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Engineering Polymeric Micelles For Solubilization of Poorly-Water Soluble Drugs: A Novel Approach For Oral Drug Delivery

par

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Engineering Polymeric Micelles For Solubilization of Poorly-Water Soluble Drugs: A Novel Approach For Oral Drug Delivery

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Abstract

Novel polysaccharide-based micelles were prepared to exploit their solubilizing potential towards poorly-water soluble drugs in order to improve their oral bioavailability. Hydrophobically-modified (HM) dextran (DEX) and hydroxypropylcellulose (HPC) copolymers were synthesized. In aqueous solution, HM DEX and HM HPC form polymeric micelles with low onset of micellization and small size. Cyclosporin A (CsA) was selected as model drug. CsA-loaded polymeric micelles were prepared by a dialysis procedure. The CsA incorporation in micelles of HM polysaccharides was significantly higher than in corresponding unmodified polysaccharides. The polymeric micelles exhibited high stability in gastric and intestinal fluids. On the cellular level, the polymeric micelles presented no significant cytotoxicity towards Caco-2 cells. The apical to basal permeability of CsA across Caco-2 monolayers increased significantly, when loaded in polymeric micelles, compared to free CsA. Meanwhile, permeability of the host polysaccharides was demonstrated, where the amount of transported HPC-based micelles was greater than that of DEX-based micelles. Targeted Vitamin B_{12}-modified polymeric micelles enhanced the permeability of CsA across the intestinal barrier compared to unmodified micelles. Therefore, Polysaccharide-based polymeric micelles are promising carriers for the oral delivery of poorly-water soluble drugs.

Keywords : Poorly-water soluble drug, Polysaccharides, Polymeric micelles, Solubilization, Oral drug delivery, Caco-2, Permeability, Vitamin B_{12}.

Résumé

Des micelles polymères à base de polysaccharides ont été préparées afin d'évaluer leur potentiel comme agent de solubilisation de principes actifs hydrophobes pour en augmenter la biodisponibilité. Des copolymères à base de dextran (DEX) et d'hydroxypropylcellulose (HPC) modifiés aux groupements hydrophobes ont été synthétisés. En solution aqueuse, DEX et HPC modifiés forment des micelles polymères de petite taille à des basses concentrations. La Cyclosporin A (CsA) a été choisi comme modèle de principe actif hydrophobe. Des micelles polymères contenant de la CsA ont été préparées par une procédure de dialyse. Le pourcentage d'incorporation de la CsA dans les micelles de polysaccharides modifiés a été significativement plus élevé que dans le cas des polysaccharides non-modifiés. Les micelles polymères ont été démontrées stables dans les fluides gastriques et intestinaux. Au niveau cellulaire, les micelles polymères n'ont pas présenté de cytotoxicité significative envers les cellules intestinales Caco-2. La perméabilité apicale-basale de la CsA à travers une monocouche de Caco-2 a augmenté significativement lorsque incorporée dans les micelles polymères en comparaison avec le principe actif seul. L'étude de la perméabilité de copolymères a démontré une augmentation de la perméabilité des micelles polymères à base de HPC par rapport aux micelles à base de DEX. Les micelles polymères greffées de vitamine B_{12} ont démontré une plus grande perméabilité de la CsA à travers les cellules intestinales en comparaison avec les micelles non-greffées. En conclusion, les micelles polymères à base de polysaccharides représentent une technologie prometteuse de vecteurs pharmaceutiques pour l'absorption orale de principe actif hydrophobe.

Mots-clés : Médicament peu-soluble dans l'eau, Polysaccharides, Micelles polymères, Solubilisation, Voie orale, Caco-2, Permeabilité, Vitamin B_{12}.

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A	Surface area
ACN	Acetonitrile
AP	Apical side
BL	Basolateral side
BCS	Biopharmaceutics classification system
°C	Degree Celsius
ΔC	Concentration gradient
C	Concentration of a substance at time t
CAC	Critical association concentration
Cbl	Cobalamin
CO_2	Carbon dioxide
C_{max}	Maximum concentration of drug in blood
CMC	Critical micelle concentration
C_s	Saturation solubility
CsA	Cyclosporin A
CYP	Cytochrome P450
D	Diffusion coefficient
dC/dt	Dissolution rate
DCM	Dichloromethane
DEX	Dextran

DL	Drug loading
DLS	Dynamic light scattering
DMEM	Dulbecco's Modified Eagle's Medium
DMSO-d_6	Deuterated dimethyl sulfoxide
DTAF	5-([4,6-dichlorotriazin-2-yl] amino)-fluorescein
$D : S$	Dose/solubility ratio
EDTA	Ethylenediaminetetraacetic acid
EPO	Erythropoietin
Et_3N	Triethylamine
FAO	Food and Agriculture Organization
FBS	Fetal Bovine Serum
FDA	United States Food and Drug Administration
g	graft
G-CSF	Granulocyte colony-stimulating factor
GI	Gastrointestinal
h	Hour
h	Thickness of dissusion boundary (or cell membrane)
HBSS	Hank's Balanced Salt Solution
Hc	Haptocorrin
HCl	Hydrochloric acid
HM	Hydrophobically-modified
^{1}H-NMR	Proton nuclear magnetic resonance

HP	Hewlett-Packard
HPC	Hydroxypropylcellulose
HPLC	High performance liquid chromatography
i.d.	Internal diameter
IF	Intrinsic factor
IFR	Intrinsic factor receptor
IL-2	Interleukin-2
I.V.	Intravenous
J	Rate of diffusion
KH_2PO_4	Monobasic potassium phosphate
LHRH	Luteinizing hormone releasing hormone
$Me_3N.HCl$	Trimethylamine hydrochloride
min	Minutes
MEM NEAA	Minimum Essential Medium Nonessential Amino Acid
mRNA	Messenger Ribonucleic Acid
MS	Molar substitution
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
M_w	Molecular weight
NaCl	Sodium chloride
Na_2HPO_4	Dibasic sodium phosphate
NaH_2PO_4	monobasic sodium phosphate
NaOH	Sodium hydroxide

NCE	New chemical entities
NEAA	Non-essential amino acids
NSAID	Non steroidal anti-inflammatory drugs
P85	Pluronic® P85
PBS	Phosphate buffered saline
PEG	Poly(ethylene glycol)
P-gp	P-glycoprotein
PGI	P-glycoprotein inhibitor
pk_a	Ionization constant
PEO	Poly(ethylene oxide)
POE	Polyoxyethylene
Py	Pyrene
R&D	Research and development
RH	Relative humidity
S.D.	Standard deviation
SDS	Sodium dodecyl sulfate
TcII	Transcobalamin II
TEER	Transepithelial electrical resistance
TEM	Transmission electron microscopy
TM	Transport medium
T_{max}	Time to reach peak concentration of drug in blood
U	Unit

UV	Ultra violet
v	Volume
vs	versus
VB_{12}	Vitamin B_{12}
w	Weight
W_c	Weight of CsA loaded in micelles
W_M	Weight of CsA-loaded micelles
WHO	World health organization
λ	Wavelength
λ_{cm}	Emission wavelength
λ_{cx}	Excitation wavelength

Please note that the abbreviations PEG, PEO and POE, all refer to the chemical structure $H(OCH_2CH_2)_nOH$.

Target The Best

& Surround Yourself With The Best

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CHAPTER ONE

INTRODUCTION – PART I

AN OVERVIEW ON THE MORPHOLOGICAL, PHYSIOLOGICAL AND PHYSICAL CHEMICAL BARRIERS FACING THE ORAL ABSORPTION OF THERAPEUTICS

Part one of this two-part review addresses the transport of therapeutics across the intestinal barrier, and focuses on the anatomical, physiological and physicochemical parameters impacting transcellular uptake of orally administered drugs. Part two will cover approaches for overcoming obstacles limiting successful oral delivery of poorly-water soluble drugs, namely polymeric micelle-based carrier system.

The average development cost of a new chemical entity (NCE) is approximately US$ 300–550 million (Frantz, 2004; Preziosi, 2004). It often costs substantially less to develop new methods of administration for an existing drug, which results in improved efficacy and bioavailability together with reduced dosing frequency to minimize side effects. Therefore, the pharmaceutical industry is under constant pressure to maximize the full potential of a drug candidate at an early stage of its life cycle. This objective can be accomplished by incorporating the drug into various drug delivery systems. Such exercise can lead to extended patent life and convenient dosage forms that overcome previously presented administration problems. For the last two decades, there has been an enhanced demand for more patient-compliant dosage forms. As a result, novel drug delivery systems evolved over a short period of time to optimize the dosage regimen of a drug without compromising its therapeutic efficacy. There are now approximately 350 drug delivery corporations and 1000 medical device companies. The demand for their technologies was approximately $14–20 billion in 1995 (Annual report on drug delivery, 1996) and, according to industry reports, this is expected to grow to $60 billion annually.

Peroral administration of therapeutics, when appropriate, remains the route of choice for drug delivery. On the one hand, from the patient standpoint, the oral route is the most popular means of delivering drugs into systemic circulation due to ease of administration (ingestion) and pain avoidance (Li, V.H.K. *et al.*, 1987). On the other hand, oral delivery systems do not require sterile conditions and are, therefore, less expensive to manufacture.

Unfortunately, formulating a drug for oral delivery is a technically challenging process. Methodologies for systematically optimizing drug stability in the gastrointestinal (GI) tract, obtaining the desired pharmacokinetic profile and biodynamics, as well as reducing absorption variabilities and side effects are not well-established routine processes. Delivery issues become even more complex when the pharmaceutical scientist is confronted with designing formulations for the ever-expanding number of poorly soluble therapeutic drug candidates that are currently being identified and evaluated for oral administration (Fasano, 1998; Charman, 2000; Saha *et al.*, 2000; Dressman, J. *et al.*, 2001).

The success of an oral drug delivery system depends on various biological and physicochemical factors. The biological considerations that will determine the success of an oral drug delivery system include intestinal mobility, the composition (*e.g.* pH, enzymes, food) of intestinal lumen and intestinal mucosal cells (Lipka *et al.*, 1996; Daugherty *et al.*, 1999; Kurosaki *et al.*, 2000). The physicochemical factors include: a) formulation factors (*e.g.* dosage form size, deaggregation and dissolution of dosage form, and rates of drug release from the drug carrier), and b) drug factors (*e.g.* solubility, chemical and enzymatic stability, lipophilicity, hydrogen bonding potential, conformation, pKa, molecular size, and affinity for endogenous transporters).

This review will address a number of issues related to transcellular intestinal transport mechanisms that might be manipulated for improved oral drug delivery.

1.1. MORPHOLOGICAL BARRIERS

Absorption of solutes occurs mainly in three sections of the small intestine (duodenum, jejunum and ileum). Each of these segments has unique anatomical and physiological properties that dictate parameters of digestion and solute absorption (Table 1.1.).

Table 1.1. Biological and physical parameters of the human intestinal tract (Wilson, 1967; Dressman, J.B. *et al.*, 1993; Charman *et al.*, 1997).

Gastrointestinal segment	Approximate surface area	Approximate segment length	Approximate residence time	Approximate pH		Prominent catabolic activities
				Fasted	Fed	
Oral cavity	100 cm^2		Seconds to minutes	6.5	6.5	Polysaccharidases
Esophagus	200 cm^2	23 – 25 cm	Seconds			
Stomach	3.5 m^2 (variable)	0.25 m (variable)	1.5 h (variable)	1 – 2	5 (1h)	Protease, lipases
Duodenum	1.9 m^2	0.35 m	0.5 – 0.75 h	5.5 – 6.5	4.5 – 5.5 (1h)	Polysaccharidases; oligosaccharidases; proteases; peptidases; lipases
Jejunum	184 m^2	2.8 m	1.5 – 2 h	6 – 7	4.5 (2h)	oligosaccharidases; peptidases; lipases
Ileum	276 m^2	4.2 m	5 – 7 h	6.5 – 7.5	6.5 – 7.5	oligosaccharidases; nucleases, peptidases; lipases
Colon and rectum	1.3 m^2	1.5 m	1 – 60 h (35 h average)	7 – 8	7 - 8	Broad spectrum of bacterial enzymes

1.1.1. Morphology of the "intelligent intestine"

Consider Alfred Binet's definition of intelligence, given at the end of the 19th century: "intelligence is the range of processes involved in adapting to the environment".

The small intestine is designed to impede the entry of pathogens, toxins and undigested macromolecules while simultaneously digesting and selectively absorbing essential nutrients, vitamins and cofactors (Walker, 1981). Unlike the skin, which has keratin to provide an overwhelming physical barrier against bacterial entry, the intestinal mucosa uses biochemical and physiological mechanisms to complement its physical barrier to prevent entry of unwanted materials. These protective mechanisms of the small intestine should be judiciously modified or circumvented for successful oral drug delivery. Such obstacles include:

- Varying pH
- The high enzymatic activity of the small intestine
- The inherently low permeability of the intestinal mucosa
- The binding capacities of resident mucous and luminal contents
- The efflux pathways back into the gut lumen following internalization into epithelial cells

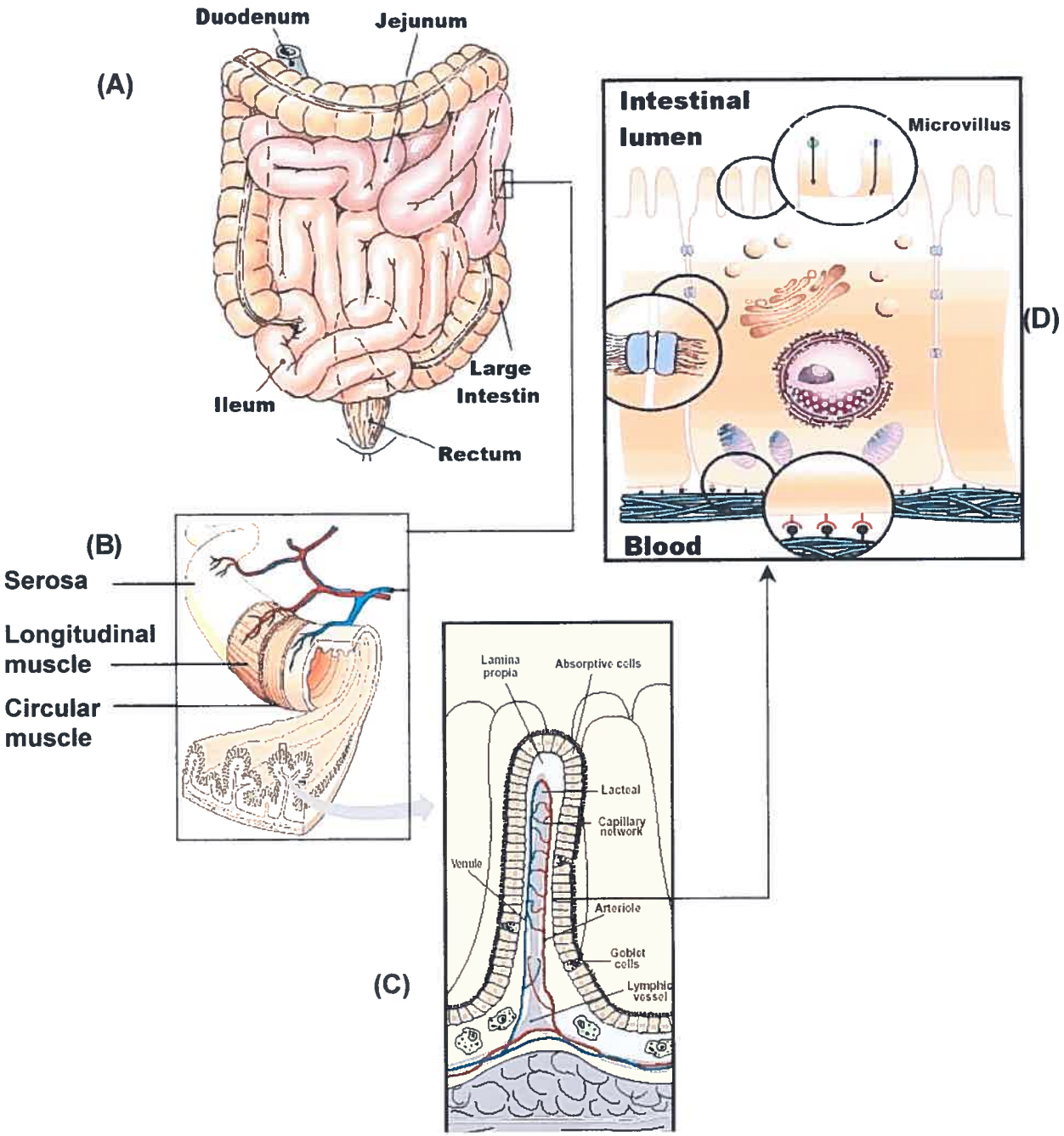


Figure 1.1. Morphology of (A) the intestinal tract showing (B) the small intestine mucosa with folds of Kerckring, (C) villi with proliferating and maturing epithelial cells traveling up the villi to be lost at the tip, and (D) microvilli on epithelial cells of the apical brush-border membrane. Adapted from (Walker, 1981; Carr *et al.*, 1984; Madara *et al.*, 1987).

The multifunctional characteristics of the small intestine make the epithelium an extremely complex living system (Figure 1.1.). The luminal mucosa is composed of a monolayer of epithelial cells, the "lamina propria", which is a connective tissue scaffold, and the "muscularis mucosa", which is a thin layer of smooth muscle. The deeper connective tissue layer underlying the mucosa is called "submucosa".

The lamina propria is a structural support for the confluent monolayer of epithelial cells and is highly folded to form "villi" which are tiny finger-like projections that increase the surface area of the mucosa by approximately 30-fold. Villi are absent in the large intestine (colon). Microvilli, which are projections of 1 mm high that extend from the apical surface of intestinal cells, further increase the surface area for digestion and absorption by approximately 600-fold. The "crypts of Lieberkühn", from which the cells originate and differentiate, reside at the base of the villi. The blood and lymphatic channels, which transport absorbed molecules, are located in lamina propria.

By contracting, the muscularis mucosa may modulate the thickness of the unstirred water layer at the surface of the mucosa, affecting the absorption of lipophilic molecules and helping to empty the luminal contents of the crypt.

The epithelial cells are a heterogeneous population of cells which include mainly: enterocytes or "absorptive cells"; "goblet cells" which secrete mucin and "M cells", which transport antigens present in the intestinal lumen to the lymph. In the crypt, the major cell type is the undifferentiated cell. As cells move up the crypt to the base of the villi, they

differentiate into absorptive cells, as well as goblet or M cells, and continue to the tip of the villi where they are extruded (Carr *et al.*, 1984; Madara *et al.*, 1987).

The most common epithelial cell is the enterocyte or the absorptive cell. This cell is responsible for the majority of the absorption of both nutrients and drugs that takes place in the small intestine (Madara *et al.*, 1987). It is highly polarized with distinct apical and basolateral membranes (Figure 1.1.C). The absorptive cells are separated by tight junctions which are apical intercellular attachement zones and comprise a meshwork of strands (Cereijido *et al.*, 1988; Denker *et al.*, 1998). The apical membrane has striking, uniform microvilli measuring approximately 1 μm in height (brush border) in which disaccharidases and peptidases reside. The apical membrane also expresses receptor-mediated transport systems (*e.g.* cobalamin (Seetharam *et al.*, 1991)), together with ion, monosaccharide, amino acid, peptide, and fatty acid transporters (Sigrist-Nelson *et al.*, 1977). In contrast to the apical membrane, the basolateral membrane has smooth contours with no sugar and peptide hydrolases. However, Na-K ATPase, glycosyltransferases, and adenyl cyclase are located in the basolateral membrane (Madara *et al.*, 1987).

Absorptive cells are covered by a mucous layer which is bound to the apical cell surface by "glycocalyx". Microvilli and the associated glycocalyx establish a brush border structure that presents a considerable catabolic barrier of enzymatic activities. The mucous coating of the epithelial surface contains mucin glycoproteins, enzymes and electrolytes. Mucous is secreted by goblet cells that are intercalated between enterocytes covering the villi, as well as from subepithelial glands. The mucous acts as a defense mechanism against

damage to the absorptive cells, and is a potential site for drug interaction and binding (Kurosaki *et al.*, 2000). The pH of the various intestinal segments ranges from slightly acidic to slightly basic. A slightly acidic microclimate at the immediate surface of enterocytes has also been described. In some cases these variations in pH might impact a drug's stability or solubility, or both.

1.1.2. Tight junction

The integrity of the epithelial cell layer is maintained by intercellular junctional complexes. At the apical side of the junctional complex (Cereijido *et al.*, 1988), the tight junction forms a continuous, circumferential, belt-like structure at the luminal end of the intercellular space, where it serves as a gatekeeper of the paracellular pathway (Cereijido *et al.*, 2000; Schneeberger *et al.*, 2004). The tight junctions allow only the passage of ions and small molecules, up to approximately 900 Da (Gumbiner *et al.*, 1988; Tang *et al.*, 2003).

However, the tight junctions appear to be a potential site for the action of absorption enhancers (calcium chelators and surfactants), which when present, open tight junctions, and after their removal the process is reversed (van Hoogdalem *et al.*, 1989). The application of enhancers is usually necessary to increase the flux of therapeutic peptides and proteins such as human calcitonin (Shah *et al.*, 2004) and enaminone anticonvulsants (Cox *et al.*, 2001).

1.2. PHYSIOLOGICAL CONSIDERATIONS IN THE DESIGN OF DRUG CARRIERS FOR ORAL DELIVERY

1.2.1. Enzymatic barrier

Drugs and other xenobiotics that gain access to the body may undergo one or more of 4 distinct fates:

1) Elimination unchanged
2) Retention unchanged
3) Spontaneous chemical transformation
4) Enzymatic metabolism

Although each of these fates is of importance, it is the enzymatic metabolism of drugs that predominates, and usually results in loss of efficacy.

The scope of drug metabolism is immense, and this is reflected in the range of chemical reaction that are involved in the metabolism of substrates, including oxidation, reduction, hydrolysis, hydration, conjugation and condensation. Typically, the process of drug metabolism is biphasic whereby the compound first undergoes a functionalization reaction (oxidation, reduction, or hydrolysis), which introduces or uncovers a functional group, *e.g.* –OH, -NH_2, -SH, suitable for subsequent conjugation with an endogenous conjugating agent.

The main site of metabolism of foreign compounds is considered to be the liver, although extrahepatic tissues, frequently at the site of entry to or excretion from the body

(*e.g.* GI mucosa, lungs, kidney) also play a role in the metabolism of drugs (Lee, V.H.L. *et al.*, 1990).

An important consideration in the oral absorption of drugs became their pre-systemic metabolism since the finding that enzymes in humans are expressed at high levels in mature enterocytes of the small intestine (Suzuki *et al.*, 2000). This level constitutes approximately 70% of cytochrome P450 (CYP) content in human enterocytes, while in the liver it is only 30% of total human hepatic CYP content. It has been estimated that an orally administered peptide or protein, during the passage across the GI tract, might encounter 40 different peptidase enzymes contained in the secretions of the stomach and pancrease, bound to the brush-border, and contained in the cytosol of the epithelial cell along with the degradative enzyme systems of the lysosome. Table 1.2. summarizes the typical peptidases in the GI tract (adapted from references (Tobey *et al.*, 1985; Guan *et al.*, 1988; Mackay *et al.*, 1997):

Table 1.2. Typical peptidases in the gastrointestinal tract.

Type	Enzyme	Site of action
Endopeptidase	Pepsin	Stomach
	Trypsin	Pancreas
	A-Chymotrypsin	Pancreas
	Elastase	Pancreas
	Endopeptidase-24.11	Brush-border
	Endopeptidase-24.18	Brush-border
	Endopeptidase-3	Brush-border
	Enteropeptidase	Brush-border
Exopeptidase	Aminopeptidase N	Brush-border
NH_2 terminus	Aminopeptidase A	Brush-border
	Aminopeptidase P	Brush-border
	Aminopeptidase	Brush-border
	Dipeptidylpeptidase IV	Brush-border
	γ-Glutamyltranspeptidase	Brush-border
Exopeptidase	Carboxypeptidase A	Pancreas
COOH terminus	Carboxypeptidase B	Pancreas
	Angiotensin-converting enzyme	Brush-border
	Carboxypeptidase P	Brush-border
	Carboxypeptidase M	Brush-border
	γ-Glutamyl Carboxypeptidase	Brush-border
Exopeptidase	Microsomal dipeptidase	Brush-border
dipeptidase		

1.2.2. P-glycoprotein efflux pump

In humans, two members of the P-glycoprotein (P-gp) gene family (MDR1 and MDR2) exist (Gottesman *et al.*, 1993). The P-gp encoded by human MDR1 functions as a drug efflux transporter, while human MDR3 P-gp is believed to be functional in phospholipid transport (van Helvoort *et al.*, 1996).

While the physiological function of P-gp is still not fully understood, the anatomical localization of the human MDR1 P-gp in the brush border region, on the apical surface of intestinal epithelial cells (Thiebaut *et al.*, 1987) suggests that P-gp plays an important role in the process of absorption. It functions as a biological barrier by extruding a wide variety of structurally and chemically unrelated compounds (drugs, toxic substances, xenobiotics, ...) out of epithelial cells (Chin *et al.*, 1993).

Furthermore, given that P-gp is located within the intestinal epithelium with the major phase I metabolizing enzyme CYP 3A and the large number of substrates and inhibitors they have in common (Wacher *et al.*, 1998), it has been proposed that these bioavailability-limiting processes can act in a complementary and synergistic manner to reduce systemic drug exposure (Tran *et al.*, 2002).

1.2.3. Mechanisms of transepithelial transport

A molecule can be transported across the intestinal epithelium *via* two routes, either between adjacent cells - termed paracellular pathway, or through adjacent cells - termed transcellular pathway. Furthermore, the movement across the epithelium can occur in both

directions: that is, apical (AP) to basolateral (BL) (movement from the intestinal lumen to the underlying tissue and circulation) and BL to AP (from the tissue out into the lumen through the P-gp efflux pathway).

1.2.3.1. Paracellular pathway

Intestinal epithelial cells differentiate by polarizing into an AP and a BL domain and by forming "junctional complexes" between the cells (as stated in section 1.1.Morphological barriers). These junctional complexes regulate the paracellular transport and contribute to the function of the transepithelial permeability barrier (Cereijido *et al.*, 2000; Tang *et al.*, 2003). In healthy intestinal epithelium, transport through the paracellular pathway is minimal due to the presence of the tight junctions. Only small hydrophilic drug molecules are allowed to pass through the paracellular route (Hayashi *et al.*, 1999), which is the aqueous route that normally absorbs nutrients, vitamins, etc (Guy *et al.*, 2001; Bronner, 2003).

1.2.3.2. Transcellular pathway

Lipophilic drug molecules are transported across the intestinal epithelium through a transcellular pathway, which can take place *via* either a passive mechanism, or a specific carrier system.

A) Passive transcellular transport

For passive flux of a molecule to occur, the drug must have the correct physicochemical properties (*e.g.* size, charge, lipophilicity, hydrogen bonding potential, solution conformation) to cross both the apical and basolateral membranes, which are lipophilic, and diffuse through the cytoplasm, an aqueous environment separating the two membranes (Lee, V. H. *et al.*, 1989).

However, the epithelial cell is limited in its ability to exert a control over transcellular passive absorption. Passive diffusion is modeled by Fick's law of diffusion, which states that "*the rate of diffusion is proportional to the concentration gradient*" (Equation 1):

$$J = D\left(\Delta C / h\right) \quad \text{.....Equation (1)}$$

where J is the rate of diffusion, D is the diffusion coefficient, ΔC is the concentration gradient and h is the cell membrane thickness.

Meanwhile, it is thought that for conventional drug molecules, a strong correlation exists between the partition coefficient and absorption. The partition coefficient measures the relative lipophilicity of a molecule and has long been recognized as one of the major determinants in membrane permeability of drugs. Most orally administered amphipathic drugs are absorbed through the passive pathway, by partitioning into and out of the cell membrane lipid bilayer (Walker, 1981).

However, compounds that are absorbed by the passive transcellular route may be substrates for the apically polarized P-gp efflux mechanism (Hunter *et al.*, 1997).

B) Carrier-mediated cell uptake

A drug designed for delivery must either be small enough to diffuse paracellularly, or lipophilic enough to pass through the cell. Compounds may also be absorbed transcellularly by utilizing naturally occurring carriers that will transport them from the lumen into the cell. Internalization occurs within minutes, and the rate of uptake is many orders of magnitude higher than would be expected from bulk phase uptake or pinocytosis (Feger *et al.*, 1994; Swaan, 1998). For example, L-dopa and oral cephalosporins (Dantzig *et al.*, 1990) are absorbed by amino acid and dipeptide transporters, respectively.

In general, macromolecules absorbed through random pinocytosis into intestinal epithelial cells are catabolized in lysosomes and multivesicular bodies. A select group of macromolecules, however, readily moves across the transcellular barrier presented by intestinal epithelia. These molecules are too large to easily slip through facilitated transport pathways and too constrained thermodynamically to readily unfold and refold in a fashion that might make traversing a lipid bilayer possible. Instead, they move through an efficient, tightly regulated transport pathway known as "receptor-mediated endocytosis" (Feger *et al.*, 1994). Epidermal growth factor, immunoglobulins (*e.g.* thyroglobulin, IgA and IgG (Mostov, 1994)), transferrin (Mazurier *et al.*, 1985) and the vitamin B_{12}–intrinsic protein complex (Seetharam, 1999) enter the intestinal cells by receptor-mediated endocytosis *via* specific receptors. For epithelial cells involved in endocytosis, the apical and basolateral

membranes undergo continuous endocytosis. During receptor-mediated endocytosis, the binding of receptor to ligand triggers the complex to be endocytosed from the cell surface into a coated pit. During this process, the endocytosed material is transported across the cell as follows:

- Specific receptors on the apical membrane recognize and bind to a select set of macromolecular ligands

- The complex is then endocytosed and transported to endosomes *via* coated vesicles.
- Ion channels and transporters in the vesicle membrane act to drop the intra-endosome pH to approximately 5.5 while maintaining proper osmotic balance and surface charge
- Most ligands are dissociated from their receptors by the low pH encountered in the endosome and then transported across the cell in a membrane vesicle.

In this fashion, the endocytosed material is somehow shuttled across the cell away from lysosomal attack and is eventually released from the cell by exocytosis.

A common feature of any of the endocytosis mechanisms described above is that there is a high degree of specificity in the transport process. Thus cells lacking the specific receptor or ligand are incapable of endocytosis of the material or of transcytosis.

a) e.g. Absorption of Vitamin B_{12} in the small intestine following receptor-mediated endocytosis

The uptake of nutrients from the intestine occurs *via* a variety of mechanisms. Most fat soluble molecules, such as vitamins A, D, E and K, are generally solubilized in mixed micelles (consisting of bile salts, phospholipids, monoglycerides, and fatty acids) from which they are subsequently absorbed (Basu *et al.*, 2003; Borel, P., 2003; Li, E. *et al.*, 2003). The small water-soluble vitamins, such as vitamin C, thiamine, nicotinic acid, riboflavin, pyridoxine, biotin, folic acid and pantothenic acid, are mainly absorbed by facilitated diffusion (Foraker *et al.*, 2003; Nabokina *et al.*, 2004b; Subramanian *et al.*, 2004; Nabokina *et al.*, 2004a; Crisp *et al.*, 2004; Said, 2004). Amino acids, on the other hand are absorbed by active transport, with separate transport systems for basic, neutral and imino acids (Webb, 1990; Yang *et al.*, 1999; Daniel, 2004). Dietary sugars are absorbed by energy-dependent transport through small pores located on the epithelial membrane of the enterocyte (Levin, 1994; Wright *et al.*, 2003).

In contrast to the vitamins mentioned above, vitamin B_{12} (VB_{12}, cobalamin, Cbl) is a large, water-soluble molecule (M_W 1356 Da) (Figure 1.2.) and cannot permeate the intestinal barrier through simple diffusion, facilitated diffusion or active transport. Its absorption occurs through a markedly different mechanism to that of the other vitamins, involving a number of transport proteins. During the process of absorption, VB_{12} must first be released from food substances by the action of pepsin in the stomach. The vitamin is then complexed to haptocorrin (Hc), a specific binding protein secreted in saliva, which is

thought to protect the VB_{12} from degradation in the acid environment of the stomach. The [VB_{12}-Hc] complex leaves the stomach and enters the duodenum where the Hc is degraded by the action of trypsin and chymotrypsin. Intrinsic Factor (IF), a VB_{12}-binding protein, subsequently binds to VB_{12}, forming an [IF-VB_{12}] complex. The complex is in turn bound to an IF receptor (IFR) located on the surface of intestinal epithelial cells. The [VB_{12}-IF-IFR] complex is then internalized by the enterocyte *via* receptor-mediated endocytosis. Once inside the cell, the VB_{12} is released from IF following the action of cathepsin L on IF. The released VB_{12} is bound to another VB_{12} binding protein, Transcobalamin II (TcII), which completes the process of transcytosis of the VB_{12} and the VB_{12}-TcII complex enters the circulation (Dix *et al.*, 1987; Dix *et al.*, 1990; Seetharam, 1999; Okuda, 1999; Brada *et al.*, 2001).

Several studies have shown that Vitamin B_{12} is capable of shuttling peptides and proteins across the intestinal epithelia (from the apical to basal surfaces), when these molecules are covalently linked to VB_{12} (Habberfield *et al.*, 1996b; Alsenz *et al.*, 2000; Russell-Jones, 2004). For example, Russell-Jones and co-workers observed a potential increase in the oral uptake of luteinizing hormone releasing hormone (LHRH) analogues (Russell-Jones *et al.*, 1995a), α-interferon, erythropoietin (EPO) (Habberfield *et al.*, 1996a) and granulocyte colony-stimulating factor (G-CSF) (Russell-Jones *et al.*, 1995b) which have been covalently linked to the VB_{12} molecule.

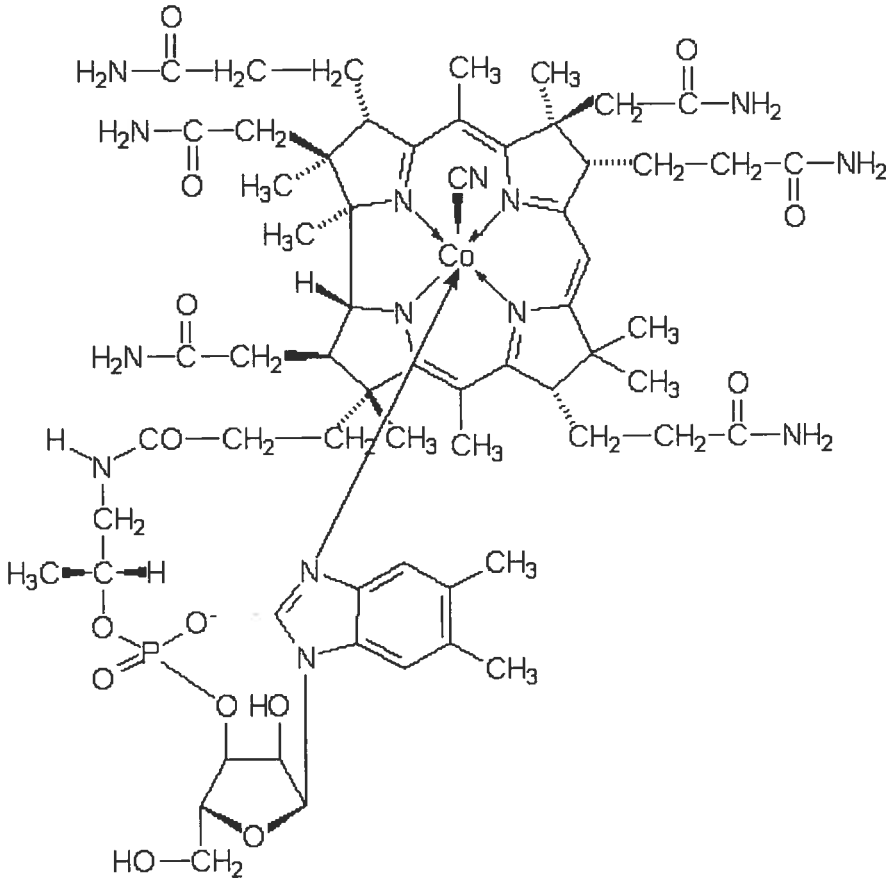


Figure 1.2. Structure of Vitamin B_{12} (VB_{12}).

1.3. PHYSICO-CHEMICAL BARRIERS

1.3.1. pH variation

According to the location in the GI tract, the pH value varies widely, as shown in Table 1.1. Typical values in the fasted stomach are pH 1-2, while in the upper small intestine the pH usually lies between 5.5 and 6.5.

Additionally, there are complex variations in pH between the fasted and fed state (Figure 1.3). Upon ingestion of a meal, the gastric pH at first increases because of buffering effects of food components. In response to food ingestion, however, gastric acid is secreted, and by 3–4 h after the meal intake, the fasted state pH has usually been reestablished (A) (Malagelada *et al.*, 1976). In the small intestine, pH at first decreases in response to a meal with the arrival of acidic chyme from the stomach, but later the fasted state pH is reestablished as a result of pancreatic bicarbonate output (B) (Malagelada *et al.*, 1976).

Note that the pH of the lumenal fluids is also dependent on other factors like age, pathophysiological conditions such as achlorhydria and AIDS, and concurrent drug therapy such as H_2-receptor antagonists and proton pump inhibitors. In the case of poorly soluble weak bases, especially the imidazole antifungals, ketoconazole and itraconazole, elevated gastric pH in AIDS patients leads to a reduced rate of drug dissolution and consequently to malabsorption(Lake-Bakaar *et al.*, 1988; Zimmermann *et al.*, 1994).

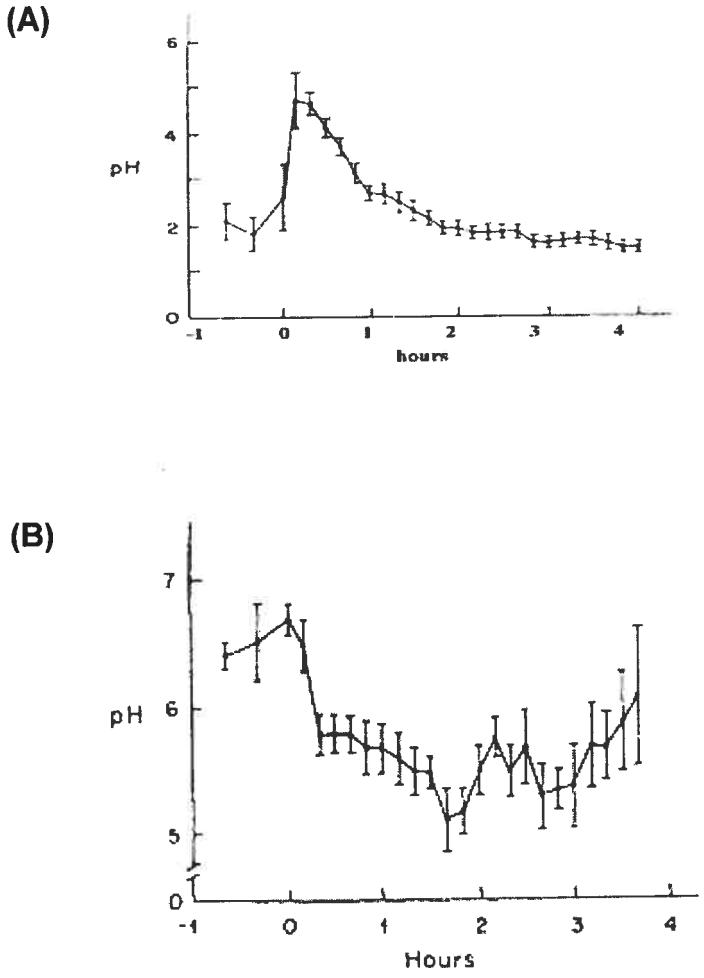


Figure 1.3. (A) Gastric pH and (B) duodenal pH in the fasted state and after food intake in 10 healthy volunteers (adapted from Malagelada, 1976).

1.3.2. Charge state

Drug substances consisting of weak acids and bases ionize in solutions to varying extent, depending on pH (Cleveland *et al.*, 1993; Avdeef *et al.*, 2001). This in turn affects the solubility and bioavailability of the drug. The characteristic thermodynamic parameter relating the pH to the charge state of a molecule is the ionization constant, pK_a (Cleveland *et al.*, 1993; Avdeef *et al.*, 2001). A widely used rule of thumb is that 50% of the compound will be in the ionized state and 50% in the neutral state when the pH equals the pK_a. As the pH goes down, the ratio (ionized form/unionized form) increases for basic moieties, and decreases for acidic ones (Avdeef, 1993).

Compounds have an intrinsic solubility in the unionized form and solubility is typically much greater in the ionized form. Because compounds are permeable in the unionized form, there is a dynamic balance between solubility and permeability throughout the pH gradient of the GI tract that affects absorption (Martinez *et al.*, 2002).

On the one hand, poorly soluble weak acids with pK_a values less than 6, *e.g.* furosemide with pK_a 3.9 and indomethacin with pK_a 4.5 (Martindale, 1982), are relatively insoluble in the preprandial gastric juice and dissolution occurs first in the upper small intestine. In the case of very weak acids, *e.g.* paracetamol with pK_a 9.5 and hydrochlorothiazide with pK_a 8.8 (Martindale, 1982; Deppeler, 1981), the variations in pH in the GI tract are irrelevant to the solubility because these compounds are always in the free acid form over the physiological pH range.

On the other hand, weak bases like itraconazole with pK_a 3.7 (Zimmermann *et al.*, 1994) and dipyridamole with pK_a 6.4 (Martindale, 1982), will be less soluble in the stomach if given immediately after food intake because the gastric fluid is less acidic. By contrast, the solubility of fluconazole,a weak base with a pK_a of 1.5, is sufficiently high (6 mg/ml, dose:solubility ratio about 17 ml) that its administration to patients with elevated gastric pH does not lead to dissolution rate limited absorption (Blum *et al.*, 1991).

Knowledge of the pK_a of a drug substance can also be used in optimizing chemical reaction or synthesis yields. For example, solvent extraction can be best applied in a pH region where the synthesized molecule is uncharged. Furthermore, knowledge of pK_a is useful to predict the absorption, distribution, and elimination of a drug following oral administration. For example, urine pH (normally 5.7 – 5.8) can be altered (with oral doses of NH_4Cl or $NaHCO_3$) to satisfy reabsorption of uncharged species to increase therapeutic efficacy, or to ease excretion of ionized species in toxicological emergencies (Kerns, 2001).

1.3.3. Particle size

An important factor determining the dissolution rate is the particle size of the drug. The dissolution rate is directly proportional to the surface area of the drug, which in turn increases with decreasing particle size. The rate of dissolution of a drug is the rate limiting step for its absorption and thus controls its pharmacological action pattern (Horter *et al.*, 2001). Micronization to particle sizes of about 3 – 5 μm is often a successful strategy for enhancing the dissolution rate of a drug.

The effective surface area also depends on the ability of the fluid to wet the particle surface. When the dissolution medium has only poor wetting properties, micronization sometimes results in a decreased dissolution rate due to agglomeration, as reported by Finholt for Phenobarbital (Solvang *et al.*, 1970).

The particle size is also an important parameter in determining sedimentation rates in biphasic formulations (*e.g.* suspensions and emulsions), since the particle size of the disperse phase is inversely proportional to the sedimentation rate.

1.3.4. Solubility

The aqueous solubility of a drug is a key determinant of its dissolution rate and, consequently, its oral bioavailability. Therefore, the solubility behaviour of a drug remains one of the most challenging aspects in formulation development.

The Nernst-Brunner and Levich modification of the Noyes-Whitney equation (Equation 4) identified the important factors to the kinetics of *in vivo* drug dissolution. these factors include drug diffusivity and solubility in the GI contents, the surface area of the solid wetted by the lumenal fluids and the GI hydrodynamics (Dressman, J.B. *et al.*, 1998).

$$\frac{dC}{dt} = AD\left(\frac{C_s - C}{h}\right) \quad \text{.....Equation (4)}$$

where dC/dt is the dissolution rate, A is the surface area available for dissolution, D is the diffusion coefficient of the drug, C_S is the saturation solubility of the drug in the dissolution medium, C is the concentration of drug in the medium at time t, and h is the thickness of the diffusion boundary layer adjacent to the surface of dissolving drug.

Several physicochemical and physiological aspects can have a great influence on the factors in Equation 4 and therefore on the dissolution rate, such as crystalline form, drug lipophilicity, particle size, viscosity of the medium, solubilization by native surfactants and co-ingested foodstuffs, and pK_a in relation to the GI pH profile (Horter *et al.*, 2001).

1.3.4.1. The Biopharmaceutics Classification System

In the past decade, a greater understanding of the molecular transport in relation to physico-chemical properties (especially solubility) has led to the evolution of a biopharmaceutics classification system (BCS), which is becoming a road map governing future drug design, development and delivery (Amidon *et al.*, 1995).

