KHRKHPG[29]	βG
Maize R protein[101]	MSERKRREKL[248]	βG/GLUC
hFGF-1a/b[102]	NYKKPKL9/2[7]	βG
Angiogenin[103]	RRRGL[35]	BSA/IgG
hFHF-1[104]	RKRPVRRR[65]	βG

[a] h, human; m, mouse.

[b] Key residues shown by mutation analysis to be essential for NLS-function are indicated in bold.

[c] Carrier proteins that have been shown to be able to be targeted to the nucleus by the NLS either as a peptide covalently coupled to the carrier or encoded within a fusion protein derivative (see Ref. [5]). PK, pyruvate kinase, BSA, bovine serum albumen, IgG, immunoglobulin, βG, b-galactosidase, CSA, chicken serum albumen, HSA, human serum albumen, GK, galactokinase, POL, polio virus VP1, a1G, a1-globin, CAT, chloramphenicol acetyltransferase, DHFR dihydrofolate reductase, GLUC, b-glucuronidase.

[d] MyoD contains two functional NLSs—see also Table IC.[90]

[e] Alternately spliced longer form of PDGF A [91].

[f] Both NLSs are required for nuclear localization [93].

[g] NLSs shown require one of the other two R protein NLSs (see also Table IC) for nuclear localization [101].

In the lack of the need for an α-subunit, they are analogous to the M9 shuttle sequence which appears to be recognized by the β-importin homolog transportin in the absence of an α-subunit (see Sec. 2-C). Intriguingly, nuclear transport of U snRNPs (small nuclear ribonucleoproteins), also appears to require β-importin and not α-importin, even though the U snRNP NLS, where the trimethylguanosine cap of the RNA and core snRNP structure (together with associated proteins) constitute a novel two component NLS, [139] is quite different from those of the proteins listed in Table II.

Table II. Selected Examples of Bipartite NLSs

Protein[a]	**NLS**[b]	**Carrier Proteins Targeted** [c]
h poly(ADP-ribose) polymerase[105]	KRK—10 a.a. spacer—KKKSKK[226]	βG
h c-fos[106]	KRRIRR—12 a.a. spacer—KRRRL[161]	PK
hARNT[107d]	RAIKRR—13 a.a. spacer—KFLR[61]	GST–GFP
hSRY[108]	KR—12 a.a. spacer—RRK[77]	IgG/βG
hHSF2[109]	KRK—9 a.a. spacer—KIR[122]	
	KRKR—9 a.a. spacer—KK[210]	
hGlucocorticoid receptor[110,111]	RK—10 a.a. spacer—RKTKK[4,95]	βG
hProgesterone receptor[112]	RK—10 a.a. spacer—RKFKK[495]	
hEstrogen receptor[112,113]	RK—11 a.a. spacer—RKDR[4,95]	
hRb (p110)[57]	KR—11 a.a. spacer—KKLR[8,69]	βG
h hnRNP K[114,115]	KR—11 a.a. spacer—KRSR[37]	
hFHF-1[104]	RQKR—13 a.a. spacer—KRRSSPSKDGR[38]	βG
hIL-5[116]	KK—12 a.a. spacer—RRR[111]	βG
mFGF3[117]	RLRR—16 a.a. spacer—RRRK[76,47e]	βG
Chicken nucleolin[118]	KRKK—10 a.a. spacer—KKKK[273]	
x NO38[71,119]	KR—11 a.a. spacer—KKTR[153]	
x nucleoplasmin[71,73,77]	KR—9 a.a. spacer—KKKKL[171]	PK/βG/IgG/CSA
x N1/N[244]	RKKRK—12 a.a. spacer—KKSK[5,51]	βG
x xnf7120	KRK—8 a.a. spacer—KKAKV[121f]	PK
Herpes ICP-81[22]	RKR—14 a.a. spacer—KK[11,88e]	PK
SWI5 (S. cer)[123,124]	KK—10 a.a. spacer—RKRGRPRK[6,55f]	βG
TGA-1A (tobacco)[125]	KK—11 a.a. spacer—RLRKK[92]	GLUC
TGA-1B (tobacco)[125]	KKKAR—11 a.a. spacer—RQRKK[3,64e]	GLUC
Opaque-2 (maize)[126]	RRK—8 a.a. spacer—KMTR	GLUC
	RKRK—7 a.a. spacer—RRSRYRK[247g]	GLUC
VirD2 protein (octopine)[127,128]	KR—11 a.a. spacer—RKRAR	βG/GLUC
VirD2 protein (nopaline)[127,128]	KR—11 a.a. spacer—RKRER	βG/GLUC
VirE2 protein129	KLR—11 a.a. spacer—RREIQKR	GLUC

[a] h, human; m, mouse; x, *Xenopus laevis.*

[b] The two arms of basic residues of the bipartite NLS are shown in bold type—substitution of the basic residues of either arm oblates NLS function (see Ref. [71]).

[c] As per legend to Table IB; GST–GFP, glutathione S-transferase-green fluorescent protein fusion protein.

[d] ARNT, aryl hydrocarbon receptor nuclear translocator.

[e] The two forms of FGF3 originate from alternative start codons [117].

[f] A homologous sequence is found in the *Xenopus* PwA protein [121].

[g] Bacterial sequence capable of nuclear targeting in plant (and yeast) cells.

3. Novel NLSs

A novel NLS of the accessory protein Tat of HIV-1 which is not recognized by either importin α or β has been recently indenfified [140]. The Tat-NLS (GRKKRRQRRRAP59)[141] is sufficient to target the 476 kDa heterologous protein β-galactosidase to the nucleus in ATP-dependent but, intriguingly, cytosolic factor-independent fashion [140]. An excess of T-ag NLS-containing peptide does not compete nuclear accumulation conferred by the Tat-NLS, indicating that it functions through a pathway distinct from that of conventional NLSs. As mentioned above, the latter are nuclear entry rather than nuclear retention signals, but the Tat-NLS appears to be both since it can confer nucleoplasmic accumulation even in the absence of an intact nuclear envelope, implying that it mediates binding to nuclear components. Intriguingly, the Tat-NLS also appears to confer binding to insoluble cytoplasmic components; nuclear accumulation in the absence of an intact nuclear envelope is blocked in the absence of ATP, as well as by nonhydrolyzable ATP and GTP analogs, demonstrating that ATP is required to effect release from cytoplasmic retention. Taken together, the results demonstrate that, dependent on ATP for release from cytoplasmic retention, the Tat NLS is able to confer both nuclear entry and binding to nuclear components [140]. These unique properties indicate that Tat accumulates in the nucleus through a novel import pathway, which may be shared by the homologous NLSs of the HIV-1 Rev (RQARRNRRRRWRERQRQ[51] Ref. [142]) and the HTLV-1 (human T-cell leukemia virus) Rex (MPKTRRRPRRSQRKRPPTP20) proteins, both of which have been shown to be functional in nuclear targeting [142–145].

B. Nuclear Retention Sequences

The discussion of the properties of the Tat-NLS above has alluded to sequences that are able to contribute to nuclear localization by conferring binding to nuclear components, called "nuclear retention sequences" (NRSs). They play a significant role in the nuclear accumulation of proteins which are able to shuttle between nucleus and cytoplasm through possession either of nuclear–cytoplasmic "shuttle" signals (see next section) or of NLSs in combination with specific nuclear export sequences (NESs—see Sec. 2-D).

C. Shuttle Sequences

Shuttle sequences are targeting sequences sufficient and necessary to confer nuclear import and export on the proteins carrying them, and hence can mediate transport in both directions through the NPC. In terms of sequence, they are distinct from both conventional NLSs and NESs. The best defined is the 38 residue M9 sequence of the human mRNA-binding protein hn-RNPA1[114,115], which is largely hydrophobic, in contrast to conventional NLSs (Tables I and II). A distinct, functionally similar sequence—the KNS sequences of hnRNP—has been recently defined. It contains some polar and charged amino acids, including a few basic residues towards its amino-terminus, but the fact that removal of the C-terminal amino acids QMAY impairs nuclear targeting activity [154] indicates that the sequence is not in any sense similar to conventional basic NLSs (Table IA, B), or NLSs

recognized exclusively by importin β (Table II). Several critical residues within the M9 sequence have been identified, including both N- and C-terminal residues, and G274P275.[114,115] M9-dependent nuclear import requires cytosolic factors, and in particular transportin (Kap104/MIP/karyopherin β2), a 97 kDa homolog of β-importin, [155–157] and Ran (see Refs. [157, 158]). A yeast homolog of transportin, Kap104, has been identified as a mediator of nuclear import of Nab2p and Hrp1p, two mRNA-binding proteins that are related to human hnRNP proteins[159] No correlate of α-importin appears to be involved, but the sequence of events in terms of M9 recognition and Ran-mediated passage through the NPC in the import direction is probably comparable to that for nuclear import mediated by the α/β-importin heterodimer and conventional NLSs.

D. Nuclear Export Sequences

The correlates of NLSs which mediate nuclear export are NESs, the short peptide sequences sufficient and necessary for nuclear export of their respective proteins. In similar fashion to NLSs, these have been identified based on the fact either that mutation or deletion of the NES renders it inactive in mediating nuclear export of the protein carrying it, or that the NES is active in effecting export from the nucleus of a carrier protein such as β-galactosidase. The detailed description of export sequences is out of the scope of this chapter and can be found elsewhere. A summary of all the nuclear entry/exit signals was shown in the figure 4.

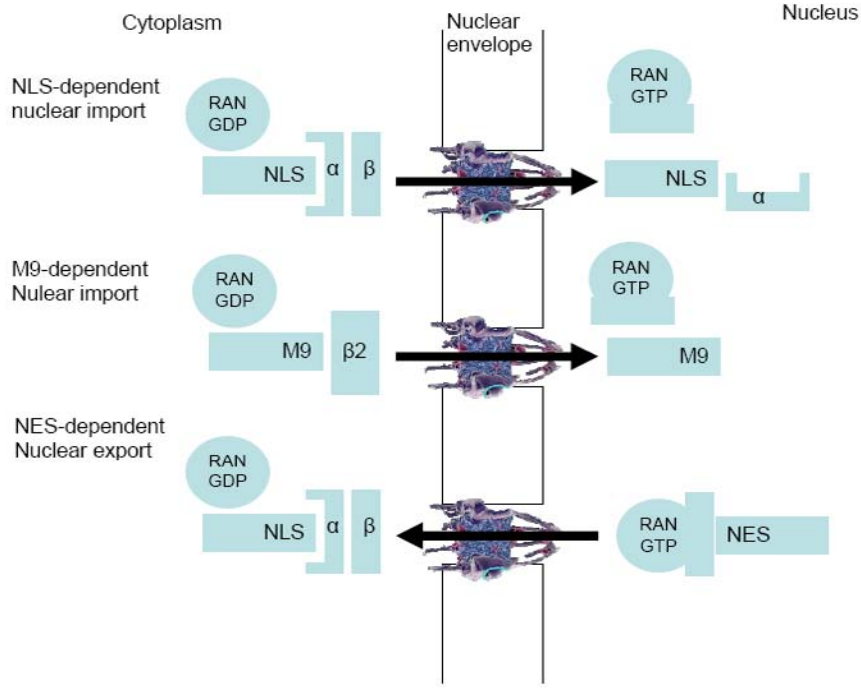

Figure 4.

3. APPLICATIONS OF TARGETING SIGNALS IN DRUG DELIVERY

A. Desirability of Targeting Drugs to the Nucleus in a Medical or Research Context

The growing knowledge with respect to nuclear targeting signals and their regulation can potentially be exploited in a medical or research context. One area beckoning the application of NLSs/prNLSs is that of gene transfer, where plasmid DNA encoding a gene of interest is introduced into relevant cell types either: (1) in a gene therapy context where expression of a particular gene is desired (a) to correct a genetically predetermined biochemical lack of that activity, or (b) to block cell division or effect cytotoxicity in tumor cells, or (2) in a research context where improved DNA transfer is desired (a) to increase expression of a gene product under study in transfected cells, or (b) to increase the probability of homologous recombination in gene knockout experiments where the aim is to oblate completely expression of a particular gene product in order to establish its function in the whole animal. In all of these areas of application, optimization of the transfer of DNA to the nucleus (where gene transcription and DNA rearrangements take place in the cell) through the considered use of NLSs/prNLSs etc.[180, 252, 253] would elevate its concentration in the nucleus and thereby increase nuclear gene expression and the likelihood of homologous recombination. A second area where NLSs/prNLSs may have application is that of drug delivery, where the therapeutic agent in question has its specific target site of action (e.g., DNA, the nuclear envelope etc.) within the nucleus. Agents that might be used in this context largely encompass anticancer drugs; the example that will be discussed below (Sec. 4-C) is that of photosensitisers[180, 254–258] which, upon photoactivation, effect oxidative damage to which the nucleus is a hypersensitive site. The concentration of drug within the nucleus could be increased through optimized nuclear targeting leading to increased cytotoxicity of tumor or other cells.

B. Use of NLSs in DNA Transfer

DNA delivery approaches currently in vogue can be divided into viral and nonviral. Viral-mediated gene delivery is widely used because the ability of viruses such as adenoviruses and retroviruses to enter and express their genomes in the host cell enables high DNA uptake and expression [259]. There are disadvantages with viral systems, however; adenovirus, for example, can be produced in relatively large quantities and efficiently infect cells, albeit transiently, but causes problems because of its immunogenicity. Retroviruses and adenovirus-related viruses can be used to transfect dividing cells stably, but are difficult to produce in high enough titers [260]. Non viral gene transfer strategies, including receptor- and cationic liposome-mediated approaches, afford more flexibility in design and construction. The latter, pioneered by Felgner and coworkers, [261] uses cationic liposomes which form complexes with DNA through charge interactions, and bind to the cell surface to trigger DNA-uptake through the cell membrane and subsequent protected delivery into the cytoplasm.[262] A number of different cationic liposomes have been developed including

lipofectin,[263] lipofectamine,[264] and DOTAP.[265] One disadvantage of non viral gene delivery is that it is generally distinguished by transient gene expression, often lasting for only a short period. Although in certain situations—for example, in vascular diseases such as restenosis after angioplasty where cellular proliferation peaks in the first few weeks after arterial injury [266–268]—this may in fact be desirable, the inclusion of DNA carrying replication origins, a centromere and telomeres can allow the maintenance of the introduced DNA through its segregation in mammalian cells (see Ref. [269]). To achieve high transfection efficiencies, it is necessary not only to be able to facilitate the transit of the DNA across the cell membrane and into the cell, but once within the cytoplasm, the DNA must be transported through NPC and into the nucleus for transcription and expression to occur. Several studies have demonstrated that this is a critical limiting step in gene transfer. Boutorine and Kostina,[270] for example, estimated that less than 1% of plasmid molecules introduced into the cytoplasm eventually reach the nucleus. Zabner *et al*. [271] used gold-labeled DNA complexed with the cationic lipid DMRIE/DOPE to demonstrate that less than 50% actually expressed the encoded reporter gene, and based on these results and those from DNA and DNA/lipid microinjection they concluded that the most critical limitation to successful gene transfer is the movement of the DNA from the cytoplasm to the nucleus. *In vitro* studies have shown that DNA entry into intact mammalian nuclei occurs through the NPC, is energy dependent, and can be inhibited by WGA. [272] Nuclear import of DNA is independent of cytosolic factors and occurs in a size-dependent manner, such that DNA greater than 2 kD in size enters the nucleus with reduced efficiency compared to a 1 kb DNA [272].

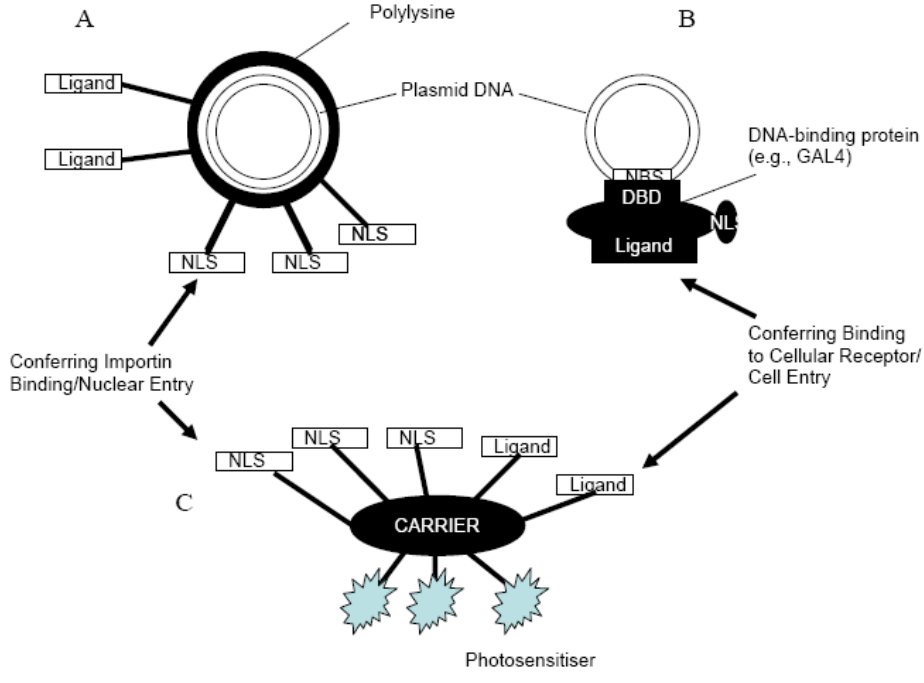

Figure 5.

Since therapeutic genes are generally larger than 2 kb, additional measures need to be taken in order to increase the efficiency of nuclear DNA uptake in mammalian cells, one of

which is the inclusion of NLSs. NLSs/prNLSs can of course not be simply attached to plasmid DNA directly, meaning that carrier molecules are required. Several approaches have been employed [see Figure 5AandB], including the use of either polylysine or DNA-binding proteins, including histones and other chromatin components, where NLSs can be either: (1) intrinsic to the DNA-binding protein or histone, (2) chemically cross-linked to the carrier, or (3) included in the coding sequence of fusion proteins encompassing part or all of a DNA-binding protein/histone etc. The available data with respect to each of these approaches in terms of nuclear targeting is depicted below (Figure 5).

1. Polylysine as an NLS

Because of its highly positive charge, polylysine has been widely used as a carrier for DNA molecules through charge interaction [273–275] and the fact that ligands and peptides can easily be coupled to it using conventional cross-linking procedures [Figure 5(A); see Ref. [252, 253]. The resemblance of polylysine to the T-ag NLS has prompted suggestions that polylysine, apart from its carrier role, could also mediate nuclear targeting [276–278]. Localization studies in several different systems, [253, 279] however, suggest that this is unlikely; polylysine microinjected into *Tetrahymena thermophilia,* for example, does not accumulate in the nucleus [279]. An ELISA-based binding assay has been used to demonstrate that polylysine can indeed be recognized by importin α/β, but that binding is absent when polylysine is complexed with plasmid DNA. In contrast, polylysine to which the T-ag prNLS is covalently linked as a peptide, however, is recognized by importin α/β even when complexed to DNA, indicating that in this case, the NLS is accessible; indeed, Tag-prNLS peptide-complexed polylysine exhibits enhanced transfection efficiencies compared to uncomplexed polylysine (manuscript in preparation). Polylysine alone does enhance transfection compared to in its absence, probably due to its ability to condense DNA into a small toroid form (c. 40 nm in diameter) [275] which is not only more easily taken up by cells, but also able to pass more easily through the NPC. Another consequence of this effect of polylysine may be to protect intracellular DNA from degradation by DNA [280].

2. Use of Specific DNA Binding Proteins

An alternative to polylysine as a carrier is to use DNA-binding proteins such as GAL4 where the specific nucleotide recognition site (a 17 base pair sequence in the case of GAL4) is included within the sequence of the plasmid DNA to be transfected [see Figure 5(B)] [137, 281, 282]. Since GAL4 contains an NLS (see Sec. 2-A.2), its amino-terminal 147 amino acids are particularly attractive as a DNA carrier, able to function within fusion proteins containing other modular domains conferring cell surface binding and internalization such as Erb-2-endotoxin [281] and invasion [282]. While GAL4 can clearly enhance DNA transfection of plasmids containing the specific recognition sequence, [137, 281, 282] recent results suggest quite clearly that these effects are almost certainly not through enhancement of nuclear targeting since binding of the GAL4 NLS by importin β competes DNA binding activity and DNA-bound GAL4 appears to lack an NLS that can be recognized by importin (see Sec. 3-B.3). [137]. That DNA binding and NLS activity on the part of GAL4 are mutually exclusive has profound implications for its use as a carrier in non viral gene delivery. The enhancement by GAL4 of DNA transfer must be attributable to other effects of GAL4, such as DNA compaction to enable more efficient cell entry, or protection of the DNA from degradation after entry into the cell [137]. Whether addition of a second NLS, such as that of T-ag, into

GAL4 may improve nuclear targeting and enhancement of transfection requires direct investigation.