The BCS sets the criteria for allowing a drug substance, in an immediate release form, to circumvent a Bioequivalence study. It classifies the drugs into four major categories (Table 1.3.) according to two main parameters: the solubility and permeability behaviours of each molecule (Lobenberg *et al.*, 2000; Zhao *et al.*, 2002).

Table 1.3. Biopharmaceutics Classification System (BCS) of drug molecules.

Biopharmaceutics Classification System	
(I) High solubility – High permeability [a]	**(II)** Low solubility – High permeability [b]
(III) High solubility – Low permeability [c]	**(IV)**Low solubility – Low permeability [d]

[a]Exhibit dissolution rate-limited absorption (generally very well absorbed)
[b]Exhibit solubility rate-limited absorption
[c]Exhibit permeability rate-limited absorption
[d]Exhibit both, solubility and permeability rate-limited absorption with very poor oral bioavailability

Recognizing a need to demonstrate the bioequivalence of drug substances in immediate release (IR) dosage forms, without performing the traditional bioequivalence study, the United States Food and Drug Administration (FDA) has issued a set of guidelines outlining what is now known as the BCS. According to the FDA guidance for industry (FDA, 2000), the two individual parameters of classification are evaluated as follows:

A) **Solubility.** The solubility class boundary is based on the highest dose strength of an immediate release (IR) product that is subject of a biowaiver request. The solubility scale is defined in terms of the volume (ml) of water required to dissolve the highest dose strength at the lowest solubility in the pH range 1.0 – 7.5 at 37°C, with 250 ml being the dividing line between "*high*" and "*low*" aqueous solubility. The volume estimate of 250 ml is derived from typical bioequivalence

volume estimate of 250 ml is derived from typical bioequivalence study protocols that prescribe administration of a drug product to fasting human volunteers with a glass (about 8 ounces) of water. A drug substance is considered highly soluble when the highest dose strength is soluble in 250 ml or less of aqueous media over the specified pH range and temperature (*i.e.* dose/solubility ($D : S$) ratio < 250 ml).

B) **Permeability.** The permeability class boundary is based indirectly on the extent of absorption (fraction of dose absorbed, not systemic bioavailability) of a drug substance in humans, and directly on measurements of the rate of mass transfer across human intestinal membrane. Alternatively, non-human systems capable of predicting the extent of drug absorption in humans can be used (*e.g.*, *in vitro* epithelial cell culture models) (FDA, 2000). To date, computational methods (*e.g.* based on polar surface area of the molecule) have not been accepted by the FDA as sufficiently reliable for this purpose. Permeability refers to human jejunal values, with "*high*" being above 10^{-4} cm/s, and "*low*" being below that value. The high permeability class boundary is intended to identify drugs that exhibit nearly complete absorption (90% or more of an administered oral dose) from the small intestine, based on a mass determination or in comparison to an intravenous

reference dose, in the absence of evidence suggesting instability in the GI tract.

The FDA's proposed biowaivers for *in vivo* bioavailability and bioequivalence testing (FDA, 2000) will dramatically reduce a pharmaceutical company's research and development (R&D) costs.

To be considered for a Biowaiver, the drug substance must be classified as being highly soluble, highly permeable, and having a new formulation with a similar dissolution profile to the original (Yu, L.X. *et al.*, 2002). The FDA is accepting *in vitro* data for the solubility and permeability components of the BCS.

Examples of molecules from the various four classes are presented in Table 1.4. According to the BCS, the dissolution rate is the limiting factor for the absorption of class II and IV drugs. Currently, 40% of the NCE fall in these two classes. Such molecules provide potential challenges to the formulation scientist. Their poor water solubility almost inevitably leads to low oral bioavailability from conventional dose forms.

Table 1.4. Example of some orally administered drugs classified in the World Health Organization (WHO) 13th model list of Essential Medicines (formerly Essential Drugs List or EDL) according to the biopharmaceutics classification system (WHO, 2003).

Drug	Pharmacological action	Solubility	Permeability	Dose (mg)	BCS class
Cyclophosphamide	Antineoplastic	High	High	25	I
Primaquine	Antimalarial	High	High	7.5; 15	I
Riboflavin	Vitamin	High	High	5	I
Carbamazepin	Antiepileptic	Low	High	100; 200	II
Ibuprofen	Pain relief	Low	High	200; 400	II
Nifedipine	Ca channel blocker	Low	High	10	II
Atenolol	β-blocker	High	Low	50; 100	III
Codeine phosphate	Antitussive/analgetic	High	Low	30	III
Paracetamol (Acetaminophen)	Pain relief	High	Low	100 – 500	III
Cyclosporin A	Immunosuppressant	Low	Low	50	IV
Furosemide	Diuretic	Low	Low	40	IV
Nelfinavir	Antiviral	Low	Low	250	IV

1.3.4.2. Poorly-water soluble drugs

Poor aqueous solubility is an industry wide issue, especially for pharmaceutical scientists in drug discovery and drug development. A poorly water soluble drug is usually associated with poor absorption and bioavailability upon oral administration (Lipinski *et al.*, 2001). Although a certain degree of hydrophobicity is necessary for a drug molecule to cross the cell membrane easily (Charman *et al.*, 1991), the overall rate of absorption is dictated by the time required for the dosage form to release its contents, and for the drug to dissolve in the GI fluid (Lipinski, 2000).

The water solubility of 'poorly soluble' drugs is usually less than 100 μg/ml (Fasano, 1998). A further parameter useful for identifying 'poorly soluble' drugs is the dose:solubility ratio of the drug. The dose:solubility ratio is defined as the volume of GI fluids necessary to dissolve the administered dose. When this volume exceeds the volume of fluids available, one may anticipate incomplete bioavailability from solid oral dosage forms. Griseofulvin provides a classic illustration of the utility of the dose:solubility ratio. With an aqueous solubility of 15 μg/ml at 37°C and a dose of 500 mg, griseofulvin has a dose:solubility ratio of about 33 liters (Katchen *et al.*, 1967). Thus the combination of its poor solubility and high dose constitutes a severe limitation to its oral bioavailability.

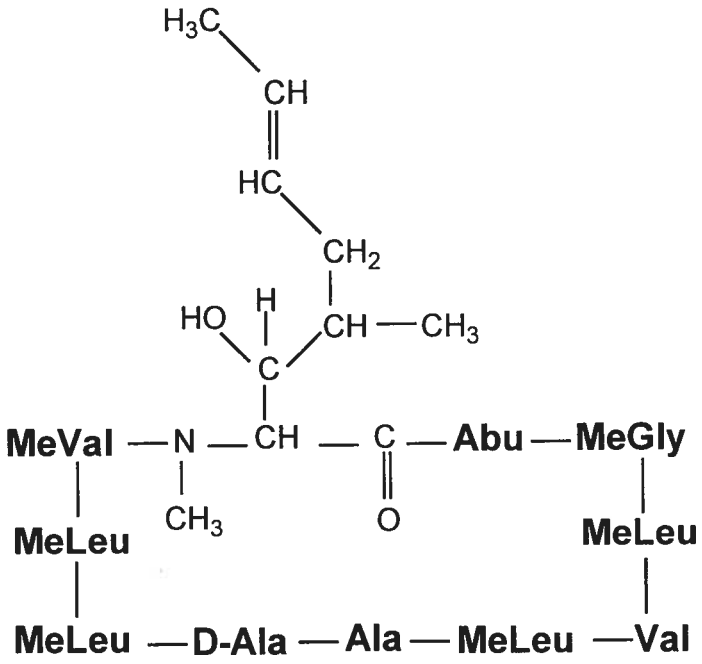
During the past decade, dramatic progress in the field of biotechnology has resulted in a sharp increase in the number of NCE. Through rational drug design, synthetic medicinal chemists have prepared new specific drugs with enormous therapeutic potential

drug design does not necessarily mean *rational drug delivery*, which strives to incorporate into a molecule the molecular properties for optimal transfer between the point of administration and the first target site in the body. Clinical requirement for such molecules in order to have a therapeutic response in humans is that it has to be dosed more than 20 mg/kg/day. Because of the poor physicochemical and biopharmaceutical properties of such molecules, their formulation for oral delivery represents one of the most frequent and greatest challenges to formulation scientists in the pharmaceutical industry. As a result, these drugs are commonly administered by injection (intravenous, subcutaneous, or intramuscular). However, parenteral drug administration, generally accepted by hospitalized patients, when conducted in an out-patient setting often results in noncompliance due to inconvenience and patient discomfort. In the case of therapeutic indications, such as HIV and cancer, it is more important to cure the disease or extend life than the compliance. For such diseases, it can be possible to have a very high dose oral formulation. However, for diseases such as arthritis, diabetes and hypertension, it is very important to have a low dose formulation because it has to be taken everyday in a long-term therapy.

Therefore, it is important that formulation scientists coordinate with the medicinal chemists for a thorough understanding of the pharmacological, biopharmaceutical and preformulation behaviour of a NCE before it enters into the development phase.

1.3.4.3. A case study : CYCLOSPORIN A

Chemically, the CsA molecule is designated as [*R*-[*R**,*R**-(*E*)]]-cyclic [(L-alanyl)-(D-alanyl)-(*N*-methyl-L-leucyl)-(*N*-methyl-L-leucyl)-(*N*-methyl-L-valyl)-(3-hydroxy-*N*,4-dimethyl-L-2-amino-6-octenoyl)-(L-(alpha)-amino-butyryl)-(*N*-methylglycyl)-(*N*-methyl-L-leucyl)-(L-valyl)-(*N*-methyl-L-leucyl)] with a molecular weight of 1202 g/mol (Wenger, 1983). This structure is rich in hydrophobic amino acids linked *via* 11 amide bonds (Figure 1.4.), seven of which are *N*-methylated, contributing to the extreme hydrophobicity associated with the peptide. CsA is naturally produced as a metabolite by the fungi species *cylindrocarpon lucidum Booth* and *tolypocladium inflatum Gams* (Borel, J.F. *et al.*, 1977; Borel, J.F. *et al.*, 1976). Although CsA is practically insoluble in water (its aqueous solubility is as low as 23 µg/ml at 20°C), it is soluble in many organic solvents such as ethanol, methanol, acetonitrile, etc. With regard to the current rules of the US Food and Drugs Administration (FDA), CsA should be produced with a purity >98.5% (United States Pharmacopeia, 1995)



$C_{62}H_{111}N_{11}O_{12}$

MW = 1202.63 g/mol

Figure 1.4. Structure of Cyclosporin A, a cyclic undecapeptide, showing 11 amino acids.

Clinically, CsA is commonly used as immunosuppressant to prevent allograft rejection in various allogenic transplantations involving kidney, liver, heart, skin, lung, small intestine and pancreas (Merion *et al.*, 1984), as well as for prophylaxis of graft-versus-host disease in patients who undergo bone-marrow transplantation (Powles *et al.*, 1978). The drug has also been shown to be effective in the treatment of systemic and local autoimmune disorders such as Freund's adjuvant arthritis, psoriasis, and so forth (Borel, J.F. *et al.*, 1986; Richardson *et al.*, 1995; Galla *et al.*, 1995).

The exact mechanism of action of CsA is not fully known (Borel, J.F. *et al.*, 1983). Experimental evidence suggests that the effectiveness of CsA is due to specific and reversible inhibition of immunocompetent lymphocytes (White *et al.*, 1979; Kahan, 1982), which play a central role in the induction of immune responsiveness. The T-helper cells (producing interleukin-2 (IL-2) following antigenic stimulation) are the main target. CsA is internalized in the cytoplasm, where it binds to a protein termed cyclophilin (Handschumacher *et al.*, 1984). The CsA-cyclophilin complex is transported into the cell nucleus, where it interacts with specific sites on the chromosome (Figure 1.5). This process interferes with the transcription of messenger ribonucleic acid (mRNA) encoding for IL-2 (Elliott *et al.*, 1984) (this might be the crucial CsA-sensitive step, because addition of the drug beyond this stage, i.e. 2-4 h after stimulation, remains ineffective). As a consequence, the translation of mRNA and subsequent synthesis and release of IL-2 are inhibited (Bunjes *et al.*, 1981; Schreier, 1984). The immunosuppressive activity of CsA is thought to be dependent on the concentration of the drug in the lymphatic system.

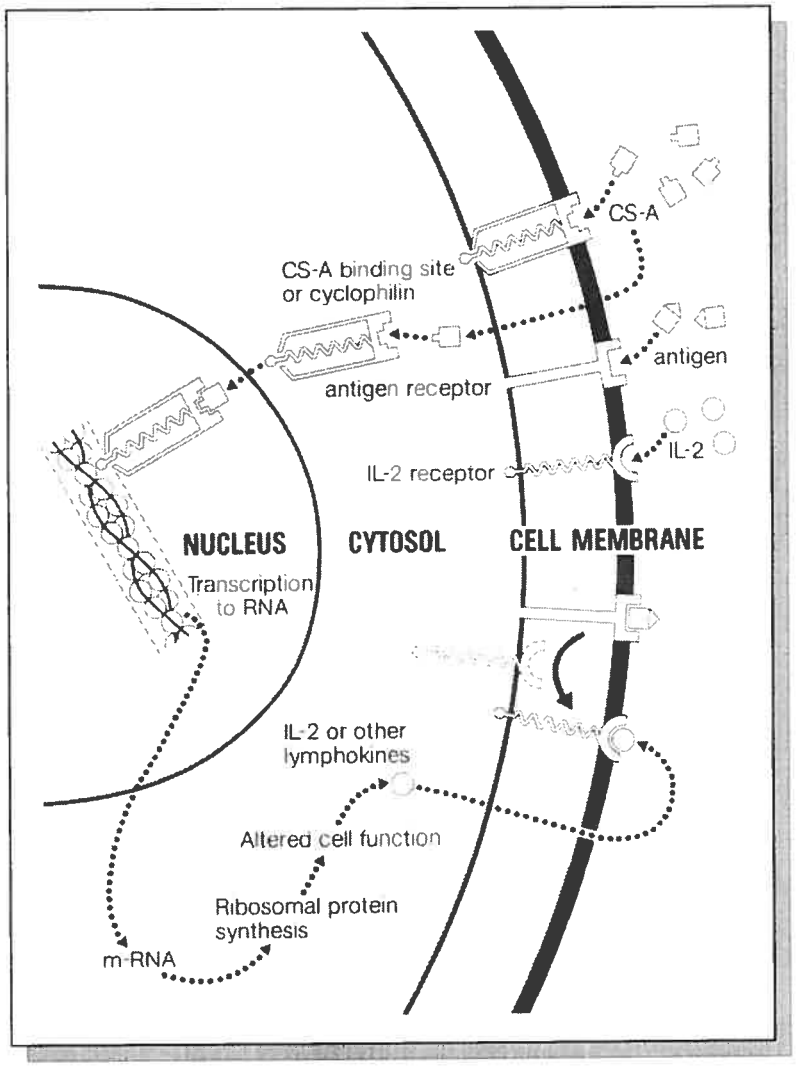


Figure 1.5. Mechanism of action of Cyclosporin A (Borel, J.F. *et al.*, 1985).

The therapeutic window of CsA is rather narrow. Consequently, there is a risk of possible organ rejection because of too low levels of CsA, while an overdose of CsA may cause a variety of adverse effects, in particular renal dysfunction and renal structural changes (Mason, 1990; Skorecki *et al.*, 1992).

Elimination of CsA is primarily biliary with only 6% of the dose (parent drug and metabolites) excreted in the urine (Wood *et al.*, 1983). Neither dialysis nor renal failure alters CsA clearance significantly (Hamwi *et al.*, 2000). Of 15 metabolites characterized in human urine, 9 have been assigned structures (Loor *et al.*, 2004). The major pathways consist of hydroxylation of the C(gamma)-carbon of 2 of the leucine residues, C(eta)-carbon hydroxylation, and cyclic ether formation (with oxidation of the double bond) in the side chain of the amino acid 3-hydroxyl- *N*,4-dimethyl-L-2-amino-6-octenoic acid and *N*-demethylation of *N*-methyl leucine residues (Christians *et al.*, 1993). Hydrolysis of the cyclic peptide chain and/or conjugation of the aforementioned metabolites do not appear to be important biotransformation pathways (Vine *et al.*, 1987).

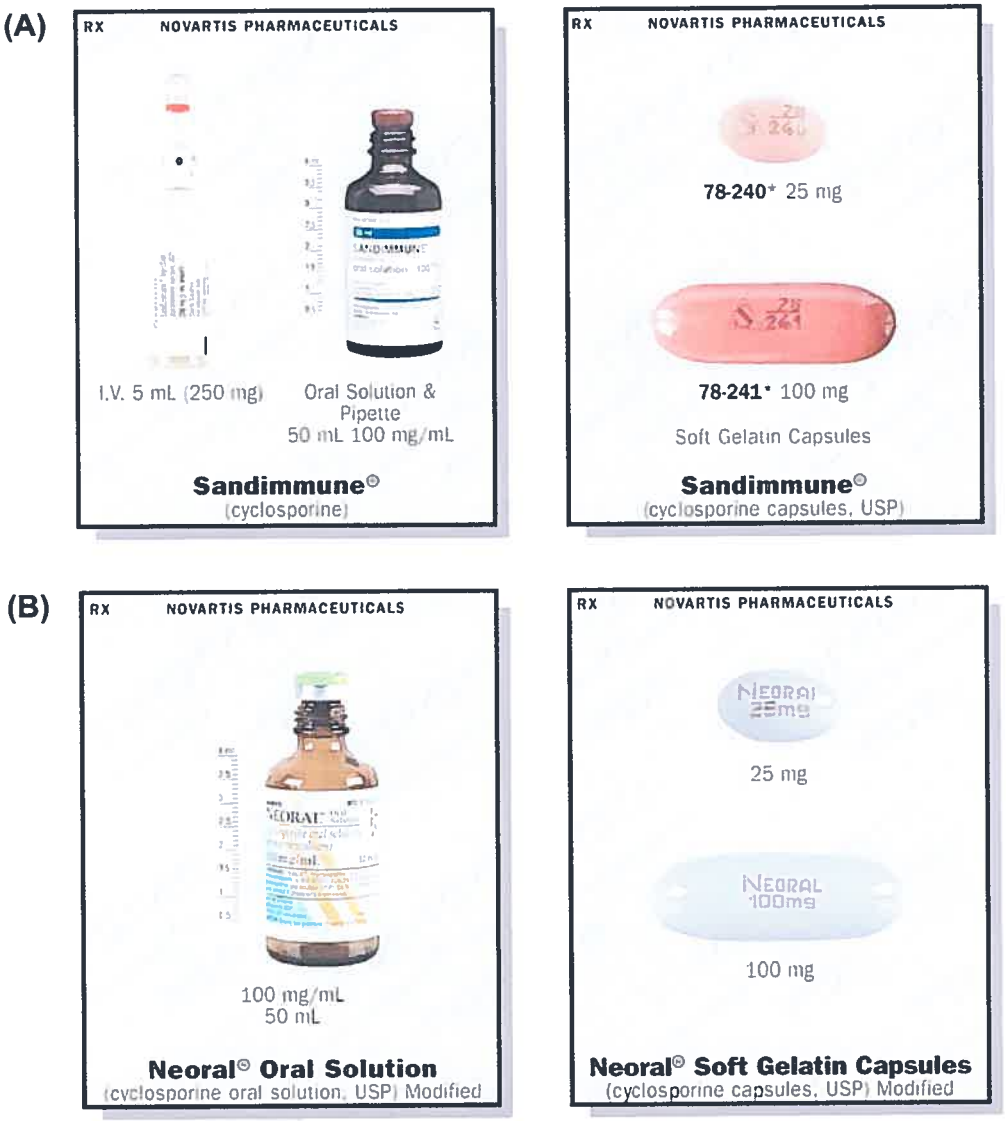


Figure 1.6. Photos showing product samples of (A) Sandimmune® ampul for intravenous infusion, oral solution (50 ml) and soft gelatin capsules (25 mg and 100 mg); and (B) Neoral® oral solution (50 ml) and soft gelatin capsules (25 mg and 100 mg) (adapted from Thomson Physicians Desk Reference® (PDR), 2004, Montvale, NJ).

A) Commercially available intravenous formulation of CsA

CsA is very often given to patients during transplantation surgery by intravenous infusion. After the acute operation period, most patients take the drug orally.

A commercially available concentrate for intravenous infusion (Sandimmune® injection, Novartis Pharma Stein AG, Stein, Switzerland) is available in a 5 ml sterile ampul. Each ml contains:

Cyclosporine, USP ..... 50 mg

Cremophor® EL ..... 650 mg

Alcohol, Ph. Helv. ..... 32.9% by volume

Nitrogen ..... qs

The concentrate is to be diluted 1:20 to 1:100 with normal saline injection (0.9% sodium chloride) or 5% dextrose injection before use.

Although Cremophor®-EL (CrEL, a polyoxyethylated castor oil, BASF Aktiengesellschaft) is one of the best anionic surfactants used to dissolve lipophilic drugs, the administration of Sandimmune® injection at high doses has been associated with anaphylactic reactions and histamine release due to the high content of CrEL (Howrie *et al.*, 1985; Friedman *et al.*, 1985).

Therefore, there was a need to find an alternative formulation for CsA. Knowing that oral drug delivery is the preferred route for drug administration, one of the greatest challenges has been the administration of CsA by the peroral route.

B) Regional Differences in CsA Absorption from GI tract

CsA has been extensively studied with respect to its site of absorption in the GI tract. Sawchuck and Awni (Sawchuck *et al.*, 1986) demonstrated that CsA was absorbed to a greater extent from the duodenum than from the jejunum in the rabbit when these two segments were simultaneously perfused with drug solution. Further evidence for the site-dependent CsA absorption comes from the work of Grevel et al. (Grevel *et al.*, 1986) who observed a short lag-time of 0.38±0.11 h and an abrupt end of absorption at 2.8±1.6 h following CsA administration in healthy volunteers. Such a short lag-time and short oral absorption phase suggested that the absorption of CsA was confined mainly to the upper part of the GI tract (Gupta *et al.*, 1989). In addition, Ritschel et al. (Ritschel *et al.*, 1990) demonstrated 4-8 fold higher peak blood levels from the rat intestine compared to the stomach, large intestine and rectum for CsA administered in microemulsion.

It should be noted that the clinical use of CsA has historically been complicated by incomplete and unpredictable absorption from the GI tract due to the presence of an important first-pass metabolism in the intestinal mucosa and in the liver (Maurer *et al.*, 1984); (Kolars *et al.*, 1991). CsA absorption is partly controlled by the metabolizing enzyme cytochrome P-450 3A4 (CYP3A4) and the multi-drug transporter p-glycoprotein (P-gp) in the small intestine as well as by hepatic CYP3A4 (Kolars *et al.*, 1991; Fricker *et al.*, 1996). CYP3A4 metabolizes the drug into more than 30 metabolites while P-gp pumps the drug from the enterocyte cytosol out in the intestinal lumen (Tjia *et al.*, 1991). Moreover, the relatively high molecular weight of CsA, very high lipophilicity and minute

solubility in aqueous fluids of the GI tract represent non-negligible absorption limiting barriers of CsA through the GI mucosa (Gupta *et al.*, 1989; Ismailos *et al.*, 1991; Hamwi *et al.*, 2000). This poor absorption of CsA is one of the main reasons for its low and variable absolute bioavailability which increases significantly the risks of both acute and chronic rejection and diminishes the predictive value of therapeutic drug monitoring (Lindholm, 1991; Lindholm *et al.*, 1988; Schroeder *et al.*, 1995).

C) Commercially available oral formulations of CsA

Several attempts were made to find an optimal oral formulation of CsA that fulfills the criteria to increase its bioavailability. An olive oil solution of 100 mg/ml CsA (olive oil:peglicol-5-oleate:ethanol = 60:30:10) was clinically used as an oral dosage form (Ueda *et al.*, 1984). In view of both the high lipophilic property of CsA and the oily dosage form, it was reasoned that the intestinal lymphatic absorption of CsA would be extensive. Peglicol-5-oleate could be dispersed in a drink of the patient's choice (milk, fruit juice, etc). Sandimmune® (Novartis Pharma S.A., Huningue, France) represents marketed oral commercial formulations (soft gelatin capsules and drink solutions) of CsA in olive oil solution.

Sandimmune® Soft Gelatin Capsules (cyclosporin A capsules, USP) are available in 25 mg and 100 mg strengths.

Each 25 mg capsule contains:

Cyclosporin A, USP..... 25 mg

Alcohol, USP dehydrated ..... max 12.7% by volume

Each 100 mg capsule contains:

Cyclosporin A, USP ..... 100 mg

Alcohol, USP dehydrated ..... max 12.7% by volume

Inactive Ingredients: *corn oil, gelatin, glycerol, Labrafil M 2125 CS (polyoxyethylated glycolysed glycerides), red iron oxide (25 mg and 100 mg capsule only), sorbitol, titanium dioxide, and other ingredients.*

Sandimmune® Oral Solution (cyclosporin-A oral solution, USP) is available in 50 ml bottles. Each ml contains:

Cyclosporin A, USP ..... 100 mg

Alcohol, Ph. Helv. ..... 12.5% by volume

dissolved in an olive oil, Ph. Helv./Labrafil M 1944 CS (polyoxyethylated oleic glycerides) vehicle which must be further diluted with milk, chocolate milk, or orange juice before oral administration.

Sandimmune® oral formulations showed large inter- and intraindividual variations of CsA pharmacokinetics. One of the major factors is an erratic absorption of the drug from the gut (effect of food, bile, etc) (Lindholm *et al.*, 1988; Mueller *et al.*, 1994). Furthermore, the clinical use of Sandimmune® was somehow limited by the occurrence of adverse events, most frequent of which are renal dysfunction, hypertension and hepatotoxicity (Skorecki *et al.*, 1992; Lorber *et al.*, 1987). It should be noted that the peak concentrations (C_{max}) in blood and plasma are achieved at about 3.5 hours following Sandimmune® administration.

The most recent formulation of CsA (Neoral®, Novartis Pharma S.A., Huningue, France) promises to alleviate some of the difficulties in CsA absorption by using the microemulsion technology to ensure uniform drug dispersion within the absorptive region of the intestinal lumen (Canadian Neoral Renal Transplantation Group, 1997; Dunn *et al.*, 1997). Microemulsions are bicontinuous systems that are essentially composed of bulk phases of water and oil separated by a surfactant/co-surfactant-rich interfacial region. These systems have an advantage over "conventional emulsions" in that they are thermodynamically stable liquid systems and are spontaneously self-forming in water with droplet size of 100 - 200 nm (Kawakami *et al.*, 2002).

The Neoral® oral formulations consist of dispersing the drug in a mixture of propylene glycol (hydrophilic solvent) and corn oil-mono-di-triglycerides (lypophilic solvent) by stirring. Once dispersed, polyoxyl 40 hydrogenated castor oil (surfactant) and an antioxidant (DL-(alpha)-tocopherol) are added (Levy *et al.*, 1994). This oil-in-water

(O/W) microemulsion preconcentrate spontaneously forms in water a homogeneous, macroscopically transparent and thermodynamically stable monophasic solution of CsA (Vonderscher *et al.*, 1994).

Neoral® Soft Gelatin Capsules (cyclosporin-A capsules, USP) are available in 25 mg and 100 mg strengths.

Each 25 mg capsule contains:

Cyclosporin A ..... 25 mg

Alcohol, USP dehydrated ..... 11.9% *v/v* (9.5% *w/v*)

Each 100 mg capsule contains:

Cyclosporin A..... 100 mg

Alcohol, USP dehydrated ..... 11.9% *v/v* (9.5% *w/v*)

***Inactive Ingredients*:** Corn oil-mono-di-triglycerides, polyoxyl 40 hydrogenated castor oil NF, DL-(alpha)-tocopherol USP, gelatin NF, glycerol, iron oxide black, propylene glycol USP, titanium dioxide USP, carmine, and other ingredients.

Neoral® Oral Solution (cyclosporin-A oral solution, USP) is available in 50 ml bottles. Each ml contains:

Cyclosporin A..... 100 mg/ml

Alcohol, USP dehydrated ..... 11.9% *v/v* (9.5% *w/v*)

***Inactive Ingredients*:** Corn oil-mono-di-triglycerides, polyoxyl 40 hydrogenated castor oil NF, DL-(alpha)-tocopherol USP, propylene glycol USP.

This formulation resulted in enhanced bioavailability in comparison to Sandimmune® oral formulations (Sketris *et al.*, 1994); relative independence from the effects of food, bile flow, and gastrointestinal dysfunction; which reduced inter-and intraindividual variability of exposure (Kahan *et al.*, 1995; Kovarik *et al.*, 1994). It is for this reason that Neoral® is administered orally from the day of surgery and it is now the preferred formulation of CsA for patients undergoing solid organ and bone marrow transplantation. Another advantage of Neoral® is that the time to peak blood CsA concentrations (T_{max}) ranged from 1.5-2.0 hours following oral administration. It has been shown however that kidney damage, characterized by serial deterioration in renal function and morphologic changes in the kidneys, is a potential consequence of Neoral® (Maeda *et al.*, 2002) and therefore renal function must be monitored during therapy (Canadian Neoral Renal Transplantation Study Group, 2001).

1.3.4.4. General approaches used to improve the solubility/dissolution of poorly soluble drugs for oral administration

As the physiological barriers cannot be changed, all the focus has been directed towards trying to disguise the drug molecule in order to deliver it to the systemic circulation across the intestinal barrier. There are a number of techniques used to improve the dissolution/solubility and hence oral bioavailability of poorly soluble drugs including:

A) Chemical modifications of drug molecule (prodrug approach, salt synthesis, etc)

B) Physical modifications of drug molecule (particle size reduction, change in physical form, etc)

C) Drug-loading in carrier systems (liposomes, emulsions, microemulsions, polymer-based nano- and microparticles, and polymeric micelles)

A) Chemical modifications

Among the various approaches to minimize the undesirable drug properties while retaining the desirable therapeutic activity, the chemical approaches using drug derivatization offer a high flexibility and have been demonstrated as an important means of improving drug efficacy.

a) Prodrug approach

The prodrug approach gained attention as a technique for improving drug therapy in the early 1970s. Numerous prodrugs have been designed and developed since then to overcome pharmaceutical and pharmacokinetic barriers in oral drug absorption (Fleisher *et al.*, 1985; Friedrichsen *et al.*, 2001; Schoffski, 2004).

The term "prodrug" was first introduced by Albert (Albert, 1958) to signify pharmacologically inactive chemical derivatives that can be converted *in vivo* to the active parent drug molecules, enzymatically or nonenzymatically, to exert a therapeutic effect. Ideally, the prodrug should be converted to the original drug as soon as the goal is reached, followed by the subsequent rapid elimination of the released derivatizing group (Stella *et al.*, 1985).

Since Albert discussed the concept of prodrugs in the late 1950s, such compounds have also been called "latentiated drugs" (Harper, 1962) and "bioreversible derivatives" (Sinkula *et al.*, 1975), but "prodrug" is now the most commonly accepted term.

Usually, the use of the term implies a covalent link between a drug and a chemical moiety. Therefore, it is very important that the NCE has a functional group(s) that allows conversion to a prodrug. The second important criterion is that the human body should have an enzyme or some other mechanism to cleave this molecule into the active entity once it is absorbed into the blood. When both these phenomena occur, then it is possible to use this approach not only to increase the solubility/dissolution of a therapeutic agent but its permeability too (Notari, 1981; Stella *et al.*, 1985; Choi *et al.*, 2004). Another advantage,

especially with non steroidal anti-inflammatory drugs (NSAID), is to reduce gastric irritation. Many of the NSAID are either strong or weak acids and they cause gastric irritation upon oral administration. By synthesizing ester prodrugs, this irritation has been significantly reduced (Bansal *et al.*, 2001; Jung *et al.*, 2003; Larsen *et al.*, 2004). However, in some cases, the interindividual differences in metabolism and drug-food interactions may cause problems.

Dalacin® C flavoured granules, an antibacterial agent (Pharmacia & Upjohn Co.) is an example of a poorly soluble drug (Clindamycin, solubility 0.2 mg/ml) commercially available in the form of a water soluble prodrug (Clindamycin Palmitate HCl, solubility 150 mg/ml) for oral administration (CPS, 2004).

b) Salt synthesis

This is the most widely used approach to increase solubility of weakly acidic or basic NCE (Gould, 1986; Gu *et al.*, 1987; Ware *et al.*, 2004). If the NCE has ionizable group(s), then it is very likely that it will form a salt. The solubility of salt is typically based on the counter ion. Selection of salt is based on many parameters such as solubility, hygroscopicity and stability of the physical form. The most important requirement is that the counter ion should be FDA approved, if not one should have enough toxicological data to support the selection of the counter ion.

When administered as a salt, the dissolution and hence the bioavailability of many NCE increase up to a great extent, compared to their weak acid/base form. Table 1.5.

represents a few examples of commercially available salts along with their aqueous solubilities.

Table 1.5. Commercially available drug salts.

Active pharmaceutical ingredient (API)	**Solubility (mg/mL)**	**Brand name**	**Pharmacological Action**	**Oral Dosage form**	**Manufacturer**
Codeine Codeine sulfate Codeine phosphate	8.3 33 445	Codeine Contin® Codeine Phosphate	Opioid analgesic Opioid analgesic-Antitussive	Tablet Tablet	Purdue Pharma Technilab
Pseudoephedrine Pseudophedrine hydrochloride	0.02 2000	Pseudofrin	Decongestant	Tablet	Trianon
Cetrizine Cetrizine hydrochloride	0.03 300	Reactine ™	Histamine H_1-receptor antagonist	Tablet	Pfizer

B) Physical modifications

a) Particle size reduction

As described by the modified Noyes-Whitney model of dissolution (Equation 4., see section 1.3.4. Solubility), surface area of the drug is directly proportional to its rate of dissolution.

Reduction in particle size is an old approach to increase the surface area and hence the dissolution rate and bioavailability of poorly soluble drugs. Several techniques are used for reduction in particle size such as jet mill, fluid energy mill and ball mill (Chaumeil, 1998), compressed or supercritical CO_2 solvent (Gosselin *et al.*, 2003a; Gosselin *et al.*, 2003b), spray drying (Kawashima *et al.*, 1975; Takeuchi *et al.*, 1987; Ahn *et al.*, 1998), spray freezing (Rogers *et al.*, 2002; Rogers *et al.*, 2003), etc. Figure 1.7. shows the increase in specific surface area and dissolution of a poorly water soluble drug following micronization (Rasenack *et al.*, 2003).

Beside the advantage of increased dissolution rate of micronized drugs, several disadvantages resulting from the preparation process exist. Handling particles in sub-micron size is difficult, and chances of airborne contamination and serious toxicity by inhalation are very high (Merisko-Liversidge *et al.*, 2003; Hu *et al.*, 2004).

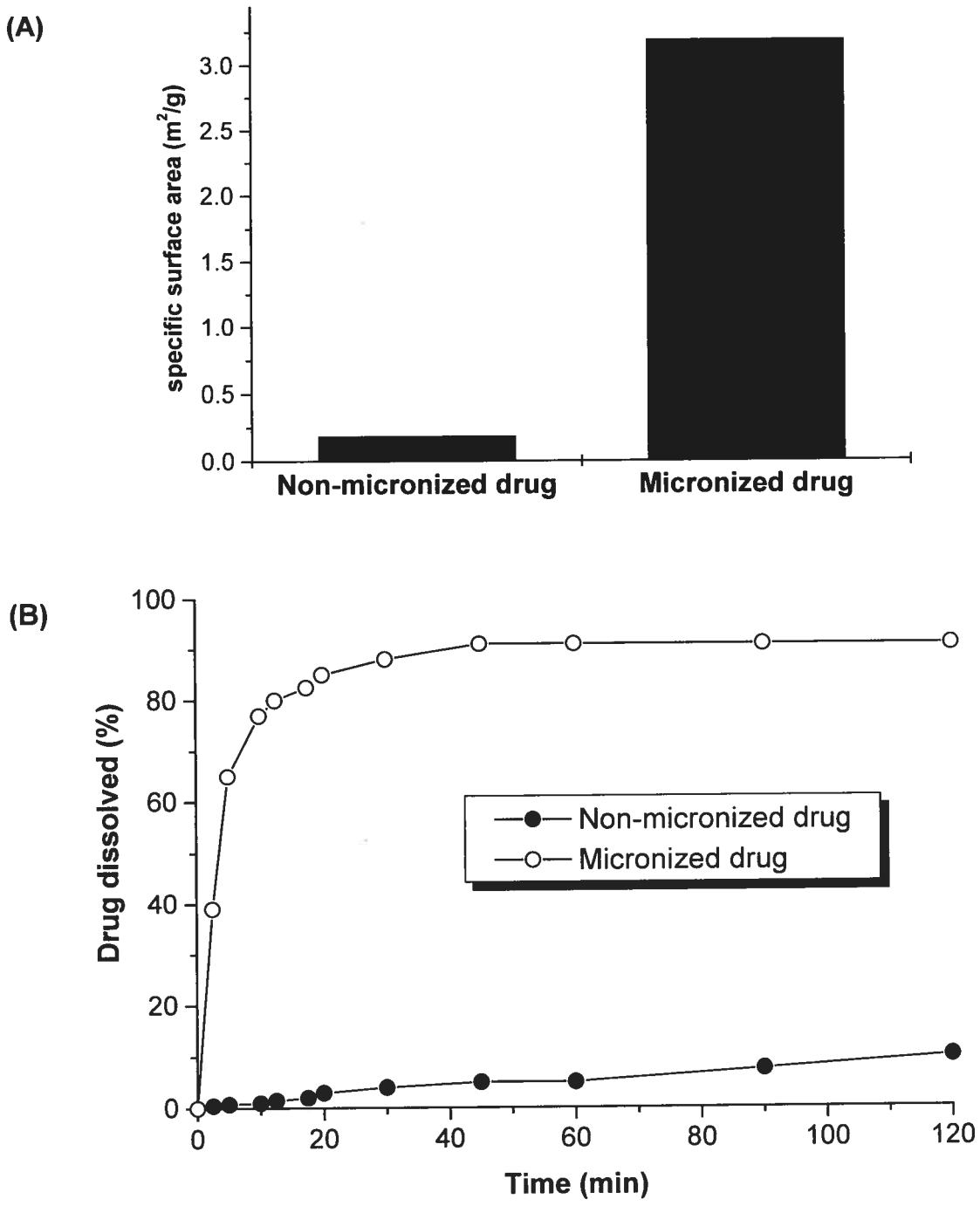


Figure 1.7. (A) specific surface area and (B) amount dissolved (%) of the new drug substance ECU-01, a poorly water soluble enzyme-inhibitor with anti-inflammatory properties for oral administration. ECU-01 is a heterocyclic derivate of a 3,5-dichloro-pyridine (adapted from Rasenack N. et al., 2003).

b) Change in physical form

The majority of drugs marketed in the past decades have been isolated and delivered to the human body in their crystalline state (Byrn *et al.*, 1994). In most instances, this state represents the lowest energy form of the drug, and the one having the greatest physical and chemical stability (Yu, L.X. *et al.*, 2003). However, amorphous forms are of substantial interest because they usually are much more soluble than their crystalline counterparts (Hancock *et al.*, 2000; Yu, L., 2001).

Polymorphic changes may have pronounced effects on tablet performance in terms of *in vitro* dissolution and consequently, *in vivo* absorption. For example:

a) Chloramphenicol palmitate is available in at least two different forms; polymorphs A and B, where the polymorph B is the more soluble. Figure 1.8. shows that an increase in the rate and extent of bioavailability of the drug occurs with increasing the content of the polymorph B in the administered dose (Aguiar *et al.*, 1967). Such profile is due to increasing rate of dissolution in presence of higher content of polymorph B.

b) In the case of ampicillin, the aqueous solubility of the anhydrate phase was reported to be 20% higher than that of the trihydrate form at 37°C (Ali *et al.*, 1981). The time for 50% of the drug to dissolve *in vitro* was found to be 7.5 and 45 min for the anhydrate and trihydrate forms, respectively. *In vivo* blood levels following separate oral administration of the two forms of the

ampicillin showed higher maximum concentration of ampicillin C_{max} in the anhydrous form, and an earlier time to reach maximum concentration T_{max} in the blood serum relative to the trihydrate form. Therefore, it has been concluded that suspensions containing ampicillin anhydrate exhibit superior bioavailability to analogous formulations made from the trihydrate form (Figure 1.9.).

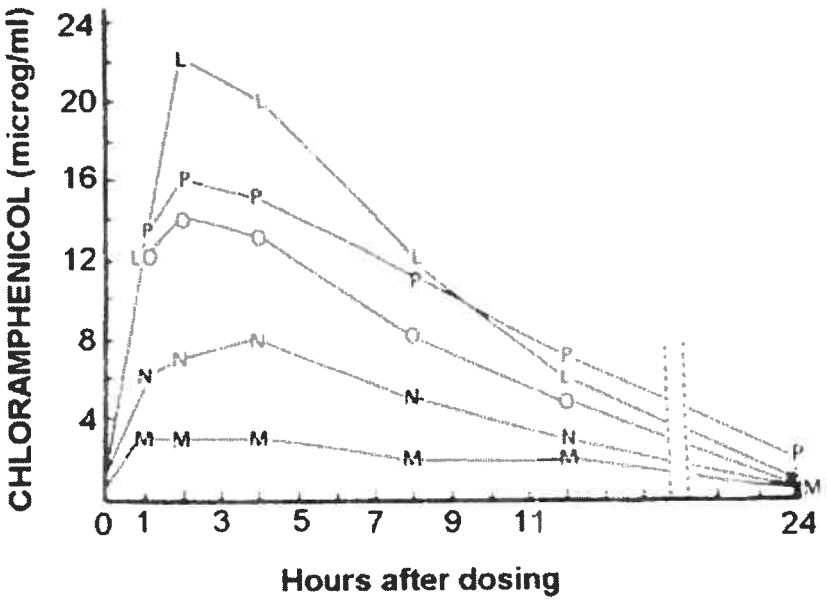


Figure 1.8. Mean blood serum levels obtained for identical suspensions of chloramphenicol palmitate with varying ratios of A and B polymorphs following oral administration. Percentage of polymorph B in the suspension:(M) 0%; (N) 25%; (O) 50%. (P) 75% and (L) 100% (adapted from Aguiar *et al.*, 1967).

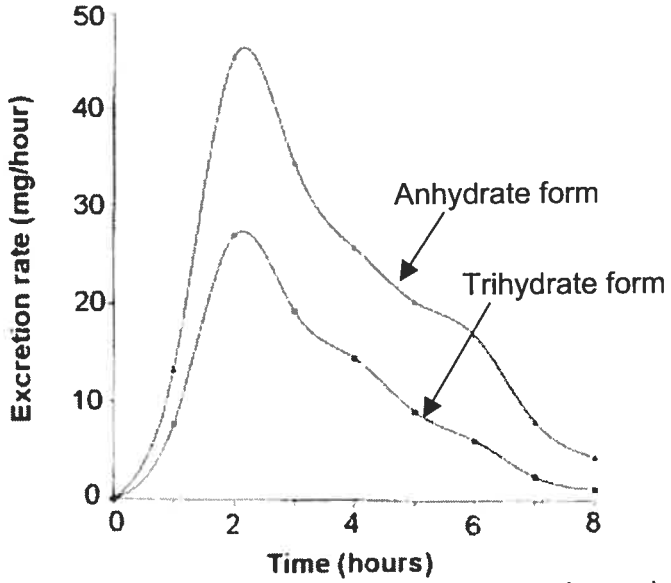


Figure 1.9. Ampicillin urinary excretion rates at various times after separate administration of the two forms : anhydrate and trihydrate (adapted from Ali *et al.*, 1981).

C) Carrier-mediated intestinal transport of drugs - Nano and microparticles

The implementation of carrier systems in the field of therapeutics may be defined as the control of human biological systems at the molecular level, using engineered micro- and nano-devices (O'Hagan, 1998; Allemann *et al.*, 1998; Haruyama, 2003). The proven commercial and clinical successes that can be achieved with particulate dosage forms and recent advances in technology transfer and manufacturing issues suggest that these novel systems should more often be considered an integral part of a formulator's armament.

Oral delivery systems with micron and submicron-sized colloidal carriers offer three distinct advantages (Delie, 1998; Jenkins *et al.*, 1994; Castelvetro *et al.*, 2004):

(i) improving the solubilization of drug in the carrier system

(ii) providing some level of protection against degradation within the GI tract (varying pH, enzymatic activity, ...)

(iii) prolongation of the drug transit time, and consequently, the duration of exposure of drug molecules to the epithelial surface.

Many of the problems associated with oral delivery of poorly soluble pharmaceuticals and strategies to overcome physiological and physical barriers in the GI tract have been discussed in details in part I. In part II, the ability of carrier systems, especially micelles, to deliver poorly water soluble pharmaceuticals through oral administration will be discussed in more details.