3. Use of Chromatin Components

Different histones, DNA-binding proteins employed in the packing of DNA into chromatin, have also been used as DNA carriers [275,283–285]. Since some histones have been shown to contain functional NLSs (e.g., Ref. 86) they can be considered comparable to GAL4 in this respect although no specific DNA recognition site is required by histones. Histone H4 in a ternary complex with DNA and transferrin–polylysine was found to increase DNA expression by 4–7-fold, [275] which was not attributable to DNA condensation as determined by electron microscopy. Histone H1 and H3 mediated about 2- and 3-fold increases in DNA expression, respectively, in the same study, [275] while total histones from calf thymus enhanced transfection up to 5-fold [285] Galactosylated histone-DNA complexes internalized via the acyloglycoprotein receptor have also been used, indicating optimal increase in gene expression for galactosylated histone H1 [284]. An engineered recombinant histone H1 containing the T-ag NLS has also been used and found to enhance transfection efficiency, although not significantly compared to histone H1 alone [283]. Cells microinjected with NLS-H1-DNA and H1-DNA also showed no difference in their foreign gene expression, but no real conclusions with respect to the effect of the NLS could be drawn from these studies since the histone H1 and recombinant H1 were from different species, and different amounts were used [283]. It can be concluded that there is little direct evidence supporting the notion that histones may enhance transfection through facilitating nuclear targeting. The chromatin component HMG-1 has also been used in non viral DNA transfer in conjunction with liposomes, and like histones, found to enhance both DNA delivery to the nucleus and transfected gene expression [286, 287]. DNA was delivered 3–5 times more efficiently in the presence of HMG-1, compared to BSA, and persisted in the nucleus of transfected adult rat liver cells for over 7 days [286]. Similar results were obtained *in vivo*, transfected gene expression persisting for over 9 days where HMG-1 was used [284]. The correlation between the increased rate of nuclear delivery of DNA and enhanced gene expression implies that the latter effect of HMG-1 in part functions through facilitation of DNA targeting to the nucleus; HMG-1 has indeed been shown to be able to cotransport a specifically reacting 170 kD monoclonal antibody to the nucleus, [150] and therefore must contain a functional NLS. Interestingly, HMG-1 has been observed to demonstrate developmentally regulated nuclear localization (see Ref. [288]) indicating that it may also be useful for regulated targeting of DNA to the nucleus.

C. Use of NLSs in Photosensitizer Delivery to Tumor Cells

Photosensitizers such as porphyrins are molecules which produce active oxygen species upon activation by visible light, and are currently being extensively used in photodynamic therapy (PDT) to treat cancer and other clinical conditions [289, 290]. Since normal cells are able to accumulate porphyrins, however, and porphyrins are only excreted slowly from the body, prolonged skin photosensitization as well as other effects can be a problem, leading to normal cell and tissue damage [291–293]. A high priority with respect to PDT is thus to increase the specificity of the uptake of photosensitizers in the desired target cells, thereby

enabling the active dose of porphyrins administered to patients to be reduced. It has been previously shown that the photosensitizer chlorin e6 has significantly higher photosensitizing activity when present in conjugates containing specific ligands such as insulin or concanavalin A, and thus was able to get internalized by receptor-expressing cells [254–256]. Photosensitization could be competed by incubating cells in the presence of an excess of unconjugated ligand, [254–256] indicating that cellular uptake was receptor-dependent. Since only cells expressing specific receptors are targeted in this approach (see Refs. [254–256]), it is clear that it enables selectivity in terms of the cell types targeted for photosensitization. Due to the fact that injury induced by singlet oxygen, comprising 80% of all of the active oxygen species generated upon porphyrin activation, is localized within less than 0.1 mm of the site of its production, the effect of photosensitizers is integrally related to their site of cellular accumulation [289]. While most porphyrins such as chlorin e6 and hematoporphyrin derivatives localized largely at the plasma membrane, [294,295] it is known that intracellular sites, and particularly the nucleus, are much more sensitive sites for photodynamic damage [294, 296, 297] Consistent with this, results from recent studies [254–256] indicate that the enhancement of photosensitization effected by internalizable conjugates is directly attributable to their ability to be internalized (and thereby damage intracellular sites), since treatments preventing internalization severely reduce photosensitization. The directed delivery of photosensitizers to particularly sensitive subcellular organelles such as the nucleus using specific targeting signals is central to performing efficient PDT. Insulin-containing conjugates to which variants of the T-ag prNLS were linked have recently been used in order to target chlorin e6 to the nucleus [257, 258]. The T-ag prNLSs were included either as peptides cross-linked to the carrier BSA [see Figure 5(C)], or encoded within the sequence of a β-galactosidase fusion protein carrier. Results for photosensitization demonstrated clearly that NLSs increase the photosensitizing activity of chlorin e6, maximally reducing the EC50 by a factor of over 2000-fold [257, 258]. Importantly, conjugates containing the T-ag NLS alone were not as efficient photosensitizing agents as those containing the T-ag NLS together with the CK2 site (see Sec. 3-A.2, 3-A.3), indicating that increased efficiency of nuclear import was a critical factor in enhancing photosensitization. NLS mutant derivatives did not enhance photosensitizing activity to the same extent as those containing an intact functional NLS.

Clearly, constructs such as those used in these studies [see Figure 5(C)] have important application in cell-type-specific PDT. Increased specificity in the administration of PDT comes from technical advances with respect to specific photoirradiation of tumor tissue through the use of optical fibres and so forth. Future work may include optimizing nuclear targeting through modification of NLS-function modulating flanking sequences such as phosphorylation sites (see below) and substituting insulin with alternative ligands in order to target photosensitizers specifically to particular tumor cell types. A recent advance [258] has been to employ adenoviruses as an endosomolytic agent to increase passage from endosomes to the cytoplasm, to enable the NLSs to function even more efficiently in their targeting role.

D. Future Prospects—Potential Use of Targeting Signals

The indications from the studies outlined above (Sec. 4-A–C), especially with respect to PDT, are that considering the use of the increasing knowledge with respect to the various

targeting signals for nuclear-cytoplasmic transport outlined in Sections 2 and 3 may be invaluable in research and clinical contexts. Where this may be desirable in either of the latter applications, targeting molecules of interest to the nucleus in a strictly regulated fashion may be achieved through the use of particular regulated NLSs. Since these regulated NLSs appear to be active in a variety of different cell types and species (see Sec. 3-A), any of the prNLSs, srNLSs, or drNLS listed in Table II could be considered a potentially useful targeting signal [124, 208] Engineered prNLSs such as the PK-A site derivative of the T-ag prNLS209 discussed in Section 3-A, where sites for kinases with well-understood regulation may be introduced into prNLSs to effect tightly regulated nuclear targeting, may also have important application. Regulated CRSs, NRSs, and even NESs (regulated NESs ?) or shuttle sequences (when the parameters modulating their directionality are understood and able to be manipulated), may also be used in conjunction with prNLSs to ensure that nuclear targeting is precisely scheduled according to need. Alternatively, prNLSs such as those of T-ag or Dorsal, where phosphorylation site serine/threonines are substituted by aspartic acid/glutamic acid residues, [200–202, 205, 206, 208] may be used as constitutive prNLSs where phosphorylation is not required for enhanced nuclear import. A further interesting possibility is the use of ligands to effect cell-specific targeting, and particularly of NLS-containing ligands such as fibroblast growth factor (FGF), [102, 117] platelet derived growth factor, [91] interleukin (IL)-1a[96] or IL-5[116] (see Refs. [180, 181]). These molecules are particularly attractive as targeting agents since they provide both receptor-specificity and nuclear targeting capability. Of such molecules, FGF-2 coupled to polylysine has thus far been successfully used, together with endosome-disrupting peptides, as an agent for DNA delivery, although no assessment has been made as to the extent to which the FGF-2 NLS plays a role [298]. A further area of development will be the application of modular sequences conferring efficient exit from endosomes in similar fashion to adenoviruses. These include sequences from various viral or bacterial proteins or synthetic peptides, [299–302] and in particular amphipathic helices such as the 20 amino acid peptide INF1 derived from influenza virus hemagglutinin [299] or the synthetic peptide GALA [302,303] which is able to disrupt membranes at acidic pH. These endosomolytic sequences, in identical fashion to NLSs or other targeting sequences, could be included in a delivery conjugate either as peptides cross-linked to carrier molecules or encoded within the sequences of fusion proteins. A further possibility in terms of exploiting the cellular nuclear transport apparatus to effect specific targeting to the nucleus is to use specific portions of the signal-binding receptor molecules themselves. For example, the amino terminus or IBB domain (see Table II) of the importin α subunit (amino acids 1–65 of *Xenopus* importin α), which binds importin β with high affinity, [59] could be used as an NLS-substitute able to be recognized by importin β and targeted to the NPC and subsequently the nucleoplasm (see Ref. [59]). Since, however, the IBB at sufficiently high concentrations can compete for cellular importin β and inhibit nuclear transport (e.g., Ref. [139]), it would be important to ensure that targeting rather than transport blocking activities were achieved, unless of course a cytostatic/cytotoxic effect through the latter was desired. Importin β mutants unable to bind Ran (e.g., importin β amino acids 45–876—see Ref. [139]) act as dominant negative mutants able to block all tested nuclear import and export pathways, [304] and these could also be used as cytotoxic agents in a given context. Thus, knowledge with respect to the nuclear transport pathways could be exploited in this fashion to inhibit the growth/proliferation of tumor or other cells; as our understanding increases, further possible applications in terms of therapeutic approaches will arise. Novel

nuclear trafficking pathways used by viral proteins and/or oncogene products can of course be exploited in terms of antiviral/anticancer therapies. Mention was made in Section 2-A.3, for example, of the unique properties of the HIV-1 Tat-NLS, [140] (possibly shared by the NLSs of other related viral proteins such as Rev and HTLV-1 Rex) where known targeting signal receptors and Ran do not appear to be required for transport, and ATP hydrolysis appears to be specifically required for release from cytoplasmic retention [140]. If the nuclear import pathway mediated by the Tat-NLS is indeed shown to be unique to other viral proteins and is essential for viral replication or maintenance, its retention/ATP release property could be used as a target for therapeutic approaches designed to specially inhibit this pathway, as opposed to nuclear protein import or export in general. Analogous possibilities may be found for other viral proteins or oncogene products.

CONCLUSION

The recent progress with respect to understanding the signals mediating the transport of proteins in both directions through the NPC, and cellular proteins interacting with the signals to affect the transport process has made possible a number of advances in terms of the use of this information in a clinical setting. In particular, our understanding of the mechanism of regulation of the process, and of how we may exploit the cellular transport machinery itself in a therapeutic situation, especially where there may be transport pathways specific to particular viruses, has advanced considerably. We appear to be slowly but surely approaching a situation where application of regulated targeting signals can be used to deliver drugs or DNA molecules to the nucleus in an efficient, precisely scheduled, and specific fashion. This should enable high concentrations of therapeutic agents in the nucleus to be achieved as desired/required, to increase their efficacy and specificity, and simultaneously reduce the dose of the agent necessary to be delivered to the patient, thereby diminishing undesired side effects. While, as indicated in this chapter, the use of targeting signals in this context has thus far been fairly restricted, it seems clear that interest and progress in this area should greatly increase over the next few years, to the point where molecules will indeed be targeted to the nucleus precisely and efficiently, as and when desired/required.

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Chapter 6

BIOMAGNETIC APPROACHES APPLIED TO DRUG DELIVERY STUDIES

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ABSTRACT

Biomagnetism involves the measurement and analysis of weak magnetic fields detected from physiological activity or magnetic sources within the human body. The implementation of biomagnetic methods in pharmaceutical studies as a non-invasive monitoring of drug delivery is currently an alternative to nuclear medicine. This chapter will present a short review of biomagnetic techniques, particularly AC Biosusceptometry (ACB) as an innovative method in pharmaceutical research.

ACB uses induction coils for recording the magnetic flux variation obtained from the response of a magnetic material ingested. This material has a high magnetic susceptibility that produces a strong response when an alternating magnetic field is applied in a biological system containing small amounts of magnetic material.

As this technique has been continuously improved, novel instrumental arrangements became a suitable tool not only for gastrointestinal (GI) motility studies as well as an alternative method for pharmaceutical purposes.

A multisensor system was initially implemented to characterize the disintegration process of tablets *in vitro* in the human stomach. Disintegration process was quantified by the transition of a magnetic marker (non-disintegrated dosage form) to a magnetic tracer (disintegrated dosage form). Moreover, ACB was employed to monitor magnetic tablets

and hypromellose capsules in human GI tract showing the disintegration in colonic region and intestinal transit.

Imaging techniques provide valuable information on the *in vivo* performance for any dosage forms. ACB was able to characterize the disintegration process through magnetic images introducing a novel concept in imaging of biological systems.

Several pharmaceutical approaches which have been used for targeting drugs to the proximal GI tract are based on gastroretentive drug delivery systems. In addition, colon-specific delivery has renewed interest in the development of therapeutic agents for treating colonic diseases providing systemic absorption of drugs susceptible to enzymatic digestion in upper GI tract.

Recently, ACB has been used in the monitoring of multiparticulate gastroretentive and colonic delivery systems in human GI tract, since multiparticulate systems are ideal for controlled drug delivery and have advantages over single unit formulations.

This magnetic method will be associated with pharmacokinetic parameters ("magnetopharmacokinetics") to predict the drug absorption for optimized pharmacotherapy, deserving the same importance as conventional techniques in pharmaceutical research. Biomagnetic techniques are harmless and have added new insights in the evaluation of solid dosage forms in human GI tract.

1.1. INTRODUCTION

Nowadays, biomagnetic techniques are capable to evaluate conventional dosage forms and also the most sophisticated drug delivery systems in the human gastrointestinal tract. A primary difference of this is the non-invasivity and radiation-free characteristics which is a potential advantage over classical methods.

In order to understand how these methods may be employed for pharmaceutical investigation, it is essential to know the complex interactions among the drug, the dosage form and the human gastrointestinal tract. Consequently, it allows optimizing the drug delivery systems to increase the effectiveness of drugs orally administered.

In this chapter, we will discuss a basic knowledge of the Superconducting Quantum Interference Devices (SQUIDs), Magnetoresistive Sensors, Hall-Effect Sensors, and Induction Coils and how these techniques can be applied in the pharmaceutical research.

1.2. HUMAN GASTROINTESTINAL TRACT – THE TARGET

Despite some disadvantages oral administration remains the most common route for drug delivery. Moreover, the gastrointestinal (GI) tract is an appropriate site for drug absorption. Absorption of an orally administered drug from the gastrointestinal tract can be regarded as a part of a serial process that includes the drug release by the disintegration of the dosage form, the dissolution of the drug, the solubility and physicochemical properties and its effective permeability coefficient.

Additionally, physiological properties of the GI tract such as the diameter, length and surface area, pH profile, the gastric emptying, the intestinal transit times and the GI motility

are the major determining factors in bioavailability of the drug and can limit the fraction dose absorbed.

Gastrointestinal tract is a complex environment and presents regional differences that must be fully investigated to provide more reliable information when a local or systemic effect is desirable.

1.2.1. Absorption from Stomach, Small Intestine and Colon

Due to its nature, the primary function of the stomach is the digestion this organ is subdivided into two compartments performing different functions. The proximal region acts as a reservoir whereas the distal stomach has a grinding and mixing capacity. The coordinated actions of these regions allow the gastric emptying to the duodenum at a required rate. The thick mucus layer with a small surface area and high electrical resistance limits the absorption process in the stomach.

In contrast, the small intestine has an extremely large surface area with low electrical resistance which facilitates the absorption of nutrients and, consequently, most of the drugs. The absorption process in the small intestine is modulated by motor activity including aboral movement of its content and by chemical digestive process.

The colon is a suitable site for the slow absorption of drugs which are targeted at the large intestine or designed to act systemically. Colonic physiology is complex and influenced by many factors including diet, bacterial metabolism, and also colonic mixing and transit time. Compared with the small intestine, the colon has a lower surface area that is compensated by the slow transit rate, which provides a significant opportunity for the absorption of drugs and other materials.

1.3. GASTROINTESTINAL VARIABLES

The dynamic interaction between the ***gastrointestinal*** tract ***variables*** and the orally administered dosage form determine the rate and extent of drug absorption. The major physiological ***variables*** are gastrointestinal motility, gastric emptying time, intestinal transit time, and pH. Moreover, the physicochemical properties of a drug such as solubility and particle size, beyond the formulation parameters including controlled-release mechanism, pH sensitivity, size, shape, and density can also affect the absorption. An understanding of the factors involved in drug absorption and how the gastrointestinal variables can interfere with this process is important to develop more reliable drug delivery system.

1.3.1. Gastrointestinal Motility

The gastrointestinal tract is structurally complex and its functions depend on coordinating actions. The gastrointestinal smooth muscle has the ability to produce coordinated motion which is called motility. Hence, the knowledge of the motility aspects is fundamental to understand the gastrointestinal physiological functions and how the interactions between

these parameters and the orally administered solid dosage forms can interfere with drug release.

Spatial and temporal patterns of contractions or relaxation of smooth muscle cells and their integration are modulated by a complex neuro-hormonal regulatory system control. Basically, gastrointestinal motility is organized into two motor patterns according to the prandial state.

After cessation of the digestive processes a cyclic motility pattern, called interdigestive migrating motor complex (MMC) initiates. This pattern is organized into alternating cycles of activity and quiescence, as illustrated in Figure 1. Phase I is characterized by a period of motor quiescence; phase II consists of an irregular phasic contractile activity; and phase III presents a short period of intense and rhythmic contractions, termed *housekeeper waves* (Quigley, 1996). During the phase III occurs the emptying of indigestible particles, including solid dosage forms, retained in the stomach. Contractile waves of the MMC propagate distally from the stomach to the terminal ileum and are interrupted by food ingestion. Phase IV is the transition period between phases III and I.

With the ingestion of food the MMC is replaced by the fed pattern characterized by contractions of variable amplitude and frequency according to gastrointestinal segment (Hasler, 1995). This pattern persists as long as the bulk of food remains in the stomach and depends on multiple factors including energy content, volume, and the nature of nutrients.

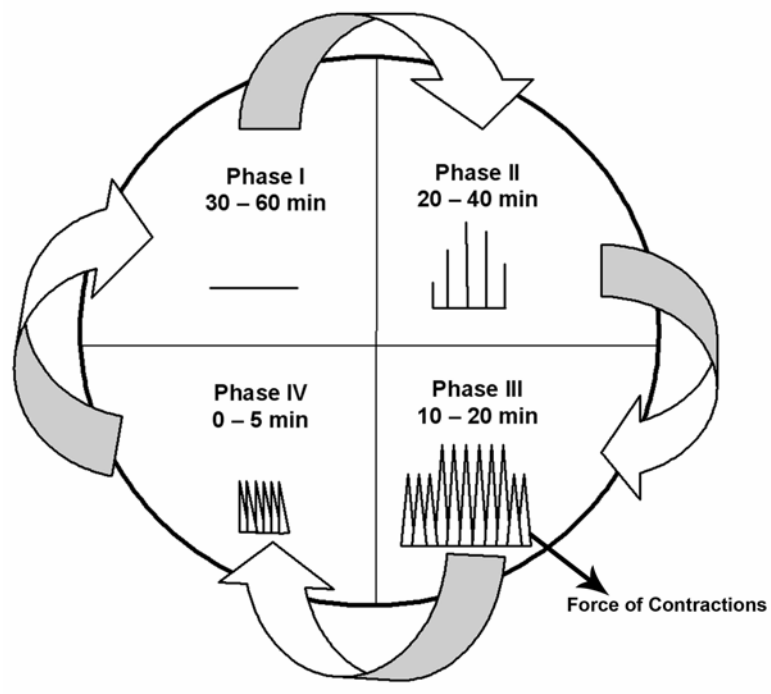

Figure 1. Interdigestive gastrointestinal motility pattern (Modified from Chawla et al, 2003, with permission).