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CHAPTER TWO

INTRODUCTION – PART II

POLYMERIC MICELLES FOR ORAL DRUG DELIVERY: WHY AND HOW

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2.1. ABSTRACT

The oral delivery of drugs is regarded as the optimal means for achieving therapeutic effects due to increased patient compliance. Unfortunately, the oral delivery route is beset with problems such as: gastrointestinal destruction of labile molecules, low levels of macromolecular absorption, etc. To reduce the impact of digestive enzymes and to ensure the absorption of bioactive agents in an unaltered form, molecules may be incorporated into microparticulate carriers. Many approaches to achieve the oral absorption of a wide variety of drugs are currently under investigation. Among the different polymer-based drug delivery systems, polymeric micelles represent a promising delivery vehicle especially intended for poorly-water soluble pharmaceutical active ingredients in order to improve their oral bioavailability. Recent findings of a dextran-based polymeric micelles study for solubilization of a highly lipophilic drug, cyclosporin A, will be discussed.

2.2. AUTHOR KEYWORDS

Micelles, oral delivery, poorly-water soluble drugs, review.

2.3. INTRODUCTION

Oral administration of therapeutic agents represents by far the easiest and most convenient route of drug delivery, especially in the case of chronic therapies (Lavelle *et al.*, 1995). While convenient from the patient's perspective, oral drug formulation presents a challenge to the chemist, who has to design delivery systems optimizing drug stability in the gastrointestinal tract, such that a desirable pharmacokinetic profile may be attained for a given drug (Daugherty *et al.*, 1999). Indeed, the gastrointestinal (GI) tract presents a variety of hurdles for a drug, from morphological barriers (mucus layer, microvilli, etc) to stringent physiological factors (a wide range of pH, enzymatic activities, specific transport mechanisms, etc), which all conspire to limit intestinal absorption of drug. In the case of poorly-water soluble drugs, the dissolution time in the gastrointestinal contents may be longer than the transit time to the intended absorptive sites (Horter *et al.*, 2001). Therefore, dissolution of drugs is quite often the rate-limiting step which, ultimately, controls the bioavailability of the drug (Charman *et al.*, 1991). This poses a major challenge for effective delivery of poorly water-soluble therapeutics *via* the oral route (Chen *et al.*, 1998).

One approach to enhance the absorption efficiency and bioavailability of highly lipophilic drugs taken orally consists in using a particulate delivery system soluble or dispersible in an aqueous environment. This strategy is based on the following premises: (a) particulate systems provide the drug with some level of protection against degradation within the GI tract; (b) they prolong the drug transit time and facilitate translocation of the

drug across epithelial barriers, thus improving drug absorption; and (c) they may be targeted to specific sites and carry functionalities that assist specific absorption pathways.

2.4. PHYSICO-CHEMICAL CHARACTERISTICS OF ORAL DELIVERY SYSTEMS

The transport of a drug across the intestinal membrane is a complex transfer process involving several mechanisms. Based on the physiology of the intestinal epithelium, three potential routes for drug transport across intestinal barriers have been considered (Figure 2.1.): (1) the paracellular route, a passive, diffusional transport pathway taken by small, hydrophilic molecules (*e.g.* mannitol) which can pass through the tight junction channels between adjacent epithelial cells; (2) Transcellular passive diffusion of lipophilic molecules through the lipid bilayer and the membrane bound protein regions of the cell membrane; and (3) Transcellular receptor-mediated transcytosis of surface-bound ligands such as intrinsic factor-cobalamin complexes. The extent of particle uptake through the absorbing membrane varies considerably depending on its size, chemical composition, hydrophobicity and charge.

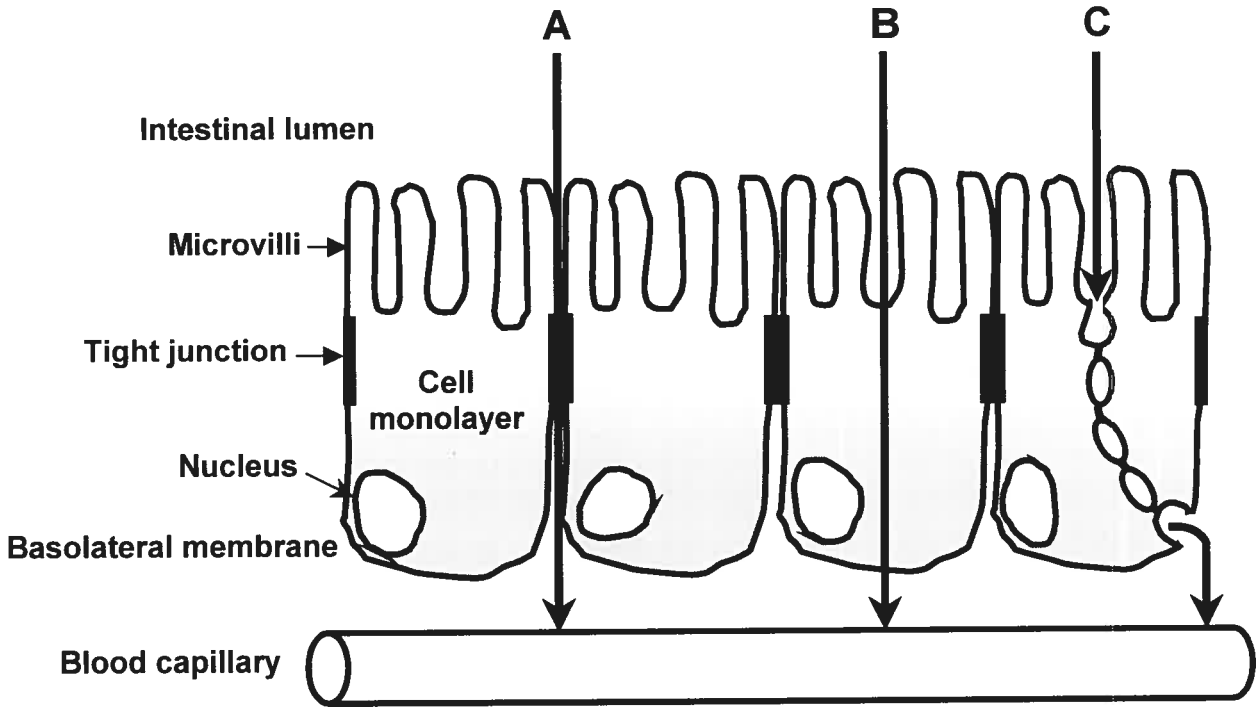

Figure 2.1. Schematic representation of intestinal epithelial cells showing potential transepithelial pathways: **(A)** paracellular route; **(B)** transcellular passive diffusion; and **(C)** transcellular receptor-mediated transcytosis.

2.4.1. Particle size

The uptake of particles within the intestine and the extent of drug absorption increase with decreasing particle size and increasing specific surface area (Florence *et al.*, 2001; Sass *et al.*, 1990; Jenkins *et al.*, 1994). Jani et al. (Jani *et al.*, 1989; Jani *et al.*, 1990) assessed the size-dependance of the uptake of nanoparticles by the rat intestine by monitoring their appearance in the systemic circulation and their distribution in different tissues. After administration of equivalent doses, 33% of nanoparticles of 50 nm in diameter and 26% of the 100 nm nanoparticles were detected in the intestinal mucosa and gut-associated lymphoid tissues, whereas, in the case of 500 nm particles, only 10% were found in the intestinal tissues. The uptake of particles larger than 1 μm in diameter was marginal. Moreover, these micronsized particles were found only in the lymph nodes of the small intestine (Peyer's patches). Similar findings were reported by Desai et al. (Desai *et al.*, 1996) for the absorption characteristics of nanoparticles prepared from poly(lactide-*co*-glycolic acid) (PLGA). The uptake of 100 nm nanoparticles in the rat intestine was significantly increased compared to larger particles of 1 and 10 μm. Therefore, size is an important parameter controlling the internalization of nanoparticles into epithelia of the GI tract, and as a rule, sizes smaller than 500 nm are required.

2.4.2. Chemical composition

A comparative study investigating a range of polymeric particles suggests that the extent of drug absorption depends on the hydrophobicity of the polymer (Eldridge *et al.*,

1990). It would appear that particles consisting of the hydrophobic polymer poly(styrene), are absorbed more readily than PLGA, a somewhat more hydrophilic polymer.

2.4.3. Particle charge

Particle charge is also an important factor that determines the extent of uptake from the GI tract (Jani *et al.*, 1989; Hillery *et al.*, 1994). Carboxylated poly(styrene) nanoparticles show a significantly decreased affinity to intestinal epithelia, compared to positively charged and uncharged poly(styrene) nanoparticles (Jani *et al.*, 1989). However, Mathiowitz et al. (Mathiowitz *et al.*, 1997) observed a highly increased adhesion of negatively charged poly(anhydride) copolymers to cell surfaces, resulting in an enhanced absorption rate of the encapsulated drug, compared to drug encapsulated in neutral particles. Kriwet et al. (Kriwet *et al.*, 1998) in their investigation of the affinity of negatively charged poly(acrylic acid) nanoparticles towards intestinal epithelia reached the same conclusion. Thus, neutral and positively charged nanoparticles have a higher affinity to intestinal epithelia than negatively charged nanoparticles, but negatively charged nanoparticles exhibit bioadhesive properties, which may also favor the transport process.

2.5. APPROACHES

Several particulate systems have been reported as effective carriers of therapeutic agents administered orally. Among them are liposomes, which have been used, for example, to administer insulin by the oral route (Damgè *et al.*, 1988). Liposome

formulations are particularly attractive, as they may carry lipid-soluble drugs in the lipid bilayer and, at the same time, water-soluble drugs in the aqueous core (Sharma *et al.*, 1997). They are prepared routinely under mild conditions which minimize drug denaturation during encapsulation. Unfortunately, most liposome formulations are readily disrupted by intestinal detergents, such as bile salts, and are subject to degradation by intestinal phospholipases. Both mechanisms trigger the premature release of the liposome-entrapped drug in the GI fluid (Okada *et al.*, 1995; Deshmukh *et al.*, 1980). In addition to liposomes, other colloidal formulations, such as emulsions and microemulsions, are effective in the oral administration of drugs with limited solubility in biological fluids (Toorisaka *et al.*, 2003; Itoh *et al.*, 2002). Microemulsions (oil/water) are bicontinuous systems composed of a continuous water phase containing nanometer sized oil droplets separated by a surfactant/co-surfactant-rich interfacial region. Compared to emulsions, they offer several advantages as drug delivery systems, such as high solubilization capacity, thermodynamic stability, and ease of preparation (Itoh *et al.*, 2002). In several cases, administration of microemulsions, especially formulations based on castor oil, has triggered adverse events, most frequently renal dysfunction, hypertension and hepatotoxicity (Vischer *et al.*, 1983; Uchida *et al.*, 2000). Recently a new class of delivery system has been under intense scrutiny. They are nanoparticles ranging in size from ~15 to 150 nm (Couvreur *et al.*, 1993; Sakuma *et al.*, 2001) such as nanospheres (Jung *et al.*, 2000), nanocapsules (Damgè *et al.*, 1988; Sai *et al.*, 1996), hydrogels (Saffran *et al.*, 1986; Edman *et al.*, 1980; Andrianov *et al.*, 1998), and silica particles coated with proteins, labile drugs and hormones. Matsuno et al. (Matsuno *et al.*, 1983) found that nearly spherical

poly(vinylpyrrolidone)-coated silica gel particles with an average diameter of 30 nm were taken up effectively by mouse enterocytes. Polymers investigated in nanocarrier formulations for oral drug delivery include poly(alkylcyanoacrylates) (Sai *et al.*, 1996), and terpolymers of methyl methacrylate, 2-hydroxyethylmethacrylate and *n*-butylacrylate (Kukan *et al.*, 1991). The methodologies reviewed briefly in this section have met success in various therapies and interested readers are referred to reviews on the subject (Kreuter, 1991; Fasano, 1998; Gershanik *et al.*, 2000; Leuner *et al.*, 2000; Vasir *et al.*, 2003). The focus of this article is, a yet different approach towards enhancing the bioavailability of highly lipophilic drugs, based on the molecular dissolution of a drug within the hydrophobic core of polymeric micelles.

2.6. MICELLES: DEFINITION AND ADVANTAGES

In the late1960's, micelles drew much attention as drug carriers due to their easily controlled properties and good pharmacological characteristics (Yokoyama *et al.*, 1992; Wiedmann *et al.*, 2002). Micelles are formed when amphiphiles are placed in water. They consist of an inner core of assembled hydrophobic segments able to solubilize lipophilic substances and an outer hydrophilic corona serving as a stabilizing interface between the hydrophobic core and the external aqueous environment (Kataoka *et al.*, 1993). Depending on the delivery purpose, one can select the size, charge and surface properties of these carriers simply by adding new ingredients to the mixture of amphiphilic substances before micelle preparation and/or by variation of the preparation method.

Micelles as drug carriers provide a set of advantages – they physically entrap sparingly soluble pharmaceuticals and deliver them to the desired site of action at concentrations that can exceed their intrinsic water solubility and thus increase their bioavailability. The stability of the drug is also increased through micelle incorporation. Furthermore, undesirable side effects are lessened, as contact of the drug with inactivating species, such as enzymes present in biological fluids, are minimized, in comparison with free drug (Yokoyama *et al.*, 1990; Lee *et al.*, 1990; Torchilin, 2001). They can be prepared in large quantities easily and reproducibly (Kataoka, 1994; Yokoyama, 1998). By far the most important feature of micellar delivery systems, which distinguish them from other particulate drug carriers, lies in their small size (~ 10 to 30 nm) and the narrow size distribution (Florence *et al.*, 2001).

Micelles made of non-ionic surfactants are widely used as adjuvants and drug carrier systems in many areas of pharmaceutical technology and controlled drug delivery (Malik *et al.*, 1975; Takada *et al.*, 1986; Lasic, 1992; Schubiger *et al.*, 1997; Redondo *et al.*, 1998; Bardelmeijer *et al.*, 2002). Such a high level of activity has brought a great deal of diversity to this field, since most groups introduced their own micelle system formed from unique hydrophilic-hydrophobic combinations. In almost all cases, the hydrophilic outer shell consists of poly(ethylene oxide) (PEO) chains, due to their high degree of hydration and large excluded volume inducing repulsive forces, which contribute to the stabilization of the micelle (Kataoka, 1994; Elbert *et al.*, 1998; Otsuka *et al.*, 2001; Otsuka *et al.*, 2003). In addition, the PEO corona prevents recognition by the reticuloendothelial system and therefore minimizes elimination of the micelle from the bloodstream. Thus,

these so-called "stealth" properties of the PEO corona result in increased blood circulation times and allow drugs to be administered over prolonged periods of time (Kwon, G. *et al.*, 1994; Kwon, G.S. *et al.*, 1995; Yamamoto *et al.*, 2001). A wide range of hydrophobic blocks have been explored resulting in different micellar systems with distinct physicochemical properties.

Surfactant micelles form only above a critical concentration, the critical micelle concentration (CMC) and rapidly break apart upon dilution, which can result in premature leakage of the drug and its precipitation *in situ*. These limitations of surfactant micelles as drug delivery carriers triggered the search for micelles of significantly enhanced stability and solubilizing power.

The use of polymer-based micelles has gained much attention because of the high diversity of polymers, their biocompatibility, biodegradibility and the multiplicity of functional groups they display for the conjugation of pilot molecules (Andrianov *et al.*, 1998).

2.7. POLYMERIC MICELLES

Like their low molecular weight counterparts, amphiphilic polymers associate in water to form "polymeric micelles" (Kwon, G.S. *et al.*, 1996), consisting of a hydrophobic core stabilized by a corona of hydrophilic polymeric chains exposed to the aqueous environment (Kataoka *et al.*, 2001). Polymeric micelles can be used as efficient carriers for compounds, which alone exhibit poor solubility, undesired pharmacokinetics and low

stability in a physiological environment. The hydrophilic shell contributes greatly to the pharmaceutical behavior of polymeric formulations by maintaining the micelles in a dispersed state, as well as by decreasing undesirable drug interactions with cells and proteins through steric-stabilization effects. The size of polymeric micelles ranges from ~10 nm to ~100 nm and usually the size distribution is narrow (Kataoka *et al.*, 2001). This topology is similar to that of surfactant micelles, hence polymeric micelles can be expected to solubilize hydrophobic drugs within their core. However, there are significant differences between the two types of assemblies from the physicochemical viewpoint. The polymer concentration at which the association first takes place, sometimes known as the critical association concentration (CAC), is lower by several orders of magnitudes than typical surfactant CMC values. Thus, polymeric micelles are more stable towards dilution in biological fluids. They can increase drug bioavailability and retention, since the drug is well protected from possible inactivation under the effect of their biological surroundings (Kwon, G.S., 2002).

Polymeric micelles have been studied extensively as delivery medium for injectable drug formulations of poorly water-soluble drugs such as paclitaxel, indomethacin, amphotericin B, adriamycin and dihydrotestosterone. Overall, they proved to be highly effective drug delivery vehicles (Zhang *et al.*, 1997; Shin *et al.*, 1998; Yu *et al.*, 1998; Jeong *et al.*, 1999; Allen *et al.*, 2000). To date, most contributions in the area of polymeric micelles for oral formulations, have been made by the group of Kabanov (Batrakova *et al.*, 1998a; Batrakova *et al.*, 1998b; Batrakova *et al.*, 1999; Alakhov *et al.*, 2001; Kabanov, A. *et al.*, 2002a; Kabanov, A.V. *et al.*, 2002b). Their work focused mostly on micelles formed

from commercially available Pluronic® triblock copolymers (also termed Poloxamer; Poly(ethylene oxide)$_x$-*b*-poly(propylene oxide)$_y$-*b*-poly(ethylene oxide)$_x$; PEO_x-*b*-PPO_y-*b*-PEO_x), and more recently, on block ionomer complexes as carriers for DNA (Lemieux *et al.*, 2000).

Our approach towards oral drug delivery through polymeric micelles was to design with care the chemical composition and architecture of the amphiphilic polymers forming the polymeric micelle vehicle. To maximize our chances of success, we chose to base the new materials on polysaccharides, which are non-toxic, naturally occurring polymers and readily available in a range of molecular weights. We selected hydroxypropylcellulose (HPC), a non-ionic water-soluble cellulose derivative, and dextran (DEX), a glucose polymer with predominantly 1,6-α-glycosidic linkages (Larsen, 1989). Both polymers are non-toxic and non-irritant polysaccharides. They are used in medicine, *e.g.* dextran as plasma substitute both locally and systemically (Couch, 1965) and hydroxypropylcellulose in oral tablet formulations, where it acts as a disintegrant (Machida *et al.*, 1974) and as a binder in granulation (Skinner *et al.*, 1999), due to its bioadhesive properties. In order to impart either dextran or HPC with an amphiphilic character, we linked to their backbone a small number of hydrophobic cetyl groups, as described below in the case of dextran. We chose to attach the hydrophobic groups to the polysaccharide hydrophilic backbone *via* a short PEO linker, yielding dextran grafted with PEO_{10}-C_{16} (DEX-*g*-PEO_{10}-C_{16}) or HPC-*g*-PEO_{10}-C_{16}; the digit next to PEO refers to the number of ethylene oxide groups in PEO, and the digit next to the carbon refers to the number of carbons in the alkyl chains (Figure 2.2.); and to exploit the solubilizing potential of this copolymer towards poorly water-soluble

drugs. In aqueous solution, hydrophobically-modified (HM) polysaccharides associate into polymeric micelles above the CAC. Thus, a lipophilic drug can be entrapped in the hydrophobic core formed by the alkyl residues, while the hydrophilic polysaccharide chains stabilize the system in the aqueous environment. In this article, we present the design, chemical characterization, and in-vitro evaluation of HM-dextran based polymeric micelles as an example of the novel polymer-based nanocarriers for oral delivery of poorly water-soluble drugs developed recently in our group.

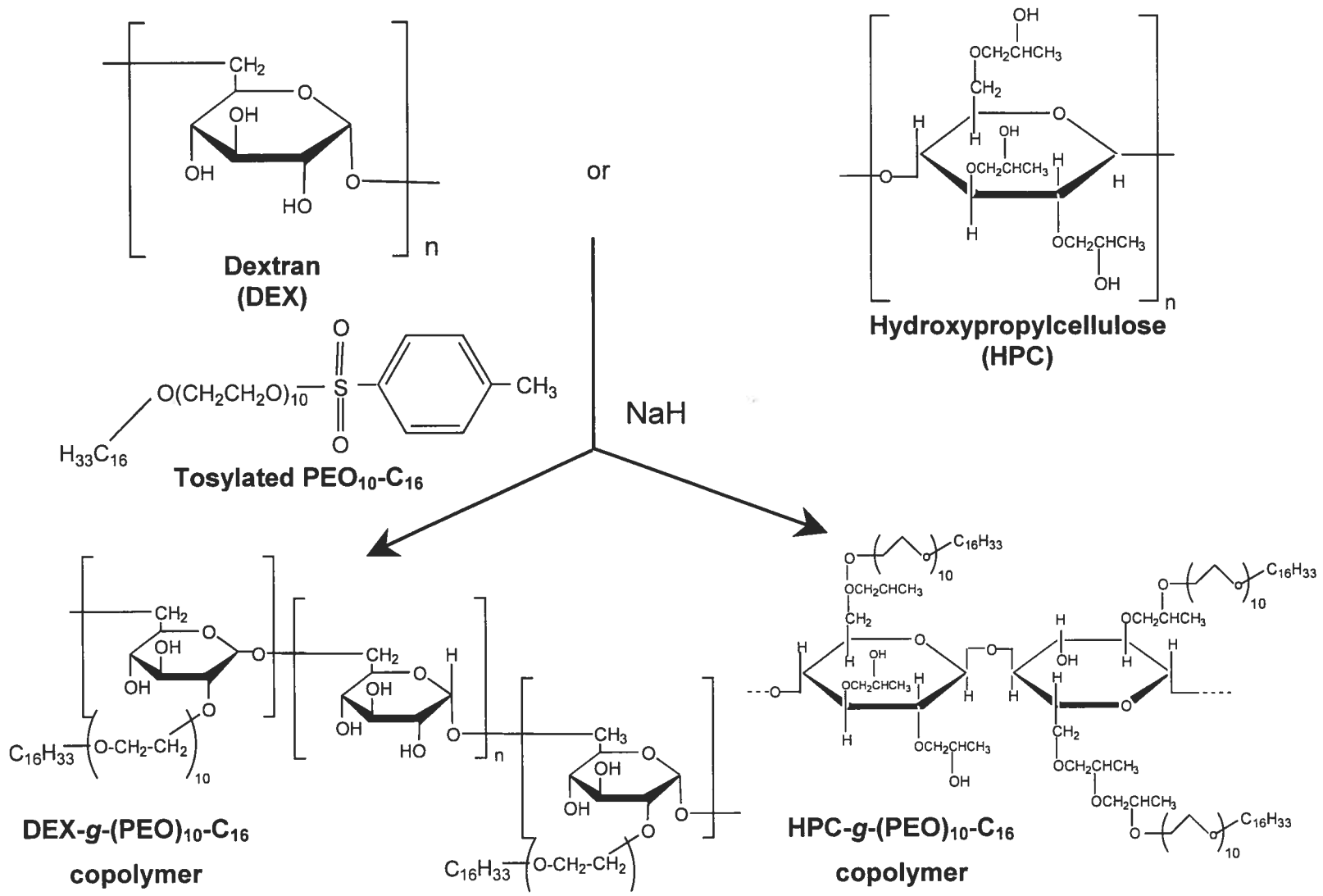

Figure 2.2. Synthesis of HM-dextran and HM-hydroxypropylcellulose copolymers.

2.8. SYNTHESIS AND CHARACTERIZATION OF HM DEXTRAN COPOLYMERS

Hydrophobically-modified DEX-*g*-PEO_{10}-C_{16} graft copolymers were synthesized following the scheme shown in Figure 2.2. (Cristea *et al.*, 2003). A tosylated poly(ethylene oxide) (10) cetyl ether (PEO_{10}-C_{16}) was coupled to dextran T10 (DEX ; M_W 10,000 Da) by a Williamson ether synthesis reaction. The resulting DEX-*g*-PEO_{10}-C_{16} copolymers were purified by soxhlet extraction with dichloromethane to remove all free PEO_{10}-C_{16}. The extent of grafting (Table 2.1.) was determined from ^{1}H-NMR spectroscopy data, using the signal due to the resonance of the terminal cetyl methyl protons ($\delta \sim 0.85$ ppm) and the signal due to the resonance of the anomeric protons of dextran ($\delta \sim 4.7$ ppm) (Figure 2.3.). Depending on the initial relative concentration of dextran and PEO_{10}-C_{16}, amphiphilic dextrans carrying from 2.3 to 15 mol % PEO_{10}-C_{16} (relative to the number of glucose units) were prepared in excellent yield and a high level of purity.

Table 2.1. Characteristics of DEX-*g*-PEG-C_n copolymers with various compositions.

Polymer composition	Grafted PEO-C_{16} (mol %)[a]	CAC[b] (mg/l)	Mean diameter[c] (nm ± S.D.)	Maximum CsA loading[d] (%*w*/*w*)
Dextran T10	0	-	-	0.6 ± 0.1
PEO_{10}-C_{16}	-	4.3 ± 1	-	17.5 ± 0.5
DEX-*g*-PEO_{10}-C_{16}	2.3 ± 0.1	7.6 ± 0.3	44 ± 4	1.1 ± 0.2
DEX-*g*-PEO_{10}-C_{16}	6.4 ± 0.1	4.2 ± 0.4	12 ± 3	6.8 ± 0.2
DEX-*g*-PEO_{10}-C_{16}	15.2 ± 0.1	3.7 ± 0.2	11 ± 5	8.5 ± 0.6

[a]Determined by ^{1}H NMR measurement.

[b]Determined by change in I_{336}/I_{333} ratio of pyrene fluorescence with log polymer concentration at 25 °C.

[c]Determined by DLS measurements at 25 °C with a scattering angle of 90 °.

[d]Determined by HPLC analysis with UV detection at 210 nm.

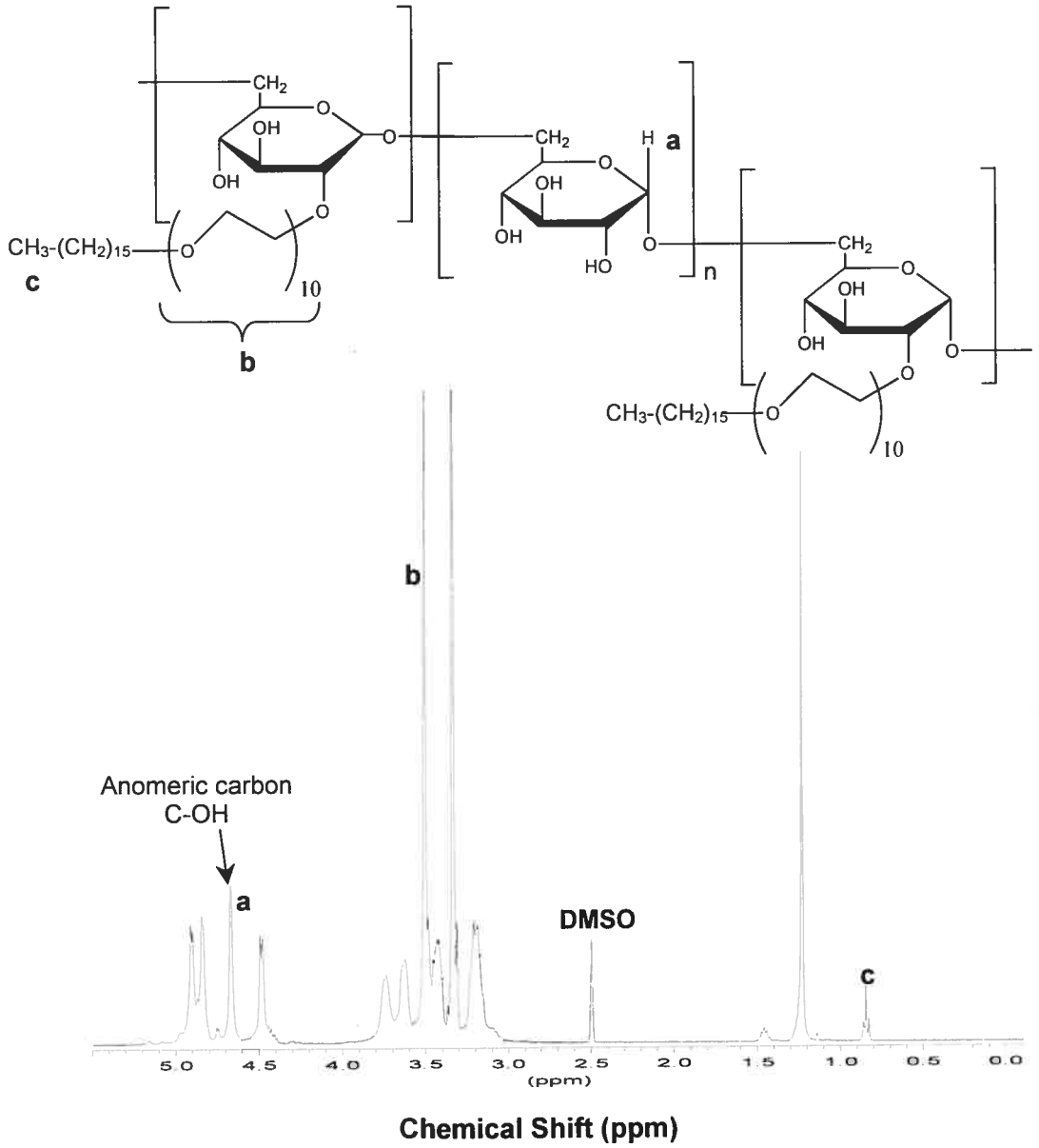

Figure 2.3. Chemical structure of DEX-*g*-PEO_{10}-C_{16} and its ^1H NMR spectrum in DMSO-d_6.

2.9. PHYSICO-CHEMICAL CHARACTERISTICS OF POLYMERIC MICELLES

As discussed in the introduction, the ability of polymeric micelles to act as effective drug delivery systems is controlled, to a large extent, by their size and their critical association concentration (CAC), defined as the copolymer concentration below which only single chains exist. In solutions of the copolymer of concentration higher than the CAC, micelles and single chains coexist. This definition is used commonly to describe polymeric micelles, in analogy with the CMC of surfactant micelles, although the mechanism of micellization may not be the same for amphiphilic polymers and surfactants (Nagarajan *et al.*, 1989b; Zana, 2002). A delivery system is subject to "sink conditions" or severe dilution in the gastrointestinal fluid upon oral administration. It is important to know the CAC value in order to select dosage compositions such that the copolymer concentration remains above the CAC immediately upon administration. The CAC of amphiphilic copolymers is determined by many factors, such as the nature and length of the core-forming segments, and the length of the hydrophilic chain. Amphiphilic copolymers which contain highly hydrophobic residues have lower CAC values in water than those which include the less hydrophobic residues. For a series of copolymers, if the corona-forming chain is kept constant, an increase in the molecular weight of the core-forming segment will decrease the CAC (Alexandridis *et al.*, 1994b). To a lesser extent, if the length of the core-forming segment is maintained at a constant length, an increase in the length of the hydrophilic chain will cause an increase in the value of the CAC (Astafieva *et al.*, 1993; Alexandridis *et al.*, 1994a).

The CAC of polymeric micelles can be estimated by fluorescence spectroscopy using pyrene, a hydrophobic fluorescence probe that preferentially partitions into the hydrophobic core of the micelle. Pyrene undergoes changes in its photophysical properties as a result of the change in the micropolarity it experiences upon diffusion from bulk water (hydrophilic environment) into the micelle core (hydrophobic environment) (Dong *et al.*, 1984; Zhao *et al.*, 1990). Two methods exist for determining the CAC of polymeric micelles with pyrene fluorescence (Winnik *et al.*, 1998). The original method, proposed by Kalyanasundaram et *al.* (Kalyanasundaram *et al.*, 1977), takes advantage of the changes in the vibronic fine structure of the pyrene emission and monitors the changes in the ratio of the intensities I_1 and I_3 of the [0,0] and [0,2] bands, respectively. More recently, it has been suggested that a more accurate determination of the CAC can be obtained by monitoring the changes in the ratio of the pyrene excitation spectra intensities (Wilhelm *et al.*, 1991) at λ = 333 nm for pyrene in water and λ =336 nm for pyrene in an hydrophobic medium (Figure 2.4.). By plotting the I_{336}/I_{333} intensity ratios versus the logarithm of the concentration of the aqueous solutions of copolymer, sigmoidal curves are obtained, where, at the CAC, a sharp increase is observed in the fluorescence intensity ratio (I_{336}/I_{333}) as the polymer concentration increases (Figure 2.4.).

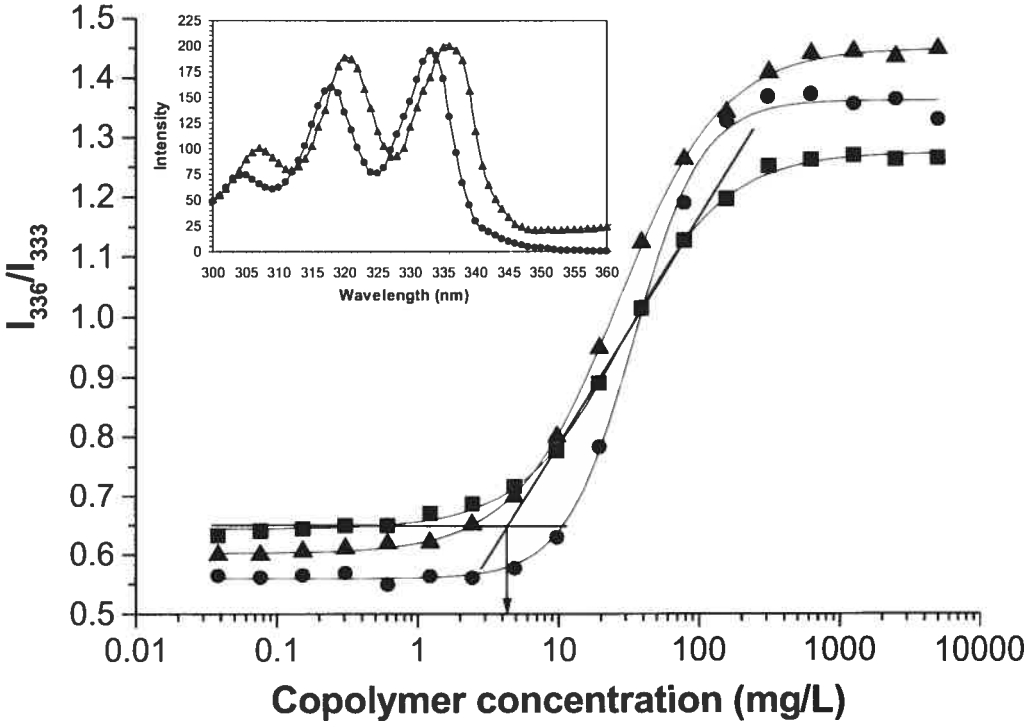

Figure 2.4. Plot of the intensity ratio I_{336}/I_{333} (from pyrene excitation spectra) as a function of concentration (0.04 – $5x10^3$ mg/l) of DEX-*g*-PEO_{10}-C_{16} copolymers containing (●) 2.3 mol%; (■) 6 mol% and (▲) 15 mol% of grafted PEO_{10}-C_{16} residues. Each value is the mean of two independent measurements. *Inset*: Excitation spectra of pyrene (2 x 10^{-7} M aqueous solution) monitored at λ_{em} 390 nm in absence (●) or presence (▲) of HM DEX copolymer.

We used the latter method to estimate the CAC of the DEX-*g*-PEO_{10}-C_{16} copolymers. As shown in Table 2.1., the CAC values decrease with increasing molar content of PEO_{10}-C_{16} residues linked to the dextran backbone. These results are consistent with recent evidence suggesting that the increase in the length of a hydrophobic residue for a given length of a hydrophilic polymer chain causes noticeable decrease in CAC value and increase in micelle stability (Nagarajan *et al.*, 1989b).

The size of micelles is controlled by the length of the core-forming segment and the length of the corona forming chain (Halperin, 1987; Nagarajan *et al.*, 1989a), but it depends also on the method of micellization selected for the preparation of micelles. For micelles produced using the dialysis method, it varies depending on the organic solvent employed (La *et al.*, 1996; Kim, S.Y. *et al.*, 1998). The choice of organic solvent also affects the yield of micellization. For example, La et al. (La *et al.*, 1996) reported that the use of dimethylsulfoxide (DMSO) as the organic solvent gave rise to PEO-*b*-poly(β-benzyl-L-aspartate) (PBLA) micelles which were only 17 nm in size, but the micelle yield was low (6%). Using dimethylacetamide (DMAc) as organic solvent resulted in a much higher micelle yield, with an average particle size of 19 nm and a narrow size distribution (La *et al.*, 1996). In this way, the dialysis method provides a means of tailoring the size and size distribution of the micelles. Note that, above all, it is important to select conditions that minimize the formation of large polymer aggregates (diameter > 300 nm), in addition to the desired polymeric micelles (Cammas *et al.*, 1997).

We determined the size of dextran-based polymeric micelles by dynamic laser light scattering (DLS) at 25 °C, with a scattering angle of 90°, and found that the hydrodynamic diameter of DEX-*g*-PEO_{10}-C_{16} micelles ranged from 10 to 45 nm (Table 2.1). In all cases the size distribution was unimodal, indicative of the absence of free polymer chains and of large aggregates. Polymeric micelles formed by amphiphilic dextrans of structure similar to that of DEX10-*g*-PEO_y-C_n, but prepared from a higher molecular weight dextran (Dextran T40; M_W 40,000 Da; DEX 40) are larger than those formed by DEX10-*g*-PEO_y-C_n, independently of the size of the hydrophobic substituent and of the level of modification (Francis *et al.*, 2003b). This observation can be taken as an indication of the steric hindrance induced by the carbohydrate chains, which are expected to take place over a larger volume for the polymer of higher molecular weight (Maksimenko *et al.*, 2001; Baldwin *et al.*, 1988). It has been shown that the uptake of particles within the intestine and the extent of drug absorption increase with decreasing particle size and increasing specific surface area (Florence *et al.*, 2001). Thus, the small size exhibited by all the polymeric micelles studied here shows a favorable trend towards oral drug delivery.

2.10. Physical Loading of a Drug In DEX-*g*-PEO_{10}-C_{16} Polymeric Micelles

The hydrophobic core of the polymeric micelles is expected to serve as the loading space for various lipophilic drugs. By design, given the nanometric size of the micelles, this space is limited. In order to exploit fully this loading space, one must manipulate the many factors that control loading capacity and loading efficiency. In devising a drug

incorporation strategy, one must try to match as closely as possible the polarity of the hydrophobic micelle core to the solubility characteristics of the drug. Nagarajan et al. (Nagarajan *et al.*, 1986) demonstrated that aromatic hydrocarbons are incorporated to a greater extent into poly(ethylene oxide-*b*-propylene oxide) and poly(*N*-vinylpyrrolidone-*b*-styrene) polymeric micelles, in comparison to aliphatic hydrocarbons. Kim et al. (Kim, S.Y. *et al.*, 1998) showed that the amount of indomethacin that can be incorporated into micelles can reach 42.2 (*w* %) in the case of PEO-*b*-poly(ε-caprolactone), while La et al. (La *et al.*, 1996) obtained an indomethacin content of only 22.1 (*w* %) for micelles formed from PEO-*b*-PBLA. An increase in the length of the core forming segment tends to enhance the loading capacity per micelle (Gadelle *et al.*, 1995). Note that a drug may reside preferably within the core of the micelle or within the core/corona interface, depending on its solubility characteristics (Gadelle *et al.*, 1995).

We report here the loading capacity of DEX-*g*-PEO_{10}-C_{16} polymeric micelles towards cyclosporin A (CsA), a highly effective immunosuppressive agent that is used for the prevention of graft rejection following organ transplantation (Laupacis *et al.*, 1982). Chemically, CsA is a neutral cyclic undecapeptide with a molecular weight of 1202 g/mol (Wenger, 1983), consisting of hydrophobic amino acids linked *via* 11 amide bonds, seven of which are *N*-methylated. Four intramolecular hydrogen bonds (Vine *et al.*, 1987) contribute to the rigidity of its skeleton. This chemical composition is responsible for the extremely low solubility of CsA in water (23 μg/ml at 20 °C). The oral administration of CsA is complicated by the presence of several metabolizing enzymes: cytochrome P-450 3A4 (CYP3A4), the multi-drug transporter P-glycoprotein (PGP) in the small intestine, and

hepatic CYP3A4 (Tjia *et al.*, 1991). These factors, together with the poor solubility of CsA in the aqueous fluids of the gastrointestinal tract, severely limit the absorption of CsA through the gastrointestinal mucosa. They account for the low bioavailability of the drug and significantly increase the risks of both acute and chronic rejection (Lindholm *et al.*, 1988). Commercial CsA oral formulations are mostly microemulsion-based. While more effective than the original emulsion-based formulations, this formulation presents a rather large interindividual variation in terms of CsA bioavailability (Kovarik *et al.*, 1994). In view of the clinical importance of CsA, much effort has been placed towards designing oral formulations leading to acceptable bioavailability. A number of innovative drug delivery approaches including mixed micelles (Takada *et al.*, 1985), charged nanoparticles (El-Shabouri, 2002), liposomes (Al-Meshal *et al.*, 1998), lipids (Ueda *et al.*, 1983), surfactants (Chang *et al.*, 1996), microspheres (Kim, S.J. *et al.*, 2002), and microemulsions (Ritschel *et al.*, 1990) have been investigated in order to improve the unfavorable absorption characteristics of CsA.

We chose to incorporate CsA into DEX-*g*-PEO_{10}-C_{16} polymeric micelles by a dialysis method, which involved treatment of an aqueous polymer solution with a solution of CsA in ethanol. Ethanol was used as solvent to prevent any cytotoxic effect that could be caused by trace organic solvent in the final formulation. The initial CsA loading varied from 2.5 – 40 % (*w/w* CsA/polymer). The mixed solutions were extensively dialyzed against distilled water. In this process, the organic solvent is slowly replaced by water so that micelles start to form and to incorporate the lipophilic drug inside their hydrophobic core (Figure 2.5.). After completion of the dialysis, the solutions were filtered to eliminate

excess CsA and the filtrate was freeze-dried yielding a free flowing powder which readily dispersed in water to form polymeric micelles of size and size distribution nearly identical to those of micelles devoid of CsA.

The amount of CsA incorporated in the micelles was determined by a high performance liquid chromatography quantitative assay of the CsA extracted from freeze-dried micelles with acetonitrile, a non-solvent of the copolymer but a good solvent of CsA. Drug loading (DL) was calculated using Equation (1):

$$DL(\%)=100(W_C/W_M) \quad \textbf{Equation (1)}$$

Where W_C is the weight of CsA loaded in micelles and W_M is the weight of micelles before extraction. The amount of CsA released upon dissolution was plotted against the CsA concentration added initially for each preparation. Both free and micelle-entrapped CsA were measured with this assay. The amount of free CsA is expected to be low, since the undissolved fraction of the drug should have been removed by filtration after dialysis.

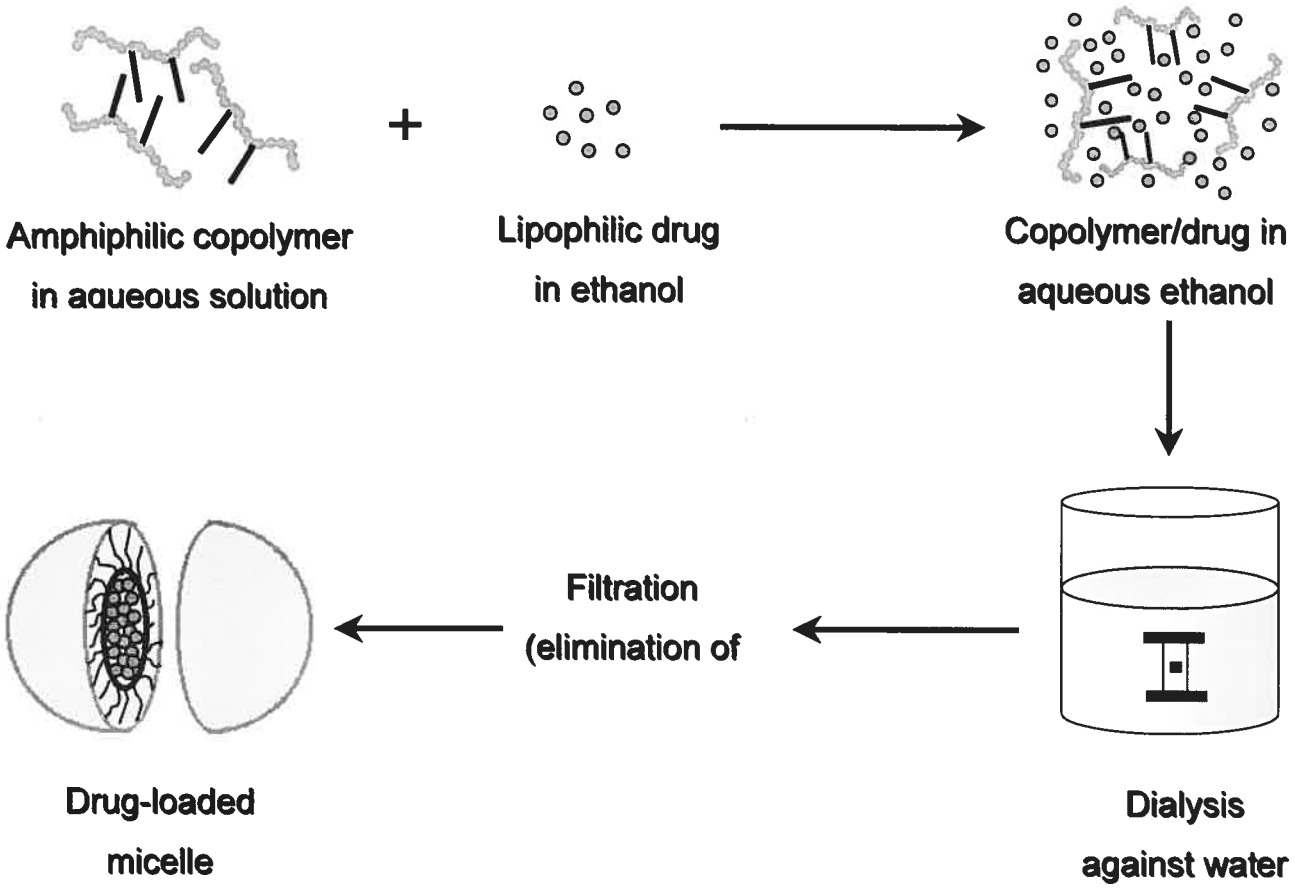

Figure 2.5. Schematic representation of drug loading in polymeric micelles using the dialysis method.

We evaluated first the ability of the PEO_{10}-C_{16} micelles to solubilize CsA and found that a high level of CsA (17.5% *w/w*) was incorporated in the surfactant micelles, an indication of the favourable solubilizing characteristics of PEO_{10}-C_{16} towards CsA. Next, we assessed the incorporation of CsA within polymeric micelles. The maximum amount of CsA loaded within the micelles was 1, 7 and 8% (*w/w*), for DEX-*g*-PEO_{10}-C_{16} containing 2.3, 6 and 15 mol% PEO_{10}-C_{16}, respectively (Figure 2.6.). In all cases, the amount of CsA incorporated was larger in the case of polymeric micelles than in the case of unmodified dextran, which proved to have a very low affinity for CsA. By comparing the highest CsA loading percentage in the various polymers, it is apparent that the CsA loading increases with increasing molar content of PEO_{10}-C_{16} grafted on the dextran backbone. Other structural features of the copolymers also affect the micellar loading capacity towards CsA. For example, micelles formed by polymers of higher molecular weights (*e.g.* DEX40) are not as effective CsA carriers as those based on DEX10 (Francis *et al.*, 2003b). Also, for a constant number of PEO_{10}-C_n units grafted per dextran chain, PEO_{10}-C_{16}-based micelles seem more effective in solubilizing CsA than PEO_{10}-C_{18}-based micelles, indicating that the micropolarity of a hydrophobic core made up of hexadecyl-PEO chains might present a better solubilizing microenvironment than that offered by the octadecyl-PEO moieties (Francis *et al.*, 2003b).

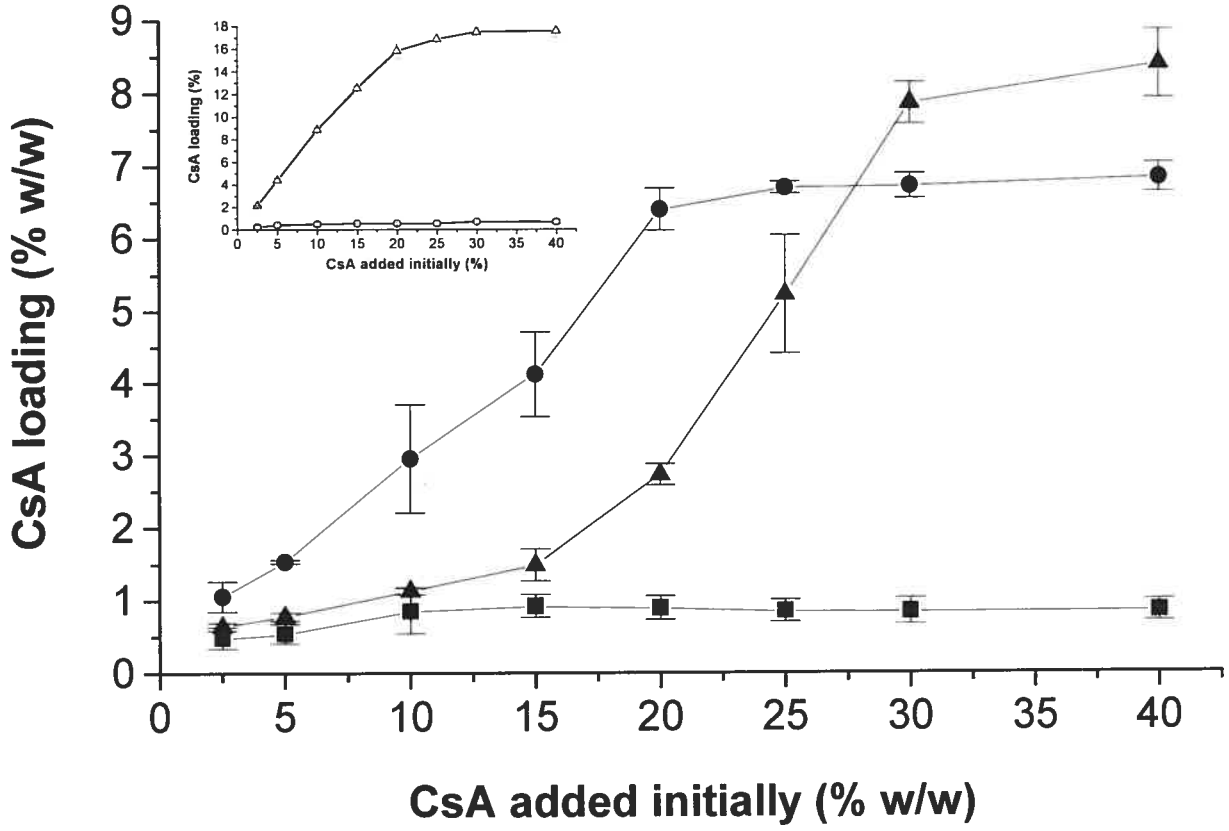

Figure 2.6. CsA final loading (*w/w* %) in micelles of DEX-*g*-PEO_{10}-C_{16} containing (■) 2.3 mol%; (●) 6 mol% and (▲) 15 mol% grafted PEO_{10}-C_{16} residues, at 2.5 – 40 (% *w/w*) of initially added CsA. *Inset*: CsA loading (% *w/w*) in presence of (Δ) free PEO_{10}-C_{16} surfactant as well as (o) unmodified DEX polymer. Mean ± S.D. ($n = 3$).

2.11. CYTOTOXICITY STUDY

The human colon adenocarcinoma, Caco-2 cells, are generally selected to estimate *in vivo* drug absorption, as they retain many features of small intestinal epithelial cells (Dantzig *et al.*, 1990; Hidalgo *et al.*, 1989). Thus, we used Caco-2 cells to study the cytotoxicity of DEX-*g*-PEO_{10}-C_{16} polymeric micelles, using the colorimetric MTT assay (Hansen *et al.*, 1989). The cell viability in the presence of micelles was compared to that in the presence of unmodified DEX as well as of free PEO_{10}-C_{16} surfactant residues (Figure 2.7.).

Cell growth was severely inhibited by PEO_{10}-C_{16}, even at surfactant concentrations below 1 g/l. Such deleterious effect is consistent with the reported propensity of poly(ethylene oxide) alkyl ethers to affect membrane integrity (Dimitrijevic *et al.*, 2000). In contrast, the polysaccharide DEX showed no toxicity up to a concentration of 10 g/l, confirming the reported inertness of dextrans. Turning now to the effect of DEX-*g*-PEO_{10}-C_{16} (15 mol%), we determined that it exhibited no significant toxicity towards Caco-2 cells, up to concentrations of 10 g/l or concentration of PEO_{10}-C_{16} (0.05 g/l) equivalent to a $PEO_{10}C_{16}$ concentration showing a significant cytototoxic effect (60% cell viability). This important result indicates 1) that the polymer purification method efficiently removed any free PEO_{10}-C_{16} from the polymer, and 2) by grafting the PEO_{10}-C_{10} residues onto dextran, we succeeded in minimizing their toxic effect on cells. The non-toxic hydrophilic dextran chains forming the polymeric micelle outer shell may be in contact with the cells and effectively insulate them from the surfactant residues assembled in the inner core.

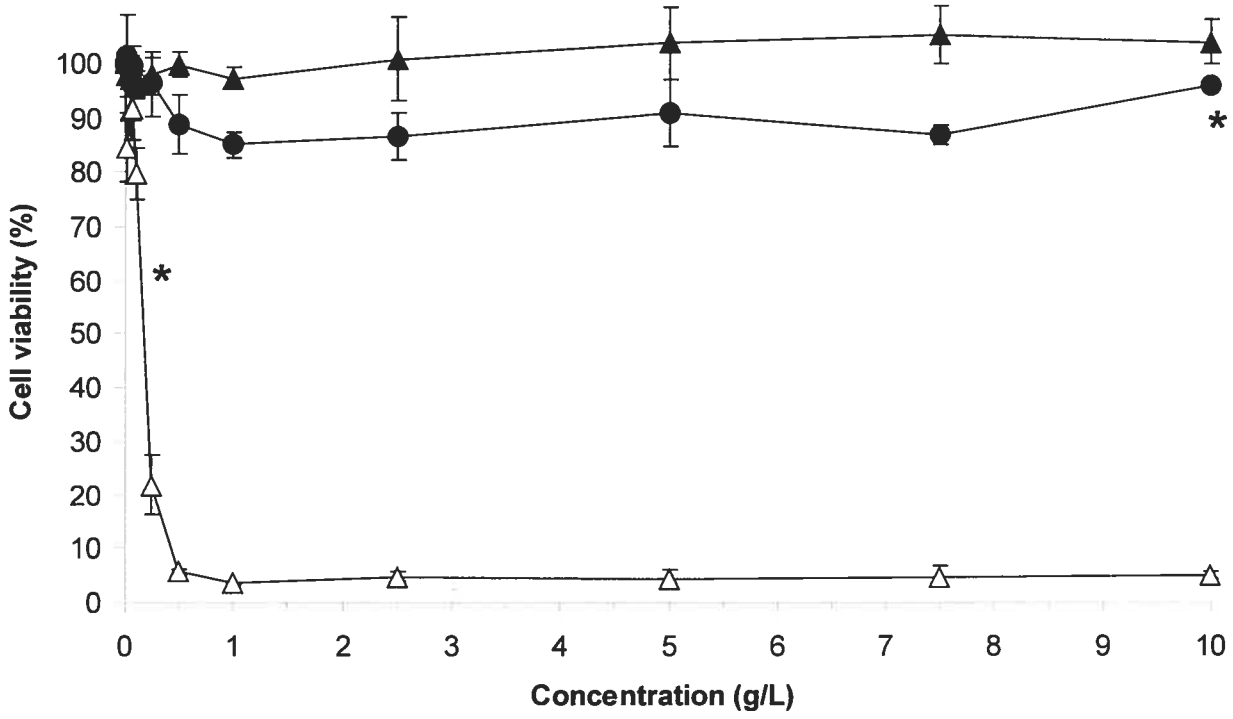

Figure 2.7. Effect of unmodified dextran (▲), free PEO_{10}-C_{16} (Δ) and DEX-*g*-PEO_{10}-C_{16} (15 mol%) (●) on Caco-2 cell viability measured by the MTT assay. Equivalent concentrations of free PEO_{10}-C_{16} and PEO_{10}-C_{16} grafted to dextran backbone are indicated in the figure by stars. Mean ± S.D. ($n = 3$).

2.12. CONCLUSION

The experimental evidence reviewed in this article led us to conclude that polymeric micelles present a number of enabling properties for oral delivery of lipophilic drugs. For example, HM-dextran form nanosized assemblies characterized by a low onset of micellization and a high solubilization power towards highly lipophilic drugs. Moreover, they present no significant cytotoxicity. Initial results indicate that the transport of a HM-dextran-entrapped lipophobic drug is enhanced, compared to free drug (Francis *et al.*, 2003a). Current research on particulate delivery systems is focused on gaining a better understanding of the mechanisms, efficiency, and reproducibility of the permeability of both the carrier and/or the drugs across the GI tract, and analysis of the effect of carrier composition on these parameters.

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THE AIM OF THE PRESENT STUDY

The ultimate goal of our study is to develop a novel oral formulation especially intended to increase the oral bioavailability of poorly-water soluble drugs. The project relies on the design, characterization and development of polymeric micelles capable of solubilizing high levels of lipophilic drugs and eventually facilitating their transfer across intestinal epithelial cells.

To reach our goal, polysaccharide-based polymeric micelles have been proposed. Cyclosporin A (CsA) was the drug of choice for encapsulation in the different proposed polymeric micelles.

In **chapter 3**, we present the physicochemical characterization and solubilization efficiency of various dextran-based polymeric micelles with varying chemical composition.
"*Solubilization of cyclosporin A in dextran-g-polyethyleneglycolalkyl ether polymeric micelles*", Eur. J. Pharm. Biopharm. (2003), 56 (3) : 337-346.

In **chapter 4**, we present the physicochemical characterization and solubilization efficiency of various hydroxypropylcellulose-based polymeric micelles with varying chemical composition.
"*Solubilization of poorly water soluble drugs in micelles of hydrophobically modified hydroxypropylcellulose copolymers*", J. Control. Release (2003), 93 (1) : 59-68.

In **chapter 5**, we investigated the permeability of CsA-loaded in polymeric micelles across Caco-2 intestinal epithelium, compared to free CsA under the same conditions.

"Engineering polysaccharide-based polymeric micelles to enhance permeability of Cyclosporin A across Caco-2 cells" Pharm. Res. (2004), in press.

In **Chapter 6**, we studied the possibility to utilize the specific uptake mechanism for Vitamin B_{12} (VB_{12}) to enhance the oral uptake of CsA loaded in VB_{12}-modified polymeric micelles.

"Vitamin B_{12}-targeted polymeric micelles for improving Cyclosporin A permeability across Caco-2 cell monolayers" Biomacromolecules (2004), to be submitted.

In **Chapters 7 and 8**, the general discussion and the perspective will be presented, respectively.

2.14. REFERENCES

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CHAPTER THREE

RESEARCH PAPER

SOLUBILIZATION OF CYCLOSPORIN A IN DEXTRAN-*g*-POLYETHYLENEGLYCOLALKYL ETHER POLYMERIC MICELLES

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3.1. ABSTRACT

Solubilization of the poorly water-soluble drug, Cyclosporin A (CsA), in aqueous dispersions of dextran-grafted-polyethyleneglycolalkyl ether (DEX-*g*-PEG-C_n) polymeric micelles was examined as a function of copolymer structure. In aqueous solution, DEX-*g*-PEG-C_n form polymeric micelles of low critical association concentrations (CAC) and small micelle sizes as determined by fluorescence spectroscopy and dynamic light scattering (DLS). Copolymers with longer polysaccharide chain showed larger CAC and mean diameter. The percentage of CsA loading into micelles was determined by high performance liquid chromatography. It was significantly larger in polymeric micelles compared to unmodified dextrans. It increased with increasing number of PEG-C_n units grafted per dextran chain and decreasing dextran molecular weight. The cytotoxicity of DEX-*g*-PEG-C_{16} polymeric micelles towards Caco-2 cells, tested by MTT cytotoxicity assay, was significantly lower than that of free PEG-C_{16} molecules. It can be concluded that the length of the hydrophilic part as well as the content and chemical nature of the hydrophobic substituents have an important effect on the ability of polymeric micelles to solubilize poorly-water soluble drugs.

3.2. AUTHOR KEYWORDS

Polymeric micelle; Poorly water soluble drug; Oral delivery; Cyclosporin A; Dextran; Polyethyleneglycolalkyl ether; Solubilization; Cytotoxicity.

3.3. INTRODUCTION

Peroral drug administration represents by far the easiest, most common and most convenient route of drug delivery, especially when repeated or routine administration is necessary (Chen *et al.*, 1998). For effective delivery *via* the oral route, a therapeutic agent must first dissolve in the gastrointestinal lumen (Florence *et al.*, 1993). This can pose major challenges in the case of poorly-water soluble drugs (Gershanik *et al.*, 2000). A drug may be defined as "poorly soluble" if its dissolution rate is slower than the transit time to its absorptive sites (Horter *et al.*, 2001). The dissolution of a poorly water-soluble drug in the gastrointestinal contents is quite often the rate-limiting step that, ultimately, controls the bioavailability of the drug at its site of action (Charman *et al.*, 1991).

One approach to enhance the solubility and bioavailability of a highly lipophilic drug is to dissolve it on the molecular level in the hydrophobic core of a delivery system, itself soluble or dispersible in the aqueous environment. In the late 1960s, surfactant micelles drew much attention as drug delivery carriers, due to their good pharmacological characteristics (Wiedmann *et al.*, 2002). They are widely used as adjuvants and drug carrier systems in many areas of pharmaceutical technology and controlled drug delivery (Lasic, 1992). Surfactant micelles form only above a critical concentration, the critical micelle concentration (CMC) and rapidly break apart upon dilution which could result in a premature leakage of the drug and its precipitation in situ.

These limitations of surfactant micelles as drug delivery carriers triggered the search for micelles of significantly enhanced stability and solubilizing power. Like their low

molecular weight counterpart, amphiphilic polymers associate in water to form "polymeric micelles" (Kwon *et al.*, 1996) consisting of a hydrophobic core stabilized by a corona of hydrophilic polymeric chains exposed to the aqueous environment (Kataoka *et al.*, 2001). The size of polymeric micelles ranges from ~10 nm to ~100 nm and usually the size distribution is narrow (Kataoka *et al.*, 2001). This topology is similar to that of surfactant micelles, hence polymeric micelles can be expected to solubilize hydrophobic drugs within their core. However, there are significant differences between the two types of assemblies from the physico chemical viewpoint. The polymer concentration at which the association first takes place, sometimes known as the critical association concentration (CAC), is lower by several orders of magnitudes than typical surfactant CMC values. Thus, polymeric micelles are more stable towards dilution. From the pharmaceutical point of view, these amphiphilic carriers can solubilize more poorly-water soluble drugs within their hydrophobic core than most surfactant micelles. They can increase drug bioavailability and retention, since the drug is well protected from possible inactivation under the effect of their biological surroundings (Kwon *et al.*, 1996).

Cyclosporin A (CsA) is a highly effective immunosuppressive agent that is used for prevention of graft rejection following organ transplantation (Laupacis *et al.*, 1982). CsA is a neutral cyclic undecapeptide with a molecular weight of 1202 g/mol (Wenger, 1983), consisting of hydrophobic amino acids linked via 11 amide bonds, seven of which are *N*-methylated. Four intramolecular hydrogen bonds (Vine *et al.*, 1987) (Figure 3.1.) contribute to the rigidity of its skeleton. This chemical composition is responsible for the extremely low solubility of CsA in water (23 μg/ml at 20 °C). The oral administration of CsA is

complicated by the presence of several metabolizing enzymes: cytochrome P-450 3A4 (CYP3A4), the multi-drug transporter P-glycoprotein (PGP) in the small intestine, and hepatic CYP3A4 (Tjia *et al.*, 1991). These factors, together with the poor solubility of CsA in the aqueous fluids of the gastrointestinal tract, severely limit the absorption of CsA through the gastrointestinal mucosa. They account for the low bioavailability of the drug and significantly increase the risks of both acute and chronic rejection (Lindholm *et al.*, 1988). In view of the clinical importance of CsA, much effort has been directed towards designing oral formulations leading to acceptable bioavailability. A number of innovative drug delivery approaches including mixed micelles (Takada *et al.*, 1985), charged nanoparticles (El-Shabouri, 2002), liposomes (Al-Meshal *et al.*, 1998), lipids (Ueda *et al.*, 1983), surfactants (Chang *et al.*, 1996), microspheres (Kim *et al.*, 2002), and microemulsions (Ritschel *et al.*, 1990) have been investigated in order to improve the unfavorable absorption characteristics of CsA.

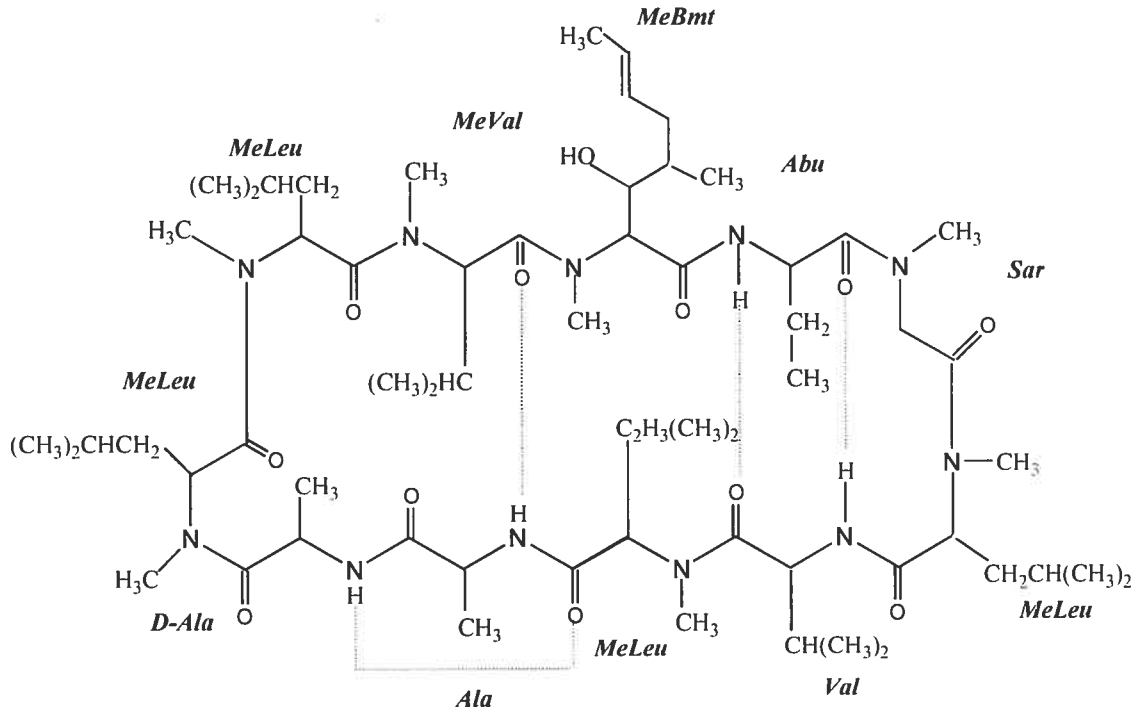

Figure 3.1. Chemical structure of cyclosporin-A showing 11 amino acids and 4 hydrogen bonds.

We set out to investigate the suitability of polymeric micelles as carriers in the oral delivery of CsA. This drug was selected, not only because of its clinical importance, but also since it is representative of a number of new, highly water-insoluble drugs in current development. The chemical composition and architecture of the amphiphilic polymers forming the polymeric micelle drug carriers was selected with care, taking into account the following requirements. The hydrophilic sections of the polymer should have high water solubility and be non-toxic, and ideally should enhance the bioavailability of the drug transported within the micelle. We selected dextrans, which are branched polysaccharides introduced in medicine in the early 1950s as non-toxic plasma substitutes (Clagett *et al.*, 1998). They are readily available in a range of molecular weights (Couch, 1965) and their chemical modification has been studied extensively (Moriyama *et al.*, 1996; Maksimenko *et al.*, 2001; Sayer *et al.*, 2002). In order to impart dextran with an amphiphilic character, we linked to its backbone a small number of polyethylene glycol *n*-alkyl ether (PEG-C_n; C_nEO_{10}) chains (Figure 3.2.). Nonionic surfactants such as polyethyleneglycol alkyl ethers are known to enhance drug absorption. In fact, the effect of PEG-C_n has been reported in several studies to be superior to those of fatty acid esters and sorbitan derivatives (Sakai *et al.*, 1986; Siegel *et al.*, 1985). However, several PEG-C_n surfactants suffer from adverse effects on mucosal integrity (Dimitrijevic *et al.*, 2000).

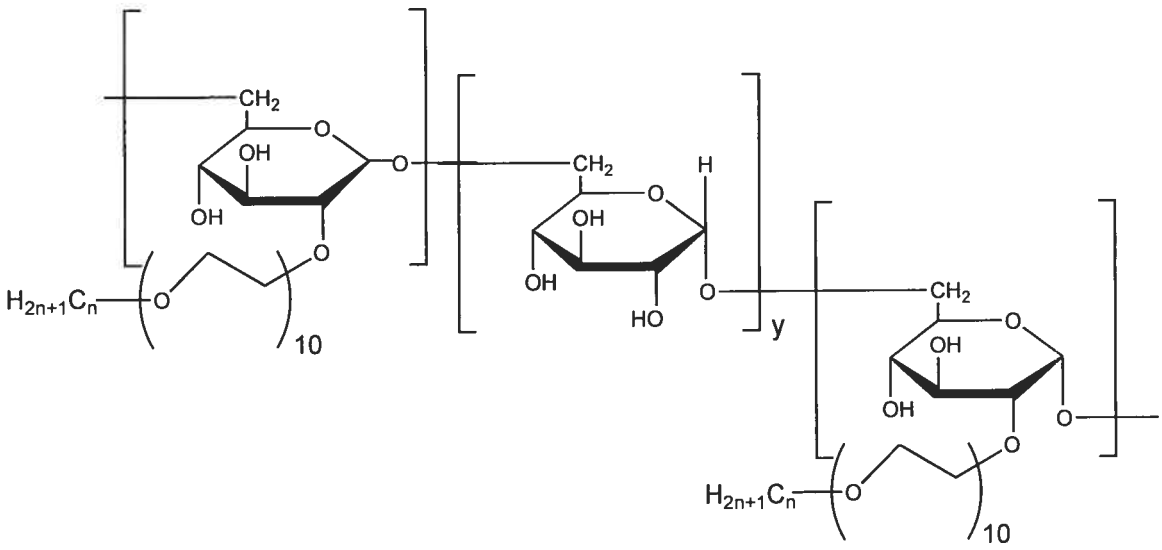

Figure 3.2. Chemical structure of DEX-*g*-PEG-C_n copolymer where n=16 and 18 for DEX-*g*-PEG-C_{16} and DEX-*g*-PEG-C_{18}, respectively.

The objectives of the work presented here were (1) to determine the physicochemical properties of the polymeric micelles formed in aqueous solutions of hydrophobically-modified dextrans (DEX-*g*-PEG-C_n); (2) to assess the solubility of CsA in DEX-*g*-PEG-C_n micelles; and (3) to evaluate the cytotoxicity of the polymeric micelles. The latter study was of particular importance in view of the known toxicity of the PEG-C_n surfactants when they are not linked to polymers. The effects of the polymer molecular weight, the level of hydrophobic substitution, and the chemical nature of the hydrophobic substituents on the properties of the polymeric micelles were examined using fluorescence spectroscopy, dynamic light scattering, CsA assay by high-performance liquid chromatography (HPLC) and MTT cytotoxicity assay.

3.4. Materials And Methods

3.4.1. Materials

Cyclosporin A (CsA), polyethyleneglycolcetyl ether (PEG-C_{16}; $C_{16}EO_{10}$; Brij 56), polyethyleneglycolstearyl ether (PEG-C_{18}; $C_{18}EO_{10}$; Brij 76), sodium chloride (NaCl), monobasic sodium phosphate (NaH_2PO_4), dibasic sodium phosphate (Na_2HPO_4), sodium dodecyl sulfate (S.D.S) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma–Aldrich (St. Louis, MO). Optical grade pyrene (99%), dichloromethane (DCM) and deuterated dimethyl sulfoxide (DMSO-d_6) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Dextran T10 (DEX10; M_w 10000 Da) and dextran T40 (DEX40; M_w 40000 Da) were supplied by Pharmacia Fine Chemicals

(Uppsala, Sweden). HPLC-grade acetonitrile (ACN) and water were obtained from Anachemia Science (Montreal, PQ, Canada). Ethanol (95%) was obtained from Commercial Alcohols Inc. (Brampton, ON, Canada). The Caco-2 cell line was purchased from American Type Culture Collection (ATCC, Rockville, MD) at passage 18. Dulbecco's modified Eagle medium (DMEM), penicillin–streptomycin (10000 U/ml penicillin G and 10000 μg/ml streptomycin), fetal bovine serum (FBS), 0.25% (w/v) trypsin – 1 mM EDTA·4Na (1×) and non-essential amino acids (NEAA) were supplied by Invitrogen Inc. (Burlington, ON, Canada).

3.4.2. Synthesis of DEX-*g*-PEG-C_n copolymers

Hydrophobically-modified DEX-*g*-PEG-C_n graft copolymers were synthesized following a procedure reported by our group (Cristea *et al.*, 2003). Briefly, the terminal hydroxyl group of PEG-C_n was tosylated in good yield using the joint action of the amines Et_3N and Me_3N HCl. Subsequently, the tosylated PEG-C_n was coupled to dextran of various molecular weights (10000 and 40000 Da) by a Williamson ether synthesis. The resulting DEX-*g*-PEG-C_n copolymers were purified by a dichloromethane soxhlet extraction to remove all free PEG-C_n. They were characterized by ^{1}H NMR spectroscopy of their solution in DMSO-d_6 using a Bruker ARX-400 400 MHz spectrometer (Milton, ON, Canada).

3.4.3. Critical association concentration (CAC) of DEX-*g*-PEG-C_n polymeric micelles

The CAC of the DEX-*g*-PEG-C_n copolymers were estimated by fluorescence spectroscopy using pyrene, a hydrophobic fluorescence probe that preferentially partitions into the hydrophobic core of the micelle. It undergoes changes in its photophysical properties as a result of the change in the micropolarity it experiences upon diffusion from bulk water (hydrophilic environment) into the micelle core (hydrophobic environment) (Dong *et al.*, 1984; Zhao *et al.*, 1990). Two methods exist for determining the CAC of polymeric micelles with pyrene fluorescence (Winnik *et al.*, 1998). The original method, proposed by Kalyanasundaram et al. (Kalyanasundaram *et al.*, 1977), takes advantage of the changes in the vibronic fine structure of the pyrene emission and monitors the changes in the ratio of the intensities I_1 and I_3 of the [0,0] and [0,2] bands, respectively. More recently, it has been suggested that a more accurate determination of the CAC can be obtained by monitoring the changes in the ratio of the pyrene excitation spectra intensities (Wilhelm *et al.*, 1991) at λ=333 nm for pyrene in water and λ=336 nm for pyrene in an hydrophobic medium. The latter method was used here and is exemplified in Figure 3.3. where we present excitation spectra of pyrene in water and in the presence of the non ionic surfactant PEG-C_{16} at a concentration higher than its CMC.

Samples for spectroscopic analysis were prepared as follows. Two-milliliter aqueous solutions of increasing polymer concentration ($0.04–5\times10^3$ mg/l) were equilibrated overnight with pyrene saturated water ([Py] ~$6–7\times10^{-7}$ M). Excitation spectra were monitored at λ_{em}=390 nm (excitation and emission bandpass: 2 nm). Fluorescence spectra

were recorded at 25 °C on a series 2 Aminco Bowman spectrofluorimeter (Spectronic Instruments Inc., Rochester, NY).

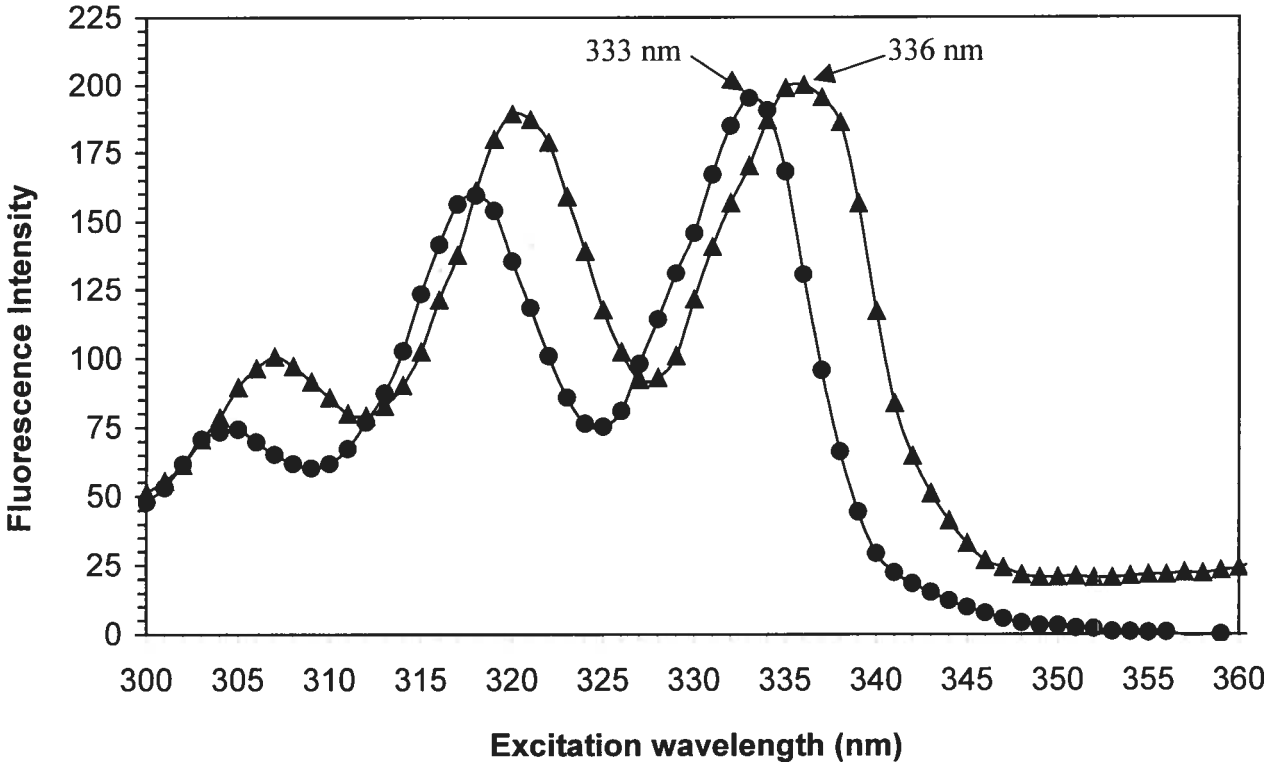

Figure 3.3. Excitation spectra of pyrene (2×10^{-7} M aqueous solution) monitored at λ_{em} 390 nm in absence (●) or presence (▲) of PEG-C_{16} at a concentration of 5×10^{3} mg/l.

3.4.4. Physical loading of CsA in DEX-*g*-PEG-C_n polymeric micelles

A dialysis method was employed to prepare CsA-loaded polymeric micelles. A DEX-*g*-PEG-C_n solution (5 mg/ml) in deionized water (Milli-Q water purification system, Millipore, Billerica, MA) and a CsA solution (5 mg/ml) in ethanol were prepared separately. Each solution was stirred 1 h at room temperature. Subsequently, different mixtures of polymer with varying CsA concentrations (2.5–40% *w/w*) were prepared by mixing the two solutions to a final volume of 4 ml.

The different mixtures were stirred for 2 h at room temperature. To remove free CsA and form CsA-loaded micelles, the mixtures were transferred into a pre-swollen semi-permeable Spectra/Por 1 dialysis membrane (molecular weight cutoff 6000–8000 g/mol, Spectrum Laboratories Inc., Laguna Hills, CA) and were dialyzed against distilled water for 48 h. During the first 2 h, the water was exchanged two times (every hour) and then six times during the following 46 h. After a total of 48 h of dialysis, each solution was filtered through a 0.22-μm pore-size nylon filter (Whatman Inc., Clifton, NJ) and the filtrate was freeze-dried in the absence of any lyoprotectant.

3.4.5. Micelle size measurement

The hydrodynamic diameter of CsA-free as well as CsA-loaded DEX-*g*-PEG-C_n polymeric micelles in aqueous solution was evaluated by dynamic laser light scattering (DLS) using a Malvern system (Autosizer 4700, Malvern Instruments Ltd, Malvern, UK) during 180 s at 25 °C, with a scattering angle of 90°. Samples were passed through 0.22-

μm pore-size filter before size measurement to remove dust particles. It was verified that the filtration step did not significantly influence the mean micelle size. The correlation decay functions were analysed by the cumulant method to determine the *Z*-average size. The constrained regularized CONTIN method was used to obtain the particle size distributions. The values in Table 3.2. represent the average particle diameter together with the polydispersity index obtained for the same sample. All measurements were performed in triplicate; the data presented are the mean±S.D.

3.4.6. HPLC analysis

The micelle-incorporated CsA was extracted from freeze-dried micelles using ACN. The resulting suspensions were sonicated for 10 min then agitated for 8 h. They were then filtered through 0.45-μm pore-size Gelman GHP Acrodisc filters (Waters, Milford, MA) and assayed by HPLC (Ugazio *et al.*, 2002) using an Agilent Technologies HP 1100 chromatography system with a quaternary pump, a UV-visible detector, a column thermostat and a HP Vectra computer (Agilent Technologies, Waldbronn, Germany) equipped with HP-Chemstation software. A symmetry octadecyl-silane C_{18} (5 μm, 250×4.6 mm i.d.) column and the corresponding guard column of similar characteristics (20×3.9 mm i.d.) (Waters) were used. The mobile phase consisted of ACN/water (80:20) with a flow rate of 1.2 ml/min. The column was thermostated at 70 °C. In all cases, the injection volume was 50 μl and the run time was 10 min. The CsA peak, monitored at 210 nm, appeared at a retention time of 6.5 min. A CsA calibration curve was prepared using standard solutions of concentrations ranging from 3.125 to 400 mg/l, with a first-order

correlation coefficient (r^2) greater than 0.99. Finally, the extent of drug loading (DL) was calculated using Equation (1):

$$DL(\%)=100(W_C/W_M) \quad \textbf{Equation (1)}$$

where W_C is the weight of CsA loaded in micelles calculated using the calibration curve, and W_M is the weight of CsA-loaded micelles before extraction with ACN.

3.4.7. Cell culture

The human colon adenocarcinoma cells, Caco-2, were grown as described previously (Hidalgo *et al.*, 1989). Briefly, Caco-2 cells were routinely maintained in DMEM with 4.5 g/l D-glucose, supplemented with 10% (*v/v*) heat-inactivated FBS, 1% (*v/v*) NEAA and 1% (*v/v*) penicillin–streptomycin antibiotics solution (100 U/ml penicillin G and 100 μg/ml streptomycin). Cells were allowed to grow in a monolayer culture in 75-cm^2 T-flasks in an incubator at 37 °C with controlled atmosphere containing 5% CO_2 and 90% relative humidity. Culture medium was changed every 48 h and cells were passaged at 80–90% confluency at a split ratio of 1:3 using 0.25% trypsin–1 mM EDTA.

3.4.8. Colorimetric MTT cytotoxicity assay

The cell viability in presence of dextran T10, PEG-C_{16} or DEX10-*g*-PEG-C_{16} (7 mol%) was evaluated using the MTT colorimetric assay. Caco-2 cells were seeded in triplicate in 96-well culture plates at a density of approximately 5×10^4 cells in 100 μl of cell culture medium per well. The cells were cultured at 37 °C in a humidified atmosphere of

5% CO_2 in air for 48 h. Subsequently, increasing concentrations of dextran T10, PEG-C_{16} or DEX10-*g*-PEG-C_{16} (0–10 g/l in culture medium) were added to the cells. Caco-2 cells were further incubated at 37 °C for 4 h. Thereafter, cell viability was determined by a MTT test according to the procedure described by Mosmann (Mosmann, 1983). The test is based on mitochondrial dehydrogenase cell activity as an indicator of cell viability. Ten microliters of 5 mg/ml MTT solution in phosphate-buffered saline (PBS; 75 mM NaCl, 53 mM Na_2HPO_4, 13 mM NaH_2PO_4, pH 7.2) were added to each well. After 4 h of additional incubation at 37 °C, 100 µl of 10% S.D.S in 0.01 N HCl solution were added to each well to stop the reaction and to ensure solubilization of formazan crystals. The plates were incubated overnight at 37 °C, after which the optical density values were measured at 570 nm using a multiwell-scanning spectrophotometer (PowerWave; Biotek Instruments, Winooski, VT).

3.5. Results And Discussion

3.5.1. Characterization of the modified dextrans

Various modified dextrans were prepared. They differed in three molecular aspects: (i) the molecular weight of dextran (10000 and 40000 Da or ca. 62 and 247 glucose units per chain, respectively); (ii) the level of grafting, i.e. the number of hydrophobic substituents linked to the chain; and (iii) the size of the hydrophobic group (hexadecyl or octadecyl). The association properties, summarized in Table 3.1., were gathered from fluorescence probe experiments that yield the critical association concentration, and from

DLS measurements that give the average size of the polymeric micelles (Table 3.2.). The ^{1}H-NMR spectroscopy data, summarized in Table 3.1., showed that DEX-g-PEG-C$_n$ copolymers with different molar content in PEG-C$_n$ (2–7 mol%) were synthesized. The degree of substitution was calculated as $I_{Me}\times 100/I_a$, where I_{Me} is the average integral of the signal due to the terminal methyl protons of the PEG-C$_n$ groups (~0.85 ppm) and I_a is the integral of the signal due to the anomeric protons of dextran (~4.7 ppm).

Table 3.1. Characteristics of DEX-*g*-PEG-C_n copolymers with various compositions.

Polymer composition	Grafted PEG-C_n [a] (mol %)	Average number of PEG-C_n units per dextran chain	Maximum CsA loading[b] (w/w%)	CAC	
				Polymer concentration[c] (mg/l)	PEG-C_n concentration (nmol/l)
PEG-C_{16}	100	-	17.5 ± 0.5	2.5	3600
PEG-C_{18}	100	-	13.1 ± 0.9	3.0	4200
Dextran T10	0	0	0.6 ± 0.1	-	-
DEX10-*g*-PEG-C_{16}	3.0	2	4.0 ± 0.1	7.5	23
DEX10-*g*-PEG-C_{16}	7.0	4	4.8 ± 0.4	6.5	49
DEX10-*g*-PEG-C_{18}	3.9	2	3.0 ± 0.2	12.5	50
Dextran T40	0	0	1.0 ± 0.02	-	-
DEX40-*g*-PEG-C_{16}	2.3	6	1.2 ± 0.1	110.0	65
DEX40-*g*-PEG-C_{16}	3.5	9	1.5 ± 0.1	18.0	16

[a] Determined by ^{1}H NMR measurement in DMSO-d_6 (Cristea *et al.*, 2003).

[b] Determined by HPLC analysis with UV detection at 210 nm.

[c] Determined by change in $I_{336\ nm}/I_{333\ nm}$ ratio of pyrene fluorescence with log polymer concentration at 25 °C.

Table 3.2. Size measurements of CsA-free and CsA-loaded polymeric micelles with different compositions, determined by DLS measurements of 5 mg/ml aqueous solutions at 25 °C with a scattering angle of 90°.

Polymer composition	Grafted PEG-C_n (mol %)	CsA-free polymeric micelles		CsA-loaded polymeric micelles	
		Mean diameter ± S.D. (nm)	Polydispersity ± S.D.	Mean diameter ± S.D. (nm)	Polydispersity ± S.D.
DEX10-*g*-PEG-C_{16}	3.0	18 ± 2	0.3 ± 0.08	20 ± 1	0.4 ± 0.01
DEX10-*g*-PEG-C_{16}	7.0	9 ± 0.3	0.2 ± 0.07	10 ± 0.3	0.3 ± 0.02
DEX10-*g*-PEG-C_{18}	3.9	21 ± 1	0.4 ± 0.04	22 ± 0.5	0.4 ± 0.03
DEX40-*g*-PEG-C_{16}	2.3	23 ± 1	0.3 ± 0.02	25 ± 1	0.5 ± 0.04
DEX40-*g*-PEG-C_{16}	3.5	30 ± 1	0.5 ± 0.03	35 ± 0.5	0.4 ± 0.03

3.5.2. Critical association concentration (CAC) of DEX-*g*-PEG-C_n micelles

A fluorescence assay described in detail in the experimental section was used to determine the polymer concentration at which micellization first takes place. The hydrophobic pyrene probe was added to polymer solutions of increasing concentration and pyrene excitation spectra were measured for all solutions. The excitation spectrum undergoes a small shift to longer wavelengths as the probe passes from a hydrophilic to a hydrophobic environment (Figure 3.3.). This shift is quantified in terms of the ratio, I_{336}/I_{333}, of the fluorescence intensities at 336 and 333 nm.