Contractions are based on electrophysiological properties of smooth muscle and triggered by a non-neural pacemaker system in the wall of the gut known as interstitial cells of Cajal. Electrical activity propagates through the gut because every region of phasic muscle has intrinsic pacemaker capability (Camilleri, 2006). However, the necessary discontinuity in the

pacemaker cell network between segments of the gastrointestinal tract generates a pacemaker frequency gradient. The frequency registered in the stomach is approximately 3 contractions per minute (cpm), reaching 11-12 cpm in the duodenum with decreasing gradient to distal ileum. In the colon two rhythms appears to occur: a frequency of 5-6 cpm and another around 3 cpm (Rao and Schulze-Delrieu, 1993; Frexinos and Delvaux, 1993). Moreover, the human colon presents a duality of the motility pattern: phasic contractions ensure the mixing and the aboral progression of colonic contents while the tonic activity acts as a brake. Thus, complexity of the electrical motor activity, differences in recording techniques and methods of data analysis might explain the discrepancies observed until now in this frequency gradient.

Motility patterns determine the rate of transit and residence time for pharmaceutical dosage forms in the human gastrointestinal tract, and hence the drug release.

1.3.2. Gastric Emptying

Gastric emptying of solid and liquids are controlled by coordinated actions promoting changes in gastric tone and the propagating gastric peristalsis. Gastric emptying depends mainly on nutrient content, viscosity and volume of the meals.

Ingested non-caloric liquids are rapidly distributed throughout the entire stomach and the emptying begins immediately in a first-order exponential process directly proportional to the volume. On the other hand, solid particles empty from the stomach by fundamentally zero-order kinetics that is independent of gastric volume. Hence, the gastric emptying of solids shows a *lag phase*, where the solids are redistributed and broken down to smaller particles able to pass through the pylorus during the *linear phase*(Siegel et al, 1988). Figure 2 gives an overview of the biphasic characteristic of the gastric emptying of solids.

Gastric emptying rate plays an important role in determining the gastric retention time of oral dosage forms. Density, size and shape of the dosage form, concomitant ingestion of food, simultaneous administration of drugs affecting gastrointestinal motility and biological factors, such as gender, posture, age, sleep, body mass index, physical activity and disease states are the most important parameters influencing gastric emptying rate.

The relationship between the tablet size and its gastric emptying varies significantly among the subjects. Indigestible solids including non-disintegrating dosage forms larger than the pyloric opening remains retained in the stomach for as long as the digestive phase is maintained and are emptied during the passage of the housekeeper wave. Multiple-unit systems containing pellets pass through the constricted pylorus with a gradual emptying profile. Pellets administered with a light meal follow an initial short lag phase and a linear emptying pattern. A delay gastric emptying occurs when pellets are administered with a heavy meal. Therefore, the prandial state is the primary consideration for modulating gastric residence time in relation to drug administration. While feeding substantially delays the gastric emptying of large single-unit preparations, little effect is observed on the emptying of multi-unit preparations which leave the stomach at rates similar to fasting.

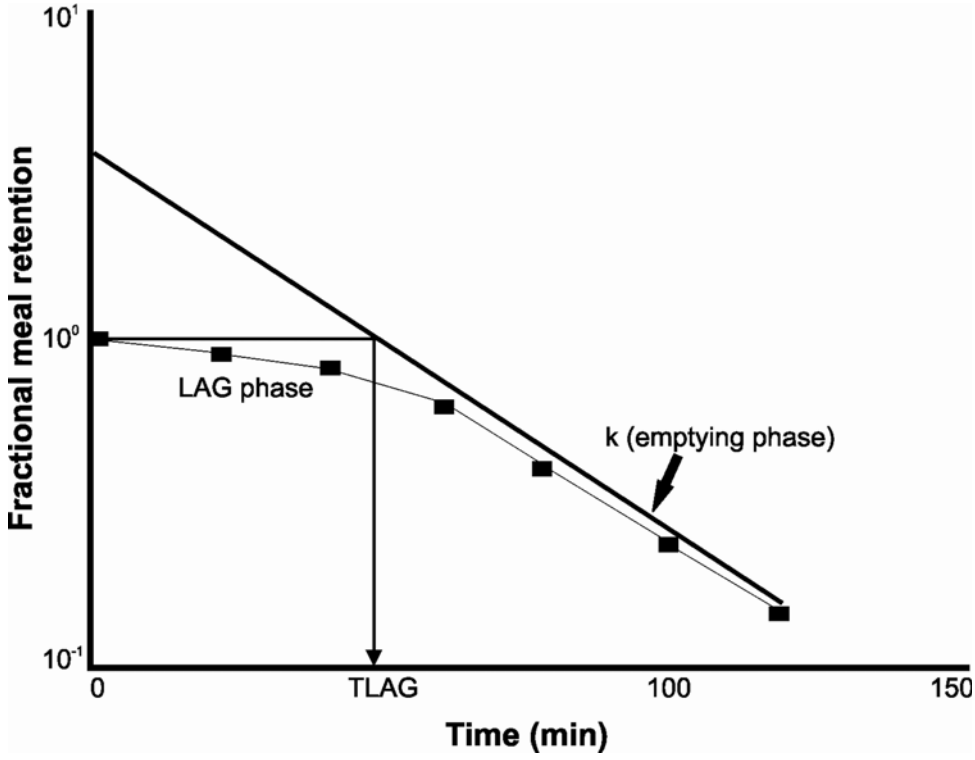

Figure 2. Solid emptying curve fitted of the function $y = 1 - (1 - e^{-kt})^{\beta}$. The *lag phase* is indicated by *TLAG* and *k* is the emptying rate. (From Siegel et al, 1988, with permission).

On the other hand, prolonged gastric retention of the dosage form provides an excellent opportunity for drug absorption, especially for drugs with an absorption window in the upper gastrointestinal tract.

1.3.3. Gastrointestinal Transit

Propulsion of the contents of the small bowel is dependent on the lumen, intestinal wall tone, and the amplitude of intestinal phasic contractions. Age and gender have no effect; however, intersubject variability is expressive.

Similar to the gastric emptying, intestinal transit is influenced by the MMC activity. After a meal, the distribution of small intestinal contents is more uniform and the transit is faster than during the interdigestive period.

For pharmaceutical purposes, the transit of a dosage form through the gastrointestinal tract determines how long a compound remains in contact with its absorptive site. The bioavailability of a drug can be affected by factors that change gastrointestinal transit.

As demonstrated by Davis et al (1986) small intestinal transit time of pharmaceutical dosage forms in humans is relatively constant at around three to four hours and appears to be independent of both the type of dosage form and prandial state.

Compared with the stomach and small intestine, the colon moves its contents slowly. The slow rate of colonic transit is an excellent opportunity to drug delivery since the drug remains

in contact with the mucosa for a longer period than in the small intestine. The residence time in any particular segment of the colon determines the absorption at that site.

Total colonic transit tends to be highly variable and is influenced by factors such as diet, mainly dietary fiber content; stress, disease, drugs and type of pharmaceutical form. When the dosage forms reach the colon, the transit depends on its size since the colonic transit of small units is slower than large ones. Hence, there may be advantages in formulating multi-unit dosage forms for colonic drug delivery to ensure that the entire drug will be released during the transit time.

1.3.4. Gastrointestinal Fluids and pH

Drug absorption and clinical response from an orally administered dosage form depends on drug solubility in the gastrointestinal contents and the surface area. Aqueous solubility, crystalline form, drug lipophilicity and pK in relation to the gastrointestinal pH profile determine the solubility of a drug. The dissolution rate of a drug is a function of the surface area and diffusion coefficient of the compound in the dissolution media. Variability in the constituents of the gastrointestinal fluids such as electrolytes, enzymes, bile acids will affect the dissolution and consequently the absorption and bioavailability of drugs.

The pH gradient in the human gastrointestinal tract is one of the most important influences on the solubility of ionizable drugs. The gastrointestinal pH is not constant rather it is influenced by factors such as diet, disease, gases, fatty acids, and other fermentation products exhibiting intra- and inter-subject variation. This variation in pH may significantly influence the performance of orally administered drugs.

The mean value of gastric pH in fasted healthy subjects is 1.1±0.15. In addition, the mean gastric pH in fed state in healthy subjects arises to 3.6±0.4 and returns to basal level in about 2 to 4 hours.

Evans et al (1988) reported that the mean pH in the proximal small intestine and terminal ileum is 6.6 ± 0.5 and 7.5 + 0.4, respectively. There is a decrease in pH to a mean of 6.4 ± 0.4 in the caecum, apparently due to the presence of short-chain fatty acids produced by bacterial fermentation of dietary fiber. The pH then rises progressively from the right to the left colon, with a final value of 7.0 ± 0.7. The variation in the pH profile is exploited as an alternative to deliver drugs in a controlled manner.

1.4. ORAL DRUG DELIVERY

Oral administration is a widely accepted route for drug delivery, mainly due to patient's acceptance. For this reason, solid dosage forms are often chosen since it affords of advantages such as stability, ease to ingestion, and convenience of dosing.

When a solid dosage form is administered, the active substance has to be released first so that it can be absorbed. If this process is slow or incomplete the bioavailability of a drug will be inadequate. Therefore, the appropriate choice of excipients and its consistency of performance have critical importance to the formulation development.

The pharmaceutical development of solid dosage forms is a key focus to assure the product quality. The challenge is to use a therapeutic agent whose clinical response can be predicted among individuals or to establish the in vitro drug release information to in vivo drug profiles.

1.4.1. Drug Release from Tablets and Capsules

Tablets and capsules are still the most popular solid dosage forms due to ease of manufacture, convenience of dosing and stability. Active substances are available for absorption after release from solid dosage form by the disintegration process. The disintegration is a time-dependent process that promotes a fast fragmentation of the dosage form under the action of disintegrants (Melia and Davis, 1989). Appropriate choice of disintegrant and its consistency of performance have critical importance to the formulation development.

Proposed mechanisms for the action of disintegrants include water uptake through wicking, swelling, deformation or particle repulsion. However, the swelling of disintegrant particles is perhaps the most widely accepted mechanism for tablet disintegration since almost all disintegrants swell to some extent. A detailed revision about the mechanism of action of disintegrants was reported by Lowenthal, 1972.

The most important quality parameter of drug formulations is bioavailability, which is limited by the extent and rate of drug release. The bioavailability is strongly influenced by the type of dosage form, the properties of its components, and the production methods. Because of its influence on the bioavailability of drugs administrated on conventional tablets, Caramela et al (1988) described a mathematical approach to analyze the disintegration process. According to this model, the disintegration force as a function of time is dependent of compression force. Hence, an increase in the compression force causes a reduction of tablet porosity and, as a consequence, a linear increase of the disintegration time.

Differences in the qualities of excipients as particle size distributions, surface properties and impurities can alter the chemical, physical, and biopharmaceutical properties of solid dosage forms.

1.4.2. In Vitro – In Vivo Correlation

A good understanding and application of the concept of the *in-vitro* and *in-vivo* correlation (IVIVC) is essential for pharmaceutical development of solid dosage forms. The concept of IVIVC refers to a mathematical model describing the relationship between *in vitro* drug dissolution characteristics and *in vivo* bioavailability parameters. Five correlation levels based on the suitable correlation reflecting the complete plasma drug profile following the dosage form administration is defined (Demírtürk et al, 2003; Emami, 2006).

Level A is the highest category of correlation and represents the point-to-point relationship between *in vitro* dissolution profile and *in vivo* absorption rate. In this correlation level, percent of drug absorbed can be calculated by methods such as Wagner-Nelson, Loo-Riegelman or numeral deconvolution. The objective of a Level A correlation is to achieve a direct measurement of *in vitro* dissolution profile able to predict the *in vivo* performance.

Level B correlation utilizes statistical moment analysis to compare the *in vitro* mean dissolution time (MDT_{vitro}) to *in vivo* mean dissolution time (MDT_{vivo}) or mean residence time (MRT_{vivo}). MDT_{vitro} is the mean time for the drug to dissolve under *in vitro* dissolution medium. MDT_{vivo} is defined as the mean time for *in vivo* drug dissolution from a dosage form and the MRT_{vivo} is the time that the drug remains in the body. This level does not reflect a point-to-point correlation and cannot alone to predict a reliable *in vivo* bioavailability data.

In the Level C correlation, one dissolution time point ($t_{50\%}$ or $t_{90\%}$) is compared to one mean pharmacokinetics parameters such as traditional area under the plasma concentration time curve (AUC), t_{max} or C_{max} as a single point correlation. The doses not represent the complete plasma drug concentration curve which is fundamental for an ideal indicative of the dosage form performance. Therefore, this level represents a partial relationship between dissolution and absorption limiting to predict *in vivo* data.

Multiple-level C relates one or several pharmacokinetics suitable parameters to the amount of drug dissolved at several points of the dissolution profile. In this level, a relationship between *in vivo* performance and *in vitro* data can be demonstrated by providing a correlation over the entire dissolution profile and one or more pharmacokinetics parameters.

Level D is a qualitative analysis, not a formal correlation, but helpful in the development of formulation or processing.

An IVIVC is developed concerning formulating parameters, dissolution conditions and pharmacokinetics/pharmacodynamics models. Based on Biopharmaceutical Classification Systems (BCS), the permeability of a drug is a key factor in establishing the feasibility of a formulation and the interpretation of the observed *in vivo* absorption profile of a dosage form (Amidon et al, 1995). Thus, BCS allows estimating the contribution of dissolution, solubility and intestinal permeability in the rate and extending of drug absorption from solid oral dosage forms.

According to knowledge of the solubility and permeability characteristics of specific drugs, it is possible to predict which variables can alter the drug absorption from orally administered dosage forms. In the BCS, pharmaceutical compounds are classified based solely on its solubility and intestinal permeability.

Class I drugs exhibit a high solubility and high permeability and are well absorbed. IVIVC is expected if dissolution rate is slower than gastric emptying. Class II compounds presents low solubility, high permeability and exhibit dissolution-rate limited absorption. IVIVC is expected if *in vitro* and *in vivo* dissolution rate are similar. Class III groups drugs that presents high solubility and low permeability, with high variability in rate and extend of absorption. This variation can be attributed to gastrointestinal transit, luminal contents and membrane permeation rather than dosage form characteristics. As absorption rate is controlled by permeability, limited or no IVIVC is expected. Class IV are low solubility and low permeability compounds with poor oral bioavailability.

Following this classification, IVIVC is generally expected for highly permeable drugs or drugs under dissolution rate-limiting conditions. The AC Biosusceptometry may be useful as a tool to correlate *in vitro* drug release information to the *in vivo* profile considering the lack of techniques able to provide this kind of analysis. The effectiveness of IVIV correlation from theory to practice has been recognized to minimize the need for additional bioavailability studies in the formulation design.

1.4.3. Formulation Strategies for Delivery Systems

Strategies to designing oral solid dosage forms and delivery systems to release drugs at optimal rate to improve the therapeutic effectiveness and to minimize adverse effects are desirable.

The enhancement of therapeutic efficacy of a drug depends on the clinical condition of the patient, its mechanism of action, including side effects, the dose–response relationship, its biopharmaceutical and physicochemical properties, and its route of administration. Modern pharmaceutical approaches for delivering drugs in a controlled manner include gastroretentive and colonic systems.

1.4.3.1. Gastroretentive Systems

A variety of drugs are characterized by a narrow absorption window in the upper gastrointestinal tract. An absorption window exists due to physiological or physicochemical factors, including the pH profile, and the presence of certain enzymes in a specific gastrointestinal region.

Pharmaceutical dosage forms with gastroretentive properties are able to delivery drugs with narrow absorption window in a controlled manner by releasing the drug for a prolonged period of time, supplying continuously its absorption site. This approach has applications for local drug delivery to the stomach and proximal small intestine ensuring optimal bioavailability (Davis, 2005). Furthermore, gastric retention provides better availability and therapeutic effectiveness of drugs with substantial benefits for patients.

Figure 3 illustrates the possibilities to achieve gastric retention based on swelling, mucoadhesive, hydrogel, floating or high-density systems. Floating dosage forms are low-density single or multi-unit systems that have sufficient buoyancy to float over the gastric contents providing continuously drug release at the desired rate. Floating systems improve the absorption of various drugs in the upper gastrointestinal tract, however cannot provide for a long gastroretentive effect. In addition, the effect is dependent on the amount of food and liquid within the stomach, which makes these systems relatively unstable.

High-density systems are based on the assumption that the dosage forms might be positioned in the lower part of the antrum and generally are made of heavy materials. Initially these systems appeared as greatly promising, but the main disadvantages are the dependence on the prandial state and the need to use relatively large and heavy structures for obtaining the desired effect.

Mucoadhesive systems are intended to extend the gastric retention by adhering them to the gastric mucous membrane by hydration, bonding, or receptor mediated.

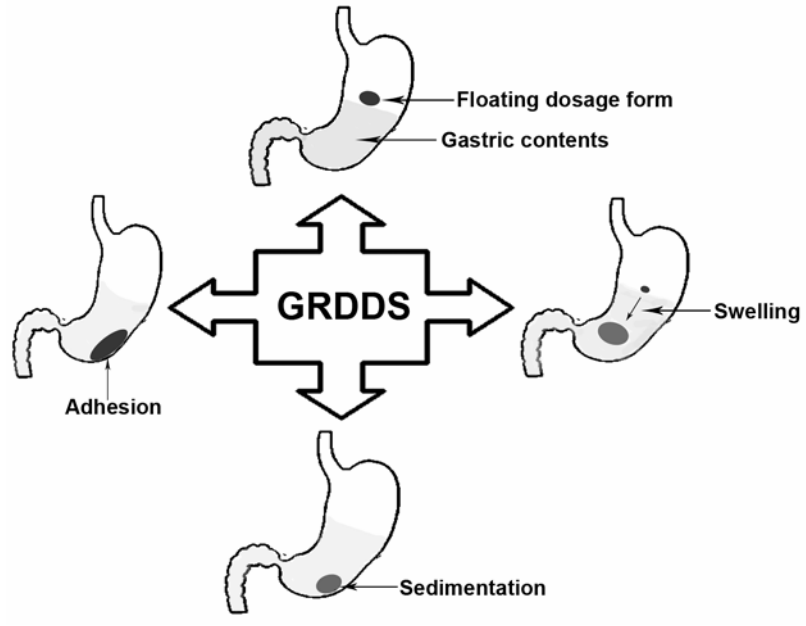

Figure 3. Gastroretentive techniques based on floating, swelling, high-density and mucoadhesive delivery systems. (Modified from Chawla et al, 2003, with permission).

Several natural or synthetic polymers have been exploited to control as well as to prolong the gastric retention. The main drawback of these systems is the susceptibility to adhere to diverse particles in the stomach. In addition, the pH-dependent behavior of bioadhesive materials can reduce acidity of the gastric juice decreasing the adhesive properties and, hence, the gastroretentive effect.

Swelling systems are constituted by polymers promoting the swelling to a size that prevents their passage through pyloric sphincter resulting in prolonged gastric retention. However, a balance between the rate and extent of swelling and the rate of erosion of the polymer is the key to achieve optimum benefits and to avoid side effects.

Indeed, the challenge to the development of successful gastroretentive system is to overcome differences in gastric physiology as well as inter-subject variability influencing substantially on the retention time and drug release.

1.4.3.2. Colonic Drug Delivery

Specific targeting of drugs to the colon is potentially useful not just for the topical treatment of intestinal diseases but also for the delivery of therapeutic peptides and proteins. Pharmaceutical approaches include the development of colonic delivery systems to maximize the effectiveness of these drugs.

Therapeutic strategies proposed for colonic targeting rely on the gastrointestinal features for their functionality such as pH, transit time, pressure or microflora. Concerning these characteristics, orally administered colonic drug delivery is based on four main mechanisms including bacterially triggered, pressure-controlled, pH-dependent, time-dependent; or CODES^{TM} systems (Chourasia and Jain, 2003; Schareef et al, 2003).

Colonic microflora is constituted by complex microorganisms whose physiological functions playing vital roles in health and disease. Microbially degradable polymers and

certain plant polysaccharides are used for colon target drug delivery systems. The linkage between the drug and the polymer is susceptible to enzymatic degradation in the colon and the drug is released. These systems appear to be promising because of their practicality and exploitation of abundant colonic microflora.

A wide range of suitable polymers are available for the use as enteric coating for tablets, capsules or multi-unit dosage forms. In the development of colonic delivery systems, the major progress was made with the introduction of semi-synthetic cellulose derivatives as well as synthetic polymethacrylates with specific solubility properties according to the pH conditions of the gastrointestinal tract. Despite of inherent limitations, pH controlled delivery systems are widely accepted due to its functionality and effectiveness ensuring excellent performance for the product.

Timed release systems are based on the delaying of the drug release until it reaches into the colon. Pulsincap® was developed using this principle; it is similar to a conventional hard gelatin capsule, however, the body is water insoluble. The drug is enclosed within the body by a hydrogel plug that is covered by a water-soluble cap. The whole unit is coated with an enteric polymer to avoid the drug release in an undesirable site. Once the cap is dissolved, the hydrogel swells and the drug is released. Time Clock® is another system proposed and it is composed of a solid core coated with hydrophobic material, surfactant, and water soluble polymer. After a predetermined time interval, the polymer is dissolved and the drug is slowly released.