Plots of the I_{336}/I_{333} ratios versus the logarithm of the concentration of the aqueous solutions of DEX-*g*-PEG-C_n of varying compositions are shown in Figure 3.4., together with those of solutions of the surfactants PEG-C_{16} and PEG-C_{18}. Sigmoidal curves were obtained for all the DEX-*g*-PEG-C_n copolymers. The CAC value was determined for each polymer solution from the intersection of two straight lines (the horizontal line with an almost constant value of the ratio I_{336}/I_{333} and the vertical line with a steady increase in the ratio value). The estimated CAC values are presented in Table 3.1. The CAC values decrease with increasing molar content of PEG-C_n residues linked to the polymer backbone, for each hydrophobic group and each dextran molecular weight. The CAC values of DEX40-*g*-PEG-C_n are larger than those of the corresponding DEX10-*g*-PEG-C_n samples, when determined in terms of weight of polymer. It is more insightful to compare the CAC in terms of the alkyl group concentration (Figure 3.4B and Table 3.1.). These results are consistent with recent evidence suggesting that the increase in the length of a

hydrophobic residue at a given length of a hydrophilic polymer chain causes noticeable decrease in CAC value and increase in micelle stability (Nagarajan *et al.*, 1989).

3.5.3. Size of the DEX-*g*-PEG-C_n micelles

The hydrodynamic diameters of polymeric micelles, determined by DLS, ranged from 10 to 30 nm (Table 3.2.). In all cases the size distributions were unimodal, indicative of the absence of free polymer chains. We note that the micelles formed by DEX40-*g*-PEG-C_n are larger than those formed by DEX10-*g*-PEG-C_n, independently of the size of the hydrophobic substituent and of the level of modification. This observation can be taken as an indication of the steric hindrances induced by carbohydrate chains, which are expected to take place over a larger volume for the polymer of higher molecular weight (Maksimenko *et al.*, 2001; Baldwin *et al.*, 1988). It has been shown that the uptake of particles within the intestine and the extent of drug absorption increase with decreasing particle size and increasing specific surface area (Florence *et al.*, 2001). Thus, the small size exhibited by all the polymeric micelles studied here show a favorable trend towards oral drug delivery.

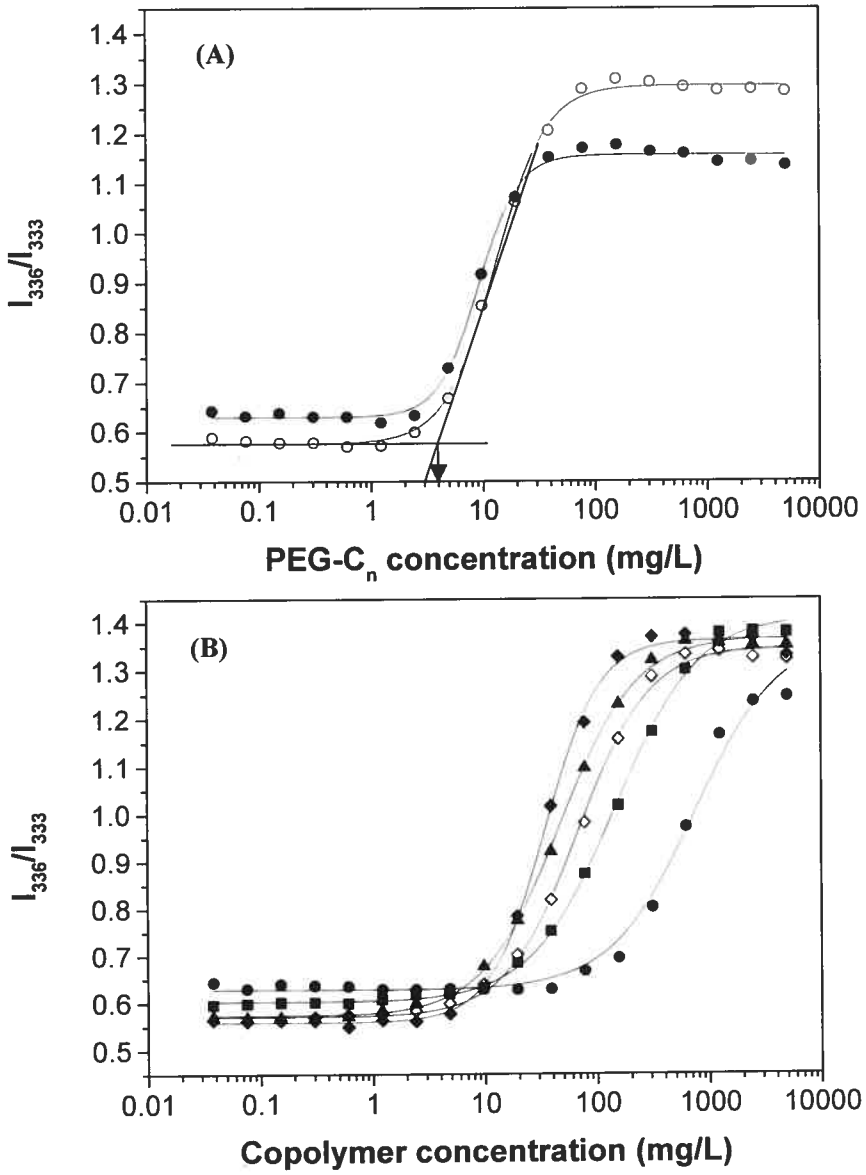

Figure 3.4. Changes in the $I_{336\ nm}/I_{333\ nm}$ ratio of pyrene fluorescence intensity with the different concentrations (0.04 – $5x10^3$ mg/l) of (A) (●) PEG-C_{16} and (o) PEG-C_{18}; and (B) (♦) DEX10-*g*-PEG-C_{16} (3 mol%); (▲) DEX10-*g*-PEG-C_{16} (7 mol%); (●) DEX40-*g*-PEG-C_{16} (2.3 mol %); (■) DEX40-*g*-PEG-C_{16} (3.5 mol%) and (◊) DEX10-*g*-PEG-C_{18} (3.9 mol%) copolymers. Each value is the mean of two independent measurements.

3.5.4. Characterization of the CsA-loaded polymeric micelles

Cyclosporin A was incorporated into the polymeric micelles by a dialysis method which involved treatment of an aqueous polymer solution with a solution of CsA in ethanol, followed by extensive dialysis of the mixed solution against water. The amount of CsA released upon dissolution of the CsA-loaded polymeric micelles was then determined by an HPLC assay and plotted against the CsA concentration added initially for each preparation. Both free and micelle-entrapped CsA were measured with this assay. The amount of free CsA is expected to be low since the undissolved fraction of the drug was removed by filtration after the dialysis procedure. We evaluated first the ability of the surfactant micelles to solubilize CsA (Figure 3.5.). Both PEG-C_{16} and PEG-C_{18} micelles were able to incorporate relatively high levels of CsA, 17.5% (*w/w*) and 13% (*w/w*), respectively.

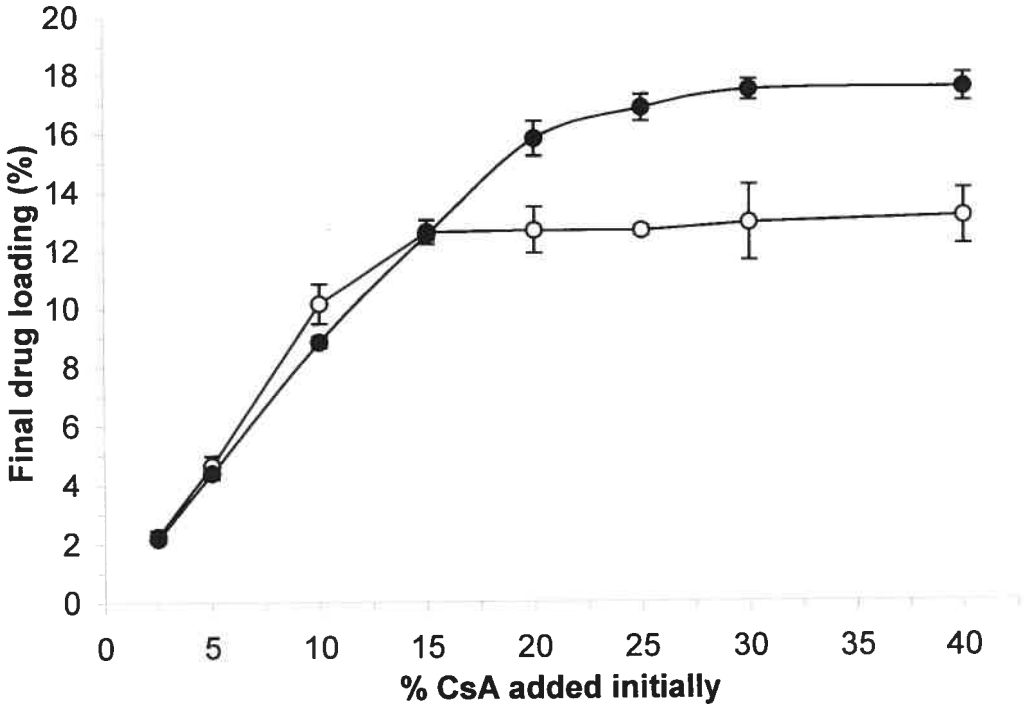

Figure 3.5. CsA loading (*w/w* %) in micelles of PEG-C_{16} (●) and PEG-C_{18} (o) at 2.5 – 40 (*w/w* %) of initially added CsA. Mean ± S.D. (n = 3).

Next, we assessed the incorporation of CsA within polymeric micelles. In the case of polymers prepared with DEX10, the maximum amount of CsA loaded within the micelles was 4 and 4.8% (*w/w*), for DEX10-*g*-PEG-C_{16} (3 mol%) and DEX10-*g*-PEG-C_{16} (7 mol%), respectively, while DEX10-*g*-PEG-C_{18} (3.9 mol%) resulted in a maximum CsA loading of 3% (Figure 3.6.A.). The micelles formed by the polymers of higher molecular weights were not as effective CsA carriers (Figure 3.6.B.). Nonetheless, in all cases the amount of incorporated CsA was larger in the case of polymeric micelles than in the case of unmodified dextrans, which have a very low affinity for CsA (Figure 3.6.). The entrapment efficiency of polymeric micelles remained low (<35%) and further work will be aimed at improving the drug loading procedure.

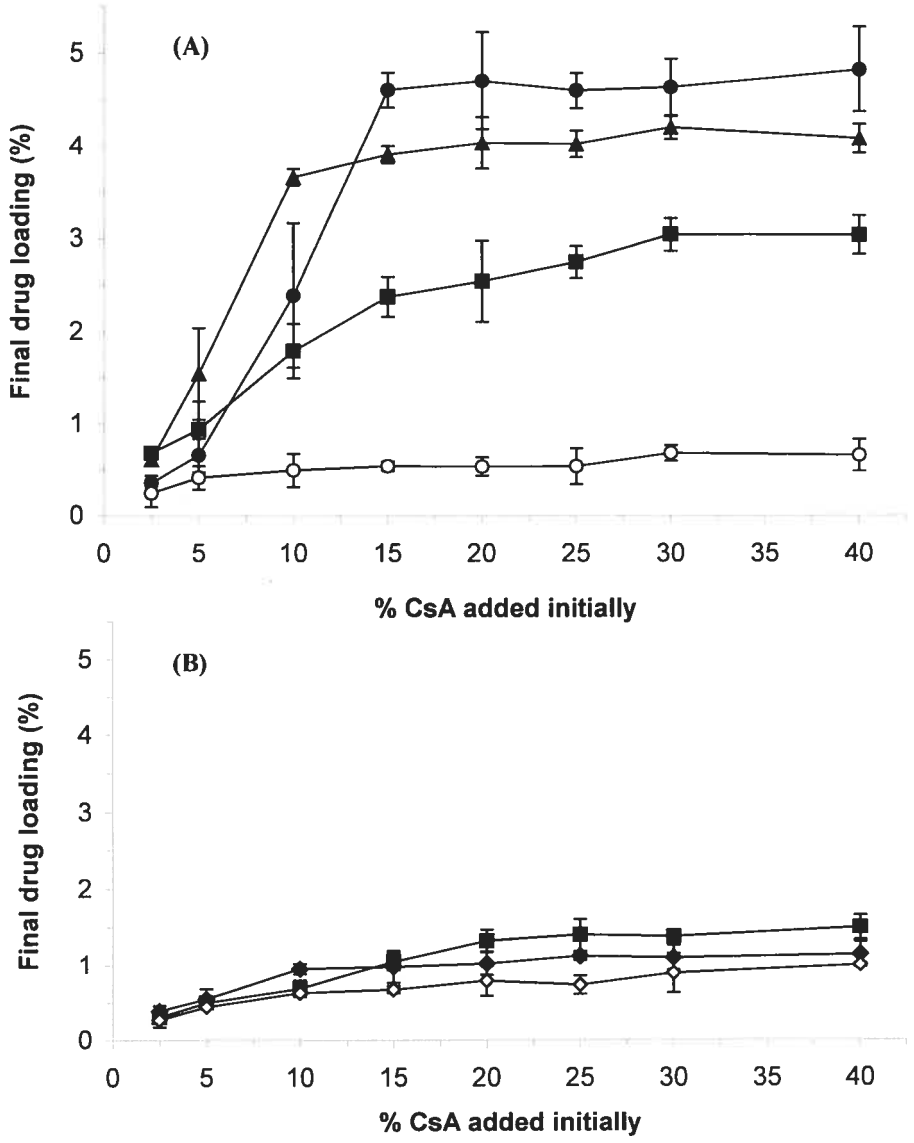

Figure 3.6. CsA loading (*w/w* %) in micelles of (A) (▲) DEX10-*g*-PEG-C_{16} (3 mol%); (●) DEX10-*g*-PEG-C_{16} (7 mol%) and (■) DEX10-*g*-PEG-C_{18} (3.9 mol%) copolymers, or micelles of (B) (♦) DEX40-*g*-PEG-C_{16} (2.3 mol%) and (■) DEX40-*g*-PEG-C_{16} (3.5 mol%) copolymers at 2.5 – 40 (*w/w* %) of initially added CsA. For comparison, CsA was incorporated in (A) unmodified dextran T10 (o) or (B) unmodified dextran T40 (◊) polymer. Mean ± S.D. (*n* = 3).

Several trends are apparent if one compares the highest CsA loading percentages determined for the various polymers (Table 3.1.). The percentage of CsA loading increases with increasing number of PEG-C_n units grafted per dextran chain. For a constant number of PEG-C_n units (~2 units/dextran chain), the CsA loading achieved with DEX10-*g*-PEG-C_{18} (3.9 mol%) is lower than that determined for DEX10-*g*-PEG-C_{16} (3 mol%). These results are consistent with the fact that PEG-C_{16} micelles are more effective in solubilizing CsA than PEG-C_{18} micelles, 17.5 and 13%, respectively (Figure 3.5.). They may be taken as an indication that the micropolarity of the hexadecyl-PEG chains core might present a better solubilizing core than that offered by the octadecyl-PEG moieties.

DLS measurements carried out on CsA-loaded micelles confirmed that the integrity of the micelles was preserved during the loading process and that the size of the micelles was not altered substantially, although a slight increase in micelle diameter was noted in all cases (Table 3.2.).

3.5.5. In vitro cytotoxicity study

The cytotoxicity of DEX10-*g*-PEG-C_{16} (7 mol%), DEX10, and PEG-C_{16} was examined using a MTT assay performed with Caco-2 cells. The dose-dependent viability of Caco-2 cells treated with the various materials for 4 h is presented in Figure 3.7. As anticipated, free PEG-C_{16} surfactant inhibited cell growth even at concentrations below 1 g/l. Such deleterious effect is consistent with the propensity of polyethyleneglycol alkyl ether surfactants to affect membrane integrity (Dimitrijevic *et al.*, 2000). Also, the polysaccharide DEX10 showed no toxicity up to a concentration of 10 g/l, confirming the

reported inertness of dextran. Turning now to the effect of DEX10-*g*-PEG-C_{16} (7 mol%), we determined that it exhibited no significant toxicity towards Caco-2 cells, up to concentrations of 10 g/l or concentration with PEG-C_{16} content (0.05 g/l) equivalent to that showing cytotoxic effect (80% cell viability) when present free in direct contact with the cell. This important result indicates that by grafting the PEG-C_n residues on dextran, we succeeded in minimizing their toxic effect on cells. The non-toxic hydrophilic dextran chains forming the polymeric micelle outer shell may be in contact with the cells and effectively insulate them from the surfactant residues assembled in the inner core.

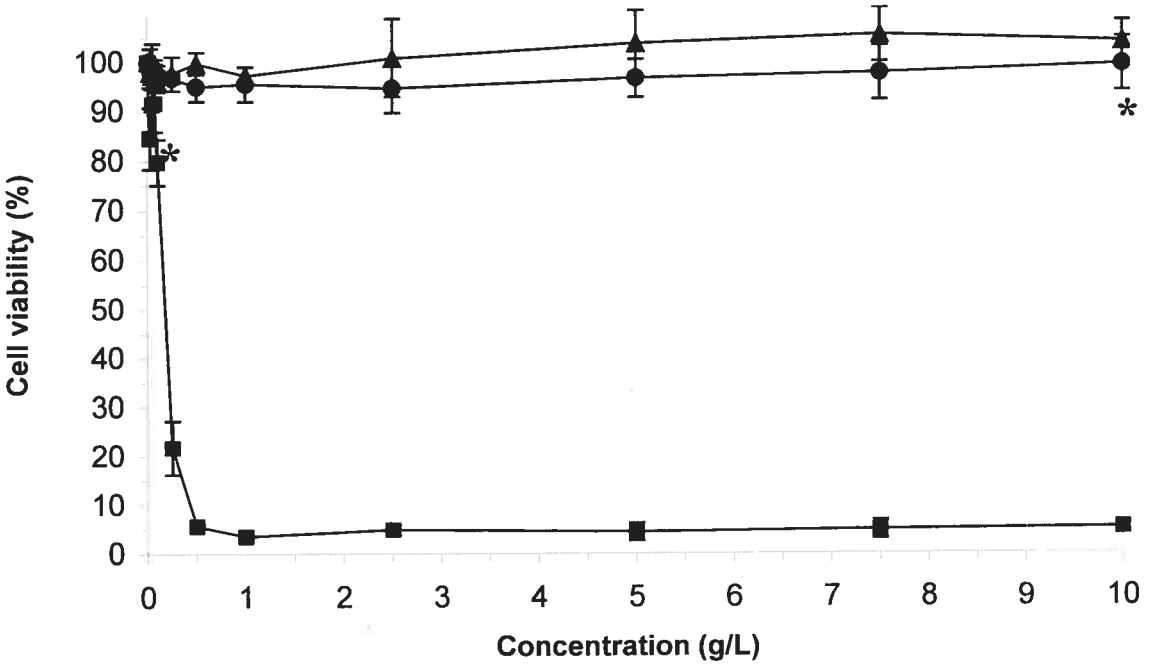

Figure 3.7. Effect of unmodified dextran T10 (▲), PEG-C_{16} (■) and DEX10-*g*-PEG-C_{16} (7 mol%) (●) concentration (0 – 10 g/l) on Caco-2 cell viability measured by MTT assay following 4 h incubation at 37 °C/ 5% CO_2. Equivalent concentrations of free PEG-C_{16} and PEG-C_{16} grafted to dextran T10 backbone are indicated in the figure by stars. Mean ± S.D. (n = 3).

3.6. CONCLUSION

In the present study, the solubilization potential of different DEX-*g*-PEG-C_n copolymers to poorly-water soluble drugs has been studied. We showed that the ability of hydrophilic, non-toxic dextran polymers to encapsulate lipophilic drugs, such as cyclosporin A, can be increased by grafting hydrophobic PEG-C_n domains on the dextran main chain. In aqueous solution, polymeric micelles are formed with low CAC values and relatively small micelle mean diameter. On the cellular level, they presented no significant cytotoxicity. Therefore, this new macromolecular system exhibits promising characteristics for the development of a novel polymeric drug carrier for the oral delivery of poorly water-soluble drugs.

3.7. ACKNOWLEDGEMENTS

This work was financially supported by the Natural Sciences and Engineering Research Council of Canada under its strategic grants program. M.F.F. acknowledges a scholarship from the Rx&D Health Research Foundation. We would like to thank Dr. Sébastien Gouin for his help in development of earlier versions of copolymer synthesis procedure.

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CHAPTER FOUR

RESEARCH PAPER

SOLUBILIZATION OF POORLY WATER SOLUBLE DRUGS IN MICELLES OF HYDROPHOBICALLY MODIFIED HYDROXYPROPYLCELLULOSE COPOLYMERS

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4.1. ABSTRACT

The main objective of this study is to exploit the solubilizing potential of hydroxypropylcellulose-*g*-polyoxyethylene alkyl ether (HPC-*g*-$(POE)_y$-C_n) polymeric micelles towards poorly water soluble drugs in order to improve their oral bioavailability. Hydrophobically modified HPC graft copolymers of various compositions were synthesized by attaching hexadecyl or octadecyl residues to the hydrophilic HPC backbone via short POE linkers of different lengths. The onset of micellization was estimated by fluorescence spectroscopy. The hydrodynamic diameter of different HPC-*g*-$(POE)_y$-C_n micelles was evaluated by dynamic light scattering (DLS). Cyclosporin A (CsA), a poorly water soluble immunosuppressant, was selected as model drug. CsA-loaded HPC-*g*-$(POE)_y$-C_n micelles were prepared by a dialysis procedure and the amount of CsA incorporated in the micelles was assayed by high-performance liquid chromatography. Following 24-h incubation with human colon adenocarcinoma, Caco-2 cells, the cytotoxicity of various HPC-*g*-$(POE)_y$-C_n copolymers was evaluated using the MTT colorimetric assay and compared to those of unmodified HPC and free $(POE)_y$-C_n. In aqueous solution, different HPC-*g*-$(POE)_y$-C_n copolymers formed polymeric micelles of low critical association concentrations (CAC) and micelle mean diameters ranging from 78 to 90 nm. CsA loading into HPC-*g*-$(POE)_y$-C_n polymeric micelles was significantly larger than in unmodified HPC. It increased with increasing number of $(POE)_y$-C_n units grafted per HPC chain. On the cellular level, unmodified HPC showed no cytotoxicity, while free $(POE)_y$-C_n molecules inhibited cell growth. Most importantly, the study revealed that HPC-*g*-$(POE)_y$-C_n exhibited no significant cytotoxic effect.

4.2. AUTHOR KEYWORDS

Polymeric micelles; Cyclosporin A; Hydroxypropylcellulose (HPC); Solubilization; Cytotoxicity.

4.3. INTRODUCTION

Oral drug delivery continues to be the preferred gateway of a drug into the bloodstream, especially when repeated or routine administration is necessary (Lavelle *et al.*, 1995). However, for effective delivery *via* the oral route, a therapeutic agent must first dissolve in the gastrointestinal lumen (Horter *et al.*, 2001; Charman *et al.*, 1991). This represents a major challenge in the field of pharmaceutical drug formulation, namely the design of an oral dosage form for highly lipophilic drugs, that would enhance their notoriously poor bioavailability (Florence *et al.*, 1993). Among various strategies investigated for oral delivery of poorly water soluble drugs, such as nanosuspensions (Muller *et al.*, 2001), microemulsions (Itoh *et al.*, 2002), lipids (Gershanik *et al.*, 2000) and liposomes (Minato *et al.*, 2003), the use of polymers has gained much attention because of their high diversity, biocompatibility, biodegradability and the multiple functional groups they display for the conjugation of various pilot molecules (Andrianov *et al.*, 1998). Among the different polymer-based drug delivery systems, "polymeric micelles" represent a promising delivery vehicle for poorly water soluble pharmaceutical active ingredients (Kataoka *et al.*, 2001; Trubetskoy, 1999). Polymeric micelles form spontaneously when amphiphilic polymers, containing both hydrophilic and hydrophobic fragments, are dissolved in water. They consist of a hydrophobic core created upon assembly of the

hydrophobic residues stabilized by a corona of highly hydrated hydrophilic polymeric chains (Jones *et al.*, 1999).

A review of past achievements readily convinces one that, in the case of injectable drug formulations, polymeric micelles are highly effective drug delivery vehicles (Zhang *et al.*, 1997; Mizumura *et al.*, 2002). They have been largely ignored, however, in oral drug delivery formulations. We present here a study of polymeric micelles as drug carriers capable of solubilizing high levels of lipophilic drugs, so as to improve their absorption from the gastrointestinal tract and, consequently, their bioavailability following oral administration.

Cyclosporin A (CsA), a highly lipophilic undecapeptide (Vine *et al.*, 1987), was selected as model drug. Its water solubility, 23 μg/ml at 20 °C, is extremely low. CsA is administered as immunosuppressant to prevent allograft rejection following various organ transplantations (Hamwi *et al.*, 2000). Its oral administration has always been complicated due to the presence of the metabolizing enzyme cytochrome P-450 and the multidrug transporter P-glycoprotein (P-gp) in the small intestine (Wacher *et al.*, 1998).

We set out to investigate the suitability of polymeric micelles as carriers in the oral delivery of CsA. Hydroxypropylcellulose (HPC), a non-ionic water-soluble polymer, was selected to form the hydrophilic shell of the micelles. HPC is widely used as an excipient in oral solid dosage forms, in which it acts as a disintegrant (Machida *et al.*, 1974), and as a binder in granulation (Skinner *et al.*, 1999). It is essentially a non-toxic and non irritant polysaccharide (Final report on the safety assessment of hydroxypropylcellulose, 1986).

Our strategy is (1) to prepare hydrophobically modified hydroxypropylcelluloses, (HPC-*g*-(POE)$_y$-C$_n$) (see Figure 4.1.) by attachment of hydrophobic cetyl (C_{16}) or octadecyl (C_{18}) groups to hydrophilic HPC via a short polyoxyethylene (POE) linker of varying length; (2) to monitor the formation and characteristics of HPC-*g*-(POE)$_y$-C$_n$ micelles in aqueous environment; (3) to exploit the solubilizing power of HPC-*g*-(POE)$_y$-C$_n$ polymeric micelles towards CsA; and (4) to evaluate the cytotoxicity of the polymeric micelles towards epithelial intestinal cells. This approach will allow us to create nanosized entities, which entrap CsA in their hydrophobic core while forming a stable aqueous suspension via the steric stabilization promoted by hydrated HPC chains.

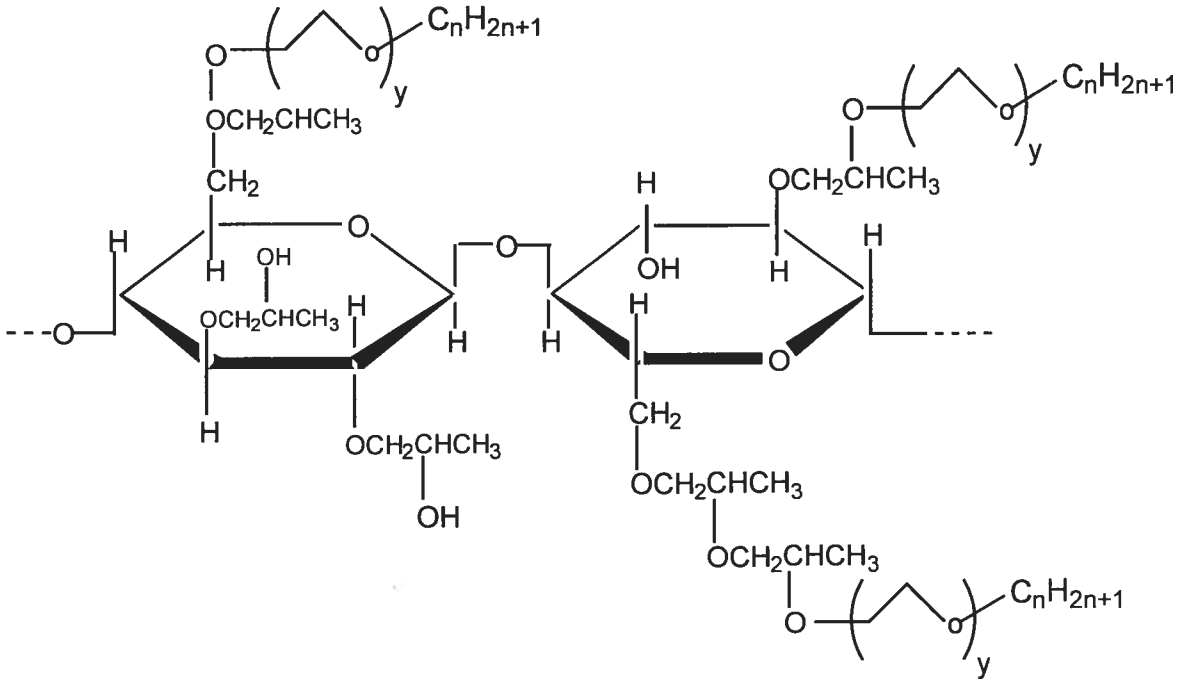

Figure 4.1. Chemical structure of HPC-*g*-$(POE)_y$-C_n copolymer where n = 16 and 18 for $(POE)_y$-C_{16} and $(POE)_y$-C_{18}, respectively, and y = 10 and 20 for $(POE)_{10}$-C_n and $(POE)_{20}$-C_n, respectively.

4.4. MATERIALS AND METHODS

4.4.1. Materials

CsA, polyoxyethylene (20) cetyl ether [$(POE)_{20}$-C_{16}; $C_{16}H_{33}(OCH_2CH_2)_{20}OH$; Brij 58®], polyoxyethylene (20) stearyl ether [$(POE)_{20}$-C_{18}; $C_{18}H_{37}(OCH_2CH_2)_{20}OH$; Brij 78®], polyoxyethylene (10) cetyl ether [$(POE)_{10}$-C_{16}; $C_{16}H_{33}(OCH_2CH_2)_{10}OH$; Brij 56®], sodium chloride (NaCl), monobasic sodium phosphate (NaH_2PO_4), dibasic sodium phosphate (Na_2HPO_4), sodium dodecyl sulphate (S.D.S), HPC (M_W 80,000 Da, molar substitution level (MS): 3.7, where MS is defined as the average number of alkylene oxide per anhydroglucose unit (Tezuka *et al.*, 1990; Wirick *et al.*, 1970)) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma-Aldrich. Deuterated dimethyl sulfoxide (DMSO-d_6), triethylamine (Et_3N) and trimethylamine hydrochloride (Me_3N·HCl) were purchased from Aldrich Chemicals. HPLC-grade acetonitrile (ACN) and water were obtained from Anachemia Science. Ethanol (95%) was obtained from Commercial Alcohols (Brampton, ON, Canada). The Caco-2 cell line was purchased from American Type Culture Collection (ATCC) at passage 18. Dulbecco's modified Eagle medium (DMEM), penicillin–streptomycin (10,000 U/ml penicillin G and 10,000 μg/ml streptomycin), fetal bovine serum (FBS), 0.25% (w/v) trypsin–1 mM EDTA·4Na and non-essential amino acids (NEAA) were supplied from Invitrogen.

4.4.2. Synthesis of HPC-*g*-$(POE)_y$-C_n copolymers

Various hydrophobically modified HPC-*g*-$(POE)_y$-C_n graft copolymers were synthesized as described elsewhere (Piredda *et al.*, 2003). Briefly, the terminal hydroxyl group of POE was activated via tosylation. Subsequently, the tosylated $(POE)_y$-C_n was coupled to HPC by a Williamson ether synthesis. The resulting HPC-*g*-$(POE)_y$-C_n copolymers were purified by a soxhlet extraction with *n*-hexane to remove all free $(POE)_y$-C_n residues. The level of $(POE)_y$-C_n grafting was determined by ^{1}H-NMR spectroscopy in DMSO-d_6 using a Bruker ARX-400 400 MHz spectrometer.

4.4.3. Critical association concentration of the HPC-*g*-$(POE)_y$-C_n polymeric micelles

The critical association concentration (CAC) of the HPC-*g*-$(POE)_y$-C_n copolymers was estimated by fluorescence spectroscopy using pyrene, a hydrophobic fluorescence probe that preferentially partitions into the hydrophobic core of the micelle. It undergoes changes in its photophysical properties as a result of the change in the micropolarity it experiences upon diffusion from bulk water (hydrophilic environment) into the micelle core (hydrophobic environment) (Zhao *et al.*, 1990; Dong *et al.*, 1984). An estimation of the CAC value was obtained by monitoring the changes in the ratio of the pyrene excitation spectra intensities (Wilhelm *et al.*, 1991) at λ =333 nm (I_{333}) for pyrene in water and λ =336 nm (I_{336}) for pyrene in the hydrophobic medium within the micelle core. The method used is exemplified in Figure 4.2. where we present (A) a series of excitation spectra of pyrene in water and in the presence of increasing concentrations of the non ionic surfactant $(POE)_{20}$-C_{18} and (B) traces of the concentration dependence of the ratio I_{336}/I_{333} in solutions

of $(POE)_{10}$-C_{16}, $(POE)_{20}$-C_{16} and $(POE)_{20}$-C_{18}. The critical micelle concentration (CMC) values are taken as the surfactant concentration corresponding to the onset of the increase in the ratio I_{336}/I_{333}. The CAC value was determined for each polymer solution from the intersection of two straight lines (the horizontal line with an almost constant value of the ratio I_{336}/I_{333} and a line approximating the steep upward section of the sigmoidal curve (see Figure 4.2.B.).

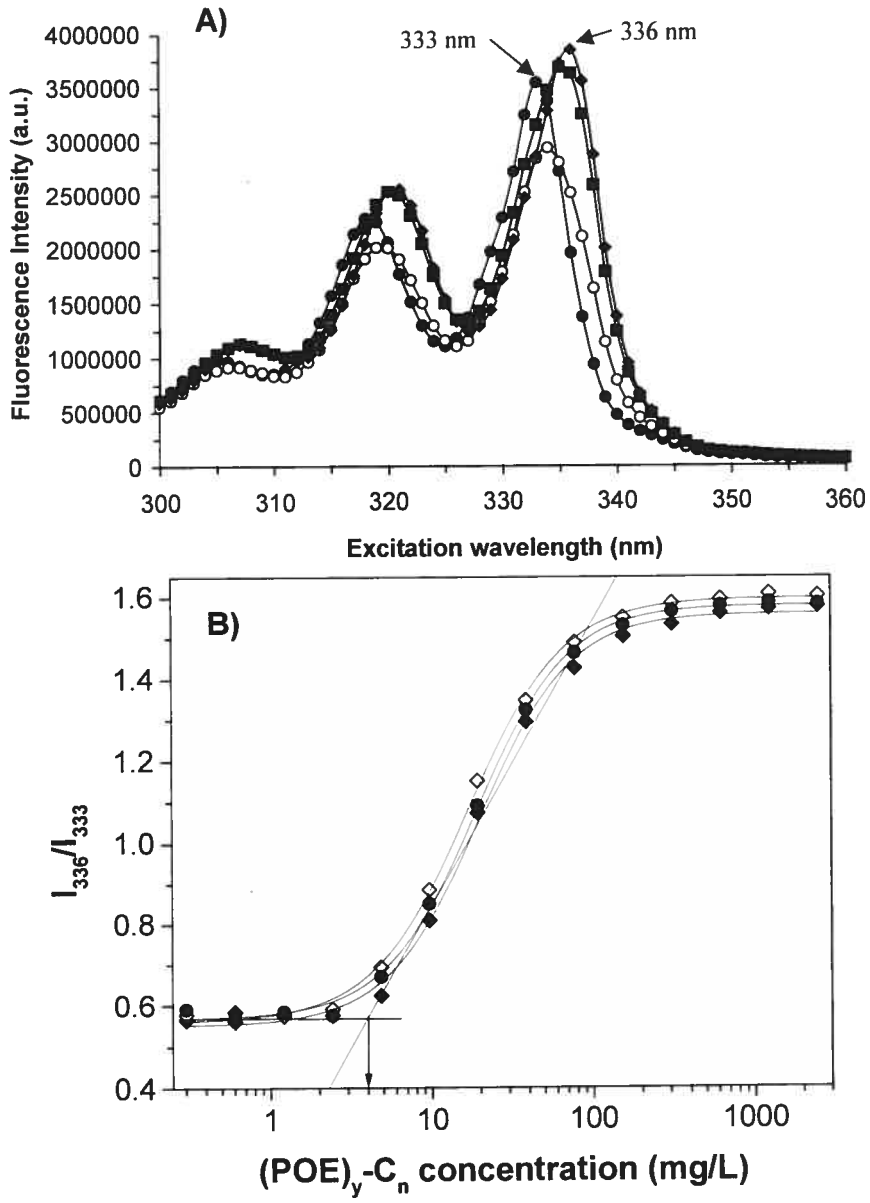

Figure 4.2. **(A)** Excitation spectra of pyrene-saturated aqueous solution monitored at λ_{em} 390 nm in absence (●) or presence of increasing concentrations of (o) 10 mg/l; (■) 20 mg/l and (♦) 40 mg/l $(POE)_{20}$-C_{18}. **(B)** Changes in the $I_{336\ nm}/I_{333\ nm}$ ratio of pyrene fluorescence intensity as a function of concentration (0.3 – 2.5 x 10^3 mg/l) for (●) $(POE)_{10}$-C_{16}, (♦) $(POE)_{20}$-C_{16} and (◊) $(POE)_{20}$-C_{18}.

Samples for spectroscopic analysis were prepared as follows: A pyrene-saturated solution in deionized water was prepared by stirring overnight a suspension of pyrene in water, followed by filtration to remove excess undissolved pyrene microcrystals. Polymer or surfactant stock solutions (10 g/l) were prepared in pyrene-saturated water. They were left to equilibrate under agitation over 24 h away from light. Subsequently, the stock solutions were diluted with pyrene-saturated water to obtain solutions of varying polymer concentrations (10^{-3}–10 g/l), which were further equilibrated under agitation for 24 h. Excitation spectra were monitored at λ_{em}=390 nm (excitation and emission slits were set at 0.5 and 1.0 mm, respectively). Steady-state fluorescence spectra were measured at 25 °C with a Fluorolog Tau-3 spectrometer (Jobin-Yvon Horiba) equipped with a GRAMS/32 (Galactic Ind.) data analysis system.

4.4.4. Physical loading of CsA in HPC-*g*-(POE)$_y$-C$_n$ polymeric micelles

A dialysis method was employed to prepare CsA-loaded polymeric micelles. A HPC-*g*-(POE)$_y$-C$_n$ solution (5 mg/ml) in deionized water (Milli-Q water purification system) and a CsA solution (5 mg/ml) in ethanol were prepared separately. Each solution was stirred 1 h at room temperature. Subsequently, different mixtures of polymer with varying CsA concentrations (2.5–40% *w/w*) were prepared by mixing the two solutions to a final volume of 4 ml, keeping the polymer concentration constant (2.5 mg/ml).

The different mixtures were stirred 2 h at room temperature. To remove free CsA and form CsA-loaded micelles, the mixtures were transferred into pre-swollen semi-permeable Spectra/Por® 1 dialysis membranes (molecular weight cutoff 6000–8000 g/mol,

Spectrum Laboratories) and were dialysed against distilled water for 48 h. During the first 2 h, the water was exchanged two times (every hour) and then six times during the following 46 h. After a total of 48 h of dialysis, each solution was filtered through a 0.22-μm pore-size nylon filter and the filtrate was freeze-dried.

4.4.5. Micelle size measurement

The hydrodynamic diameter of freeze-dried CsA-free and CsA-loaded HPC-based polymeric micelles in aqueous solution (5 mg/ml) was evaluated by dynamic laser light scattering (DLS) at 25 °C using a Brookhaven system with an Uniphase μBlue laser at wavelength of 532 nm and a scattering angle of 90°. All measurements were performed in triplicate; the data presented are the mean±S.D.

4.4.6. HPLC analysis

The micelle-loaded CsA was extracted from freeze-dried micelles using acetonitrile (ACN). The resulting suspensions were sonicated in an ultrasonic bath for 10 min, then agitated for 8 h. They were then filtered through 0.45-μm pore-size Gelman GHP Acrodisc filters and assayed by high performance liquid chromatography (HPLC) (Ugazio *et al.*, 2002) using an Agilent Technnologies HP 1100 chromatography system with a quaternary pump, a UV-visible detector, a column thermostat and a HP Vectra computer equipped with HP-Chemstation software. A symmetry® octadecyl-silane C_{18} (5 μm, 250×4.6 mm I.D.) column and the corresponding guard column of similar characteristics (20×3.9 mm I.D.) were used. The mobile phase consisted of ACN/water (80:20) with a flow rate of 1.2

ml/min. The column was thermostated at 70 °C. In all cases, the injection volume was 50 µl and the run time was 10 min. The CsA peak, monitored at 210 nm, appeared at a retention time of 6.5 min. A CsA calibration curve was prepared using standard solutions of concentrations ranging from 3.125 to 400 mg/l, with a first order correlation coefficient (r^2) greater than 0.99. Finally, the extent of drug loading (DL) was calculated using Equation (1):

$$DL(\%)=100(W_C/W_M) \quad \text{Equation (1)}$$

where W_C is the weight of CsA loaded in micelles calculated using the calibration curve and W_M is the weight of CsA-loaded micelles before extraction with ACN. It should be noted that the minimum amount of CsA detectable by this assay is 0.05% *w/w*.

4.4.7. Cell culture

The human colon adenocarcinoma cells, Caco-2, were grown as described previously (Hidalgo *et al.*, 1989). Briefly, Caco-2 cells were routinely maintained in DMEM with 4.5 g/l D-glucose, supplemented with 10% (*v/v*) heat-inactivated FBS, 1% (*v/v*) NEAA and 1% (*v/v*) penicillin–streptomycin antibiotics solution (100 U/ml penicillin G and 100 µg/ml streptomycin). Cells were allowed to grow in a monolayer culture in 75 cm^2 T-flasks in an incubator at 37 °C with controlled atmosphere containing 5% CO_2 and 90% relative humidity. Culture medium was changed every 48 h and cells were passaged at 80–90% confluency at a split ratio of 1:3 using 0.25% trypsin–1 mM EDTA.

4.4.8. Colorimetric MTT cytotoxicity assay

The cell viability in presence of unmodified HPC, free $(POE)_{20}$-C_{16}, $(POE)_{20}$-C_{18} and $(POE)_{10}$-C_{16} surfactants, or various HPC-*g*-$(POE)_y$-C_n copolymers was evaluated using the MTT colorimetric assay. Caco-2 cells were seeded in triplicate in 96-well culture plates at a density of approximately 5×10^4 cells in 100 µl of cell culture medium per well. The cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 in air for 48 h. Subsequently, increasing concentrations of unmodified HPC, free $(POE)_y$-C_n residues or various HPC-*g*-$(POE)_y$-C_n copolymers (0–10 g/l in culture medium) were added to the cells. Caco-2 cells were further incubated at 37 °C for 24 h. Thereafter, cell viability was determined by a MTT test according to the procedure described by Mosmann (Mosmann, 1983). The test is based on mitochondrial dehydrogenase cell activity as an indicator of cell viability. Ten microliters of 5 mg/ml MTT solution in phosphate-buffered saline (PBS; 75 mM NaCl, 53 mM Na_2HPO_4, 13 mM NaH_2PO_4, pH 7.2) were added to each well. After 4 h of additional incubation at 37 °C, 100 µl of 10% S.D.S in 0.01 N HCl solution were added to each well to stop the reaction and to ensure solubilization of formazan crystals. The plates were incubated overnight at 37 °C, after which the optical density values were measured at 570 nm using a multiwell-scanning spectrophotometer (PowerWave; Biotek Instruments).

4.5. RESULTS AND DISCUSSION

4.5.1. Characterization of the modified HPC copolymers

Various hydrophobically modified HPC copolymers were synthesized (Figure 4.1. and Table 4.1.). They differed in three molecular aspects: (i) the level of grafting, *i.e.* the number of $(POE)_y$-C_n substituents linked to the HPC backbone; (ii) the size of the $(POE)_y$ moiety; and (iii) the size of the hydrophobic alkyl group (C_n; hexadecyl group or octadecyl group). They were purified thoroughly to ensure complete removal of unreacted $(POE)_y$-C_n and residual solvent. Analysis by GPC confirmed that the molecular weight of HPC was not affected significantly by the chemical modification (data not shown). The degree of grafting was determined by ^{1}H-NMR spectroscopy, comparing the relative intensity of signals due to the POE and C_n protons to the intensity of signals due to protons linked to the polysaccharide backbone (Piredda *et al.*, 2003). The association properties of the amphiphilic polymers in water, summarized in Table 4.2., were gathered from fluorescence probe experiments, which yield the CAC, and from DLS measurements, which give the average size of the polymeric micelles, as discussed in detail in the following sections.

Table 4.1. Molecular and physicochemical characteristics of $(POE)_y$-C_n surfactants and HPC-*g*-$(POE)_y$-C_n copolymers.

Polymer composition	Grafted $(POE)_y$-C_n [a] (mol %)	Average number of $(POE)_y$-C_n units per HPC chain	CAC	
			Polymer concentration[b] (mg/l)	$(POE)_y$-C_n concentration ($x10^6$ mol/l)
HPC	0	0	-	-
$(POE)_{10}$-C_{16}	-	-	4.3 ± 1.0[c]	6.3 ± 1.4[c]
$(POE)_{20}$-C_{16}	-	-	4.6 ± 0.6[c]	4.1 ± 0.5[c]
$(POE)_{20}$-C_{18}	-	-	3.7 ± 0.5[c]	3.2 ± 0.4[c]
HPC-*g*-$(POE)_{10}$-C_{16}	0.9 ± 0.1	5	75 ± 14	1.8 ± 0.3
HPC-*g*-$(POE)_{10}$-C_{16}	4.7 ± 0.1	19	17 ± 3	2.1 ± 0.4
HPC-*g*-$(POE)_{20}$-C_{16}	1.1 ± 0.1	5	65 ± 12	1.9 ± 0.3
HPC-*g*-$(POE)_{20}$-C_{16}	3.9 ± 0.1	18	15 ± 5	1.5 ± 0.5
HPC-*g*-$(POE)_{20}$-C_{18}	1.1 ± 0.1	5	135 ± 10	3.9 ± 0.3
HPC-*g*-$(POE)_{20}$-C_{18}	3.1 ± 0.1	16	22 ± 6	1.8 ± 0.4

[a] Determined by ^{1}H-NMR measurement in DMSO-d_6 (Piredda *et al.*, 2003).

[b] Determined by change in $I_{336\ nm}/I_{333\ nm}$ ratio of pyrene fluorescence with log polymer concentration at 25 °C.

[c] These values refer to the critical micelle concentrations (CMC) of the $(POE)_y$-C_n surfactants.

Table 4.2. Size of CsA-free and CsA-loaded HPC-*g*-$(POE)_y$-C_n polymeric micelles and maximum CsA loading capacity (% *w*/*w*) of the micelles.

Polymer composition	Grafted $(POE)_y$-C_n (mol %)	Maximum CsA loading[a] (%*w*/*w*)	Mean diameter[b] ± S.D. (nm)	
			CsA-free polymeric micelles	CsA-loaded polymeric micelles
HPC-*g*-$(POE)_{10}$-C_{16}	0.9 ± 0.1	2.3 ± 0.2	85 ± 2	65 ± 2
HPC-*g*-$(POE)_{10}$-C_{16}	4.7 ± 0.1	5.3 ± 0.3	80 ± 1	50 ± 3
HPC-*g*-$(POE)_{20}$-C_{16}	1.1 ± 0.1	2.5 ± 0.3	90 ± 1	66 ± 2
HPC-*g*-$(POE)_{20}$-C_{16}	3.9 ± 0.1	6.7 ± 0.4	78 ± 1	44 ± 4
HPC-*g*-$(POE)_{20}$-C_{18}	1.1 ± 0.1	1.7 ± 0.3	85 ± 1	75 ± 4
HPC-*g*-$(POE)_{20}$-C_{18}	3.1 ± 0.1	4.8 ± 0.3	83 ± 2	74 ± 3

[a] Determined by HPLC analysis with UV detection at 210 nm.

[b] Determined by DLS measurements of 5 mg/ml aqueous solution at 25 °C with a scattering angle of 90°.

4.5.2. Critical association concentration of HPC-*g*-$(POE)_y$-C_n micelles

The polymer concentration corresponding to the onset of micellization was deduced from a fluorescence assay, based on the changes in the photophysics of pyrene, a hydrophobic probe added in minute amounts to a polymer solution. This probe partitions preferably in hydrophobic microenvironments, as described in detail in the experimental section where we present the approach to determine the CMC values of $(POE)_y$-C_n. Aqueous solutions of different HPC-*g*-$(POE)_y$-C_n copolymers exhibited a shift in the pyrene excitation spectra, similar to that observed with solutions of $(POE)_y$-C_n. Plots of the I_{336}/I_{333} ratios versus the logarithm of the concentration of the aqueous solutions of HPC-*g*-$(POE)_y$-C_n of varying compositions are shown in Figure 4.3. The CAC values of the copolymers in water are given in Table 4.1. in units of mg/l of polymer and 10^{-7} mol/l of $(POE)_y$-C_n units. We notice that, for a given type of hydrophobic group grafted on HPC, the CAC (mg/l) decreases with increasing molar content of $(POE)_y$-C_n residues, reflecting the increase in hydrophobicity of the polymer and consequent enhanced stability of the polymeric micelle. The major driving force for the assembly of amphiphilic copolymers in water is the removal of hydrophobic fragments from the aqueous surroundings resulting in the formation of micelles consisting of a hydrophobic core stabilized by hydrated hydrophilic chains exposed into water (Gao *et al.*, 1993). The CAC values are reported also in terms of $(POE)_y$-C_n molar concentrations (Table 4.1.). We notice that, as anticipated, the CMC of the free surfactants with longer (POE) segments is lower than the CMC of the corresponding surfactant with a shorter (POE) head group (see for example $(POE)_{20}$-C_{16} vs.

$(POE)_{10}$-C_{16}). Conversely, for the same POE segment length (20 units), the surfactant bearing a C_{18} group has lower CMC than the surfactant having C_{16} group. The CAC values of the polymeric micelles, reported in units of alkyl group concentration, are nearly identical, within experimental errors, independently of the $(POE)_y$-C_n grafting level. Overall, though, the onset of micellization of polymeric micelles occurs at lower alkyl group concentrations than the CMC of the corresponding surfactants.

4.5.3. Size of the HPC-*g*-$(POE)_y$-C_n polymeric micelles

The hydrodynamic diameters of polymeric micelles, determined by DLS, ranged from 40 to 90 nm (Table 4.2.). In all cases, the size distributions were unimodal, indicative of the absence of free polymer chains and of large polymeric aggregates. We note that, for each $(POE)_y$-C_n substituent, the size of micelles decreases with increasing level of grafting on the HPC backbone. Moreover, CsA-free HPC-*g*-$(POE)_y$-C_n micelles are generally larger than the corresponding CsA-loaded micelles independently of the size of the hydrophobic substituent and of the level of modification. It should be pointed out that the uptake of particles within the intestine and the extent of drug absorption increase with decreasing particle size and increasing specific surface area (Florence *et al.*, 2001). Thus, the small size exhibited by all the polymeric micelles studied here shows a favorable trend towards oral drug delivery.

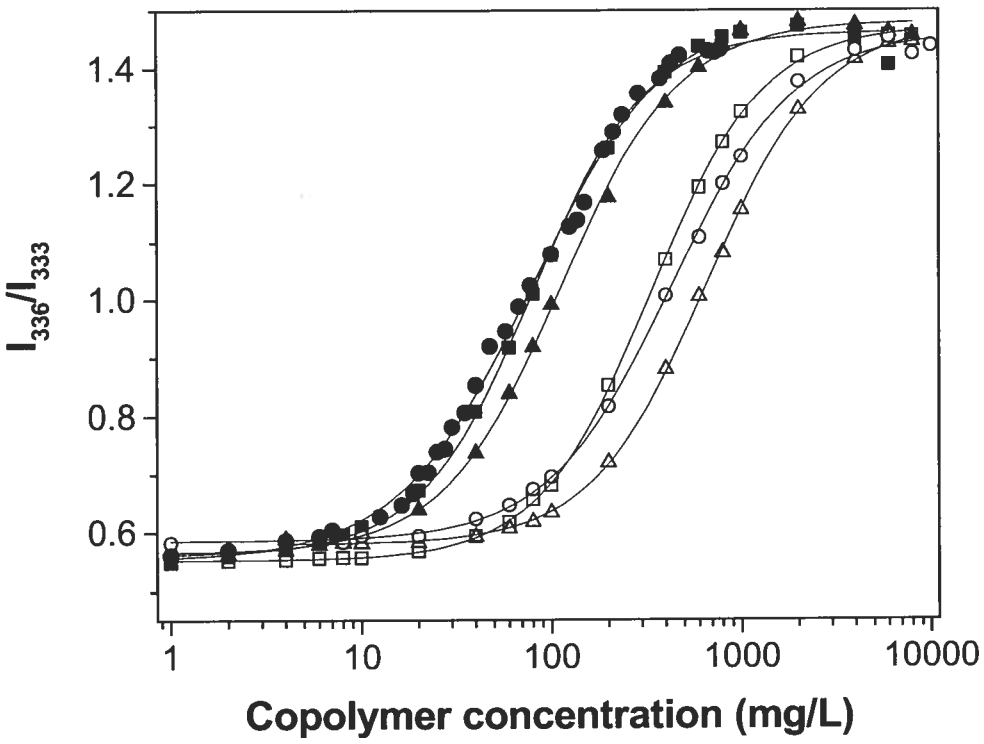

Figure 4.3. Changes in the $I_{336\ nm}/I_{333\ nm}$ ratio of pyrene fluorescence intensity as a function of concentration (1 – 1 x 10^4 mg/l) for the (o) HPC-*g*-$(POE)_{10}$-C_{16} (1 mol%); (•) HPC-*g*-$(POE)_{10}$-C_{16} (4 mol%); (□) HPC-*g*-$(POE)_{20}$-C_{16} (1 mol%); (■) HPC-*g*-$(POE)_{20}$-C_{16} (4 mol%); (Δ) HPC-*g*-$(POE)_{20}$-C_{18} (1 mol%) and (▲) HPC-*g*-$(POE)_{20}$-C_{18} (3.5 mol%) copolymers.

4.5.4. Characterization of the CsA-loaded polymeric micelles

Cyclosporin A was incorporated into the polymeric micelles by a dialysis method which involved treatment of an aqueous polymer solution with a solution of CsA in ethanol, followed by extensive dialysis of the mixed solution against water. The amount of CsA released upon dissolution of the CsA-loaded polymeric micelles was then determined by an HPLC assay and plotted against the CsA concentration added initially for each preparation. The amount of free CsA is expected to be low since the undissolved fraction of the drug was removed by filtration after the dialysis procedure. We evaluated first the ability to solubilize CsA in $(POE)_y$-C_n micelles and by unmodified HPC (Figure 4.4.). $(POE)_{10}$-C_{16}, $(POE)_{20}$-C_{16} and $(POE)_{20}$-C_{18} micelles were able to incorporate relatively high levels of CsA; 17.5%, 21% and 15.5% *w/w*, respectively. Interestingly, CsA has some affinity for unmodified HPC, with a level of CsA incorporation of 1.4% *w/w*. This effect may reflect the presence of some "hydrophobic pockets" within the HPC structure. The structure of HPC is known to be heterogenous with some longer isopropoxyl side chains (Klug, 1971). Next, we assessed the incorporation of CsA within polymeric micelles (Table 4.2. and Figure 4.5.). For all copolymer samples, the amount of CsA incorporated in the micelles was larger than in the case of unmodified HPC (Figure 4.4.). The maximum amount of CsA loaded within the different micelles ranged from 4.8% to 6.7% (*w/w*) depending on the level of grafting and on the type of grafted hydrophobic modifier.

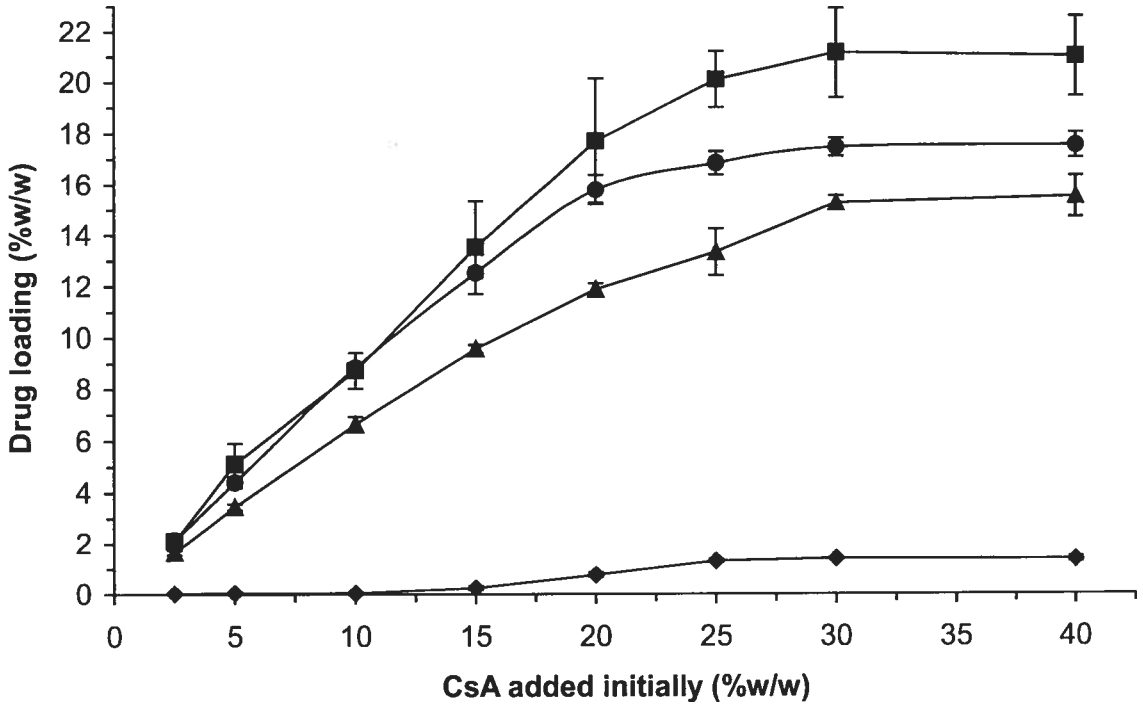

Figure 4.4. CsA loading (% *w/w*) in micelles of $(POE)_{10}$-C_{16} (●), $(POE)_{20}$-C_{16} (■) and $(POE)_{20}$-C_{18} (▲) surfactants as well as unmodified HPC polymer (♦) at 2.5 – 40 (*w/w* %) CsA initial loading in 2.5 mg/ml micelles. Mean ± S.D. (n = 3).

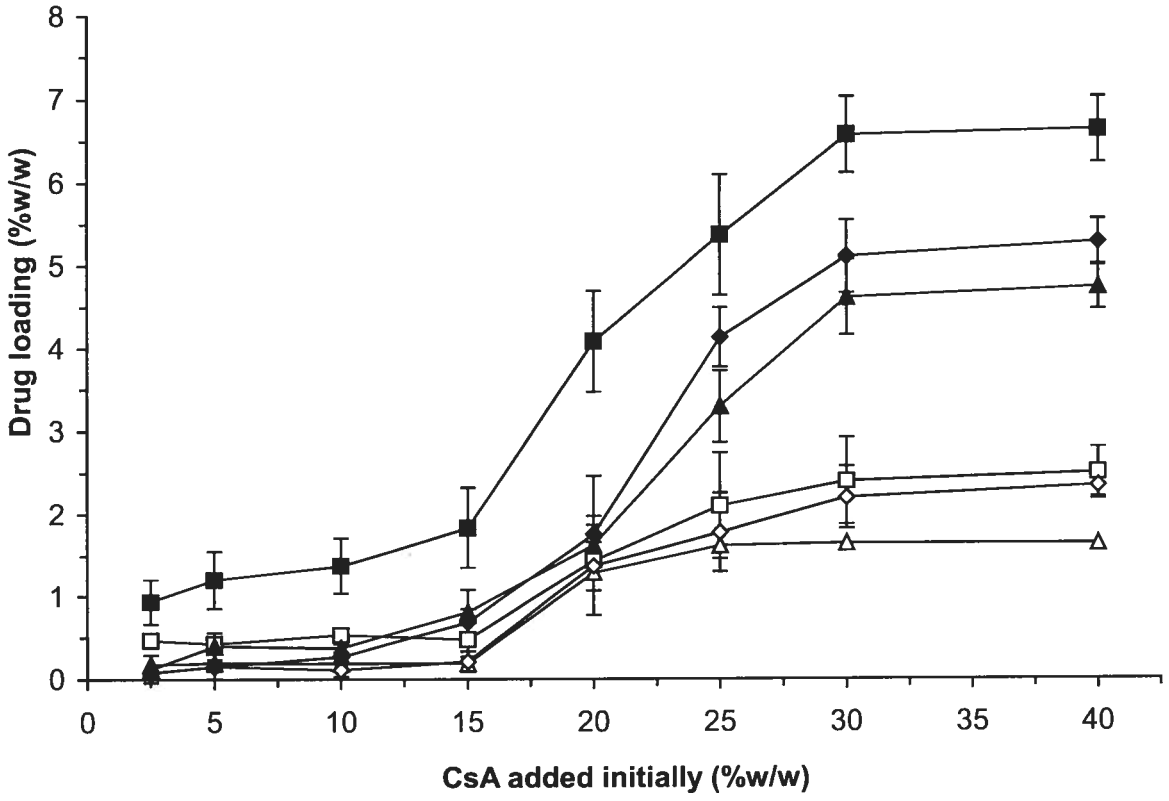

Figure 4.5. CsA loading (*w/w* %) in micelles of (◊) HPC-*g*-$(POE)_{10}$-C_{16} (1 mol%); (♦) HPC-*g*-$(POE)_{10}$-C_{16} (4 mol%), (□) HPC-*g*-$(POE)_{20}$-C_{16} (1 mol%), (■) HPC-*g*-$(POE)_{20}$-C_{16} (4 mol%), (Δ) HPC-*g*-$(POE)_{20}$-C_{18} (1 mol%) and (▲) HPC-*g*-$(POE)_{20}$-C_{18} (3.5 mol%) copolymers at 2.5 – 40 (*w/w* %) CsA initial loading in 2.5 mg/ml micelles. Mean ± S.D. (n = 3).

Several trends are apparent if one compares the highest CsA loading percentages (at 40% *w/w* CsA added initially) determined for the various polymers (Table 4.2.). The percentage of CsA loading increases with increasing grafting level of $(POE)_y$-C_n on HPC backbone. For a constant number of $(POE)_y$-C_n units (~5 units per HPC chain), the CsA loading achieved with HPC-*g*-$(POE)_{20}$-C_{18} (1.7% *w/w*) is lower than those determined for HPC-*g*-$(POE)_{10}$-C_{16} (2.3% *w/w*) and HPC-*g*-$(POE)_{20}$-C_{16} (2.5% *w/w*). These results are consistent with the fact that $(POE)_{10}$-C_{16} and $(POE)_{20}$-C_{16} micelles are more effective in solubilizing CsA than $(POE)_{20}$-C_{18} micelles, 17.5%, 21% and 15.5% (*w/w*), respectively (Figure 4.5.). This may be taken as an indication that the micropolarity of the hexadecyl-POE chains core might present a better solubilizing environment for CsA than that offered by the octadecyl-POE moieties. It is interesting to note also that, at low CsA initial concentrations (<15% *w/w*), HPC-*g*-$(POE)_y$-C_n polymeric micelles exhibit a solubilizing/loading trend different from that displayed by $(POE)_y$-C_n micelles (Figure 4.4. and Figure 4.5.). While the degree of CsA incorporation within $(POE)_y$-C_n micelles increases steadily with increasing initial CsA concentration, the level of CsA incorporation in polymeric micelles remains low and nearly constant with initial CsA concentration <15%, then increases rapidly as the initial CsA concentration exceeds 15%.

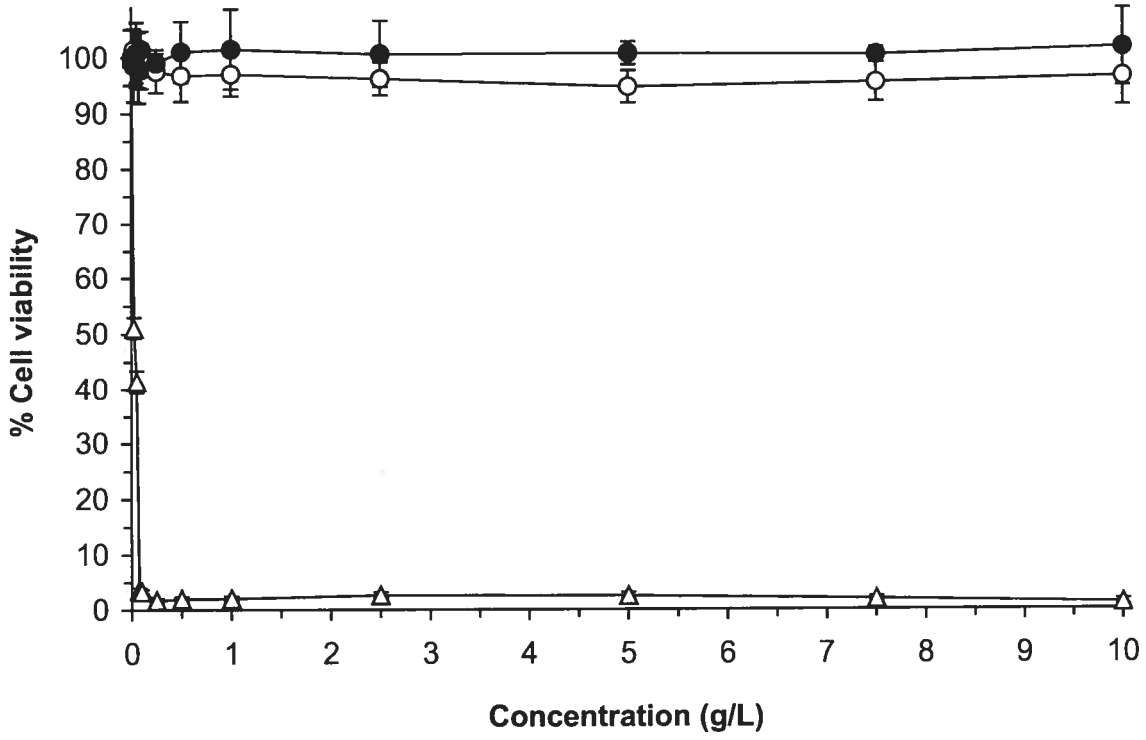

Figure 4.6. Effect of (•) unmodified HPC, (Δ) free $(POE)_{20}$-C_{16} surfactant and (o) HPC-*g*-$(POE)_{20}$-C_{16} (4 mol%) copolymer concentration (0 – 10 g/l) on Caco-2 cell viability measured by MTT assay following 24 h incubation at 37 °C/ 5% CO_2. Mean ± S.D. (n = 3).

4.5.5. In vitro cytotoxicity study

The cytotoxicity of HPC, $(POE)_{20}$-C_{16}, $(POE)_{20}$-C_{18}, $(POE)_{10}$-C_{16} and various HPC-*g*-$(POE)_{v}$-C_{n} copolymers was examined using a MTT assay performed with Caco-2 cells. This study is of particular importance in view of the known toxicity of the $(POE)_{v}$-C_{n} surfactants when they are not linked to polymers. The dose-dependent viability of Caco-2 cells treated with unmodified HPC, free $(POE)_{20}$-C_{16} surfactant and HPC-*g*-$(POE)_{20}$-C_{16} (4 mol%) copolymer over 24 h is presented in Figure 4.6. Similar cell viability patterns were observed in the case of free and HPC-grafted $(POE)_{10}$-C_{16} as well as $(POE)_{20}$-C_{18} (results not shown). As anticipated, free POE-C_{n} surfactants inhibited cell growth even at concentrations below 1 g/l. Such deleterious effect is consistent with the propensity of polyoxyethylene alkyl ether surfactants to affect membrane integrity (Dimitrijevic *et al.*, 2000). The polysaccharide HPC showed no toxicity up to a concentration of 10 g/l, confirming the inertness of HPC (Obara *et al.*, 1992). It may also be pointed out that the World Health Organization (WHO) has not specified an acceptable daily intake for HPC since the levels consumed were not considered to represent any hazard to health (FAO/WHO, 1990). Of interest to our study is the fact that none of the HPC-*g*-$(POE)_{v}$-C_{n} copolymers exhibited any significant toxicity towards Caco-2 cells, up to concentrations of 10 g/l. This important result indicates that by grafting the $(POE)_{v}$-C_{n} residues on HPC, we succeeded in eliminating their toxic effect on cells. The non-toxic hydrophilic HPC chains forming the polymeric micelle outer shell are in contact with the cells and effectively insulate them from the surfactant residues assembled in the inner core.

4.6. CONCLUSION

In summary, we have demonstrated that aqueous solutions of HPC-*g*-$(POE)_y$-C_n copolymers of various compositions increase the solubility of the lipophilic drug, cyclosporin A, through solubilization of the drug in the hydrophobic core of polymeric micelles, while the hydrophilic, non-toxic HPC outer shell stabilizes the system in the aqueous milieu. The molar contents, as well as the length of the hydrophobic substituent, have an important effect on the solubilizing power of HPC-*g*-$(POE)_y$-C_n polymeric micelles towards hydrophobic drugs. The small size of the polymeric micelles, the very low concentration of micellization onset and the absence of cytotoxic effects towards intestinal cells represent promising characteristics for the development of a novel polymeric drug carrier for the oral delivery of poorly water soluble drugs.

4.7. ACKNOWLEDGEMENTS

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CHAPTER FIVE

RESEARCH PAPER

ENGINEERING POLYSACCHARIDE-BASED POLYMERIC MICELLES TO ENHANCE PERMEABILITY OF CYCLOSPORIN A ACROSS CACO-2 CELLS

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5.1. ABSTRACT

Purpose. To assess and compare the effectiveness of two types of polysaccharide-based micelles as delivery vehicles for poorly-water soluble drugs by monitoring their permeability across Caco-2 cell monolayers. **Methods**. Dextran (DEX) and hydroxypropylcellulose (HPC) were hydrophobically-modified (HM) by grafting polyoxyethylene cetyl ether (POE-C_{16}, 15 mol % and 5.4 mol %, respectively). The onset of micellization and mean diameter of polymeric micelles formed by HM-DEX and HM-HPC were determined by fluorescence spectroscopy and dynamic light scattering, respectively. Cyclosporin A (CsA)-loaded polymeric micelles were prepared by a dialysis procedure and the amount of incorporated CsA was assayed by high performance liquid chromatography (HPLC). The micelles stability in simulated gastric and intestinal fluids was studied as a function of contact time, and their cytotoxicity towards Caco-2 cells was evaluated using the MTT colorimetric assay. The bidirectional transport across Caco-2 cells monolayers of CsA entrapped in HM-DEX and HM-HPC micelle and of the polymers themselves was evaluated in the presence and absence of P-glycoprotein inhibitor. **Results.** The amount of CsA incorporated in HM-HPC and HM-DEX micelles reached 5.5 and 8.5 %*w/w*, respectively (entrapment efficiency of 22 % or more). The polymeric micelles exhibited high stability in gastric and intestinal fluids and no significant cytotoxicity towards Caco-2 cells. The apical to basal permeability of CsA across Caco-2 cells increased significantly when loaded in polymeric micelles, compared to free CsA. **Conclusions**. Polysaccharide-based polymeric micelles are promising carriers for the oral

delivery of poorly-water soluble drugs. *In-vitro* tests indicate that, overall, HM-HPC micelles are more effective, compared to HM-DEX micelles.

5.2. KEY WORDS

Cyclosporin A; polymeric micelles, oral delivery, Caco-2, transport; P-glycoprotein, dextran, hydroxypropylcellulose.

5.3. INTRODUCTION

The cyclic undecapeptide cyclosporin A (CsA) is a potent immunosuppressive agent used to prevent allograft rejection following organ transplantations (Merion *et al.*, 1984; Klompmaker *et al.*, 1993). It is also effective in the treatment of patients with selected autoimmune diseases, such as rheumatoid arthritis (Galla *et al.*, 1995). CsA is currently available for oral administration in the form of a microemulsion containing, among other components, polyoxyethylated castor oil (Cremophor EL) (Foradori *et al.*, 1994; Sketris *et al.*, 1994). Cremophor EL is a non-ionic surfactant present in various oral lipophilic drugs formulations, due to its solubilizing properties and its ability to inhibit intestinal P-glycoprotein (P-gp) efflux (Woodcock *et al.*, 1990; Seeballuck *et al.*, 2003). Cremophor EL, however, is known to induce undesirable side effects, such as nephrotoxicity (Mason, 1990; Skorecki *et al.*, 1992) and induction of anaphylactic reactions in sensitized patients (Friedman *et al.*, 1985; Howrie *et al.*, 1985). Consequently, there has been much effort devoted to the design and development of novel dosage forms of CsA, aimed at decreasing

the side effects of the current formulation while preserving the bioavailability and therapeutic effects of the drug. Alternative approaches investigated include incorporation of the drug within microspheres, nanoparticles, and liposomes (Allen *et al.*, 1999; Chen *et al.*, 2002; Sanchez *et al.*, 1995; Itoh *et al.*, 2003; Al-Meshal *et al.*, 1998). Given the limited stability of liposomes *in vivo*, they have not been widely applied in clinical use (Ozpolat *et al.*, 2003; Nacka *et al.*, 2001; Taira *et al.*, 2004). Other particulate vehicles, however, have been developed and shown promising properties in terms of controlled drug release and distribution (Bonduelle *et al.*, 1996; Molpeceres *et al.*, 2000; Ugazio *et al.*, 2002; Daia *et al.*, 2004).

It is often observed that the gastrointestinal (GI) uptake of microparticles is affected significantly by particle size. For example, Desai and coworkers (Desai *et al.*, 1996) reported that the uptake efficiency of particles ~ 100 nm in diameter by the GI tract is 15 to 250-fold higher than that of micron-sized particles. Thus a major thrust of current research towards more efficient oral formulation lies in the design of nanoparticles able to entrap hydrophobic drugs with high efficiency, to transport them through the GI tract, and to enhance their absorption through the intestinal epithelial cells. The main materials investigated over the last few years as nanoparticulate carriers for CsA include hydrophobic biodegradable polymers, such as poly(caprolactone) (Varela *et al.*, 2001), poly(D,L-lactic acid) (Gref *et al.*, 2001), positively charged chitosan hydrochloride (De Campos *et al.*, 2001), and hydroxypropyl methylcellulose phthalate (HPMCP) (Sertsou *et al.*, 2002).

We reported recently the synthesis and physico-chemical properties of amphiphilic polymers consisting of a polysaccharide main chain decorated with lipidic side chains made-up of *n*-hexadecyl or *n*-octadecyl chains (Francis *et al.*, 2003a; Francis *et al.*, 2003b). When placed in an aqueous environment, these polymers spontaneously assemble in the form of micelles with a hydrophobic core surrounded by a hydrophilic corona made up by highly hydrated polysaccharide chains. Unlike surfactant micelles, which tend to disintegrate upon dilution triggering lysis of cell membranes (Lasic, 1992; Hofland *et al.*, 1992), polymeric micelles are remarkably stable towards dilution and tend to exhibit minimal cytotoxicity (Yu *et al.*, 1998; Kim, S.C. *et al.*, 2001; Jevprasesphant *et al.*, 2003). Several considerations need to be taken into account in designing amphiphilic polymer formulations, the most important factor being the choice of the polymer backbone. In our studies, we opted to construct the polymeric micelles from non-toxic polysaccharides, either dextran (DEX-C_{16}) or hydroxypropylcellulose (HPC-C_{16}) (Figure 5.1.). Dextran, consists of β-D-glucose units, predominantly linked by 1-6 glycosidic linkages (Larsen, 1989). It possesses excellent biocompatibility and has been used in medicine as plasma substitute, due to its known innocuousness both locally and systemically (Couch, 1965). More recently, dextran-based gels and prodrugs have been investigated as carriers for a variety of bioactive agents (Draye *et al.*, 1998; Kim, I.S. *et al.*, 2000; Zhang *et al.*, 2002; Mehvar, 2000). Hydroxypropylcellulose is a common excipient in oral formulations, due to its excellent processability, disintegrant and bioadhesive properties. The latter feature is of interest in our application as well, since it may promote the contact of HPC-based micelles with the intestinal epithelial cells and their transport through the cell membranes.

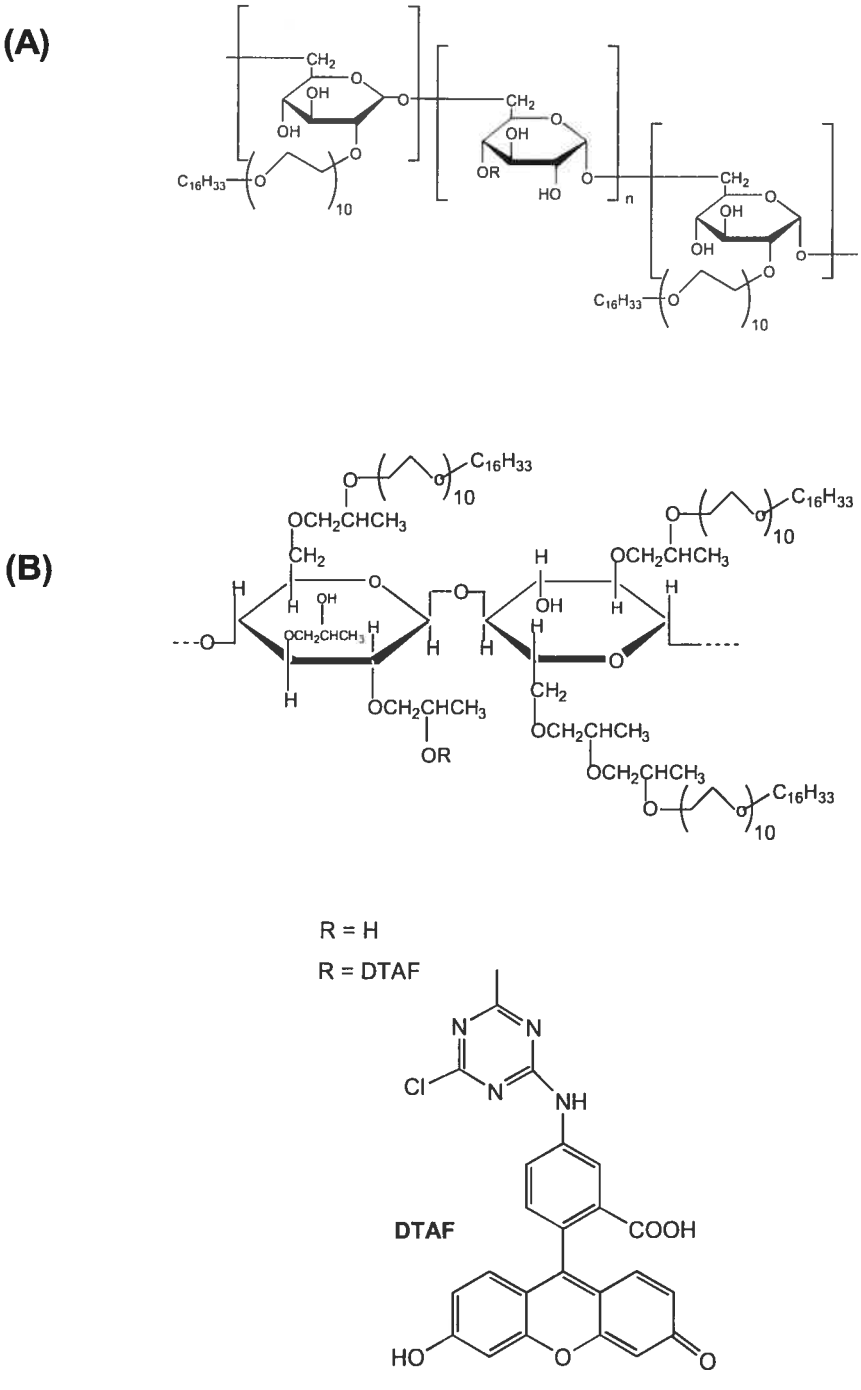

Figure 5.1. Chemical structures of unlabelled and Fluorescein-labelled (A) DEX-C_{16} (15 mol %) and (B) HPC-C_{16} (5.4 mol %) copolymers.

Two main factors contribute to the bioavailability of orally-absorbed drugs: (1) their resistance to the *in vivo* metabolic environment, in particular changes in pH and presence of enzymes; and (2) their intestinal absorption which is mainly controlled by the solubility of the drug in the intestinal lumen and by the drug permeability across the intestinal barrier. There are two routes of transepithelial drug transport by passive diffusion (Pade *et al.*, 1997): the transcellular route through the cell membrane and the paracellular route from the tight junction to the lateral space. In the case of hydrophobic drugs, the contribution of the transcellular route predominates. In order to assess, whether polymeric micelles may promote drug bioavailability it is necessary to quantify the transport of the micelles through the intestinal barrier by an *in vitro* assay (Audus *et al.*, 1990). The Caco-2 cell line, which is derived from human colon adenocarcinoma, undergoes spontaneous differentiation in culture, forming monolayers of polarized enterocytes that possess morphological and functional similarities to the small intestine (Hidalgo *et al.*, 1989; Cogburn *et al.*, 1991; Delie *et al.*, 1997; Artursson *et al.*, 2001). In particular, Caco-2 cells express on the apical (AP) surface the P-gp efflux pump(Hosoya *et al.*, 1996), an absorption barrier limiting the oral bioavailability of hydrophobic drugs from the gastrointestinal tract (Hunter *et al.*, 1997). The permeability assay through Caco-2 monolayers has emerged as a leading method for determining the apparent permeability coefficient of drugs and to investigate their absorption mechanisms (Delie *et al.*, 1997; Artursson *et al.*, 1997; krishna *et al.*, 2001; Kamm *et al.*, 2000; Faassen *et al.*, 2003), since a strong correlation was observed between *in vitro* permeability across Caco-2 cells and *in*

vivo human absorption for a variety of compounds (Artursson *et al.*, 1991; Yee, 1997; Yamashita *et al.*, 1997).

The aim of this work is to investigate, using the Caco-2 cell model, the transepithelial transport of CsA incorporated within dextran- and HPC-derived polymeric micelles. We monitor (1) the permeability of CsA through Caco-2 cell membranes in the absence and presence of Pluronic P85 used as a P-gp inhibitor and (2) the transport of the polymer micelle itself. We use a radiolabelled CsA to track the drug as it crosses the epithelial barrier and, to assess the fate of the polymers, we use a fluorescent marker covalently linked to the polymers. We assess as well the stability of HPC–C_{16} and DEX-C_{16} micelles exposed to simulated biological fluids. This study will add to our knowledge of the fate of nanoparticles as they traverse the intestinal membrane and will allow us to compare the properties of HPC- and DEX-based polymeric micelles.

5.4. MATERIALS AND METHODS

5.4.1. Materials

Cyclosporin A (CsA), polyoxyethylene (10) cetyl ether (POE-C_{16}; $C_{16}H_{33}(OCH_2CH_2)_{10}OH$; Brij 56®), hydroxypropylcellulose (HPC; MW 80,000 Da, molar substitution level (MS): 3.7, where MS is defined as the average number of alkylene oxide per anhydroglucose unit (Tezuka *et al.*, 1990; Wirick *et al.*, 1970)), monobasic potassium phosphate (KH_2PO4), sodium chloride (NaCl), (5-([4,6-dichlorotriazin-2-yl]amino)-fluorescein (DTAF), *N*-(2-hydroxyethyl)-piperazine-*N*'-(2-ethanesulfonic acid) (HEPES)

and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO). Optical grade pyrene (99 %), dichloromethane (DCM), deuterated dimethyl sulfoxide (DMSO-d_6), triethylamine (Et_3N) and trimethylamine hydrochloride ($Me_3N.HCl$) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Dextran T10 (DEX; MW 10,000 Da) was supplied by Amersham Biosciences (Uppsala, Sweden). HPLC-grade acetonitrile (ACN) and water were obtained from Anachemia Science (Montreal, PQ, Canada). Ethanol (95 %) was obtained from Commercial Alcohols Inc. (Brampton, ON, Canada). The Caco-2 cell line was purchased from American Type Culture Collection (ATCC, Rockville, MD) at passage 18. Dulbecco's modified Eagle medium (DMEM), penicillin-streptomycin (10,000 U/ml penicillin G and 10,000 μg/ml streptomycin), fetal bovine serum (FBS), 0.25 % (w/v) trypsin – 1 mM EDTA.4Na (1X) and non-essential amino acids (NEAA) were supplied from Invitrogen Life Technologies (Burlington, ON, Canada). Poly(ethylene oxide)$_{27}$-*b*-poly(propylene oxide)$_{39}$-*b*-poly(ethylene oxide)$_{27}$, also known as Pluronic P85® or P85, was provided by BASF Corp. (Parispany, NJ). [^{3}H] Cyclosporin A (8.00 Ci/mmol) was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Ultrapure water, used for all aqueous solutions, was from a Milli-Q Filtration system (Millipore, Bedford, MA).

Table 5.1. Characteristics of the polymers used in the present study.

Polymer composition	**Grafted POE-C_{16}[a] (mol %)**	**Number of POE-C_{16} units per polymer chain**	**DTAF concentration[b] (mol DTAF/g polymer)**
DEX-C_{16}	15.0 ± 0.5	9	-
HPC-C_{16}	5.4 ± 0.5	25	-
DTAF-DEX-C_{16}	15.0 ± 0.5	9	$5.45 \pm 0.05 \times 10^{-6}$
DTAF-HPC-C_{16}	5.4 ± 0.5	25	$5.70 \pm 0.05 \times 10^{-6}$

[a] from ^{1}H NMR spectra (Francis *et al.*, 2004)

[b] from UV/Visible spectra (see experimental section)

5.4.2. Synthesis of Fluorescein-labelled HM-polysaccharides

The labelled polymers were prepared by modification of HPC-C_{16} and DEX-C_{16} (Figure 5.1.), which were synthesized following the general procedure reported elsewhere (Francis *et al.*, 2004). The composition and molecular characteristics of these polymers are listed in Table 5.1. The solubility characteristics of the two polymers are different, therefore the labelling procedure had to be adapted to each sample as follows:

Preparation of DTAF-HPC-C_{16}: Hydroxypropylcellulose-grafted-polyoxyethylene (10) cetyl ether (HPC-C_{16}, 300.0 mg) was dissolved in a 1/1 *v/v* of water/acetone mixture (50 ml). The pH of the solution was adjusted to 10 using aqueous NaOH (5 N). A solution of 5-DTAF (8.0 mg, 0.015 mmol) in aqueous NaOH (15 ml, pH=10) was added in five portions to the polymer solution at time intervals of 30 min. At the end of the addition, the reaction mixture was kept at room temperature for 17 h. Subsequently, it was dialyzed extensively against distilled water (membrane MW cut-off: 6,000-8,000 dalton, Spectrum Laboratories Inc., Rancho Dominguez, CA) and isolated by freeze-drying; yield: 107.2 mg. The degree of DTAF substitution was determined by quantitative UV/Visible spectrophotometry (Hewlett-Packard 8452A photodiode array spectrometer, Hewlett-Packard, Palo Alto, CA). The labelled polymer was dissolved in aqueous solution (pH 9). DTAF was used as reference (molar extinction coefficient ε_{492nm}: 70 000 $cm^{-1}.mol^{-1}$ at λ = 492 nm) (Blakeslee, 1977).

Preparation of DTAF-DEX-C_{16}: Dextran-grafted-polyoxyethylene (10) cetyl ether (DEX-C_{16}, 300 mg) was dissolved in water (50 ml). The pH of the solution was adjusted to

10 with aqueous NaOH (5 N). A solution of 5-DTAF (29.6 mg, $5.6x10^{-5}$ mol) in aqueous NaOH (15.0 ml, pH=10) was added portion-wise over 2 h. to the polymer solution. At the end of the addition, the reaction mixture was stirred at room temperature for 17 h. It was dialyzed extensively against water (membrane MW cut-off: 3,500 dalton). The dialyzed polymer solution was further purified by ultrafiltration through an Amicon YM3 ultrafiltration membrane (Amicon, Beverly, MA). The labelled polymer was isolated by freeze-drying; yield: 132 mg and its degree of labelling was determined by UV/Visible spectrophotometry.

5.4.3. Characterization of HM DEX and HPC in solution

The critical association concentrations (CAC) of DEX-C_{16} and HPC-C_{16} in aqueous solution were estimated by steady-state fluorescence spectroscopy using polymer solutions of increasing concentration in pyrene-saturated water ([Py] = 7 $x10^{-7}$ M) and monitoring the changes in the ratio of the pyrene excitation spectra intensities (Zhao *et al.*, 1990) at λ = 333 nm (I_{333}) for pyrene in water and λ =336 nm (I_{336}) for pyrene in the hydrophobic medium within the micelle core. The hydrodynamic diameter of drug-free and drug-loaded polymeric micelles in water was evaluated by dynamic laser light scattering (DLS) at 25°C with a scattering angle of 90°.