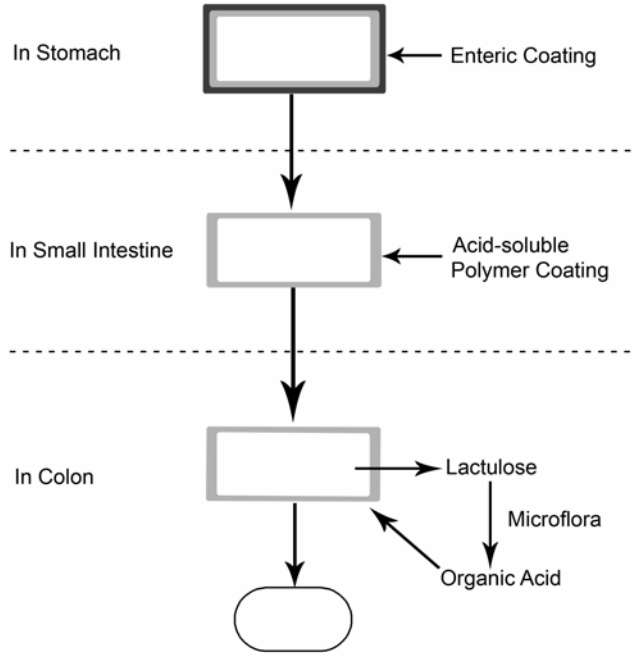

Figure 4. Schematic representation of CODES™ drug delivery system in the human gastrointestinal tract. (Modified from Yang et al., 2003, with permission).

Pressure controlled delivery systems are based on relatively strong colonic contraction waves that leads to luminal pressure. These systems are constituted by capsules coated with

water-soluble polymers (cellulose derivatives). Colonic peristalsis causes the rupture of the system and release the drug.

CODES^{TM} technology was developed as a colonic specific drug delivery to avoid the limitations associated with pH- or time-dependent systems. CODES^{TM} systems use polysaccharides that are degraded by colonic microflora in combination with three layers of pH-dependent polymers: the inner layer is an acid-soluble polymer, the outer layer is an enteric coated barrier and the core contains the drug, as shown in Figure 4 above.

Significant benefits were achieved with the development of technologies for colonic target of drugs. Colon-specific delivery systems based on the mechanisms exploited above are sufficiently reliable for most applications and also, in some cases, they are relatively inexpensive and easy to manufacture.

1.5. EVALUATING DRUG DELIVERY IN MAN

The variation of absorption profiles along the human gastrointestinal tract and the ability to target drugs by adequate dosage forms to distinct sites is the challenge in the pharmaceutical development of solid dosage forms.

Experimental determination of drug release may be assessed by well-established in vitro methodologies. However, these tests are often not predictive of the more complex in vivo behavior of drug products.

Biomagnetic methods has gained acceptance in pharmaceutical applications due to its ability to evaluate non-invasively solid dosage forms in the human gastrointestinal tract.

Biomagnetism embraces the measurement, analysis, interpretation and application of extremely weak magnetic fields, which are spontaneously generated by physiological electric currents that flow in excitable tissues or as a result of ferromagnetic particles. This property can be exploited for quantifying physiological iron storage, for detecting ferromagnetic impurities or the behavior of magnetically marked dosage forms.

1.5.1. Biomagnetic Fields

For more than 200 years, biomagnetic fields have been associated with the normal functioning of biological tissues. Briefly, Galvani discovered that muscles are activated by electric currents produced by nerve cells and later Örstedt demonstrated that when an electric current flows in a conductor, it generates a magnetic field around it. In the early 19th century, Biot and Savart calculated the magnetic field generated by a current carrying wire. Maxwell unified the theory of electromagnetism describing the behavior of the electric and magnetic fields, as well as their interactions with the matter. Maxwell's equations are the most elegant and concise methods to demonstrate the fundamentals of electricity and magnetism (Hobbie, 2001).

Nowadays, it is well known that all excitable cells base their functioning on the exchange of ions. The displacement of these electrically charged particles produces biomagnetic fields that are several orders of magnitude smaller than the Earth’s magnetic field.

This section focuses mainly on the biomagnetic fields generated by the gastrointestinal activity. Smooth muscle layers of the gastrointestinal tract exhibit a periodic cyclic electrical activity also called electrical control activity or slow waves, originated by rhythmic depolarization and repolarization of the cell membrane (Irimia and Bradshaw, 2004). This electrical activity shows specific frequencies according to the gastrointestinal regions and can be detected by mucosal, serosal, or surface electrodes.

The source of the externally recorded magnetic field is the intracellular current associated with the membrane potential, since the extracellular current flows over the body cavity which could be consider a homogeneous volume conductor. Bioelectric fields depend on tissue conductivity and are therefore attenuated by the abdominal wall. On the other hand, magnetic fields depend basically on the permeability of biological tissues instead of the abdominal wall.

Biomagnetic fields in the human gastrointestinal tract can be measured as a result of electrical currents associated to the ions movement, magnet or by ferromagnetic sources in response to an applied external magnetic field. The magnetic field due to the distribution of electric currents in the abdomen, can determine the location, orientation, and time evolution of electric current dipoles.

The precise location of the magnetic marker is useful in studies measuring frequency of gastrointestinal motility. In transit studies, the position of the marker is tracked along the gastrointestinal tract. Detection of magnetic fields generated by magnetically marked dosage forms provides an opportunity for pharmaceutical research involving functional analysis of drug delivery mechanisms.

1.5.2. Biomagnetic Instrumentation – Fundamentals and Applications

Biomagnetic fields may be generated as a result of the electric activity of the excitable tissues or due to magnetic markers and they are extremely weak. Thus, potential applications of biomagnetism in pharmaceutical research require sensitive magnetic sensors.

Currently, the most important sensors for biomagnetic measurements are Superconducting Quantum Interference Devices (SQUIDs), Magnetoresistive Sensors, Hall-Effect Sensors, and Induction Coils.

1.5.2.1. SQUID System

SQUIDs are a class of the most sensitive detector of magnetic flux and consist basically of a superconducting loop interrupted by one or two weak links. These weak links are Josephson Junctions (JJ) that can be formed between two superconductors as a single or multiple point contacts, microbridges, and tunnel junctions. The components of the detection system must be maintained at a low temperature that generally is achieved by immersion in liquid helium (4.2 K) contained in a special insulated vessel known as a Dewar. Figure 5 summarizes a simple single channel SQUID.

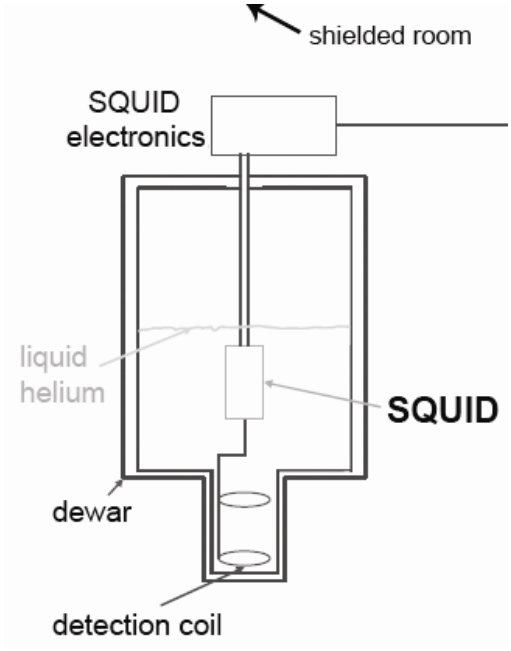

Figure 5. Schematic diagram of a single channel SQUID system. (Modified from Pizzella et al., 2001, with permission).

The physical phenomena of flux quantization and Josephson effect are the basis of the SQUIDs. Magnetic flux quantization is the state that the magnetic flux through a superconducting loop is always quantized in units of the flux quantum. While Josephson effect involves the coherent tunneling of a pair of electrons (Cooper pair) through a thin barrier separating two superconductors.

The SQUID can be used to detect a magnetic field directly, but this is not the most practical arrangement for biomagnetic studies due to the need to minimize the detection of ambient noise. It is advantageous to sense the field with a detection coil magnetically coupled to the SQUID which forms a closed circuit that is known as a flux transformer. As with an induction coil, the application of a magnetic field induces a current; but because the flux transformer is a superconducting circuit, the current is proportional to the magnetic flux applied to the coil. As this current passes through the input coil it imposes a field on the SQUID and it is the field to which the SQUID responds.

An electronic circuit outside the Dewar monitors the response of the SQUID, and it includes a sensitive preamplifier as close as possible to the SQUID to enhance its signal. The preamplifier and control unit constitute the active portion of the circuit, and its output voltage is proportional to the magnetic flux which is applied to the detection coil. A complete review of the principles of SQUIDs was reported by Williamson and Kaufman, 1981; Romani et al 1982 and Pizella et al, 2001.

The high sensitivity of SQUIDs devices makes these sensors suitable for a wide range of applications. These include magnetocardiography, magnetoencephalography and magnetogastrography for the study of ionic currents generated by nervous and muscular tissue of the human body (Sternickel and Braginski, 2006).

In the pharmaceutical research, SQUIDs systems have been applied as an alternative to investigate the behavior of solid dosage forms in the human gastrointestinal tract. For this purpose, the technique called Magnetic Marker Monitoring (MMM) uses the dosage forms marked as a permanent magnetic dipole by incorporating small amounts of ferromagnetic material and subsequent magnetization (Weitschies et al, 2005).

For magnetic monitoring of a dosage form, it is essential labeling the dosage form as a permanent magnetic dipole. The ferromagnetic iron oxides are nontoxic and insoluble color pigments for food and drugs often used.

The dosage form magnetically marked is magnetized in a homogenous magnetic field in order to align all magnetic particles in the direction of the magnetization field. This alignment produces a net magnetic moment generating a measured dipolar magnetic field. Hence, any process that can disturb the alignment of the particles reduces its net magnetic moment.

This property is the basis for investigating the disintegration process of the magnetically marked dosage forms. During the disintegration the magnetic material is released in the fluid medium. Consequently, the particles lose their orientation resulting in a decrease of the net magnetic moment over time, as illustrated in Figure 6.

Another application of the magnetic measurement is the location of a solid dosage form in the human gastrointestinal tract. After the ingestion, components of the magnetic field can be continuously measured at different fixed positions above thorax or abdomen. Therefore, it is possible to monitor the esophageal transit, the gastric residence and the intestinal transit and to reconstruct location and orientation of the marker. In this case, considering that the dosage form does not disintegrate, the magnetic moment of the marker is constant and can be determined during its passage in the different gastrointestinal regions.

Due to its low permeability and transient nature, the esophagus is not a common site for drug delivery. Magnetic marker monitoring allows investigating the effects of exogenous and endogenous factors that influences the orogastric transit of solid dosage forms.

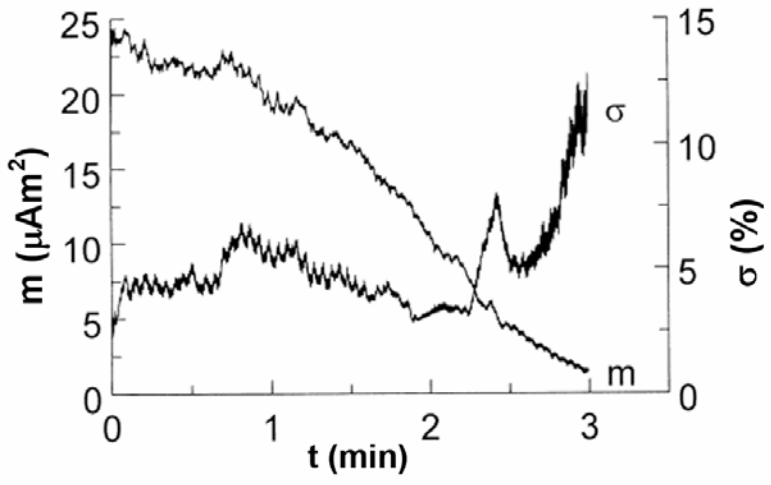

Figure 6. Magnetic signals of the disintegration of capsules *in vivo*. Magnetic moment (m) and the standard deviation (σ) are represented. (From Weitschies et al, 2001, with permission).

Figure 7 shows the vertical component (z) and the horizontal components (x, y) in a typical example of the esophageal monitoring of a solid dosage form. The intestinal transit

time of magnetically marked dosage forms is characterized by an irregular sequence that alternates periods of quiescence, slow and rapid movements, as shown in Figure 8. The release rates from the dosage form and its location, as well as, the residence time in the different segments of the gastrointestinal tract are important variables. Magnetic marker monitoring in combination with pharmacokinetic data provides a better understanding of the release properties of formulations and the plasma drug levels.

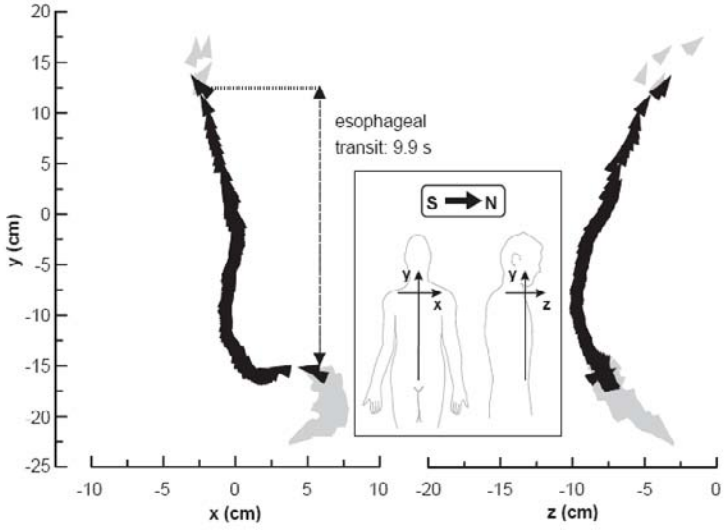

Figure 7. Magnetic monitoring of esophageal transit of a capsule. In the *inset* is represented the coordinate system and the orientation of the magnetization. (From Weitschies et al, 2005, with permission).

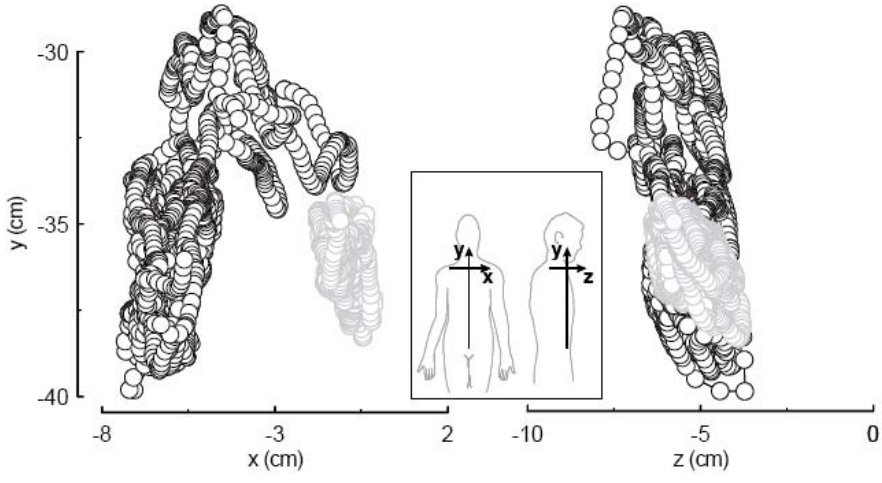

Figure 8. Magnetic monitoring of a non-disintegrating capsule in the small intestine. (From Weitschies et al., 2005, with permission).

Despite of magnetic dipolar moments obtained for a magnetically marked dosage form be very weak, SQUID systems can measure easily since the sensors are in a magnetically shielded room in order to reduce environmental noise.

1.5.2.2. Magnetoresistive Sensors

The magnetoresistive effect is the change of the electrical resistivity of a material due to a magnetic field. The sensors known as Anisotropic Magnetoresistive (AMR) require thin ferromagnetic films (e.g., permalloy) with a magnetic anisotropy. There is a hard axis with a high requirement of magnetization energy in one direction in the plane of the film, and, orthogonal to the hard axis in the plane of the film, which indicates the magnetic preference direction. AMR sensors have a high sensitivity at weak magnetic fields.

The method developed by Andrä et al (2000) is based on the measurement of the magnetic field of a permanent magnet which is repeatedly aligned by a vertically oriented pulsed magnetic field.

The permanent magnet is a homogeneously magnetized sphere, and then the magnetic field is a dipole determined by both two angles of orientation and three spatial coordinates of its magnetic moment. This property provides a real-time monitoring of the marker in the human gastrointestinal tract, since the three dipole field components are measured at multiple positions outside the body.

In the pharmaceutical assays, the position of a solid dosage form in a specific segment of the human gastrointestinal tract is essential for drugs with a narrow absorption window. Consequently, the development of a drug delivery system should take into consideration the time delayed in the region preceding and around the window. Non-disintegrating dosage forms are monitored during the gastrointestinal transit time, since the magnetic dipole remains stable during the gastrointestinal passage. Hence, the magnetic moment has a constant value in the localization procedure. In Figure 9, the positions of the marker in the intestinal phantom simulated the propulsion and retropropulsion motion.

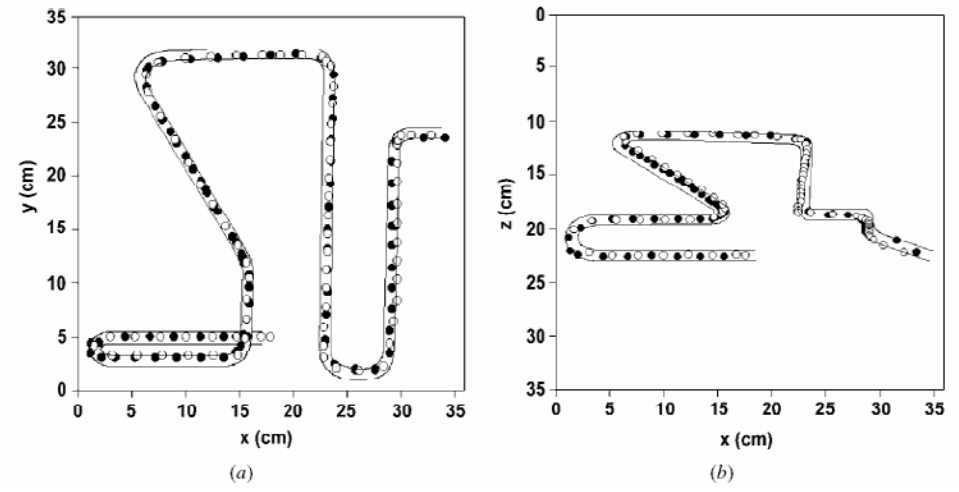

Figure 9. Magnetic monitoring of the marker positions in the intestinal phantom simulating the propulsion (open circles) and retropropulsion (full circles) motion . (*a*) top view and (*b*) side view. (From Andrä et al., 2000, with permission).

Alternatively, the disintegration process of magnetically marked tablets in relation to the temporal development of the magnetic moments also can be investigated (Weitschies et al, 2001). In this case, the magnetic moments of the magnetic particles of the tablets are aligned during magnetization into the direction of the magnetic field applied. Thus, a magnetically

marked tablet is an ensemble of particles with a stable magnetic dipole moment. When the disintegration occurs, the particles are released from the tablet core and hence this dipole moment is reduced due to the disarrangement in the alignment of the particles, as shown in Figure 10.

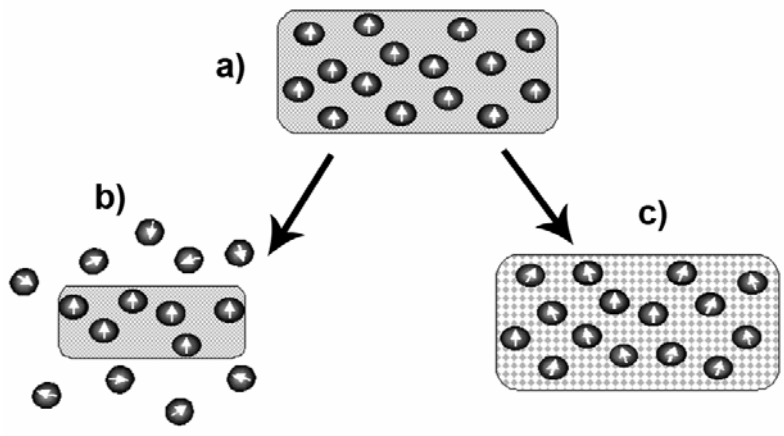

Figure 10. Representations of the magnetic dipole moments of magnetic particles. a) tablet after magnetization; b) disarrangement in the magnetic moment; c) decrease of the magnetic moment. (From Weitschies et al., 2001, with permission).