5.4.4. Physical loading of CsA in HM DEX and HPC polymeric micelles

A dialysis method was employed to prepare CsA-loaded polymeric micelles. Polymer solutions (5 mg/ml) in deionized water, and a CsA solution in ethanol (5 mg/ml)

were prepared separately. Subsequently, different mixtures of polymer with varying CsA initial concentrations (2.5 – 40 % *w/w*) were prepared. Following 48 h of dialysis, each solution was filtered through a 0.22-μm pore-size filter and the filtrate was freeze-dried. CsA was extracted from freeze-dried micelles using acetonitrile (ACN). The resulting suspensions were sonicated in an ultrasonic bath for 10 min then agitated for 8 h. They were then filtered and assayed by HPLC using a symmetry® octadecyl-silane C_{18} column. The mobile phase consisted of ACN : water (80 : 20) with a flow rate of 1.2 ml/min. The column was thermostated at 70 °C. The CsA peak, monitored at 210 nm, appeared at a retention time of 6.5 min. A CsA calibration curve was prepared using standard solutions of concentrations ranging from 3.125 - 400 mg/l, with a first order correlation coefficient (r^2) greater than 0.99. Drug loading (DL) was calculated using Equation 1:

$$DL\ (\%) = 100\ (W_c/W_M) \quad \ldots\ldots (1)$$

Where W_c is the weight of CsA loaded in micelles and W_M is the weight of micelles before extraction.

5.4.5. Stability study

The stability of CsA-loaded polymeric micelles in gastrointestinal fluid was monitored by measuring the release of CsA entrapped within micelles as a function of time when in contact with simulated gastric and intestinal fluids. Simulated gastric fluid was

prepared according to USP XXIV, using a solution of NaCl (2.0 g/l, pH 1.2 by addition of HCl). Simulated intestinal fluid was also prepared according to USP XXIV. Monobasic potassium phosphate (6.8 g) was dissolved in 250 ml distilled water, and 190 ml of 0.2 N NaOH and 400 ml of purified water were added. This solution was adjusted to a pH of 6.8 with 0.2 N NaOH and diluted to 1000 ml. Dialysis bags (MW cut-off: 6,000-8,000 dalton) containing a solution of CsA-loaded micelle (15 mg, CsA concentration 5 % *w/w*) in simulated gastric or intestinal fluid (3 ml) were placed into flasks containing 180 ml of the corresponding simulated fluid. The flasks were shaken at 100 rpm and the temperature was maintained at 37°C during 8 h. At specific time intervals, 10 ml aliquots were taken from the release medium (dialysate) and replaced by the corresponding fresh simulated fluid (10 ml) in order to keep the system under sink conditions. At the end of the experiment, the dialysis bags were cut open and their content was allowed to leak into the release medium. An aliquot of this solution was sampled to determine the concentration corresponding to 100 % release. The aliquots were freeze-dried. The CsA content of the residue isolated was assayed by the HPLC method described above. Release of free CsA was also performed as a control. All stability tests were performed in triplicates; the data presented are the mean ± S.D.

5.4.6. Cell Culture

The human colon adenocarcinoma cells, Caco-2, were routinely maintained in Dulbecco's modified Eagle medium with 4.5 g/l D-glucose, supplemented with 10 % (*v/v*) heat-inactivated fetal bovine serum, 1 % (*v/v*) non-essential amino acids and a 1 % (*v/v*)

penicillin-streptomycin antibiotics solution (100 U/ml penicillin G and 100 μg/ml streptomycin). Cells were allowed to grow in a monolayer culture in an incubator at 37 °C with controlled atmosphere containing 5 % CO_2 and 90 % relative humidity. Cells were passaged at 80 – 90 % confluency at a split ratio of 1:3 using 0.25 % trypsin – 1 mM EDTA.

5.4.7. Cytotoxicity Assay

Caco-2 cells were seeded in triplicate in 96-well culture plates at a density of approximately 5 x 10^4 cells in 100 μl of cell culture medium per well. The cells were cultured for 48 h at 37 °C in a humidified atmosphere of 5 % CO_2 in air. Thereafter, Caco-2 cells were exposed to high concentration (10 g/l) of POE-C_{16}, DEX, HPC, DEX-C_{16} or HPC-C_{16}, followed by incubation periods of 4 h and 24 h. Cell viability was evaluated using the MTT colorimetric assay as previously described (Mosmann, 1983; Hansen *et al.*, 1989). The assay is based on the reduction of MTT by mitochondria in viable cells to water insoluble formazan crystals. The absorbance was measured with a multiwell-scanning spectrophotometer (PowerWave; Biotek Instruments, Winooski, VT) at 570 nm.

5.4.8. Transport studies of CsA-loaded micelles of HM polysaccharides across Caco-2 cell monolayers

Transport of CsA-loaded polymeric micelles across Caco-2 cells was evaluated as follows: cells were seeded onto polycarbonate filter inserts in 6 well Transwell® dishes (Corning Costar Co., Cambridge, MA) at a density of approximately 8 x 10^4 cells/cm^2. Cells were incubated for 21 - 25 days to allow confluency, full maturation, including P-gp expression (Hosoya *et al.*, 1996) and increased transepithelial electrical resistance (TEER) due to the formation of tight junctions in the cell monolayer (Bailey *et al.*, 1996). The culture medium was replaced (1.5 ml apical (AP) side and 2.6 ml basolateral (BL) side) every other day for the first week and daily thereafter. The integrity of the Caco-2 monolayers was evaluated both before and immediately after the study using a Millicel® Electrical Resistance System equipped with STX-2 electrodes (Millipore Corp., Bedford, MA). Typical TEER values exceeded 300 Ω/cm^2. Prior to experiments, the culture medium of Transwell®-grown Caco-2 cell monolayers was replaced with prewarmed (37 °C) transport medium consisting of Hanks' balanced salt solution (HBSS) supplemented with 25 mM glucose and 10 mM HEPES (pH 7.4). The cell monolayer was equilibrated for 30 min at 37 °C before undertaking the transport studies. The AP and BL chambers received 1.5 and 2.6 ml of transport medium, respectively. After equilibration, TEER values of monolayers were determined in triplicates. The amounts of CsA and polymer transported across Caco-2 cell monolayers were determined as depicted in Figure 5.2.

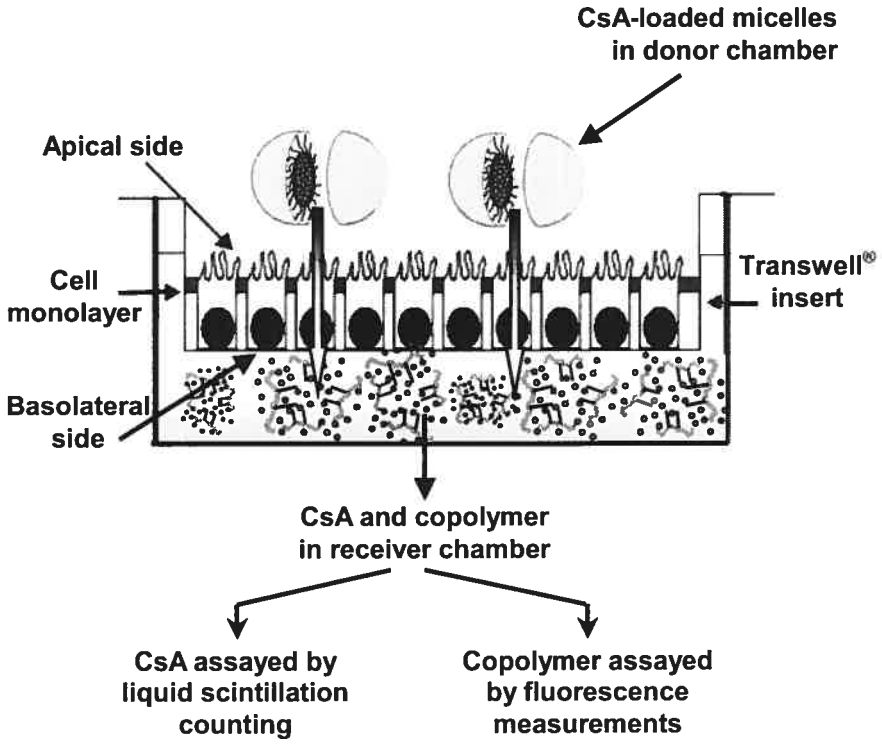

Figure 5.2. Schematic representation of the procedure used to study the transport of CsA-loaded polymeric micelles across Caco-2 cell monolayer.

5.4.9. Transport of CsA

CsA–loaded fluorescently labelled polymeric micelles or free CsA were placed in HBSS transport medium in amounts such that the final concentration of CsA was 1 μM (0.6 μCi [^{3}H] CsA together with unlabeled CsA). It should be noted that the CsA (1μM)-containing polymeric micelles were prepared from solutions containing 25 % (*w*/*w*) of CsA, a concentration for which the final CsA loading in polymeric micelles (5 % *w*/*w*) is the same, within experimental uncertainty, for DEX-C_{16} and HPC-C_{16} (see Figure 5.3.). The solutions were loaded in the AP or BL (donor) compartments. At predetermined time points over a 4-h period, aliquots (400 μl) were withdrawn from the opposite (receiver) chamber. After sample withdrawal, an equivalent volume of the transport medium was added to the receiving compartment to maintain a constant receiver fluid volume. Studies of CsA transport in presence of P-glycoprotein inhibitor (PGI) were conducted using Pluronic P85® unimers (P85, 30 μM in transport medium) (Nerurkar *et al.*, 1996; Batrakova *et al.*, 1998a; Batrakova *et al.*, 1998b). P85 solution was added to the AP side of monolayers, and following sampling, equal volumes were replaced using P85-containing solution. At the end of the experiment, TEER values were measured in triplicates to assess the integrity of the cell monolayers. The aliquots removed during the testing periods were placed in scintillation vials (along with the pipet tip used for sampling) and diluted with 10 ml of scintillation liquid (Ultima Gold®, Packard BioScience, Meriden, CT). The amount of transported CsA was determined by liquid scintillation counting using a Tri-Carb® liquid scintillation analyzer (Packard Instrument Co., Meriden, CT) after correction for changes of

volume and concentrations by the replacement media. At the end of the study, the Caco-2 monolayers were solubilized using a 1 % Triton X-100 solution. The total protein content was determined using the bicinchoninic acid (BCA) protein assay (Pierce Chemical Co., Rockford, IL). Transport results were normalized for total protein content of the cells in each well. All experiments were performed in triplicates; the data presented are the mean ± S.D.

5.4.10. Calculation of apparent CsA permeability coefficients (P_{app})

The apparent permeability coefficients (P_{app}, cm/s) of CsA expressed in cm/s, were calculated using Equation 2:

$$P_{app}\,(\text{cm/s}) = \frac{1}{ACo}.\frac{dQ}{dt} \qquad \text{.......... (2)}$$

Where dQ/dt is the rate of appearance of CsA on the receiver side (pmol/s), A is the surface area of the monolayer and C_o is the CsA concentration (pM) on the donor side at t = 0.

5.4.11. Transport of HM polysaccharides

The polymer permeability across Caco-2 cells was determined by a fluorescence assay using the intrinsic fluorescence of the labelled HM-polysaccharides. Solutions of CsA-loaded fluorescein-labelled polymeric micelles (CsA content of 1 μM) in HBSS transport medium (24 x 10^{-3} mg/ml) were placed within the AP or BL (donor) compartments. For permeability studies in presence of PGI, P85 solution (30 μM) in the transport medium was placed on the AP side of the cell monolayers. The transport of CsA

and polymer was allowed to proceed for 4 h under the same conditions as above. The fluorescence intensity of the solutions recovered from the AP and/or BL (receiver) compartments was determined using a Cary Eclipse fluorescence spectrophotometer (Varian Scientific Instruments Inc., Mulgrave, Victoria, Australia). Samples were excited at λ_{ex} = 493 nm, and the emission intensity was monitored at λ_{em} = 519 nm. The amount of transported polymer was calculated using a predetermined standard curve and calibrated with the protein content of the cells in each well, as described above.

5.4.12. Statistical analysis

All experiments were performed in triplicate; the data presented are the mean ± S.D., standardized on individual well protein concentrations. The differences between the mean values were analyzed for significance using ANOVA test. Results were considered statistically significant from the control when $P<0.05$.

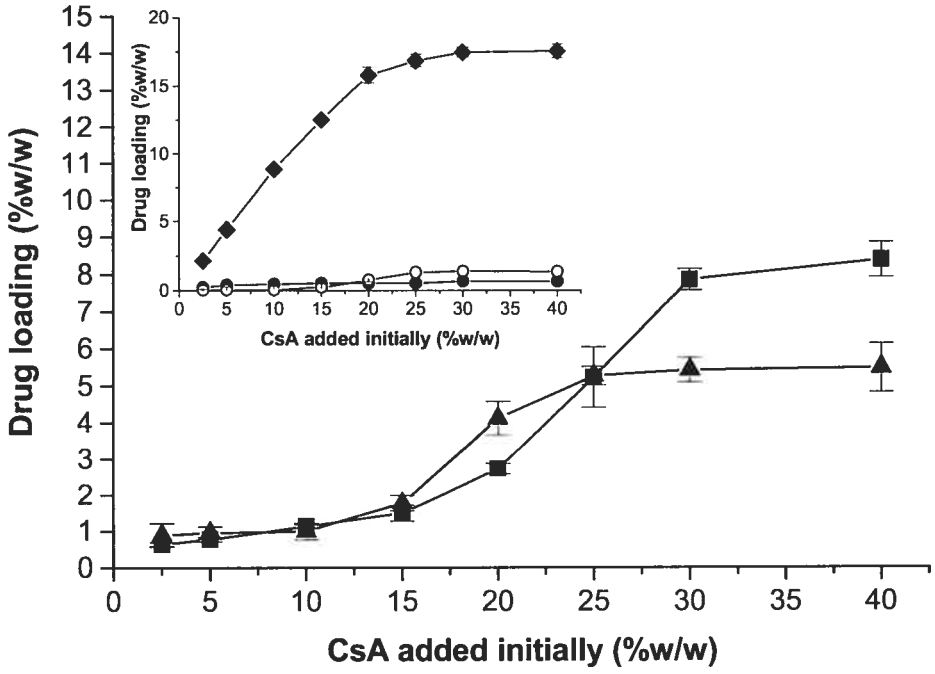

Figure 5.3. CsA loading (% *w/w*) in micelles of (■) DEX-C_{16} (15 mol %) copolymer and (▲) HPC-C_{16} (5.4 mol %) copolymer at 2.5 – 40 (w/w %) CsA initial loading. *Inset*: CsA loading (% *w/w*) in presence of (♦) free POE-C_{16} surfactant as well as unmodified (●) DEX and (o) HPC polymers. Mean ± S.D. (n = 3).

5.5. RESULTS

5.5.1. Cytotoxicity assay

The toxicity of DEX-C_{16}, HPC-C_{16}, DEX, HPC, and POE-C_{16}, a neutral surfactant analogous in structure to the hydrophopic chains linked to DEX or HPC, towards Caco-2 cells was evaluated by the MTT cytotoxicity assay following 4-h and 24-h periods of incubation at 37°C/5 % CO_2. As anticipated, DEX and HPC proved to be non-toxic towards Caco-2 cells, even when added at a concentration as high as 10 g/l. The study also revealed that neither DEX-C_{16} nor HPC-C_{16} exhibited any significant cytotoxicity towards Caco-2 cells after incubation periods as long as 24 h and at concentrations as high as 10 g/l. (Figure 5.4.). The cell viability dramatically decreased in the presence of POE-C_{16} even at concentrations below 1 g/l (data not shown), confirming prior reports (Francis *et al.*, 2003a; Francis *et al.*, 2003b). Thus linking POE-C_{16} to non-toxic polymers effectively alleviates their inherent toxicity. They are confined within the hydrophobic core of the micelles. For them to escape from the micellar assembly, it is necessary to break an ether bond, known to be stable against hydrolysis over a wide range of pH values.

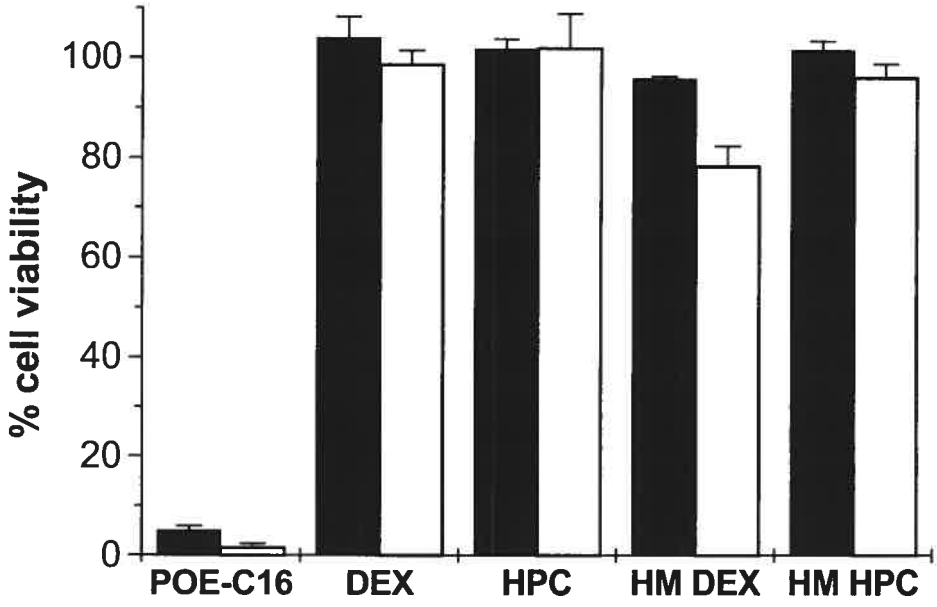

Figure 5.4. Caco-2 cell viability determined by MTT colorimetric assay following 4 h (closed columns) and 24 h (open columns) incubation periods in presence of 10 g/l of free POE-C_{16} surfactant, unmodified DEX and HPC polysaccharides as well as HM copolymers of DEX-C_{16} and HPC-C_{16}. Mean ± S.D. ($n = 3$).

5.5.2. Characterization of CsA-free polymeric micelles

In aqueous solution, DEX-C_{16} and HPC-C_{16} form micelles that can entrap up to 8.5 % *w/w* of CsA, incorporated within the hydrophobic core of the micelle by a dialysis process. The size of the micelles, which can be determined readily by dynamic laser light scattering, varies depending on the polymer structure. Micelles with average hydrodynamic diameters of ~ 12 nm and ~ 72 nm are formed in aqueous solutions of DEX-C_{16} and HPC-C_{16}, respectively (Table 5.2.). In both cases, the micelle size distributions were unimodal, indicative of the absence of free polymer chains and of large aggregates. The onset of micellization (CAC) takes place in solutions of very low polymer concentration, as determined by a fluorescence assay based on the changes in the fluorescence of pyrene, a hydrophobic probe added in minute amounts (~ 7 x 10^{-7} mol/l) in the polymer solutions. In terms of polymer weight concentrations, the CAC of DEX-C_{16} (4 mg/l) is significantly lower than that of HPC-C_{16} micelles (17 mg/l) (Table 5.2.). This may be accounted for by the fact that the average number of glucose units per alkyl chain is significantly smaller for DEX-C_{16} compared to HPC-C_{16}, rendering the former polymer more hydrophobic (Table 5.1.). We note that CAC values for the two polymers, reported in units of hexadecyl group concentration, are nearly identical, taking a value significantly lower than the critical micelle concentration (CMC) of POE-C_{16} (Table 5.2.).

Table 5.2. Characteristics of CsA-free and CsA-loaded DEX-C_{16} and HPC-C_{16} micelles.

Sample	CAC[a] (mg/l)	POE-C_{16} concentration at CAC (x10^6 mol/l)	Maximum CsA loading[b] (%)	Mean diameter[c] (nm) ± SD	
				CsA-free micelles	CsA-loaded micelles
POE-C_{16}	4.3 ± 1[d]	6.3 ± 1.4[d]	17.5 ± 0.5	-	-
DEX	-	-	0.6 ± 0.1	-	-
HPC	-	-	1.3 ± 0.1	-	-
DEX-C_{16}	3.8 ± 0.2	2.4 ± 0.1	8.5 ± 0.6	11 ± 5	14 ± 6
HPC-C_{16}	17 ± 2	2.6 ± 0.3	5.5 ± 0.6	76 ± 2	55 ± 1

[a]Determined by change in $I_{336\ nm}/I_{333\ nm}$ ratio of pyrene fluorescence with log polymer concentration at 25 °C.

[b]Determined by HPLC analysis with UV detection at 210 nm.

[c]Determined by DLS measurements at 25 °C with a scattering angle of 90 °.

[d]These values refer to the critical micelle concentration (CMC) of free POE-C_{16} surfactant.

5.5.3. Characterization of CsA-loaded polymeric micelles

The extent of CsA incorporation, *via* a solvent exchange dialysis process, in DEX-C_{16}, HPC-C_{16} and POE-C_{16} micelles as well as in DEX and HPC used as controls, was assessed as a function of the initial CsA/polymer weight ratio, keeping the polymer concentration constant (2.5 mg/ml) and varying the amount of CsA added at the onset of dialysis (2.5 – 40 % *w/w*). The loading of CsA within either DEX-C_{16} or HPC-C_{16} micelles depends strongly on its initial concentration (Figure 5.3.): it remains low (~ 1 % *w/w*) under conditions where it represents less than 15 % of the total weight content of the dialysis mixture. The % CsA incorporation increases sharply when its initial concentration exceeds 15 % (*w/w*) and reaches a saturation value of ~ 8.5 % and ~ 5.5 % in the case of DEX-C_{16} and HPC-C_{16}, respectively. CsA-loaded polymeric micelles were isolated in the powder form by lyophilization. The dry powder readily solubilized in water, without alteration in the size of the micelles and without premature release of CsA, as confirmed by light scattering measurements.

We note (Figure 5.3.) that for an initial concentration of CsA equal to 25 % (*w/w*), the drug incorporation within DEX-C_{16} and HPC-C_{16} micelles is similar (~ 5.4 % *w/w* or 22 % entrapment efficiency). These conditions were selected to prepare all the CsA-loaded micelles employed in the stability and transport tests described below, ensuring that in all the comparative studies the CsA/polymer weight ratio in the micelles is kept constant. It should be mentioned that the solubilizing ability of the modified polymers towards CsA is higher by a factor of ~ 9, compared to that of either DEX or

HPC, for which the maximum CsA loading level was of 0.6 and 1.3 % *w/w*, respectively (Figure 5.3., inset).

5.5.4. Stability of CsA loaded micelles in simulated gastric and intestinal fluids

The release rates of CsA from polymeric micelles in a simulated gastric fluid (pH 1.2) and in a simulated intestinal fluid (pH 6.8) were monitored by an *in vitro* release assay, in which CsA-loaded micelles (5.4 *w/w* %) captured in dialysis bags were placed in contact with simulated fluids during 8 h. An identical amount of free CsA was placed in contact with the fluids, serving as control. The amount of free CsA in the dialysate was monitored as a function of contacting time. The release data recorded for each type of fluid presented the same features (Figure 5.5.): a small fraction of CsA, 4 % and 12 % for HPC-C_{16} and DEX-C_{16}, respectively was released from the micelles after 4 h; but this amount was much lower than that recorded in control experiments (~ 85 %). A possible explanation for the high stability of micelles in simulated biological fluids of acid pH, is that the POE-C_{16} residues are linked to the polysaccharide backbone through ether linkages, which, unlike commonly-used ester linkages, are stable towards pH changes and enzymatic degradations.

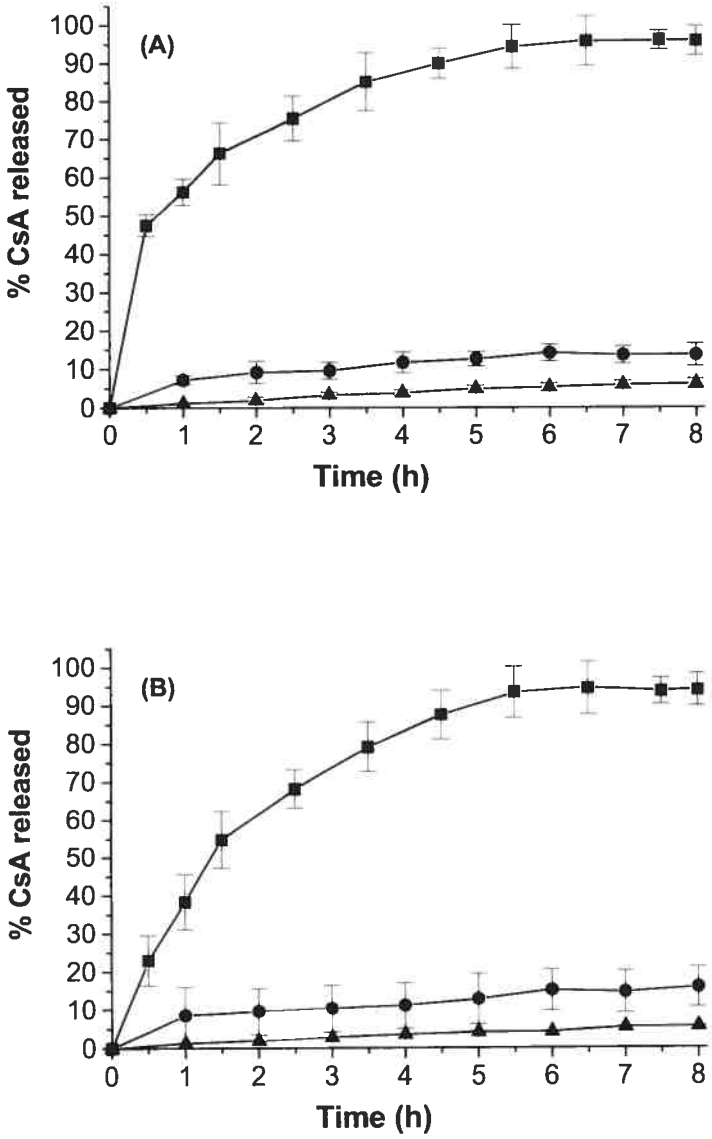

Figure 5.5. Release profile of CsA at 37 °C in (A) simulated gastric fluid at pH 1.2 and (B) simulated intestinal fluid at pH 6.8, from (■) free CsA solution (control); (●) CsA-loaded DEX-C_{16} polymeric micelles and (▲) CsA-loaded HPC-C_{16} polymeric micelles. Mean ± S.D. (n = 3).

5.5.5. Transport study

Experiments were designed that would allow to detect the transport of (1) CsA across Caco-2 cell monolayers (radioactivity measurements) and (2) the host polymeric micelles (fluorescence measurements). Moreover, as CsA is a good substrate for P-gp (Augustijns *et al.*, 1993; Fricker *et al.*, 1996; Chiu *et al.*, 2003), AP to BL and BL to AP transport measurements were carried out in the presence and absence of P85. Nerurkar et al. reported that the P85 free unimers were responsible for inhibiting P-gp efflux transport (Nerurkar *et al.*, 1996). In this study, P85 was added to the AP compartment at a concentration of 30 μM, a value lower than the P85 CMC (67 μM) (Miller *et al.*, 1997; Batrakova *et al.*, 1998b) to ensure that P85 copolymer is in the form of unimers in all measurements. In all cases, TEER values were monitored throughout the experiments: the addition of micelles and/or P85 to either the AP or BL side of Caco-2 monolayers for up to 4 h did not affect TEER values significantly, confirming that the integrity of the cell monolayers was preserved.

Monitoring first the transport of CsA across Caco-2 cell monolayers (Figure 5.6.), we note that after a 240-min incubation, the AP-BL permeability of micelle-loaded CsA increased by factors of 1.5 and 3 (compared to free CsA) when loaded in DEX-C_{16} and HPC-C_{16} micelles, respectively. In all cases, the transport was biphasic with respect to incubation time: slow or insignificant transport was detected during the first 30-min contact, followed by a nearly linear increase in transport upon prolonged incubation.

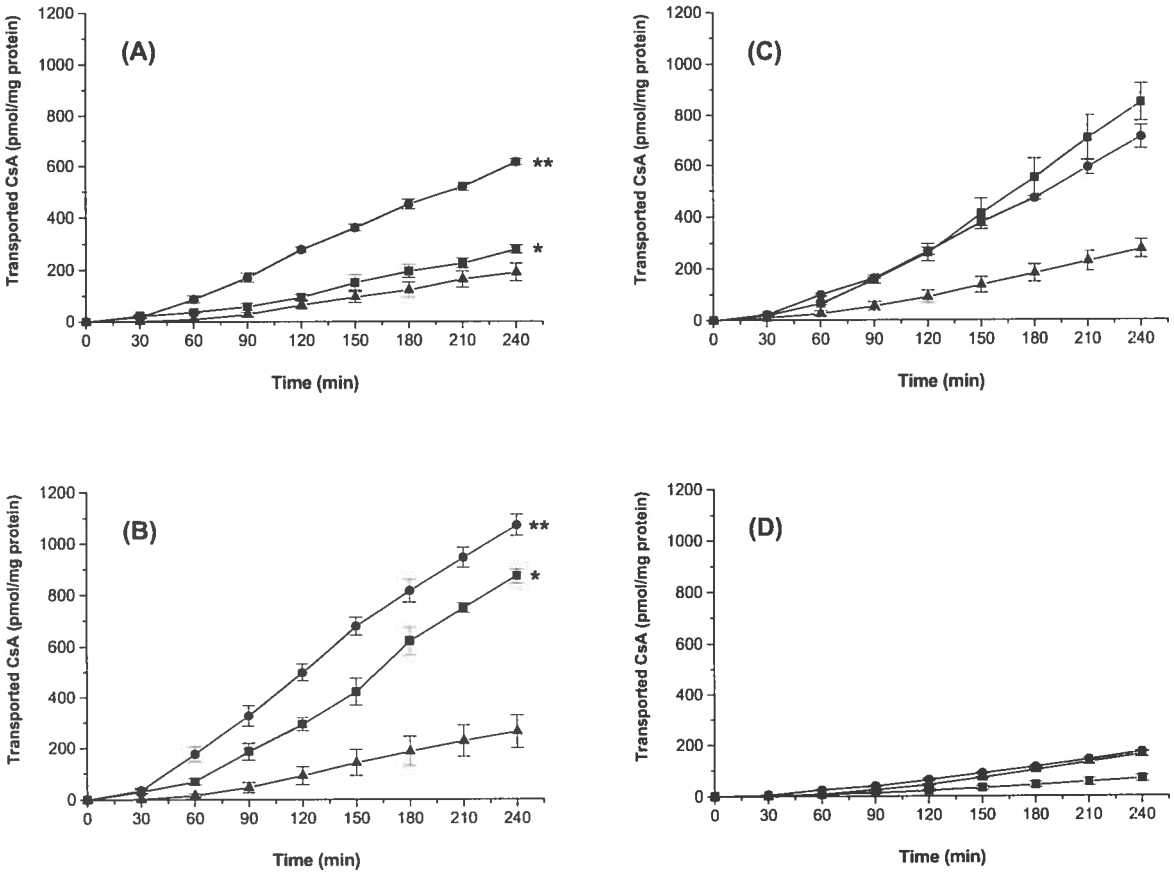

Figure 5.6. CsA (pmol/mg protein) transported across Caco-2 monolayers after 240 min-incubation in the AP-BL direction in absence (A) and presence (B) of P85, and in the BL-AP direction in absence (C) and presence (D) of P85 for free CsA (▲) and CsA loaded in DEX-C_{16} (■) and HPC-C_{16} (●) polymeric micelles. Mean ± S.D. (n = 3). (*) Statistically significant compared to free CsA and (**) statistically significant compared to both free CsA and CsA-loaded in DEX-C_{16} polymeric micelles.

Moreover, in the presence of P85, CsA transport was significantly enhanced in the AP-BL direction, but nearly precluded in the BL-AP direction (Figure 5.6.B and Figure 5.6.D), in agreement with recent reports on the major role of the P-gp efflux mechanisms in determining CsA transport in Caco-2 cells (Augustijns *et al.*, 1993; Wacher *et al.*, 1998; Batrakova *et al.*, 1999; Seeballuck *et al.*, 2003). The permeability coefficient (P_{app}) of CsA transported in the AP-BL direction was higher in the case of CsA loaded in polymeric micelles compared to free CsA, especially in the presence of P85 (Figure 5.7.).

Next, we investigated the bidirectional permeability across the Caco-2 cell monolayers of the host polysaccharides, using fluorescein-labelled copolymers, in order to assess whether CsA is transported across the Caco-2 cells in free form or entrapped within micelles. Indeed, the polymers are transported across Caco-2 monolayers, as indicated by the detection of fluorescence in the receiver compartment. Under all circumstances (AP-BL, BL-AP, without P85, with P85) the amount of transported HPC-C_{16} was greater than that of DEX-C_{16} (Figure 5.8.). The permeability of both polysaccharides was higher in the BL-AP direction, compared to the AP-BL permeability. These results are comparable to those reported for the *in vitro* permeability of polyamidoamine (PAMAM) water soluble dendrimers (Wiwattanapatapee *et al.*, 2000; El-Sayed *et al.*, 2002; El-Sayed *et al.*, 2003).

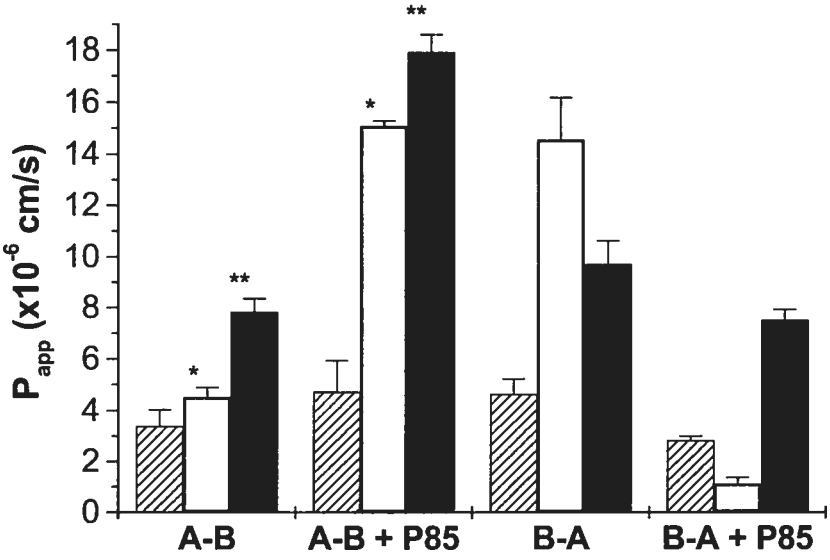

Figure 5.7. Permeability coefficient (P_{app}, cm/s) of free CsA (dashed columns), and CsA-loaded in polymeric micelles of DEX-C_{16} (open columns) and HPC-C_{16} (closed columns). Mean ± S.D. (n = 3). (*) Statistically significant compared to free CsA and (**) statistically significant compared to both free CsA and CsA-loaded in DEX-C_{16} polymeric micelles.

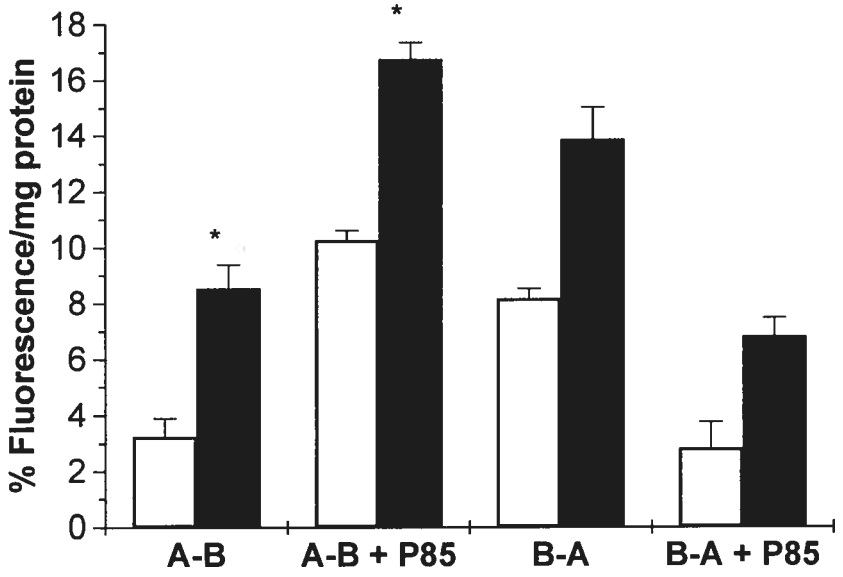

Figure 5.8. Permeability of fluorescein-labelled polysaccharides for CsA-loaded micelles of DEX-C_{16} (open columns) and HPC-C_{16} (closed columns) across Caco-2 monolayers following 4h incubation with Caco-2 cells. Mean ± S.D. (n = 3). (*) Statistically significant compared to DEX-C_{16} polymeric micelles.

5.5.6. Structure of the polymeric micelles and its effect on their transport through Caco-2 cells monolayers

The polysaccharides employed in this study were prepared based on design guidelines we had established through a systematic evaluation of key structural parameters controlling the effectiveness of HM-DEX and HM-HPC as CsA carriers (Francis *et al.*, 2003b; Francis *et al.*, 2003a). The molar content of (POE)-C_n residues is the determining factor, within a family of polymers, dextran or HPC, which affects the physico-chemical properties of the polymeric micelles. Within a series of polymers, the following trends emerge: (1) CAC values decrease with increasing (POE)-C_n chains; (2) the number of (POE)-C_n chains must remain below a limiting value of ~ 20 mol % and 8 mol %, for DEX and HPC respectively, in order to preserve micellar solubility in water; and (3) the CsA loading efficiency of polymeric micelles increases with increasing (POE)-C_n. The size of the polymer framework plays a role as well: micelles formed by DEX-C_{16} constructed from a dextran ~ 40,000 dalton in size were less effective CsA carriers than those based on the shorter dextran (10,000 dalton) (Francis *et al.*, 2003a).

For particulate drug formulations, the carrier size is one of the key parameters that determine the extent of drug absorption and much has been debated on the optimal size of micro- and nano-particles in relation to their uptake by the intestine. It is generally assumed that the uptake is inversely proportional to particle size, and most published data support this hypothesis (Jani *et al.*, 1990; Ebel, 1990; Simon *et al.*, 1995; Carr *et al.*, 1996). However, several studies on the uptake of nanoparticles, such as

dendrimers 2 to 5 nm in diameter, point to the possible existence of an optimal colloidal size for the efficient entrapment of particles in the mucous and subsequent transport through intestinal epithelial cells. (Florence *et al.*, 2001). The HPC-C_{16} and the DEX-C_{16} micelles investigated differ by a factor on nearly 10 in term of diameter, but both types are substantially larger than dendrimers, and their size is within the range considered to be ideal for mucosal uptake.

Our *in-vitro* study indicates that both DEX-C_{16} and HPC-C_{16} micelles are effective carriers for CsA. A comparison of the properties of the two carriers (Table 5.3.) points to the enhanced performance of HPC-C_{16} micelles as judged by the Caco-2 permeability assay employed here. Unmodified dextran dissolves rapidly in water and has a low affinity for the mucous layer (Miyazaki *et al.*, 2003). By linking hydrophobic chains to dextran, one succeeds in decreasing its solubility in water and in solubilizing sufficient amounts of CsA, but one does not affect the low affinity for the mucous of the polymer itself. The superior properties of HPC-C_{16}, compared to DEX-C_{16} may be attributed to the bioadhesive properties of HPC (Ponchel *et al.*, 1998; Vasir *et al.*, 2003). HPC-C_{16} micelles readily adhere to the Caco-2 cell monolayers, allowing the slow diffusion of the encapsulated drug to the basal side, while DEX-C_{16} micelles tend to remain suspended in the apical side.

Table 5.3. Comparison between Hydrophobically-Modified (HM) DEX and HM HPC copolymers.

	DEX-C_{16}	**HPC-C_{16}**
Leakage of loaded CsA in simulated gastric fluid (%)	13.5	6.2
Leakage of loaded drug in simulated intestinal fluid (%)	15.8	5.6
AP to BL transport of CsA (pmol/mg protein)	280	620
AP to BL transport of CsA in presence of P85 (pmol/mg protein)	870	1070
BL to AP transport of CsA (pmol/mg protein)	850	750
BL to AP transport of CsA in presence of P85 (pmol/mg protein)	70	170
AP to BL transport of polymer (mg/ml polymer per mg protein)	0.75	2
BL to AP transport of polymer (mg/ml polymer per mg protein)	2	3

5.6. CONCLUSION

The coupling of hydrophobic groups to water soluble polysaccharides significantly promotes the solubilizing power of either dextran or hydroxypropylcellulose towards CsA. Moreover the bioadhesive characteristics of HPC enhance the association of polymer micelles towards Caco-2 cell monolayers and facilitate the internalization of the polymer and the transport of the drug. The polysaccharide-based polymeric micelles offer unique opportunities for the oral delivery of lipophilic drugs. They are non-toxic and stable in biological fluids. Their size is optimal for effective drug delivery and they possess a high encapsulation. They effectively carry their cargo through model intestinal cell walls. Collectively, the results of this research will aid in understanding the relationship between structural features of polysaccharide-based carriers, their ability to solubilize lipophilic drugs and their intestinal permeability, with the prospect of designing novel polymeric carriers for oral drug delivery.

5.7. ACKNOWLEDGMENTS

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5.8. NOTATIONS

AP	Apical side
BL	Basolateral side
CsA	Cyclosporin A
DEX	Dextran T10
HPC	Hydroxypropylcellulose
HPLC	High Performance Liquid Chromatography
POE-C_{16}	Polyoxyethylene (10) cetyl ether
P-gp	P-glycoprotein
PGI	P-glycoprotein inhibitor
P85	Pluronic® P85

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CHAPTER SIX

RESEARCH PAPER

EXPLOITING THE VITAMIN B_{12} PATHWAY TO ENHANCE ORAL DRUG DELIVERY *VIA* POLYMERIC MICELLES

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6.1. ABSTRACT

Vitamin B_{12} (VB_{12})-modified dextran-*g*-polyoxyethylene cetyl ether (DEX-*g*-POE-C_{16}) was synthesized by linking VB_{12} residues to a DEX-g-POE-C_{16} copolymer via a 2,2'-(ethylenedioxy)bis(ethylamine) spacer. The level of VB_{12} substitution on DEX-*g*-POE-C_{16} copolymer reached 1.68% (w/w). Cyclosporin A (CsA), a poorly-water soluble immunosuppressant, was selected as model drug. CsA-loaded polymeric micelles were prepared by a dialysis procedure. CsA permeability across Caco-2 cells in VB_{12}-modified and unmodified polymeric micelles was monitored in the presence and absence of intrinsic factor (IF). Following 24 h of transport, the apical to basal permeability of CsA loaded in VB_{12}-modified DEX-*g*-POE-C_{16} polymeric micelles was significantly higher than that in unmodified micelles. In the case of VB_{12}-targeted micelles, the amount of transported CsA increased by 1.8 and 2.3 times in absence and presence of IF, respectively, compared to unmodified micelles. Therefore, VB_{12}-modified polymeric micelles enhanced the permeability of CsA across intestinal cells, a promising feature for the development of novel targeted polymeric drug carriers for the oral delivery of poorly-water soluble drugs.

6.2. KEYWORDS.

Polymeric micelles; Vitamin B_{12}; synthesis; Poorly-water soluble drugs; Cyclosporin A; Oral, Caco-2, Permeability.

6.3. INTRODUCTION

Over the last few years, we have assessed the applications of polysaccharide-based micelles as vehicles for the oral delivery of poorly-water soluble drugs (Francis *et al.*, 2003b; Francis *et al.*, 2004; Francis *et al.*, 2005a). The micelles were formed by self-assembly of hydrophobically-modified polymers consisting of a dextran (DEX) or hydroxypropylcellulose (HPC) backbone to which were linked at random a small number of hexadecyl groups. Unlike surfactant micelles, which tend to disintegrate upon dilution triggering lysis of cell membranes (Lasic, 1992; Hofland *et al.*, 1992), the polymeric micelles were remarkably stable towards dilution and exhibited minimal cytotoxicity (Yu *et al.*, 1998; Kim *et al.*, 2001; Jevprasesphant *et al.*, 2003). Their ability to act as delivery agents for hydrophobic drugs was demonstrated in the case of cyclosporin A (CsA), a notoriously water insoluble drug with poor bioabsorption properties. The micelles exhibited high entrapment efficiency for CsA (22% *w/w*), small particle size (11 nm), and very low onset of micellization (4 mg/l), all promising characteristics of carriers for the oral delivery of poorly-water soluble drugs (Francis *et al.*, 2005a; Francis *et al.*, 2003a). Additionally, the permeability of CsA through model intestinal cell membranes increased significantly, compared to the free drug (Francis *et al.*, 2005a).