Once a magnetic dosage form can be located within the gastrointestinal tract, it would be useful to monitoring the drug release (Richert et al, 2005). With the magnetic marker monitoring, the drug release from a solid dosage form is achieved by magnetization losses or demagnetization by the use of decaying alternating magnetic field cycles.

1.5.2.3. Hall-Effect Sensors

Hall sensors are semiconductor active devices that produce a voltage signal proportional to the sensed magnetic field. Hall sensors feature low cost, small size, excellent linearity, and rotational measurement repeatability. Additional information on this sensor was reported by Popovic et al (2001) and Schlageter et al (2002).

For physiological or pharmaceutical purposes, Hall sensors are useful for tracking the position of a magnetic pill. The tracking system consists in an array of cylindrical sensors with integrated flux concentrators in order to convert locally the magnetic field and to increase the sensitivity (Stathopoulos et al, 2005).

The sensors measure the magnetic field in five coordinates, x, y, z (in mm) and two angles of rotation θ and φ (in degrees), as illustrated in Figure 11. The position of the magnetic marker is calculated with an iterative algorithm. The same approach is employed by SQUIDs and Magnetoresistive sensors.

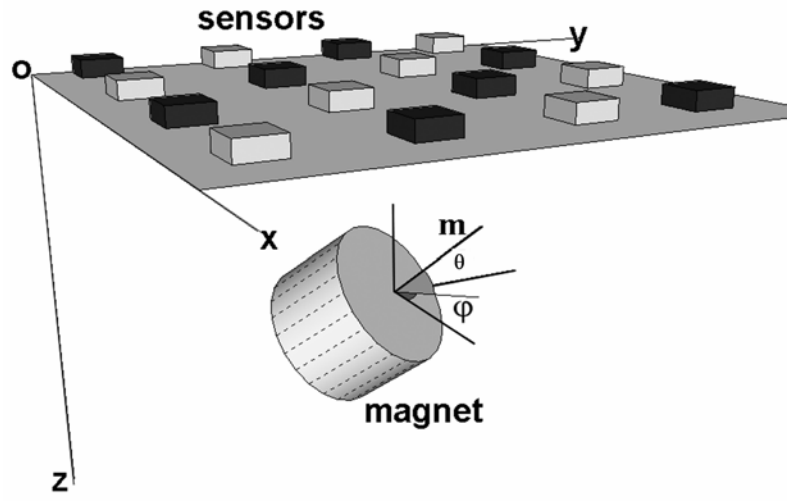

Figure 11. Magnetic tracking system. (Modified from Schlageter et al., 2002, with permission).

The magnetic tracking system seems to be an alternative for the gastrointestinal motility measurements. The trajectory of the magnet pill can be accomplished throughout entire digestive system from the oesophagus to the sigmoid colon. The system is sensitive to small variations in the position and orientation of the magnetic marker and therefore, is able to characterize the frequency of the rhythmic movements of the gastrointestinal tract.

Magnetic tracking offers an opportunity to study the effects of nutrients and drugs on specific segments of the gastrointestinal tract.

1.5.2.4. AC Biosusceptometry

Alternating Current Biosusceptometry (ACB) is an interesting and valuable tool for physiological and pharmaceutical research (Baffa et al, 1995; Corá et al, 2005). The AC Biosusceptometry bases its functioning on induction coils for recording the magnetic flux variation obtained from the response of a magnetic material. This material with a high magnetic susceptibility produces a strong response when an alternating magnetic field is applied on the biological system.

The principle behind Biosusceptometry is Faraday's Induction Law. Briefly, the induction occurs when two coils are close and a current is applied in one of the coils to generate a magnetic flux. Changing the current over time will generate a voltage in the other coil proportional to the rate of this change. The flux through the coil will change if the coil is in a magnetic field that varies with time, if the coil is rotated in a uniform field, or if the coil is moved through a nonuniform field. The flux changes generate an alternated electric current that produces its own magnetic field.

Essentially, an AC Biosusceptometer has two pairs of induction coils separated by a fixed distance (baseline), where each pair of coils is composed of an excitation coil (outer) and a detection coil (inner), in a first-order gradiometric configuration (Corá et al, 2003). The sensor is mounted as a couple of magnetic flux transformers with an air nucleus in which the pair (excitation/detection) that is located more distant from the magnetic material that will be detected acts as a reference transformer and the pair closest of the sample as a measurement transformer. Figure 12 illustrates the basic characteristics of the AC Biosusceptometer.

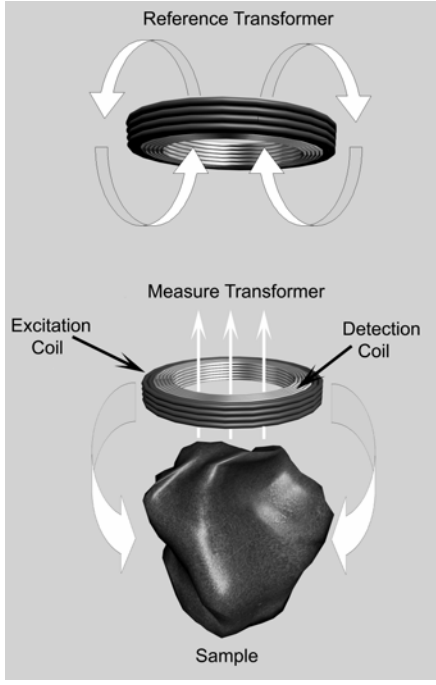

Figure 12. Functional diagram of the single sensor AC Biosusceptometer.

The excitation coil induces equal magnetic flux in the detection coil which is arranged in a first-order gradiometric configuration, to minimizing the output signal when there is no magnetic material near to the measure transformer. With the proximity of a magnetic sample an imbalance in the voltage occurs, due to the change in the differential flux between the detection coils. Hence, the gradiometric system detects the magnetic flux variation between the detection coils.

These concepts were then applied to a multisensor AC Biosusceptometry system that was developed to improve the spatial resolution and the sensitivity for pharmaceutical applications. The multisensor system has a pair of excitation coils and seven pairs of detection coils in a hexagonal configuration for acquisition of magnetic signals in distinct points, as shown in Figure 13.

The signal detected by the Biosusceptometer depends on the area of the detection coil, the number of turns, the rate of change of the magnetic flux through this coil (i.e. applied field), the amount of ferromagnetic material, and the distance between the sensor and the magnetic material.

The AC Biosusceptometry system does not require magnetically shielded environment and due to its simplicity, has reasonably low implementation cost, when compared to the other biomagnetic techniques presented above. When applied to pharmaceutical dosage forms, the AC Biosusceptometry provides non-invasive real-time data on the drug delivery process. Moreover, for Biosusceptometry measurements, the magnetic markers are not previously magnetized.

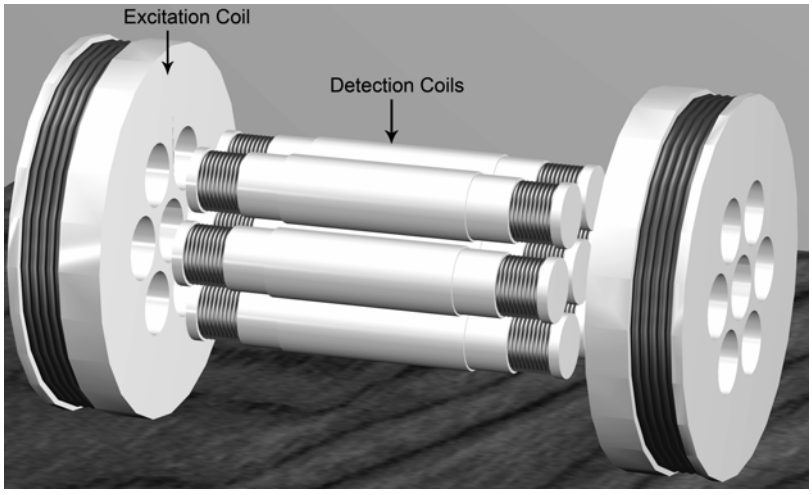

Figure 13. Multisensor AC Biosusceptometer system with the pairs of detection coils in hexagonal symmetry.

In this section, we will discuss a wide range of applications of the AC Biosusceptometry in the pharmaceutical research.

Disintegration

The disintegration of the solid dosage forms promotes the drug release to the absorption in the gastrointestinal tract. Several approaches are currently developed by the pharmaceutical industry to deliver drugs at specific sites along the gastrointestinal tract aiming a more effective therapy. This includes the development of polymers or coating systems ensuring excellent performance for a product.

All these innovations require methods able to characterize the behavior of a pharmaceutical product in humans, since the gastrointestinal physiology exerts a critical influence on its performance. The AC Biosusceptometry is innovative in this research field and due to its versatility, has become an alternative to the conventional methods.

The Biosusceptometry was firstly proposed to evaluate the disintegration process of magnetic tablets in human stomach (Corá et al, 2003). Figure 14 shows typical examples of the magnetic signals recorded during the disintegration process of a tablet *in vitro* and in the human stomach.

The disintegration process for magnetic dosage forms is characterized by the transition between a magnetic marker (non-disintegrated dosage form) to a magnetic tracer (disintegrated dosage form). Generally, the ferrites ($MnFe_2O_4$) are the inert ferromagnetic material that is incorporated into the dosage forms since they present absence of toxicity and lack effects on the gastrointestinal tract (Frei et al, 1968).

Hence, when a volunteer ingests a magnetic dosage form, the biomagnetic signals generated from the response of the dosage form to an applied magnetic field are detected by the multisensor system.

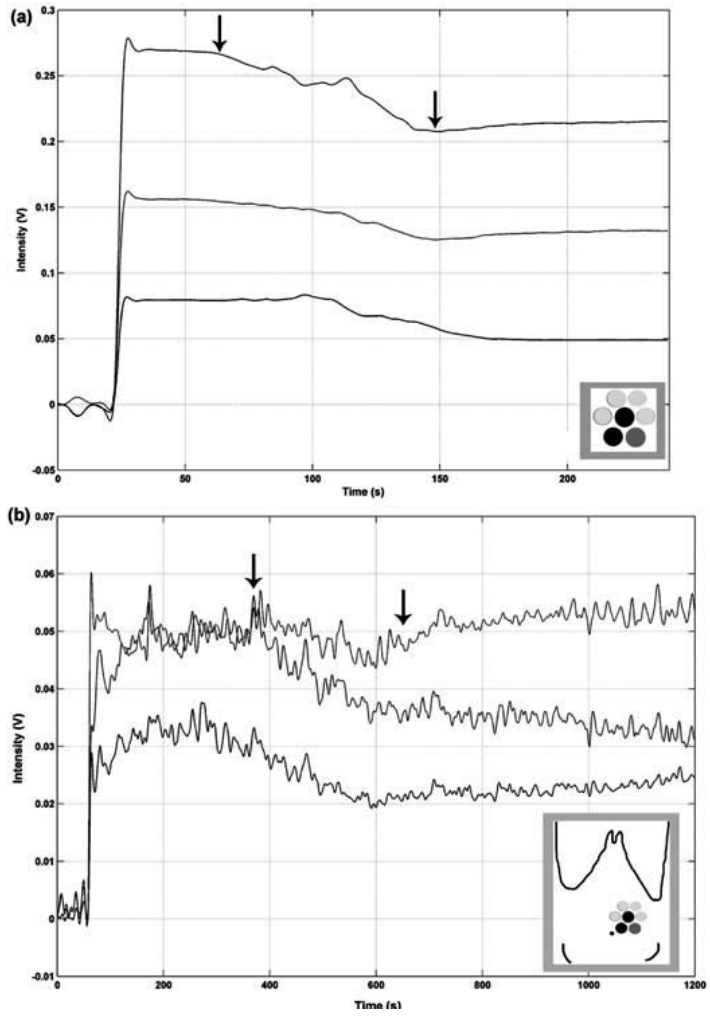

Figure 14. Magnetic signals of the disintegration process of a tablet recorded by the multisensor AC biosusceptometer. (a) *in vitro* measurement; (b) *in vivo* measurement. The *insets* represent the positioning of the multisensor system related with the magnetic signal recorded. The arrows indicate the onset and complete disintegration process, respectively.

AC Biosusceptometer is very sensitive to the movements of the gastric wall, and then the amplitude of the biomagnetic signals decreases when a gastric contraction occurs; it changes the distance between the sensors and the dosage forms within the organ. Simultaneously, in these biomagnetic signals there are information about the gastric activity contraction and the disintegration of the dosage form. This technique allows to monitor the disintegration process in real-time identifying the region where it is occurred.

- *Gastrointestinal Transit*

The development of colonic delivery systems represents one of the most prominent approaches for the pharmacological treatment of inflammatory bowel diseases (IBD). However, because of the distal location of colon, a delivery system must overcome several

physiological barriers along the gastrointestinal tract and prevent the drug release in the stomach and small intestine.

The knowledge of the gastrointestinal transit and how the physiological variables may interfere with the drug release and absorption is the major challenge for successful design of colon-specific delivery systems.

More reliable data are obtained when human studies are carried out, since the bioavailability of drugs from colonic dosage forms is dependent on gastric emptying, small intestinal transit time and drug release profile. AC Biosusceptometry has also gained importance in this kind of analysis (Corá et al, 2006a,b).

The motility pattern during the interdigestive period determines the residence time of a solid dosage form in a specific segment of the human gastrointestinal tract. Figure 15 illustrates the biomagnetic signals in the interdigestive activity following the administration of an enteric-coated dosage form.

It is interesting to notice that the colonic arrival time for a dosage form is influenced mainly by the gastric retention time that plays a dominating role in the overall transit of the dosage form. Conversely, the small intestine transit is not influenced by any physiological parameter or related to the pharmaceutical form. Figure 16 represents a gastrointestinal transit profile showing the gastric emptying time, the small intestinal transit time and the colonic arrival for a magnetic enteric-coated dosage form.

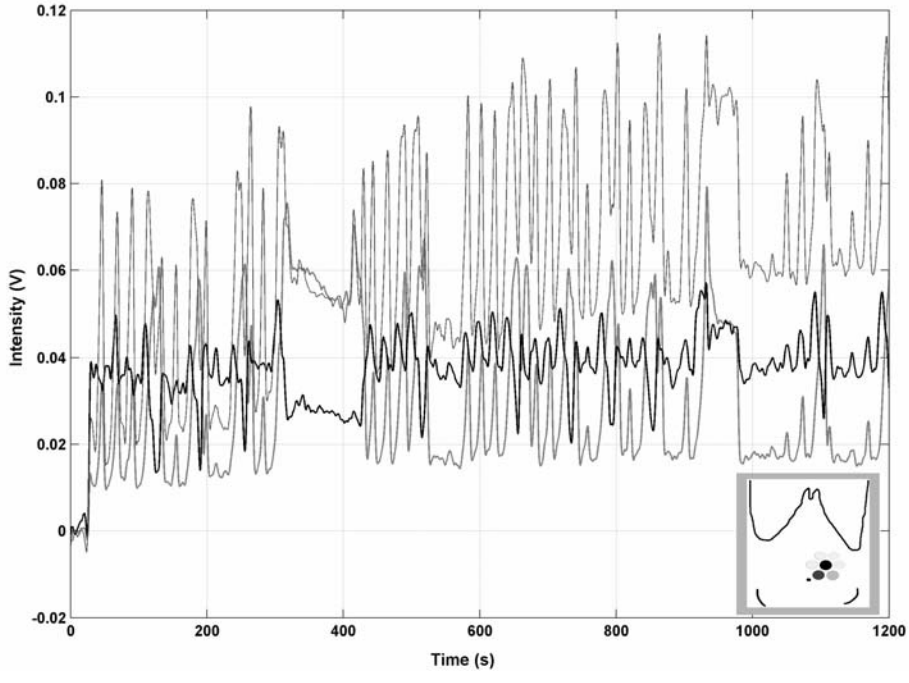

Figure 15. Magnetic signals recorded in the fasting state following the ingestion of a enteric-coated dosage form. The *inset* indicates the position of the multisensor AC Biosusceptometers in the human abdominal wall. (Modified from Corá et al., 2006.)

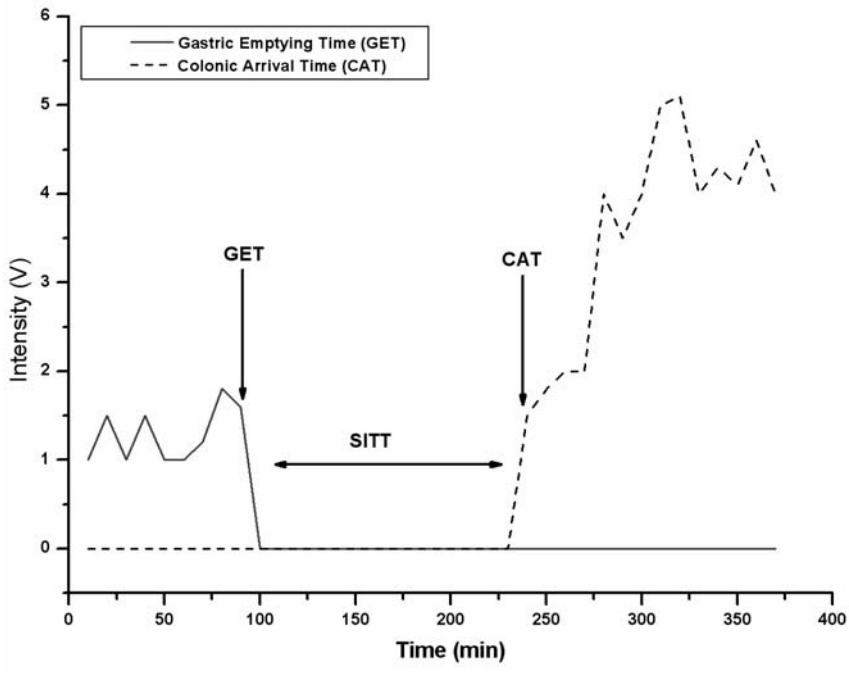

Figure 16. Gastrointestinal transit profile for a magnetic enteric-coated tablet. (Modified from Corá et al., 2005).

- *Magnetic Images*

When applied to pharmaceutical dosage forms, medical imaging techniques provide non-invasive real-time data on the drug delivery process. Recently, the AC Biosusceptometry technique introduced a novel concept in imaging of the pharmaceutical processes.

Biomagnetic signals recorded by the multisensor AC Biosusceptometry system are represented by a time series matrix. From these signals it is possible to generate magnetic images of the solid dosage forms. Corá and cols. (2005) provides a more detailed description of the principles of biomagnetic images for pharmaceutical applications.

Figure 17 shows a sequence of biomagnetic images of a tablet in the human stomach from the ingestion to it complete disintegration. In comparing the second image to the first, this corresponds to an increase of 50% in the number of pixels in the segmented image area. The disintegration process of solid dosage forms in the biomagnetic images is quantified when an increase of 50% in the number of pixels in the image area occurs.

These studies may have many roles, according to their intended, and may be applied to a wide range of pharmaceutical technologies, including enteric-coated, gastroretentive and modified-release dosage forms. In Figure 18 an example of biomagnetic images of an enteric-coated hypromellose capsule and the release of the magnetic material in the human colon can be seen.

This monitoring for biomagnetic images is useful to the quality control to demonstrating the effectiveness of these formulations in the release of drugs in specific areas of the human gastrointestinal tract.

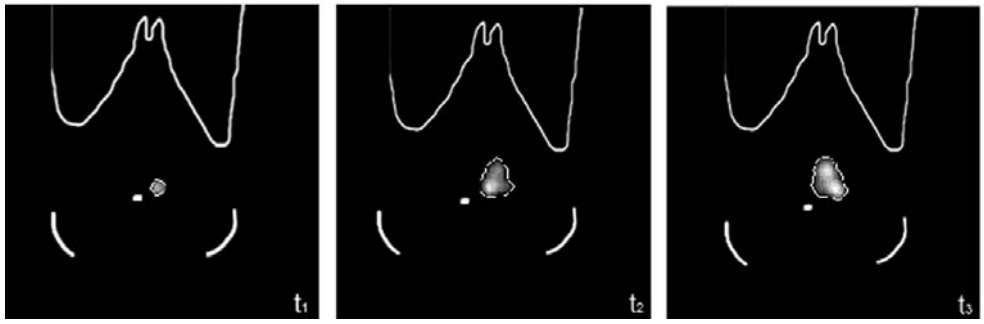

Figure 17. Magnetic images of the disintegration process of a tablet in the human stomach. 50% disintegration (t_{50}) is located between t_1 and t_2.

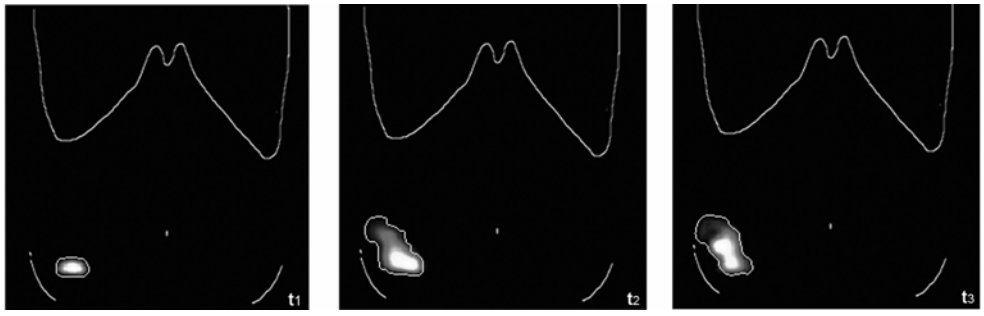

Figure 18. Magnetic images of the disintegration process of an enteric coated hypromellose capsule. In the instant t_1 is represented the arrival of the capsule at the colon; from t_2 occurs a gradual increase in the image area characterizing the spreading of the magnetic material by the disintegration process.

- *Controlled Drug Delivery Systems*

Several formulation strategies have been proposed for controlled drug delivery at specific sites of the human gastrointestinal tract. Controlled delivery systems are intended for release of drugs at an appropriate rate to avoid fluctuations in plasma drug concentrations. For controlled drug delivery technology, multiparticulate systems, such as microspheres or pellets, have several advantages in comparison to the conventional single unit.

Drug targeting to the stomach is attractive for several reasons, particularly when there is an absorption window in the upper gastrointestinal tract. Gastroretentive dosage forms based on floating delivery systems are expected to prolong the gastric residence time increasing the bioavailability of these drugs.

AC Biosusceptometry is an interesting tool to investigate the in vivo performance of gastroretentive drug formulations. Figure 19 shows a typical example of the magnetic images of a floating delivery system.

Likewise, the challenge of targeting drugs to the colonic region is to overcome the physiological variables and to provide a more reliable drug delivery. Due to their small particle size and the more uniform dispersion in the gastrointestinal tract, the multiparticulate dosage forms can reach the colon quickly and to remain retained longer in the proximal colon. Figure 20 shows the biomagnetic images (a) and the gastrointestinal transit profile (b) of a multiparticulate formulation and its dispersion over time on the colonic region.

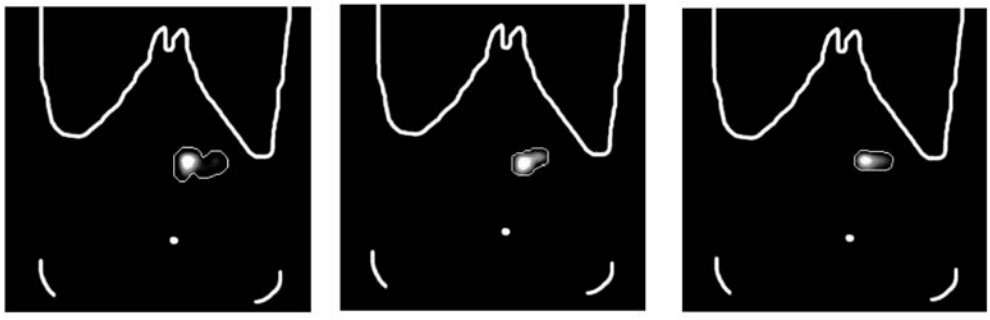

Figure 19. Magnetic gastroretentive delivery system based on floating property.

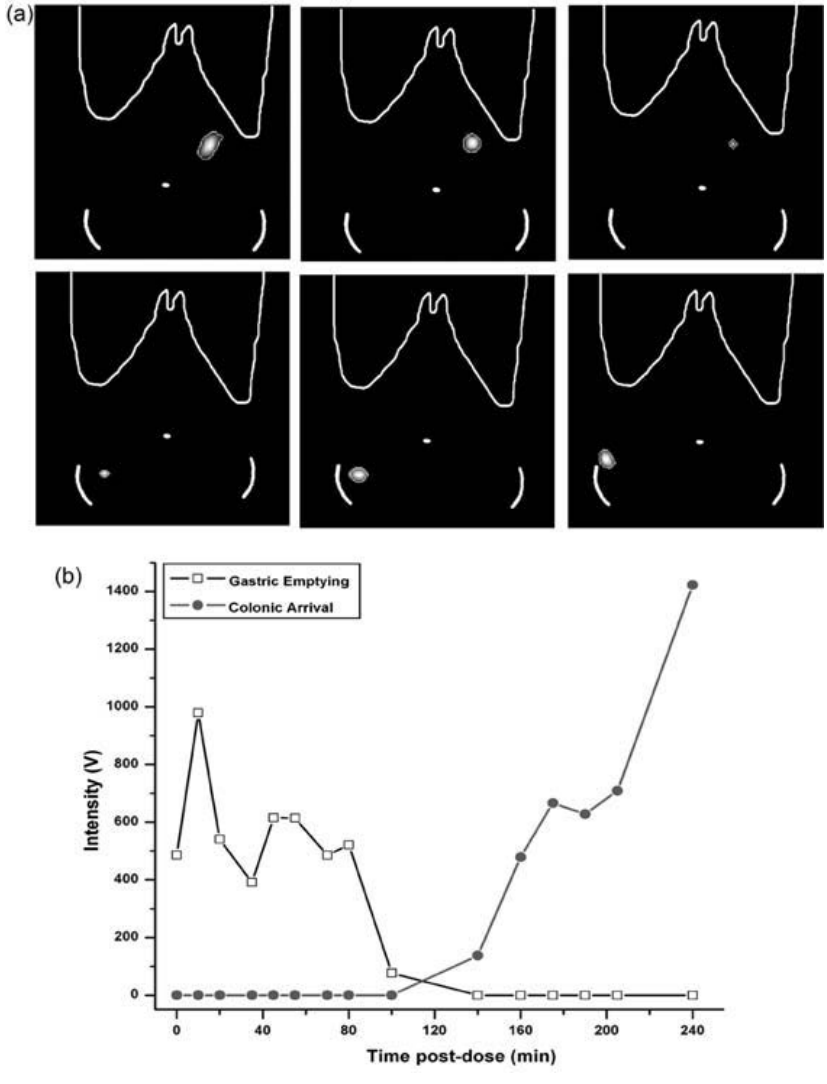

Figure 20. a) Magnetic images of a multiparticulate delivery system in the human stomach and ileocolonic region, respectively. b) Gastric emptying and colonic arrival profile for the multi-units.

CONCLUSIONS

The idea of this chapter was to integrate the basic knowledge on the main biomagnetic techniques and the pharmaceutical research. The development of new technologies for delivery drugs requires techniques able to provide more detailed information about the behavior of solid dosage forms in the human gastrointestinal tract.

In this context, biomagnetism plays an eminent role in a number of clinical research, and more recently in the pharmaceutical field. Biomagnetism involves the detection of magnetic fields produced by the human body or due to magnetic markers previously ingested. As these magnetic fields are extremely weak, it is essential the use of highly sensitive sensors enable to record these fields.

Biomagnetic instrumentation based on SQUIDs systems allows to monitoring magnetically marked dosage forms with a high temporal and spatial resolution. Moreover, the three dimensional localization and orientation as well as the strength of the magnetic source can be reconstructed as a function of time.

On the other hand, the development of real time tracking systems based on magnetoresistive or hall-effect sensors allows the monitoring of strong magnetic markers. These systems are cheaper than SQUID and can be operated without magnetic shielding environment.

In contrast to the above mentioned techniques, AC Biosusceptometry is promising to evaluate accurately solid dosage forms in the human gastrointestinal tract. AC Biosusceptometry system represents a novel imaging tool able to characterize a number of parameters related to drug delivery, deserving the same importance as conventional techniques in pharmaceutical research.

Additionally, the AC Biosusceptometer system provides a more effective monitoring of the dosage form that can be accomplished in real-time and simultaneously in distinct points than those obtained employing SQUIDs, magnetoresistive or hall-sensors.

Considering this, the Biosusceptometry is versatile not only for human studies as well as to be used as a tool in quality control for pharmaceutical products. Moreover, this biomagnetic method is especially powerful when combined with classical pharmacokinetic data allowing monitoring the dosage form to correlate directly the drug release at specific sites along the gastrointestinal tract with the drug absorption (“magnetopharmacokinetic”).

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Chapter 7

SALMONELLA AS A VACCINE DELIVERY VEHICLE

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ABSTRACT

Live attenuated mutants of *Salmonella* are under evaluation as vectors for the delivery of heterologous antigens to humans via the oral route. To produce such recombinant *Salmonella* strains, live attenuated vaccine strains of *Salmonella* are genetically manipulated to express heterologous antigens. They are able to produce a limited infection *in vivo*, producing the heterologous antigen during replication. A range of technologies have been developed to allow the controlled and stable delivery of antigens. This has allowed the optimisation of delivery of various vaccine antigens, stimulating appropriate humoral and cellular immune responses. As a result, several live attenuated *Salmonella*-based vaccines are now in human clinical trials.

INTRODUCTION

Live attenuated *Salmonella* strains were first developed as candidate vaccines for preventing infection of humans with *Salmonella enterica* serovar Typhi (previously known as *Salmonella typhi*), the causative agent of typhoid fever. A live attenuated oral vaccine containing *S. enterica* var. Typhi strain Ty21a [1], developed in 1975 by chemical mutagenesis, has been licensed and used successfully to immunise against typhoid fever for many years. However, the basis of attenuation is undefined in Ty21a, and an additional problem associated with the vaccine is the theoretical possibility of its reversion to virulence, although no revertants have been isolated to date [2]. In efficacy trials, Ty21a has been shown

to be safe and effective but only modestly immunogenic [3-7] so it is usually administered initially as 3-4 capsules on alternate days, and requires boosters every 5 years [8]. For these reasons, new genetically defined attenuated strains of *S. enterica* var. Typhi have been constructed and evaluated as candidate live oral vaccines.

One problem associated with the development of improved typhoid vaccines for use in humans is that *S. enterica* var. Typhi is not known to be virulent in any animal model by the oral route of infection. However, the closely-related pathogen *S. enterica* serovar Typhimurium (previously known as *S. typhimurium*) can infect mice via the oral route to produce a typhoid-like disease. More recently, a model of intranasal immunisation of mice has been developed in which the immunogenicity of attenuated *S. enterica* var. Typhi vaccines can be assessed [9].

One of the most widely studied classes of defined attenuated *Salmonella* vaccine candidates are auxotrophs. In early studies, *S. enterica* var. Typhimurium or var. Typhi derivatives with mutations in the *aroA* gene encoding 5-*enol*pyruvylshikimate-3-phosphate synthase (part of the shikimate pathway involved in the synthesis of aromatic amino acids) were shown to be attenuated and to be immunogenic in mice. Immunisation with these mutants stimulated immune responses that were protective against a subsequent challenge with homologous *Salmonella* [10,11]. Such *aroA* mutants undergo only limited replication *in vivo*, leaving the host immune to wild-type *Salmonella* infection [11]. However, for additional safety from reversion of such mutants to virulence (which could occur if the mutants acquired DNA from wild-type bacteria), *Salmonella* vaccine strains have been produced which have two or three defined mutations. For example, double mutants have been constructed combining the *aroA* deletion with deletions in other genes in the shikimate pathway genes such as *aroC* (encoding chorismate synthase) or *aroD* (encoding 3-dehydroquinase) [12, 13]. A triple mutant containing deletions in the *aroA*, *aroC* and *aroD* genes has also been constructed [14]. In mice, *S. enterica* var. Typhi strains CVD 906 (*aroC aroD*) or CVD 908 (*aroC aroD*) were attenuated and highly immunogenic [14]. In Phase I clinical trials only CVD 908 was immunogenic without causing febrile adverse effects [15]. However, one potential problem associated with CVD 908 was the silent vaccinaemias observed in healthy volunteers. Therefore CVD 908 has been further modified by the introduction of a deletion into the *htrA* gene [16], a homologue of an *Escherichia coli* gene coding for a heat stress protein [17]. In a Phase I clinical study, doses of 5×10^7 - 5×10^9 cfu of CVD 908-*htrA* have been well tolerated in volunteers, with only one case of fever and three cases of diarrhoea in 36 subjects following high doses of the vaccines [16]. Importantly, there were no cases of vaccinaemia and the vaccines were immunogenic. *S. enterica* var. Typhi CVD 908-*htrA* is a promising typhoid vaccine candidate that has completed Phase II clinical trials [18] and is currently in further clinical trials.

Other *Salmonella* vaccine candidates include strains with deletions in genes involved in the regulation of *Salmonella* virulence. For example, mutations in genes encoding adenylate cyclase (*cya*) and camp receptor protein (*crp*) affect the expression of genes involved in carbohydrate and amino acid metabolism and in the expression of fimbrae and flagella [19, 20]. *S. enterica* var. Typhimurium single mutants of *cya* or *crp*, or a double mutant of *cya* and *crp*, all show attenuated virulence and can protect mice against oral challenge with the wild-type strain [21]. Alternatively, vaccine strains with mutations in the *phoP* and *phoQ* genes, involved in the two-component regulatory system for phosphate sensing, have been constructed and assessed as candidate vaccines against murine or human typhoid. [22-24].

SALMONELLA AS A VACCINE DELIVERY VEHICLE

Although originally developed as candidate typhoid vaccines, attenuated *S. enterica* strains have subsequently been exploited as vaccine delivery vehicles, carrying protective antigens against heterologous pathogens to the immune system. Attenuated *Salmonella* strains are attractive for use as vaccine delivery vehicles since they can be administered to humans by the natural route of infection, *i.e.* orally, and are capable of eliciting both systemic and mucosal immune responses, and both humoral and cellular immune responses. The bacteria are easily genetically manipulated and are able to express heterologous antigens of bacterial, viral and eukaryotic origin. Such heterologous antigen-expressing attenuated *Salmonella* may potentially be used as a bivalent vaccine, affording protection against both typhoid fever and another infection.

Recombinant *Salmonella* have generally been constructed by transformation with plasmid DNA encoding the heterologous antigens under the control of a bacterial promoter, allowing the antigens to be produced within the *Salmonella*.

However, over the years, various technical problems associated with the development of effective recombinant *Salmonella* vaccines have arisen. More recent advances in *Salmonella* vaccine vector technology have provided solutions to at least some of these problems.

Gene Stabilisation

The unstable expression of heterologous antigens in recombinant *Salmonella* may occur as a result of instability of the genes encoding the antigens. For example, early experiments showed that a plasmid expressing the *E. coli* heat-labile enterotoxin subunit B was lost *in vivo* when the *Salmonella* carrier colonised the mouse RES [25]. Since then, various strategies have emerged to address the problem of genetic instability. Chromosomal integration of the antigen-encoding genes is an obvious solution to enable stable expression of the genes. Hone *et al.* developed a system whereby DNA encoding a heterologous antigen could be integrated by recombination into the chromosome of a *Salmonella* carrier [26]. Briefly, a *hisOG* deletion was introduced into the chromosome of *S. enterica* var. Typhimurium LT2H1 (*galE*). Subsequently, a suicide vector was used to incorporate the wild-type *hisOG* operon and DNA encoding *E. coli* K88 fimbrae into the *Salmonella* chromosome by homologous recombination of flanking regions, identified by selection of his^+ bacteria. This system has since been used to insert DNA encoding various heterologous antigens into the *Salmonella* chromosome. Although this approach addresses genetic instability, the integrated gene is present as a single copy which may lead to low levels of antigen expression and, as a result, low immunogenicity.

An alternative approach to solving genetic instability is via the use of plasmid stabilisation systems. In balanced-lethal systems the foreign gene is inserted into a plasmid containing a gene that complements a metabolic defect in the *Salmonella* carrier strain. Thus, the loss of these plasmids results in bacterial cell death. The best studied balanced-lethal system involves the use of the *asd* gene encoding aspartate β semialdehyde dehydrogenase, required for the production of diaminopimelic acid which is an essential component of

bacterial peptidoglycan [26-28]. Using this system, cloning of heterologous antigens into an Asd^+ expression vector allows stable expression of the antigen in Δ*asd* mutants of *Salmonella*. Similarly, an expression plasmid carrying the thymidylate synthetase (*thyA*) gene has been used to complement a *Salmonella* vaccine strain with a thymidine requirement, caused by a mutation in the chromosomal *thyA* gene [29]. Using these systems, plasmid-less bacterial cells do not survive. Since the chromosomal gene is complemented on a multicopy plasmid, the metabolic burden associated with expressing the gene likely contributes to the instability of plasmids expressing genes such as *asd.*

In an alternative plasmid stabilisation approach, the naturally-occurring *hok-sok* post-segregational killing system is exploited [30]. In this system *hok* encodes a lethal pore-forming Hok protein, whose synthesis is blocked by hybridisation of antisense mRNA (called *sok*) to the *hok* mRNA. Bacterial cells are forced to maintain an expression plasmid carrying *hok-sok* in order to allow constitutive transcription which, in turn, maintains an intracellular environment which protects *sok* mRNA from degradation by nucleases. *Salmonella* cells which do not carry the plasmid are post-segregationally killed since levels of protected *sok* mRNA drop and levels of the more stable *hok* mRNA quickly lead to Hok synthesis and cell death. This plasmid stabilisation system has the advantage that plasmids stabilised by carrying *hok-sok* can be introduced into any live *Salmonella* vector strain without the requirement for additional genetic manipulation. However, in *Salmonella* the plasmid stabilisation effect may be ineffective during extended culture since plasmid-free segregants that escaped Hok killing are reportedly able to proliferate [30].

More recently, an approach to plasmid stabilisation has been developed which addresses the problems associated with the existing technologies and requires no antibiotics or other selectable markers to maintain the recombinant plasmid [31]. Briefly, the technology, based on operator-repressor titration (ORT) technology, requires the essential bacterial gene *dapD* to be controlled by the operator/repressor of the *E. coli* lactose (*lac*) operon. Thus, the LacI repressor binds to the chromosomal *lac* operator, preventing cell growth unless an inducer such as isopropyl-β-D-thiogalactopyranoside (IPTG) is present. However, a multicopy plasmid possessing the *lac* operator is able to titrate the repressor away from *lacO,* enabling gene expression and growth. Thus, maintaining the plasmid is essential for bacterial viability. This technology has been applied to *Salmonella* vaccines expressing the protective F1 antigen of *Yersinia pestis* without the need for a selectable marker gene [31].

Controlling Antigen Expression

In order to express heterologous antigens at levels sufficient to elicit potent immune responses, high copy number plasmids are often used. However, this approach can result in toxicity of the over-expressed protein towards the *Salmonella* carrier. Controlling heterologous antigen expression from *Salmonella* may solve the toxicity associated with high level expression. One possible solution involves the use of promoters that allow expression of heterologous antigens at particular cellular sites, so-called *in vivo*-inducible promoters (IVIPs). Using IVIPs, the level of antigen expression is low until the *Salmonella* carrier recognises an *in vivo*-induced environmental stimulus, increasing antigen expression. For example, the use of the *nirB* promoter, which is induced in anaerobic environments [32]

including the entry of the *Salmonella* into cells [33], has led to the stimulation of superior immune responses to expressed antigens compared to constitutive promoters [34-36]. Similarly, the macrophage-inducible *pagC* promoter, controlled by the PhoP/PhoQ two-component regulatory system, has also been shown to provide stable, high-level expression of heterologous antigens in *Salmonella* [36-39]. Other promoters used include the *phoP* and *ompC* promoters [38], the *htrA* and *groE* promoters [33, 40], the *katG* promoter [36], the *dmsA* promoter [41], and the stationary phase-inducible promoters *spv* and *dps* [42].

Various studies have compared IVIPs directly for optimising the controlled expression of a given heterologous antigen [35-38, 40, 43]. For example, the *htrA*, *pagC* and *nirB* promoters have been evaluated for expression of the Hc fragment of the type F botulinum neurotoxin in *S. enterica* serovar Typhimurium [43]. Immunisation of mice with recombinant *Salmonella* expressing Hc from the *htrA* promoter resulted in the greatest level of protection towards a subsequent challenge with botulinum neurotoxin. Overall, the results of these studies suggest that the choice of IVIP for any given antigen is not straightforward. In addition, it has been suggested that the type of immune response induced against an antigen is influenced by the choice of promoter [38], presumably by altering the site in the gastrointestinal tract where expression is achieved. It is likely that the choice of IVIP may require selection on a case-by-case basis for individual genes.

Localising Expressed Antigen

Recombinant DNA technology may be used to localise expressed heterologous antigens appropriately within the *Salmonella* carrier to influence the magnitude and type of immune response generated to the antigen following immunisation. Since both humoral and cellular immunity may be elicited against antigens that are expressed on the surfaces of carrier strains, some antigens that are normally expressed in the cytoplasm have been directed onto the cell surface using signal sequences from exported proteins such as the periplasmic binding protein MalE [44]. Alternatively, the outer membrane proteins OmpA [45, 46] or LamB [44, 47] have been genetically fused to heterologous antigens to carry and relocate them in the *Salmonella* cell membrane.

In some instances, it may be appropriate to localise the expressed antigen in a similar way to the natural expression in the parent bacterium. The *Yersinia pestis* F1 antigen, encoded by the *caf* operon, naturally forms a capsule-like structure around the *Y. pestis* bacterium. In initial studies, the *caf1* gene encoding the F1 structural subunit was expressed in the live *aroA* attenuated *S. enterica* serovar Typhimurium strain SL3261, resulting in the intracellular accumulation of F1 antigen and the induction of a protective immune response following oral inoculation of mice [48]. However, an improved vaccine candidate was produced by incorporating expression of the entire *caf* operon, which includes *caf1* and other genes involved in the export and assembly of F1 antigen in *Y. pestis*. This allowed stable expression of the F1 antigen on the surface of SL3261 and significantly enhanced the protection afforded against *Y. pestis* infection [49].

The export or secretion of expressed antigens from *Salmonella* is another approach that may improve upon the immunogenicity of the carried antigen. Certainly, for two naturally secreted proteins, Hly and p60 of *Listeria monocytogenes*, secretion from *Salmonella* was shown to improve their immunogenicity [50]. These two protective antigens were expressed

in *S. enterica* var. Typhimurium *aroA* in either secreted or cytoplasmically-located forms. Vaccination of mice with *Salmonella* secreting Hly or p60 induced immunity that afforded protection against *L. monocytogenes* infection. In comparison, *Salmonella* somatically displaying the antigens was insufficient for induction of protective immunity. The same workers found that mice immunised with *Salmonella* secreting the naturally somatic antigen superoxide dismutase were also protected against *L. monocytogenes* challenge [51]. It is possible that some naturally secreted antigens may require cell surface expression in order to fold correctly and to adopt a conformation which is immunogenic.

Various export systems have been developed to transport expressed antigens from the normal cytoplasmic environment of the *Salmonella* carrier to cell surface locations. Most frequently, the haemolysin A (HlyA) secretion system of *Escherichia coli* [52] and the type III secretion system of *Salmonella* [53] have been used to export *Salmonella*-delivered antigens. More recently, the potential of the cytolysin A (ClyA) export system of *S. enterica* serovar Typhi for exporting heterologous antigens from *Salmonella* has been demonstrated [54, 55]. The strategy of secreting expressed vaccine antigens from *Salmonella* carriers is hypothesised to provide an appropriate balance between achieving levels of immunogenic antigen expression with minimal metabolic burden placed upon the bacteria [56].

Enhancing Immunogenicity

The fusion of heterologous antigen expressed from *Salmonella* to proven carrier antigens may increase the immunogenicity of some antigens. For example, expression in *Salmonella* vaccine strains of the *Schistosoma mansoni* [57] or *Schistosoma haematobium* [58] 28-kDa glutathione *S*-transferases as fusions to the tetanus toxin fragment C (TetC) enhanced their immunogenicity. It is thought that TetC promotes the immune response against fused antigens by providing additional T cell helper epitopes [59]. Similarly, *Streptococcus sobrinus* antigens [60] or the *E. coli* heat-stable enterotoxin [61] have been expressed in *Salmonella* as fusions to the *E. coli* heat-labile toxin subunit B (LT-B), which is also known to have immune-enhancing effects. LT-B and CT-B, the cholera toxin B subunit, are well-known non-toxic adjuvants which contain structural features that provide adjuvant and immunogenic stimulation in the intestinal mucosa [62].

Vector Strain Selection

In order to modulate the immune response appropriately for expression of a given heterologous antigen, it may be necessary to select an appropriate *Salmonella* mutant. Various studies have demonstrated the differences in immune responses generated due to immunisation with different strains of *S. enterica* serovar Typhimurium. For example, the oral administration of a *phoP* mutant in mice induced strong innate immune responses whereas an *aroA* mutant elicited more potent humoral and cellular immune responses [63]. In another study, oral immunisation with *S. enterica* serovar Typhimurium mutants of the SPI-2 system expressing β-galactosidase stimulated different types of antigen-specific immune responses [64]. Additionally, *asd* mutants of *Salmonella* with different genetic backgrounds

stimulated different strengths of immune response to the carried heterologous antigen [65]. Such differences in immune response strength and type likely reflect differences in the ability of the different *Salmonella* strains to colonise host tissues [66]. Thus, *Salmonella* harbouring mutations in different virulence or metabolic genes are controlled by distinct host mechanisms [67] which should be considered for the appropriate delivery of heterologous antigens.

FUTURE PERSPECTIVES

There are various considerations to be taken into account when devising a recombinant *Salmonella* vaccine for use in humans. The vaccine candidates need to be tested in at least two animal models and in clinical trials prior to licensing. However, there is already a precedent for the use of live attenuated *Salmonella* vaccines in humans, since the attenuated *S. enterica* serovar Typhi strain Ty21a is currently licensed as a typhoid vaccine that has been shown to be safe and effective in efficacy trials. More recently, several genetically-defined attenuated mutants of *S. enterica* serovar Typhi have been shown to be safe in human clinical trials (reviewed by Garmory *et al.* [68] and Levine *et al.* [69]). In addition, some clinical studies have been carried out using recombinant *Salmonella* expressing heterologous antigens.

To date a limited number of bivalent *S. enterica* var. Typhi vaccines have been evaluated in clinical trials. Of these, several volunteer studies have shown that the vaccine candidates were poorly immunogenic against the heterologous antigens [70-72]. However, more promising results have been demonstrated in other studies. For example, in the first report of a human trial published, a vaccine based on strain CVD 908 was constructed in which a peptide of the circumsporozoite protein (CSP) of *Plasmodium falciparum* was expressed from the *tac* promoter [73]. The vaccine was well tolerated by volunteers given two doses of 5 x 10^7 cfu, eight days apart, and 3 of 10 volunteers developed immune responses against CSP. Similarly, CVD 908-*htrA* expressing TetC was used to immunise 21 adult volunteers in a dose escalation study in which 3 of 9 volunteers who received 10^8 cfu or higher developed increased levels in serum antitoxin titres. Furthermore, the vaccine was shown to be capable of inducing protective levels of antitoxin [73]. It is not clear why some *S. enterica* var. Typhi-based vaccines have been able to induce detectable immune responses against carried antigens whilst others have not, but it is possible that some problems may arise as a result of unstable expression. Tacket *et al.*, for example, plan to improve their CVD 908-*htrA*-based vaccine expressing TetC by incorporating a plasmid stabilisation system and a promoter that will delay expression of the antigen until the carrier is *in vivo* [73].

A recent volunteer study administering a *S. enterica* serovar Typhi Ty21a-based vaccine expressing *H. pylori* urease antigens to Ty21a-vaccinated human volunteers demonstrated aimed to address the issue of prior immunity to *Salmonella* on the immune responses generated against carried antigens. The study found that there was no negative effect of prior exposure upon the antigen-specific immune response generated [74], in comparison to studies in mice that have argued that pre-exposure has deleterious [75, 76] or even advantageous [77] effects upon the subsequent immune response to *Salmonella*-delivered antigens. It is possible that the route of administration [78], time of administration post-exposure [79], or type of

Salmonella vector used [80] has an effect upon the outcome of immunisation with prior immunity.

CONCLUSION

A number of genetically-defined *S. enterica* var. Typhi strains are well-tolerated and immunogenic in humans and, in addition, a range of recombinant DNA technologies have been developed that may be selected to develop recombinant *Salmonella* vaccines that are efficacious in animal models. Preliminary results from clinical trials demonstrate that protective immunity against heterologous antigens is achievable. The challenge ahead involves optimising recombinant *Salmonella* vaccines for greater efficacy in humans. Given the progress made in understanding approaches to improving the immunogenicity of such vaccines in recent years, the future for recombinant *Salmonella* vaccines is promising.

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Chapter 8

NEW TECHNOLOGY FOR BONE TISSUE REGENERATION USING CYTOKINES AND THEIR DRUG DELIVERY SYSTEMS

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ABSTRACT

Regenerative therapy of bone tissue by cytokines is now in clinical application. The most effective cytokines for bone tissue reproduction are the bone morphogenetic proteins (BMPs). Cytokine therapy using BMPs has been tested in clinical trials and is now being used for clinical treatment; however, because of obstacles, such as high cost, it is not yet widely used. To optimize this new method of treatment, it is important to allow BMPs to act efficiently on the locus where bone growth is necessary. To achieve this, we first have to examine the effect of combination therapies to increase or activate cells reacting to BMPs. In addition, it is necessary to develop new drug delivery systems (DDS) for BMPs and determine the most suitable use of BMPs and DDS for the various clinical situations. The field is being advanced by the development of new DDS for BMPs using nanotechnology. If the technology of bone tissue regeneration using BMP

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progresses with these new developments, orthopedic treatment systems can be expected to change dramatically.

INTRODUCTION

There are many clinical situations requiring bone tissue healing. About 1,500,000 bone grafts are performed in the U.S.A. each year. In orthopedic surgery there are many needs for restoration of bone tissue, such as to treat severe fractures, for spinal fusion, and to repair bone defects after bone tumor resection. The need for bone restoration also extends to other areas such as craniofacial surgery or oral surgery. In these cases, autologous bone graft and allogenic bone graft have generally been used [1]; however, it is thought that autologous bone grafting is the most superior method for bone repair. The safety of autologous bone grafting is high because a patient's own tissue is used. Autologous grafts show superior bone conduction ability because of their ideal scaffold architecture and superior bone induction ability for living bone cells; therefore, autologous bone grafts have a high potential for bone repair. However, there is a limit to the quantity of autologous bone that can be collected. In addition, collection is associated with the risk of complications such as pain, deformity, and infection.

In contrast, there is no limitation on the quantity of allogenic bone graft. There is no harvesting of bone from a normal site; however, disease transfer and immunogenicity become problems because of the use of bone from another person. In addition, the maintenance of a bone bank is expensive. Many clinical studies have shown that the ability to restore bone tissue using allogenic bone grafts is clearly lower in comparison with autologous bone grafts [2]; therefore, autologous and allogenic bone grafting both have advantages and disadvantages.

Recently, a new bone graft technique that compensates for the weak points of either conventional grafting technique has been tried experimentally. This new technique consists of allogenic bone grafting together with transplantation of undifferentiated mesenchymal stem cells gathered from the patient's own bone marrow. This bone/cell complex has a scaffold of allogenic bone and new bone formation potential by including undifferentiated mesenchymal stem cells [3,4]. This new bone graft is a potential new therapy.

Compared to these bone graft therapies, cytokine therapy aimed at healing of bone tissue may be superior because it is non invasive, there is no issue of disease transfer, and there is limitation of bone source. Bone tissue repair by cytokine therapy is an important technology that should immediately be incorporated in clinical practice, considering the high demand for bone transplantation.

BMPS

The most effective cytokines for bone tissue regeneration are BMPs. BMPs possess the ability to let undifferentiated mesenchymal stem cells proliferate and differentiate to bone forming cells, resulting in bone tissue induction *in vivo*. When a bone fracture occurs, BMPs act as key factors for fracture healing. The fracture site, undifferentiated mesenchymal stem cells migrate from circumferential periostium, bone marrow and muscle to the fracture site,

proliferate, and differentiate into bone forming cells, forming new bone tissue and repairing the fracture. BMPs contribute to this fracture healing process and induce serial cascades in bone repair [5]. In addition, ectopic bone formation can be seen when BMPs with appropriate DDS are implanted intramuscularly. Furthermore, callus-like bone formation can be caused when we trigger BMPs in the periostium. Based on these findings, it has been hypothesized that BMPs can be used for regenerative therapy of bone tissue [6, 7].

BMP was discovered by Urist in 1965 [8] as the bioactive substance that formed neonatal bone ectopically from a demineralized bone *in vivo*. BMP was cloned by Wozney in 1988 [9], and the amino acid sequence was determined shortly thereafter. BMPs belong to the TGF β superfamily [10], and about 20 BMPs have been identified to date. Of these BMPs, BMP-2, -4, and -7 (also osteogenic protein-1; OP-1) have strong bioactivity to induce bone. As for BMP-2 and 7, recombinant human BMPs (rhBMPs) are commercially available [11]. Many animal experiments have been performed using these rhBMPs and clinical application has begun.

THREE METHODS OF BONE TISSUE REGENERATION USING BMPS

Three methods are available to use cytokines or growth factors such as BMPs (Figure 1). The first method is cytokine therapy in which implantation of BMPs with DDS induces local bone formation or regeneration. Through clinical experience, it is becoming clear that the degree of bone repair using this BMP therapy is similar to that of autologous bone grafting. However, problems with this method exist. Details of this therapy are discussed later.

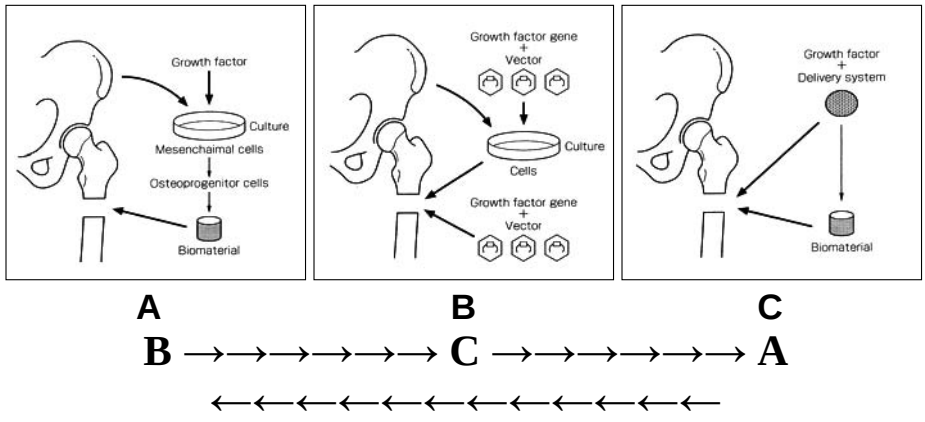

Figure 1. Three types of therapies for bone tissue regeneration using growth factors such as BMPs. A: Cytokine therapy. B: Cell therapy. C: Gene therapy. (Reprinted from Saito N, Horiuchi H, Takaoka K. Bone regenerative therapy using growth factors, M. B. Orthop. 15: 56-61, 2002).

The second method is cell therapy, in which undifferentiated mesenchymal stem cells are obtained from the patient's own bone marrow. These cells proliferate and differentiate to bone forming cells using *in vitro* BMP [12]. These bone forming cells can be compounded with porous biomaterials, inducing new bone tissue on the biomaterials. These hybrid materials are implanted in the body and repair bone defects. In this therapy, time and effort

are needed for obtaining the cells and incubating them *in vitro*. At the present time, this therapy is in the experimental phase. However this method has the advantage of using autologous cells.

The third method is gene therapy [13]. The BMP gene is put into vectors such as viruses or liposomes, and they are used to infect target cells *in vivo* or *in vitro*. These cells act as BMP producing cells, and induce bone tissue formation *in vivo*. In recent years, the techniques of gene therapy have progressed rapidly, but some problems still remain such as the safety of vectors for clinical use. However, if these problems are solved, BMP gene therapy can be realized in the future.

CLINICAL TRIALS OF BONE REPAIR BY CYTOKINE THERAPIES

There have been three large-scale clinical trials of cytokine therapies using BMPs. One was reported in 2001 and used rhOP-1 [14]. It was a randomized controlled trial (RCT) to determine the efficacy of rhOP-1 in non-union of tibial fracture and included 124 patients. In this trial, type I collagen was used as DDS for rhOP-1 and implantation of rhOP-1/type I collagen composites were compared to autologous bone grafting. Comparison of results at 9 months after surgery showed that there was no significant difference between the rhOP-1 group and autologous bone graft group with respect to union rates based on radiography and clinical evaluations, indicating that rhOP-1 therapy was efficacious. Based on these results, the United States Food and Drug Administration (FDA) authorized clinical application of rhOP-1 in 2001. Stryker Biotech company (Hopkinton, MA) manufactures this rhOP-1/collagen implant, and now it is used clinically.

The second large-scale clinical trial was an RCT of lumbar spine vertebral fusion in 279 patients by rhBMP-2, which was reported in 2001 [15]. Type I collagen was also used as the DDS for rhBMP-2, and the control group received an autologous ileum bone graft. The rhBMP-2/collagen composite or autologous bone was put in a cage implant. One level of interbody vertebral fusion was performed with the cage implant and postoperative simple radiographs and computed tomographic (CT) images were used for evaluation. The rhBMP-2 group had a significantly shorter operation time and less bleeding during the operation. In addition, the union rate after 24 months was significantly higher in the rhBMP-2 group than in the autologous bone graft group. The final clinical results of both groups were similar. Based on these results, the FDA authorized the use of rhBMP-2 therapy for spinal interbody fusion in 2001. The Medtronic Sofamor Danek company (Minneapolis, MN) manufactures the interbody spinal fusion implant system, which consists of the cage, rhBMP-2, and type I collagen.

The third large-scale clinical trial was an RCT of rhBMP-2 use for open tibial fractures in 450 patients and was reported in 2002 [16]. The rhBMP-2 group was divided into two subgroups based on the quantity of rhBMP-2 (12 mg and 6 mg] and was compared to the control group, which did not receive rhBMP-2. The 12-month results of the 12 mg rhBMP-2 group compared to the control group demonstrated lower risks for producing non-union or requiring a second operation was significantly low. Furthermore, the time to union was significantly shorter. In addition, there was a little damage of implant for fracture fixation,

and a lower infection rate and early wound cure in the group receiving 12 mg of rhBMP-2 in comparison with the control group. Based on these results, it was clearly shown that rhBMP-2 is effective for the treatment of open tibial fractures. On the other hand, the results for the 6 mg rhBMP-2 group were not as good, so that it became clear that a larger dose of BMP is necessary.

Based on these large-scale investigational results, subsequent clinical trials using BMPs were undertaken. Recently, the BESTT-ALL (the BMP-2 evaluation in surgery for tibial trauma-allograft) study group used rhBMP-2 together with allogenic bone grafting for the treatment of tibial fractures with bone defects [17]. In this RCT, an autologous bone graft alone was performed as the control treatment. Bone union was evaluated by radiography and clinical results. Thirteen of fifteen patients in the allogenic bone grafting with rhBMP-2 group achieved bone union. In contrast, 10 out of 15 patients in the allogenic bone grafting alone group achieved bone union. In this trial, the dose of rhBMP-2 used was also 12 mg.

PROBLEMS IN CLINICAL APPLICATION OF BMPS

Based on the above mentioned clinical studies, it is clear that BMPs are effective for clinical bone tissue regeneration; therefore cytokine therapy using BMPs has entered routine clinical use. It is hoped that more clinical studies demonstrating the efficacy of BMPs will increase the range of application of BMP therapy in the near future. However, some problems with the clinical application of BMPs have already become clear. First, BMPs are expensive and the medical care expenses associated with BMP therapy may be too high. Another problem is that the bone regenerative ability of BMPs in humans is considerably lower than has been demonstrated in animal studies. Therefore, effective results may not be achieved unless large quantity of BMPs are used. It is therefore necessary to develop new methods to use BMP at as low dose as possible.

One of the ways to solve these problems is to increase and activate cells reacting to BMPs in local sites. For this purpose, BMP therapy should be combined with other cytokines or drugs, or with other procedures such as cell therapy or gene therapy. Another means is to develop more efficient DDS for BMPs or scaffolds for bone regeneration.

COMBINATION OF BMP THERAPY AND OTHER THERAPIES

No matter whether BMPs have superior bone induction ability, bone tissue is not formed if target cells that have receptors for BMPs are not present. In addition, specific reactions do not occur if the signals that are initiated through BMP receptors are not transmitted effectively in these cells. Furthermore, bone formation is not achieved if other key growth factors contributing to the serial cascade are lacking [7]. It is necessary to supply the cells reacting to BMPs with other cytokines or drugs potentiating the effects of BMPs. Furthermore, incorporation of other techniques to promote signal transduction in the cells is required.

Many ideas have been suggested to achieve these goals. One is to combine BMP therapy with cell therapy, such as transplantation of target cells, which have been incubated, isolated, and proliferated *in vitro*. The other idea is to combine BMP therapy with gene therapy to synthesize other necessary proteins by genes introduced into cells by *in vivo* or *in vitro* methods. It will be necessary to develop simple, easy, and efficient methods for improving bone formation ability of BMPs based on these techniques.

DRUG DELIVERY SYSTEMS FOR BMPS

Appropriate DDS for BMPs are required to induce bone formation safely and effectively in a specific locus. The roles of DDS for BMPs are not only related to releasing BMP at specific sites and discharging it afterwards but also for becoming a scaffold for new bone formation. Therefore, we can control formed bone quantity and configuration based on changes in the DDS. The requirements that DDS for BMPs should satisfy are: a high degree of biocompatibility, no immunogenicity or carcinogenicity, no inhibition of the BMPs bone formation potency and bone repair ability, easily manipulated and sterilized, and easily and inexpensively produced [18].

Almost all of the clinical applications of BMPs have used type I collagen as DDS. This is atelocollagen in which antigenicity is decreased by chemical modification of collagen obtained from the skin or calcanean tendon of a cow or pig. When BMPs are mixed into a porous sheet of this type I collagen and implanted into the body, local bone formation is efficiency induced (Figure 2). Because the collagen is degraded gradually by enzymatic reactions and absorbed *in vivo*, it is not necessary to remove it from the body later [19]. However, bacause this type I collagen is a xenogeneic material, there is a possibility of disease transfer or immunogenic reaction [20]. In addition, it is difficult for collagen to add other characteristics, such as intensity to support weight bearing.

Therefore, synthetic biocompatible polymers that are safe to humans need to be developed as DDS for BMPs instead of collagen. Synthetic biocompatible polymers have no immunogenic risk compared to xenogeneic materials such as collagen DDS. In addition, it is possible to alter synthetic polymer characteristics, such as intensity, degradability, and stickiness to match particular clinical needs. It is not necessary to remove biodegradable synthetic polymers from the body after completing bone formation *in vivo*. Based on the polylactic acid polymers which had been used in the manufacture of surgical suture, many synthetic biocompatible polymers have been evaluated as DDS for BMPs [21].

We have developed a new synthetic polymer; poly-D,L-lactic acid-p-dioxanone-polyethylene glycol block copolymer (PLA-DX-PEG). This is thought to be one of the most suitable synthetic polymer DDS for BMPs. This polymer exhibits promising degradation characteristics for BMP-delivery systems and good biocompatibility under test conditions. The PLA-DX-PEG/rhBMP-2 composite implants can induce ectopic new bone formation more effectively than the collagen/rhBMP-2 composite when tested *in vivo*, and can repair large bony defects orthotopically [22-24] (Figure 3). This novel polymer DDS represents a significant advance in bone repair.

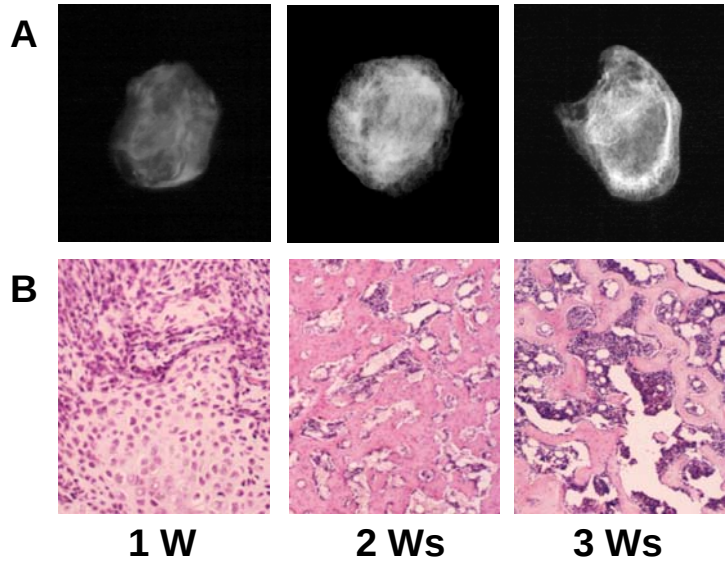

Figure 2. Ectopic bone formation mediated by BMP and type I collagen DDS. rhBMP-2 (10 μg) and type I atelocollagen were implanted into the back muscle of a mouse. They were resected 1, 2, or 3 weeks later. Cartilage tissue was mainly seen at 1 week, but woven bone tissue was recognized at 2 weeks. At 3 weeks, mature bone tissue with hematopoietic bone marrow was formed. A: Soft radiographic images. B: Histology. (Reprinted from Saito N, Horiuchi H, Takaoka K. Bone regenerative therapy using growth factors, M. B. Orthop. 15: 56-61, 2002).

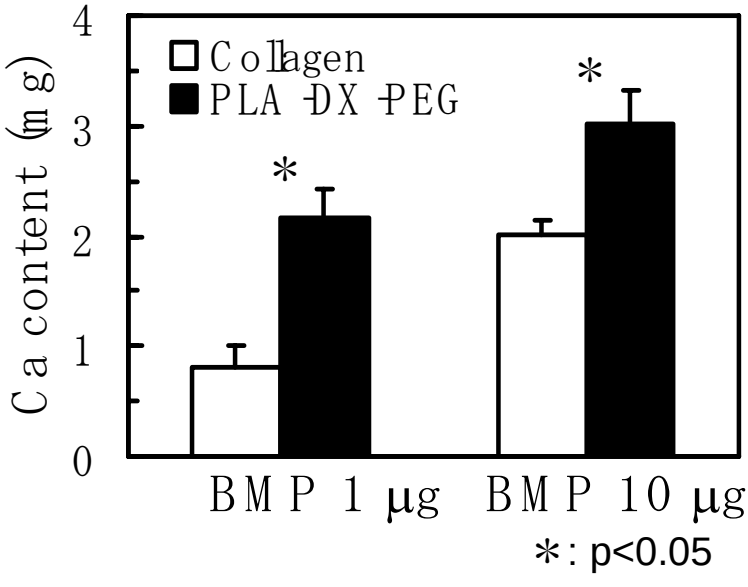

Figure 3. Comparison of the calcium content of new bone in rhBMP-2/PLA-DX-PEG implants and rhBMP-2/collagen implants. The calcium content of new bone obtained using PLA-DX-PEG DDS was significantly higher than using collagen DDS for either 1 μg or 10 μg of rhBMP-2. (Reprinted from Saito N, Okada T, Horiuchi H, Murakami N, Takahashi J, Nawata M, Ota H, Nozaki K, Takaoka K. A biodegradable polymer as a cytokine delivery system for inducing bone formation, Nat. Biotechnol. 19: 332-335, 2001).

DEVELOPMENT OF TECHNOLOGY FOR BONE REPAIR USING BMPS AND SYNTHETIC POLYMERS FOR CLINICAL USE

BMPs are already in clinical use, but the situations in which they are used are considerably limited. However, as BMP therapy increases, BMPs may be used for various indications in the future. Below, we will discuss several clinical situations and review the usage of BMPs and synthetic polymer DDS.

1. Repair of Bone Defects Using Composites of BMPs and Synthetic Polymer DDS

As the result of developments in synthetic biodegradable polymers, it is thought possible to control the size and shape of newly formed bone if an appropriate delivery system is used. For this purpose, a hard gel type of PLA-DX-PEG is suitable. To test whether this novel polymer functions in large bone defects *in vivo*, we implanted PLA-DX-PEG/rhBMP-2 composites in 4-mm diameter (critical-size) rat iliac bone defects and examined the defects using radiographic and histologic methods. The bone defect was repaired in an rhBMP-2 dose-dependent and time-dependent manner. Histologic analysis of the specimens revealed that the defects that had received 10 μg of rhBMP-2 were filled with dense trabecular bone with no evidence of polymer remnants at 4 weeks postoperatively. At the host-defect interfaces, new bone formed adjacent to the host bone. These results suggest that rhBMP-2 in the PLA-DX-PEG polymer delivery system should be suitable to elicit bone formation and healing in large bone defects.

2. Injectable Polymeric Delivery Systems for BMPs

Injectable delivery systems for rhBMP-2 could provide a less invasive method for repair of bone defects without requiring extensive surgery. The new synthetic biodegradable polymer, PLA-DX-PEG, features an exquisitely temperature-dependent liquid-semisolid transition and works well as an injectable rhBMP-2 delivery system [25]. The thermosensitive property of the PLA- DX-PEG/BMP composite means that it can be injected percutaneously when heated. The fluidity of this composite decreases as it cools to body temperature and the resultant semi-solid form provides a scaffold for bone formation through the gradual local release of the BMP. Because rhBMP-2 is heat-stable, PLA-DX-PEG of a certain molecular weight could be a suitable delivery system for the injectable delivery of rhBMP-2. This polymer combined with rhBMP-2 and heated to 60°C, could be injected as a liquid, which then turns into a semisolid form in the body at 37°C. The properties of this polymer would allow the retention of the BMP for a period of time that would be sufficient to elicit new bone formation and thereby provide a scaffold for further bone growth. Eventually, it would be completely replaced with new bone and surgery would not be required for removal because of this polymer's biodegradability.

To demonstrate the efficacy of this polymer further, 25 mg of PLA-DX-PEG combined with 10 μg of rhBMP-2 was heated at 60°C for 5 min and injected into the muscle on the

surface of the murine femur using a 14-gauge needle. Three weeks after injection, new bone was found at the injection site, which was attached to the surface of the femur (Figure 4). This new type of injectable osteoinductive material will enable a less invasive approach to surgeries involving the restoration or repair of bone tissues [26].

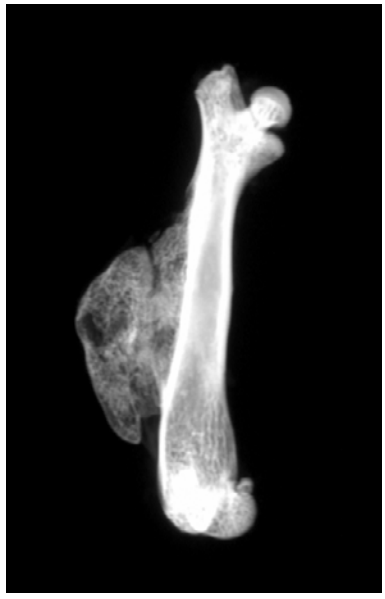

Figure 4. Soft radiograph of new orthotopic bone formed by injection of 10 μg rhBMP-2/PLA-DX-PEG polymer composite in the muscle pouch on the abraded surface of the femur of a mouse 3 weeks after injection. (Reprinted from Saito N, Okada T, Horiuchi H, Ota H, Takahashi J, Murakami N, Nawata M, Kojima S, Nozaki K, Takaoka K. Local bone formation by injection of recombinant human bone morphogenetic protein-2 contained in polymer carriers, Bone 32: 381-386, 2003).

3. Combination of the BMP/Synthetic Polymer Composites with Other Materials

Due to the hydrophobic nature of the PLA-DX-PEG polymer, it swells on contact with water. This physical property provides additional advantages for the practical use of the polymer in combination with porous biomaterials. When the solid implant with its pores filled with the rhBMP-2/PLA-DX-PEG composite is implanted, the composite will swell, ooze out of the pores, and form a layer of the composite. Based this property, a combination of the rhBMP-2/PLA-DX-PEG composite with porous hydroxyapatite (HA) was tested [27]. The rhBMP-2/PLA-DX-PEG composite was placed in the pores of an HA block, and this combination was placed into the back muscle of a mouse. After 3 weeks, new bone was formed and surrounded the HA. Histologic examination showed that new bone also was formed on the inside of the pores of HA (Figure 5).

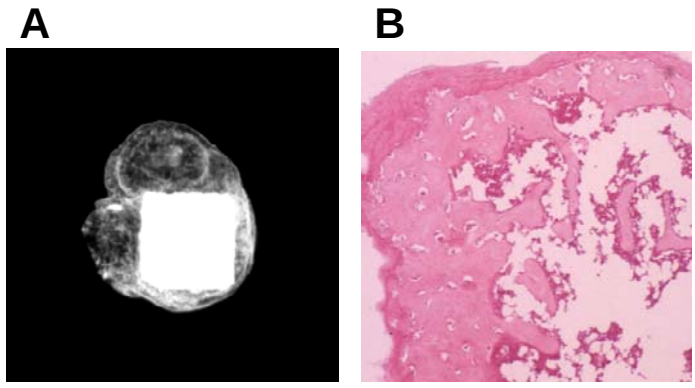

Figure 5. Ectopic bone formation by hydroxyapatite (HA) with the rhBMP-2/PLA-DX-PEG composite. The rhBMP-2/PLA-DX-PEG composite was put into the pores of an HA block, and it was placed in the back muscle of mouse. A: Soft radiograph showing the new bone formed surrounding the HA after 3 weeks. B: Histologic examination showing new bone inside the pores of HA. (Reprinted from Saito N, Okada T, Horiuchi H, Murakami N, Takahashi J, Nawata M, Ota H, Miyamoto S, Nozaki K, Takaoka K, Biodegradable poly-D,L-lactic acid-polyethylene glycol block copolymers as a BMP delivery system for inducing bone, J. Bone Joint Surg. Am. 83 Suppl 1: 92-98, 2001).

Next, rhBMP-2 (120 μg) was mixed with the polymer (120 mg) and impregnated in titanium fibermesh cylinders. Three 5-mm cylinders were placed end-to-end to fill a 15-mm defect created in the humeri of adult rabbits and stabilized with an intra-medullary rod. In control animals, the titanium fibermesh cylinders were combined with the polymer but without rhBMP-2. Six weeks after implantation, new bone had formed on the surface of the implant and had bridged the defect. The defects treated with control implants were not repaired [28] (Figure 6).

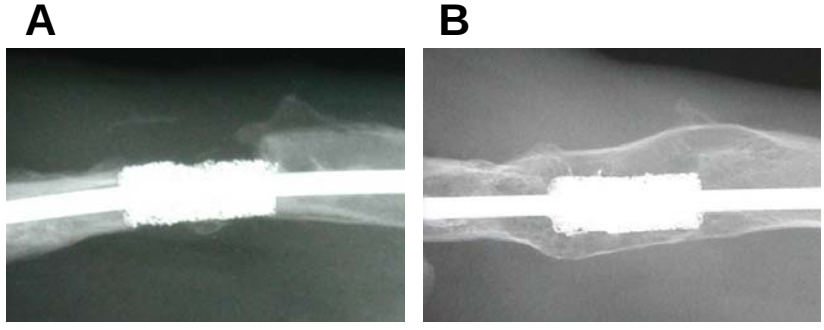

Figure 6. Repair of a bone defect with a titanium syringe implant containing an rhBMP-2/PLA-DX-PEG composite. A 1.5-cm bone defect was created in the humerus of the rabbit, and three 5-mm implants were placed in it. They were stabilized with an intra-medullary rod. A: The bone defect was not repaired in the control group. B: The defect was restored in the 120 μg rhBMP-2/PLA-DX-PEG group after 5 weeks. (Reprinted from Murakami N, Saito N, Horiuchi H, Okada T, Nozaki K, Takaoka K. Repair of segmental defects in rabbit humeri with titanium fiber mesh cylinders containing recombinant human bone morphogenetic protein-2 (rhBMP-2) and a synthetic polymer, J. Biomed. Mater. Res. 62: 169-174, 2002).

These results provide strong evidence that these new composite implants, combining rhBMP-2, synthetic degradable polymers and compatible biomaterials, provide enhanced regenerative potential for the repair of large bone defects. Using these techniques, it is possible to repair weight bearing bone, which requires strength. The combination of the

rhBMP-2/PLA-DX-PEG composite and HA or titanium creates a new osteoinductive material with strength properties.

4. Development of New Implants for Joint Arthroplasty That Restores a Bone Defect

Total hip arthroplasty (THA) has become a routine procedure for the treatment of various hip lesions. However, one of the limitations of this surgery has been the mechanical loosening of the prosthesis resulting from peri-prosthetic bone loss, a condition termed peri-prosthetic osteolysis. Consequently, at the time of revision surgery, various grades of bone defects, both in the proximal femur and the acetabulum are often noted, which present challenges for the solid fixation of a new prosthesis. Alternative approaches aimed at overcoming this problem have included a special design of the revision prosthesis and use of allo- or autogeneic bone grafting in combination with or without biomaterials such as hydroxyapatite. If such bone loss can be repaired with use of rhBMP-2, revision surgery may become more feasible and efficacious.

In a further attempt to address the loosening of the prosthesis, we have developed a new prosthesis combined with the rhBMP-2/PLA-DX-PEG composite. We tested the efficacy of the rhBMP-2-containing prosthesis to reconstruct a bone defect in a canine model. In this model, the medial half of the proximal femur was surgically resected to create a bone defect that was repaired with the rhBMP-2/PLA-DX-PEG composite. Twelve weeks after implantation, the original bone defect in the rhBMP-2 treatment group was repaired [29]. Thus, this type of ‘hybrid’ prosthesis may provide a new modality to repair bone defects or restore lost bone mass encountered in revision arthroplasty.

NEW TRIAL APPLICATIONS OF NANOTECHNOLOGY TO BONE TISSUE REGENERATION

Recently, nanotechnology has been tried to apply biomaterials and new treatment techniques in various biomedical fields. Below we will review the application of carbon nanotubes (CNTs), which are materials of nanosize, to bone tissue regeneration.

1. Application of CNTs to Biomaterials

There is a great deal of research and development regarding the use of carbon nanotubes (CNTs) as new structural or electronic material in various fields due to their unique characteristics [30-34] In addition, the performance of various materials can be improved greatly by combining them with CNTs. In the medical field, it is expected that biomaterials will be developed using CNTs, which will be applied for clinical treatment [35,36,37]. However, studies using CNTs as biomaterials are still in the preliminary stages. The most important concern for the use of CNTs as a biomaterial is biocompatibility. However, there have been several reports about the biocompatibility of CNTs. According these studies, CNTs

may be safe for use in the human body, but this has not been confirmed. It is also necessary to perform toxicity and carcinogenicity tests for CNTs prior to clinical use.

There are many uses of biomaterials adjacent to bone tissue, such as prostheses for arthroplasty, plates and screws for fracture treatment, and artificial bone. It is expected that CNTs will be applied to biomaterials used for bone, for example, to make high mechanical strength prostheses for arthroplasty that will last for a long time without failure. In addition, there are some studies applying CNTs as DDS and/or scaffolds for bone tissue regeneration [38]. Bone tissue compatibility is extremely important for the application of CNTs to biomaterials in contact with bone, but there have been no reports related to this point. Furthermore, it is also very important to determine the effects of CNTs on bone formation for the application to biomaterials related to bone. However, there have been no reports of such studies. We are beginning to study bone tissue compatibility of CNTs and their influence on bone formation to obtain a basis for application of CNTs as biomaterials in contact with bone and DDS and/or scaffold in bone tissue regeneration.

2. Studies to Apply CNTs to DDS for BMPs

Studies aimed at applying CNTs to DDS or scaffold for bone tissue regeneration are expected. It has been reported that differentiation and proliferation of osteoblasts were promoted when they are incubated on CNTs *in vitro* [38]. In addition, a recent report indicated that hydroxyapatite was formed and crystallized on CNTs in simulated body fluid and that CNTs acted as the core for the initial crystallization of hydroxyapatite [39,40]. These properties of CNTs may be advantageous when they are used as DDS for BMPs and scaffolding for bone formation. We have developed novel DDS for BMPs compounding CNTs either alone or with other biomaterials. It is hoped that the studies applying nanotechnology to bone tissue regeneration will be performed in the future.

CONCLUSION

BMPs have been used clinically, while regenerative therapy of bone tissue is in a new phase. One of the current problems is how to combine BMP therapy with other therapies to supplement cells reacting to BMPs or other cytokines interacting with them. The other problem is how to develop more effective DDS than conventional type I collagen. Furthermore, expanding the indications for the usage of BMPs for various locations and disorders is required. Studies to apply nanotechnology to promote bone tissue regeneration by BMPs bring the promise of completely new methods. These studies using nanotechnology can possible create large breakthroughs in bone tissue regenerative therapy with BMPs. Molecules and the action mechanisms controlling regeneration of bone tissue will become clearer in the next several years. With these advances, it is expected that the technology of bone tissue regeneration using BMPs will improve drastically and orthopedic treatment systems will change dramatically in the near future.

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