In this communication we describe the use of the endogenous intestinal pathway for vitamin B_{12} (VB_{12}) absorption in order to enhance the absorption of hydrophobic drugs entrapped within polysaccharide micelles. Compared to other vitamins, VB_{12} is

unusually large and cannot be taken up by the intestine by simple diffusion. Instead, it is transported through the intestine by receptor-enhanced endocytosis. The process is initiated by complexation of VB_{12} with intrinsic factor (IF), a protein produced in the stomach (Nicolas *et al.*, 1995; Okuda, 1999). Upon reaching the small intestine, this complex binds to IF receptors located in the luminal surface of the intestine (Levine *et al.*, 1984; Tang *et al.*, 1992), stimulating internalization of VB_{12}, which after several hours appears in the portal circulation bound to transcobalamin II (TCII), another VB_{12} binding protein (Bose *et al.*, 1997; Brada *et al.*, 2001). This complex, but highly effective, mechanism has been used previously to lure proteins and peptides though the intestinal wall. For example, the oral absorption of α-interferon, luteinizing hormone releasing hormone analogues, erythropoietin, or granulocyte colony-stimulating factor (G-CSF) (Russell-Jones *et al.*, 1988; Russell-Jones *et al.*, 1995b; Habberfield *et al.*, 1996; Russell-Jones, 1998) was substantially enhanced by linking them to VB_{12}. The approach reported here does not require chemical modification of the drug, instead it relies on the decoration with VB_{12} residues of the corona of micelles entrapping in their hydrophobic core the drug to be transported. Compared to the prodrug approach employed previously, this methodology is amenable to delivery of drugs of suitable solubility characteristic without chemical modification. Note that the amenability of this mechanism to enhance the transport of nanoparticles was demonstrated previously by Russell-Jones et al. (Russell-Jones *et al.*, 1999). The focus of our study is a demonstration of the transport of the encapsulated drug itself.

In the following sections, we describe the synthesis and micellization of VB_{12}-modified DEX-*g*-PEO-C_{16} (Figure 6.1.), and we assess the ability of VB_{12}-modified micelles to entrap CsA. The permeability of CsA loaded within VB_{12}-micelles through intestinal enterocytes, was evaluated *in vitro* using the human colon adenocarcinoma, Caco-2, cells. Monolayers of Caco-2 cells, derived form human colorectal adenocarcinoma, are widely accepted as effective to predict intestinal drug permeability in humans (Delie *et al.*, 1997; Artursson *et al.*, 1997). Caco-2 cell monolayers morphologically resemble small intestinal absorptive cells and express typical small intestinal enzymes and receptors associated with the brush border (Hauri *et al.*, 1985; Matsumoto *et al.*, 1990). It has been shown that, after reaching confluency, Caco-2 cells express both the IF receptor in the apical membrane, and the transporter protein TCII which facilitates the transport of VB_{12} out of the enterocyte to the portal circulation (Dix *et al.*, 1987; Ramanujam *et al.*, 1991). From a comparison of the properties and performance of VB_{12}-modified micelles to those of their precursor, we conclude that the VB_{12} pathway can be used effectively for polymeric micelles and provide options for the delivery of hydrophobic drugs.

6.4. EXPERIMENTAL SECTION

6.4.1. Reagents and materials

Vitamin B_{12} (cobalamin, VB_{12}) 99% was obtained from Amersham Biosciences. Cyclosporin A (CsA) and intrinsic factor from porcine gastric mucosa (IF) were purchased from Sigma. Ethylenediamine (amine 1) 99+% (d = 0.899), 2.2'-(ethylenedioxy)bis(ethylamine) 98%, 1,1'-carbonyldiimidazole (CDI), 4-(dimethylamino)pyridine 99% (DMAP), ammonium hydroxide 28-30%, dimethylsulfoxide (DMSO) 99.9%+ and hydrochloric acid 37% were purshased from Aldrich Chemical Co. The modified dextran, DEX-*g*-PEO-C_{16} was synthesized as described previously (Francis *et al.*, 2003b; Francis *et al.*, 2003a). It has a molecular weight of 8602 g/mol and the level of C_{16} incorporation is 15 mol%. Isopropanol, acetone and silica gel 60 were purchased from Merck KGaA. The Spectra/Por® 1 dialysis membrane (molecular weight cutoff 6,000-8,000 dalton) was obtained from Spectrum Laboratories, Inc. The Caco-2 cell line was purchased from American Type Culture Collection (ATCC) at passage 18. Dulbecco's modified Eagle medium (DMEM), penicillin-streptomycin (10,000 U/ml penicillin G and 10,000 µg/ml streptomycin), fetal bovine serum (FBS), 0.25% (w/v) trypsin – 1 mM EDTA.4Na (1X) and non-essential amino acids (NEAA) were supplied from Invitrogen Life Technologies. Poly(ethylene oxide)$_{27}$-*b*-poly(propylene oxide)$_{39}$-*b*-poly(ethylene oxide)$_{27}$ (Pluronic P85®, P85) was provided by BASF Corp. [^{3}H] Cyclosporin A (8.00

Ci/mmol) was purchased from Amersham Pharmacia Biotech. Ultrapure water, used for all aqueous solutions, was from a Milli-Q Filtration system (Millipore).

6.4.2. Synthesis of Dextran-*g*-PEO-C16-amine-VB12 copolymer

Dimethylaminopyridine (DMAP) (55 mg) was added to a solution of DEX-*g*-PEO-C_{16} (275 mg) in DMSO (25 ml), kept at room temperature under N_2. The resulting mixture was stirred for two hours. A 2,2-(Ethylenedioxy)bis(ethylamine)-modified VB_{12} (70 mg) was added in one portion to the solution. The reaction mixture was kept at room temperature under N_2 for three days. Dilute aqueous HCl (0.1M) was added drop wise until neutral pH was attained. The reaction mixture was transferred into a dialysis membrane (MW cutoff 3,500 dalton) and extensively dialyzed against distilled water. The product was isolated by freeze-drying. The degree of VB_{12} substitution was determined spectrophotometrically using a calibration curve prepared with standard solutions of VB_{12} in DMSO (concentration: $6.3x10^{-3} - 5x10^{-2}$ mg/ml, $\lambda = 360$ nm, $r^2 > 0.99$).

6.4.3. Instrumentation

^1H NMR spectra were recorded with Bruker ARX-400 400 MHz spectrometer. Fluorescence spectra were obtained using a SPEX Industries Fluorolog 212 spectrometer equipped with a GRAMS/32 data analysis system. UV-visible spectra were measured with with a Hewlett Packard 8454 A Photodiode array spectrometer,

equipped with a Hewlett-Packard 89090 temperature controller. Light scattering measurements were carried out using a Malvern system (Malvern Instruments Ltd).

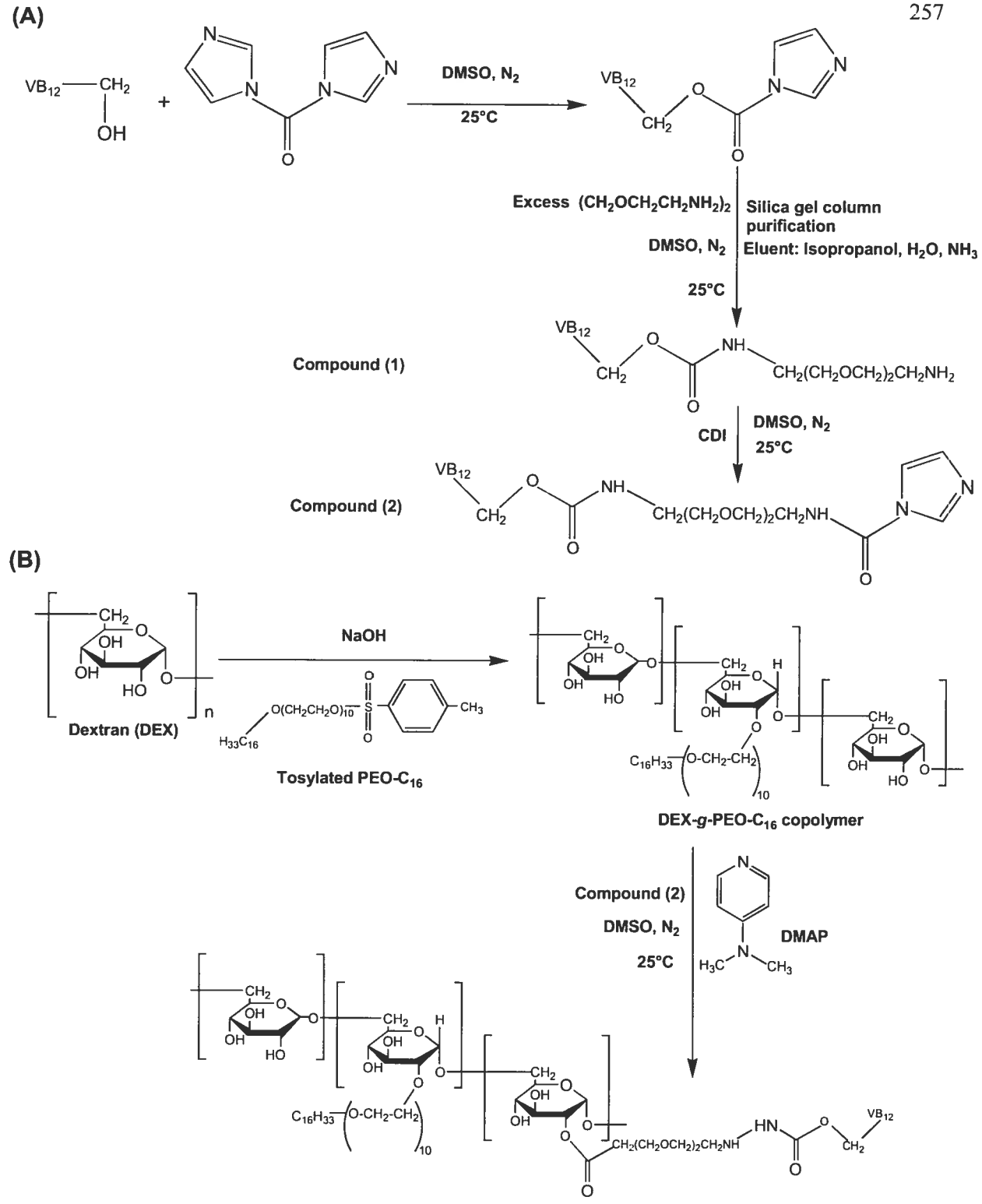

Figure 6.1. Synthetic sequence for the preparation of VB_{12}-modified DEX-*g*-PEO-C_{16} copolymer.

6.4.4. Characterization of VB_{12}-DEX-*g*-PEO-C_{16} copolymer

The critical association concentration (CAC) was estimated by a fluorescence assay (Zhao *et al.*, 1990) using pyrene as a probe, as describe previously in the case of DEX-*g*-PEO-C_{16} micelles (Francis *et al.*, 2003a). Polymer solutions of increasing concentration were prepared in pyrene-saturated water ([Py] ~ 7 x 10^{-7} M). They were equilibrated overnight prior to spectroscopic analysis. The changes in the ratio of the pyrene excitation spectra intensities was monitored at λ = 333 nm (I_{333}) for pyrene in water, and λ = 336 nm (I_{336}) for pyrene in the hydrophobic medium within the micelle core. The hydrodynamic diameter of drug-free and drug-loaded polymeric micelles in water was evaluated by dynamic laser light scattering (DLS) at 25°C with a scattering angle of 90°. Micelle aqueous solutions (3 mg/ml) were prepared for analysis.

6.4.5. Physical loading of CsA in VB_{12}-DEX-*g*-PEO-C_{16} polymeric micelles

CsA was incorporated into VB_{12}-DEX-*g*-PEO-C_{16} micelles by a dialysis process (Francis *et al.*, 2003a; Francis *et al.*, 2005b). The amount of CsA within polymeric micelles was determined by an HPLC assay (Ugazio *et al.*, 2002; Francis *et al.*, 2003a; Francis *et al.*, 2005b).

6.4.6. Cell Culture

The human colon adenocarcinoma cells, Caco-2, were routinely maintained in Dulbecco's modified Eagle medium with 4.5 g/l D-glucose, supplemented with 10%

(*v*/*v*) heat-inactivated fetal bovine serum, 1% (*v*/*v*) non-essential amino acids and 1% (*v*/*v*) penicillin-streptomycin antibiotics solution (100 U/ml penicillin G and 100 μg/ml streptomycin). Cells were allowed to grow in a monolayer culture in 75 cm^2 T-flasks in an incubator at 37 °C with controlled atmosphere containing 5% CO_2 and 90% relative humidity. Culture medium was changed every 48 h. Cells were passaged at 80 - 90% confluency at a split ratio of 1:3 using 0.25% trypsin – 1 mM EDTA.

6.4.7. Permeability of CsA loaded in VB_{12}-DEX-*g*-PEO-C_{16} polymeric micelles across Caco-2 cells

The amount of CsA incorporated into VB_{12}-DEX-*g*-PEO-C_{16} micelles that is transported across a Caco-2 cell monolayer (Figure 6.2.) was measured using tritiated [^{3}H] CsA, following a protocol described previously for DEX-*g*-PEO-C_{16} micelles (Francis *et al.*, 2005a). The apparent permeability coefficients (P_{app}) of CsA expressed in cm/s, were calculated using the following Equation:

$$P_{app}\ (\text{cm/s}) = \frac{1}{ACo}.\frac{dQ}{dt} \qquad \text{.....Equation (1)}$$

where dQ/dt is the rate of appearance of CsA on the receiver side (pmol/s), A is the surface area of the monolayer and C_o is the initial CsA concentration (pM) on the donor side at $t = 0$. All experiments were performed in triplicate. The data presented are the mean ± S.D., standardized on individual well protein concentrations. The differences between the mean values were analyzed for significance using ANOVA test. Results were considered statistically significant from the control when $P < 0.05$.

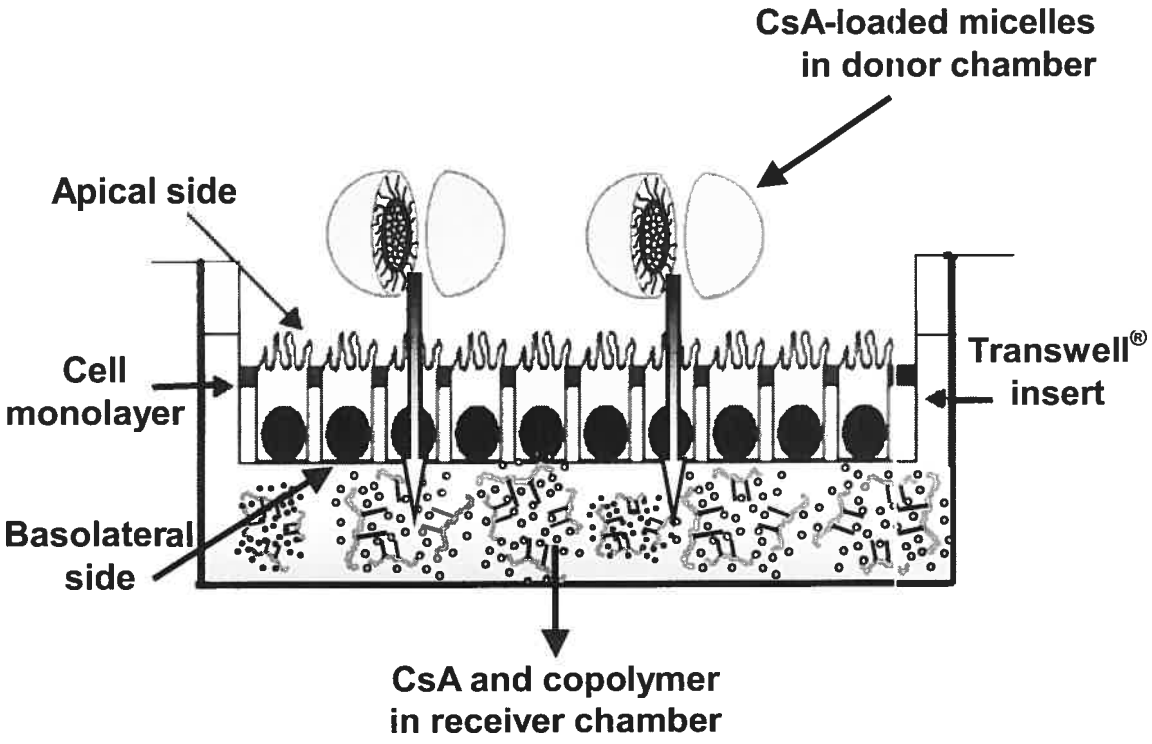

Figure 6.2. Schematic representation of the procedure used to stucy the transport of CsA-loaded polymeric micelles across Caco-2 cell monolayer.

6.5. RESULTS AND DISCUSSION

6.5.1. Preparation and micellization of VB_{12}-DEX-*g*-PEO-C_{16}

The 5'OH group of the ribose moiety VB_{12} was selected as linking site, following a procedure developed by Jones et al. (Russell-Jones *et al.*, 1995a; McEwan *et al.*, 1999). The synthesis consists in two steps, first covalent linkage of a bifunctional spacer moiety to the 5'OH group of VB_{12}, followed by reaction of the linker terminal position to DEX-*g*-PEO-C_{16}. Ethylenedioxybis(ethylamine) was selected as linker, in view of its water solubility and chain flexibility. It was added in large excess to carbonyldiimidazole (CDI) activated VB_{12}. Next, the terminal amine of the linker also activated with CDI was bound to hydroxyl groups of DEX-*g*-PEO-C_{16}, yielding VB_{12}-DEX-*g*-PEO-C_{16} (Figure 6.1.). The level of VB_{12} incorporation along the polymer chain, determined by absorbance spectroscopy, was 1.68 w/w %, or 0.33 mol %, relative to the number of glucose units. The level of VB_{12} was kept low in order to preserve the micellization properties of DEX-*g*-PEO-C_{16} and the ability of the micelles to entrap CsA, two important properties that needed to be confirmed.

Table 6.1. Characteristics of unmodified and VB12-modified DEX-g-PEO-C16 polymeric micelles.

Sample	CAC[a] (mg/l)	Maximum CsA loading[b] (%)	Mean diameter[c] (nm) ± SD	
			CsA-free micelles	CsA-loaded micelles
Unmodified DEX-*g*-PEO-C_{16}	3.8 ± 0.2	8.5 ± 0.6	11 ± 5	14 ± 6
VB_{12}-modified DEX-*g*-PEO-C_{16}	7.4 ± 0.4	6.5 ± 0.4	31 ± 2	33 ± 3

[a]Determined by change in $I_{336\ nm}/I_{333\ nm}$ ratio of pyrene fluorescence with log polymer concentration at 25 °C.

[b]Determined by HPLC analysis with UV detection at 210 nm.

[c]Determined by DLS measurements at 25 °C with a scattering angle of 90 °.

Dynamic light scattering measurements carried out with aqueous solutions of VB_{12}-DEX-*g*-PEO-C_{16} confirmed the presence of patricles ~ 31 nm in size, a diameter nearly three times larger than that of the DEX-*g*-PEO-C_{16} micelles (~ 11 nm). In both cases, the size distribution was unimodal, indicative of the absence of detectable amounts of either free polymer chains or large aggregates. The onset of micellization in solutions of VB_{12}-DEX-*g*-PEO-C_{16}, or critical association concentration (CAC), was determined by a fluorescence probe technique, using the changes in pyrene photophysical characteristics upon solubilization of Py within the hydrophobic core of micelles (Zhao *et al.*, 1990). The CAC of VB_{12}-DEX-*g*-PEO-C_{16} turned out to be higher than that of the parent copolymer, but it remained sufficiently low to prevent demicellization to occur upon dilution (Table 6.1.). Taken together, the light scattering results and the fluorescence measurements indicate that the conjugation of VB_{12} onto modified dextran did not preclude micellization, but the bulk and hydrophilicity of the VB_{12} moiety led to the formation of larger micelles and an increase in the polymer concentration needed for micellization to occur.

6.5.2. Incorporation of CsA in VB_{12}-DEX-*g*-PEO-C_{16} micelles

The extent of CsA incorporation *via* a solvent exchange dialysis process into the polymeric micelles was assessed as a function of the initial CsA/polymer weight ratio, keeping the polymer concentration constant (2.5 mg/ml) and varying the amount of CsA added to the initial dialysis mixtures (2.5 – 40 % *w/w*). In both cases, the level of CsA incorporation increased rapidly with increasing initial CsA concentration above a

minimal threshold value of ~15 w/w % (Figure 6.3.). The amount of CsA incorporation reached a saturation value of ~ 8.5 % in the case of VB_{12}-modified micelles, a value slightly lower than that measured for CsA loading in unmodified micelles, possibly reflecting the increased hydrophilicity of the VB_{12}-modified micelles. In all cases, however, the CsA loaded micelle isolated in the dried form by lyophilization were readily dispersible in water, forming micelles slightly larger in size than virgin micelles (Table 6.1), with no trace of unbound CsA.

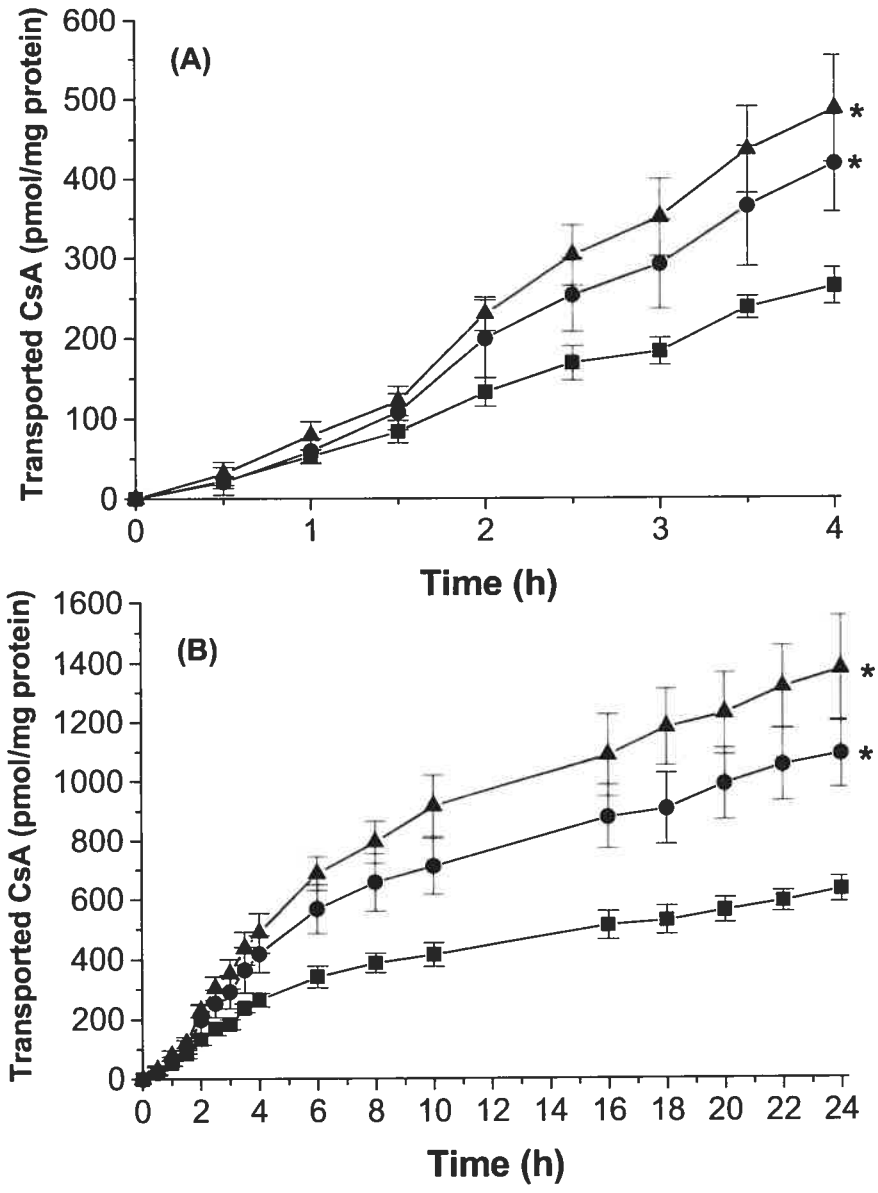

Figure 6.3. Permeability study in the AP-BL direction across Caco-2 monolayers following (A) 4-h and (B) 24-h incubation with CsA loaded in (■) DEX-*g*-PEO-C_{16} polymeric micelles, and VB_{12}-modified DEX-*g*-PEO-C_{16} polymeric micelles in absence(●) and presence (▲) of intrinsic factor. Mean ± S.D. (n = 3). (*) Statistically significant compared to CsA-loaded in DEX-*g*-PEO-C_{16} polymeric micelles.

6.5.3. Uptake and transport of CsA incorporated within VB_{12}-conjugated micelles by Caco-2 cells

Receptor-mediated endocytosis and transport of radiolabeled CsA in VB_{12}-modified micelles were assessed next *in vitro* using Caco-2 monolayers, as depicted schematically in Figure 6.2. The VB_{12}-modified micelles were added alone or in the presence of intrinsic factor (IF) to the apical side of Caco-2 cell monolayers, which were incubated for 4 h or 24 h at 37 °C after addition of the micelles. Experiments were conducted under identical conditions with CsA-loaded micelles devoid of VB_{12}. Subsequently, the amount of CsA transcytosed was determined *via* radioactivity measurements. The results presented in Figure 6.4. point to a significant enhancement of the amount of transported CsA when it is incorporated within VB_{12}-conjugated micelles, compared to the amount of CsA transported by unmodified micelles. The data also suggest that the transcytosis is even more efficient when extrinsic IF is added to the medium, further supporting our hypothesis that the VB_{12}-specific pathway is involved in the transport mechanism of VB_{12}-micelles. Other mechanisms may be in effect as well, since some level of CsA transport through the Caco-2 cell monolayer occurs even in the absence of VB_{12} conjugation, through an intrinsic factor-independent pathway (Muthiah *et al.*, 1987; Russell-Jones *et al.*, 1999). This could be possibly due to the expression of some TCII receptors on the apical surface of Caco-2 cells, as suggested by Bose et al (Bose *et al.*, 1997).

Table 6.2. Permeability coefficient (*P*app, cm/s) values of CsA across Caco-2 monolayers, in absence and presence of intrinsic factor (IF).

CsA-loaded polymeric micelles	***P*app (cm/s) ± SD**	
	4-h incubation	**24-h incubation**
Unmodified DEX-*g*-PEO-C_{16}	4.0 ± 0.2	1.4 ± 0.1
VB_{12}-modified DEX-*g*-PEO-C_{16}	6.5 ± 1.1*	2.6 ± 0.3*
VB_{12}-modified DEX-*g*-PEO-C_{16} + IF	7.7 ± 1.0*	3.3 ± 0.4*

*Statistically significant compared to CsA-loaded in DEX-*g*-PEO-C_{16} polymeric micelles.

We monitored the amount of CsA transported through the Caco-2 cell monolayer for up to 24 h after injection of CsA loaded micelles and determined the CsA concentration in the basal compartment several times (Figure 6.4.B). It is worth noting that the CsA transport trends are the same for the three experiments, featuring a rapid increase in the amount of transported CsA over the first 4 to 5 hours following micelle injection, followed by a continuous, but slower, increase in CsA transport, with no sign of leveling-off after 24 h. Following 24 h of transport, the amount of transcytosed CsA is twice as large in the case of VB_{12} –modified micelles, compared to the naked micelles. These observations are confirmed quantitatively in terms of the apparent permeability of CsA, P_{app}, through Caco-2 cell membranes (Table 6.2.).

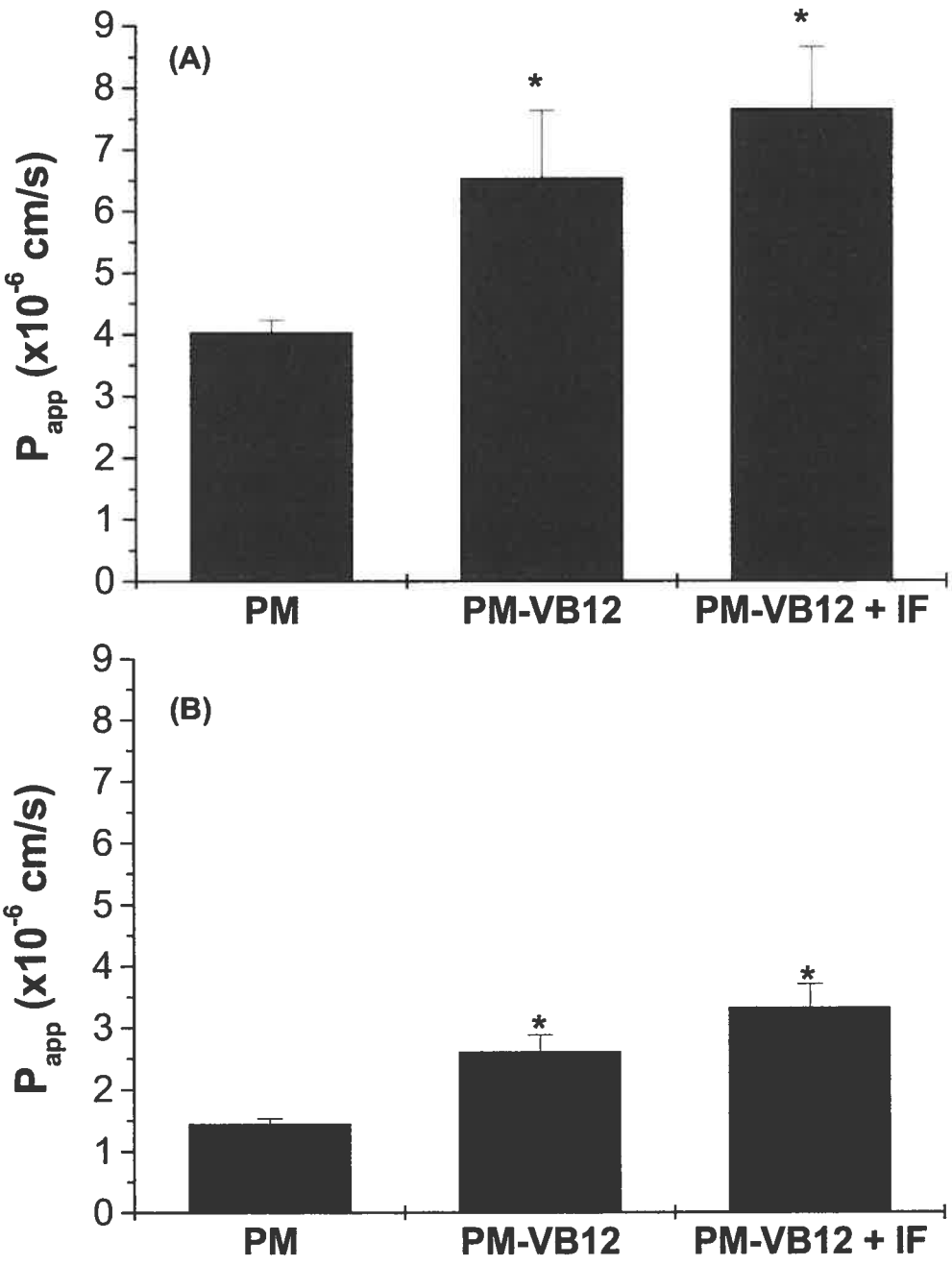

Figure 6.4. Permeability coefficient (*P*app, cm/s) of CsA-loaded in polymeric micelles of DEX-*g*-PEO-C_{16} and VB_{12}-modified DEX-*g*-PEO-C_{16} polymeric micelles in absence and presence of intrinsic factor, following (A) 4-h and (B) 24-h of incubation with Caco-2 cells. Mean ± S.D. (n = 3). (*) Statistically significant compared to CsA-loaded in DEX-*g*-PEO-C_{16} polymeric micelles.

To gain further insight into the transport mechanism, we measured the amount of CsA internalized in the cells, but not expelled in the basal side, as well as the amount of CsA trapped in the filters used in the set-up. Following a 4-h incubation (Figure 6.5.A), the amount of internalized CsA, in the case of CsA entrapped in VB_{12}-micelles is significantly larger that the amount of CsA transported through the membranes. The trend is reversed after a 24-h incubation: the amount of CsA transported through the cell membrane exceeds by a factor of nearly 2, the amount of CsA kept within the cells. We note also that in the case of CsA entrapped in unmodified micelles, there is no difference, after a 4-h incubation, in the amounts of CsA within the cells and in the basal side, whereas after 24-h the transported CsA exceeds the amount of CsA within the cell. Under all conditions, some CsA remained trapped in the filter, but in negligible amounts.

It is generally recognized that there exist a delay of approximately 3 to 4 h (Ramanujam *et al.*, 1991; Rothenberg *et al.*, 1978), between the time of formation of the IF/VB_{12} complex, internalization, the release of VB_{12} in the enterocyte, and the intracellular formation of the TCII/VB_{12} complex eventually released by the cell (Quadros *et al.*, 1999; Pons *et al.*, 2000; Alsenz *et al.*, 2000). Our data reflect this delay, providing further strength to the effectiveness of the VB_{12} pathway in the transport of VB_{12}-decorated micelles.

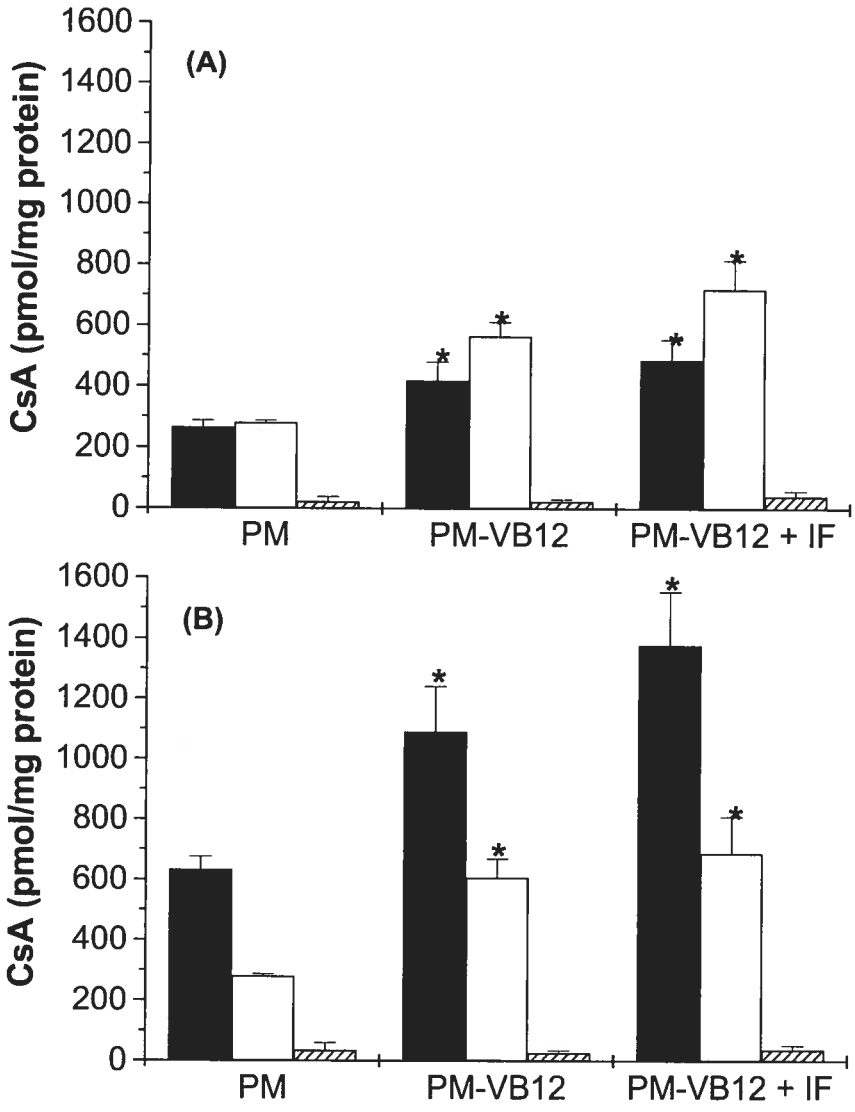

Figure 6.5. Cumulative amount of CsA (pmol/mg protein) loaded in polymeric micelles of DEX-*g*-PEO-C_{16} and VB_{12}-modified DEX-*g*-PEO-C_{16} polymeric micelles in absence and presence of intrinsic factor, following (A) 4-h and (B) 24-h of incubation with Caco-2 cells. The extent of transported CsA (closed columns), internalized CsA (open columns) and CsA bound to filter membrane (dashed columns) are presented in the figure. Mean ± S.D. (n = 3). (*) Statistically significant compared to CsA-loaded in DEX-*g*-PEO-C_{16} polymeric micelles.

Finally, the addition of micelles and/or IF to the apical side of Caco-2 monolayers for up to 24 h did not affect TEER values significantly, suggesting that the integrity of the monolayers was maintained all over the permeability experiment.

6.6. CONCLUSIONS

This investigation demonstrates that linking the large VB_{12} molecule to hydrophobically-modified dextran does not preclude the formation of polymeric micelles of size and stability amenable to drug delivery applications. Moreover we have presented strong evidence that the VB_{12} pathway is effective in the transport of drug loaded VB_{12}-modified micelles through model intestinal cell monolayers, significantly enhancing the amount of drug transported. Overall, the approach offers a promising and realistic option for oral delivery of poorly absorbed hydrophobic drugs.

6.7. ACKNOWLEDGMENT

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CHAPTER SEVEN

GENERAL DISCUSSION

Drug research has evolved and matured through several phases beginning with the botanical phase of the early human civilizations, through to the synthetic chemistry age in the middle of the 20th century, and finally the biotechnology era at the dawn of the 21st century. Although drug discovery and development have undergone a paradigm shift from serendipity to a more rational approach, they remain costly (US$ 400–650 million), time consuming (10 – 15 years) and risky processes (Chess, 1998; Dickson *et al.*, 2004). As a consequence, the pharmaceutical industry is now heading towards a better appreciation and integration of novel drug delivery systems, which are developed at 20% of the cost and in half the time, allowing pharmaceutical companies to maximize the return on their investment through optimization of the dosage regimen without compromising the therapeutic efficacy, giving a second life to old drugs with improved patient compliance (Sahoo *et al.*, 2003; Allen *et al.*, 2004).

There have been great strides in the development of successful commercial peroral novel drug delivery systems for hydrophilic molecules, however, delivery of poorly-water soluble molecules remains a goal difficult to reach. A number of approaches have emerged (Orellana *et al.*, 1998; Sastry *et al.*, 2000) to promote the oral absorption of such molecules, either by use of sorption promoters, or by solubilization in carrier systems as a way for enhancing drug concentration and stability in the harsh environment of the GI tract (Bay *et al.*, 2000).

7.1. SOLUBILIZATION APPROACH

Solubilization is defined as the preparation of a thermodynamically stable solution of a solute that is normally insoluble or very slightly soluble in a given solvent, by the introduction of one or more amphiphilic component(s) (Attwood *et al.*, 1983). The solubilization idea has been in use in the pharmaceutical field since 1868 when it was reported that cholesterol was solubilized to an appreciable extent in the presence of soap (Weissmann *et al.*, 1966). Since then, surfactants have been widely used in development of pharmaceutical formulations for the solubilization of many drugs (Malik *et al.*, 1975; Buckingham *et al.*, 1995; Nerurkar *et al.*, 1996; Patist *et al.*, 1998; Dimitrijevic *et al.*, 2000; Li *et al.*, 2001; Zana, 2002). In addition to strategies investigated for solubilization of poorly-water soluble drugs, such as nanosuspensions (Muller *et al.*, 2001), microemulsions (Itoh *et al.*, 2002) and liposomes (Minato *et al.*, 2003), the use of polymers as active agents in drug formulations has gained much attention. Polymers have long been part of drug formulations as passive ingredients, but it is only recently that polymers have been endowed with specific functions in order to facilitate or target the delivery of drugs. This approach exploits the high diversity of polymers in terms of structure and functionalities that enable conjugation of various pilot molecules (Sakuma *et al.*, 2001).

In the present work, we chose to study "polymeric micelles" which represent a promising delivery vehicle for poorly-water soluble pharmaceutical active ingredients (Halperin, 1987; Kataoka *et al.*, 2001; van Nostrum, 2004).

Hydrophobically-modified (HM) polysaccharides form polymeric micelles in water. The size, stability, and colloidal properties of these micelles depend on their chemical composition, the number of saccharide units and the architecture of the hydrophobic grafts. While a number of fundamental studies of HM-polysaccharides have been reported (Akiyoshi *et al.*, 2000; Pelletier *et al.*, 2001), their use as nanometric carriers of poorly-water soluble drugs has been largely overlooked.

Therefore, we initiated a study of polysaccharide-based micelles as oral drug delivery vehicles using two polysaccharides, dextran and hydroxypropylcellulose, selected as starting materials for the following reasons :

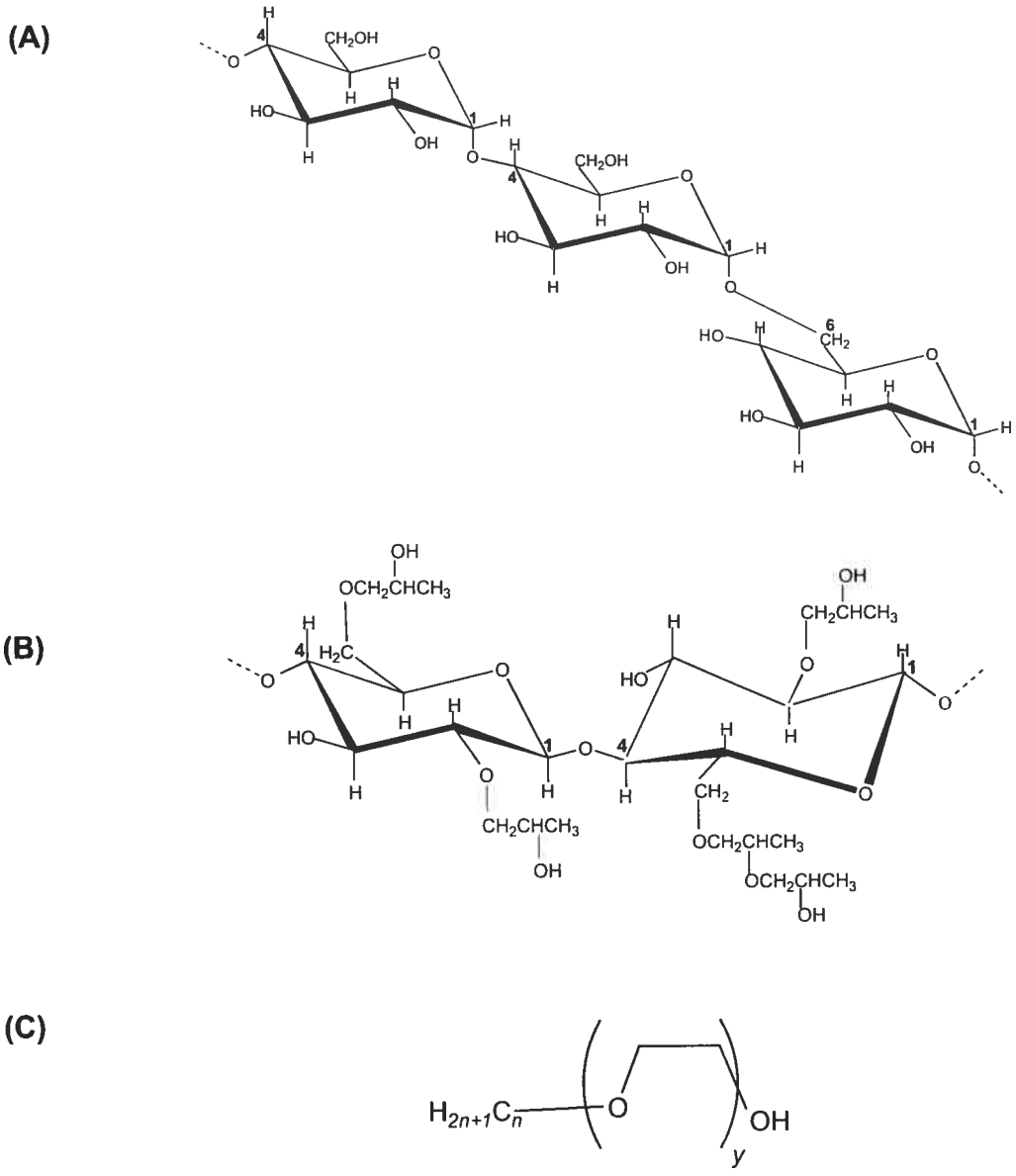

Figure 7.1. Chemical structure of (A) dextran (DEX) showing α(1-6) and α(1-4) glycosidic linkages, (B) Hydroxypropylcellulose (HPC) showing β(1-4) glycosidic linkages, and (C) polyoxyethylene alkyl ether $(POE)_y$-C_n).

7.2. DEXTRAN (DEX)

Dextrans are glucose polymers produced by bacteria growing in sucrose-containing media. The term "Dextran" describes a glucopyranose polymer wherein the α(1-6) glycosidic linkage predominates, although there are also α(1-3) and α(1-4) linkages (Gelin *et al.*, 1961) (Figure 7.1.A).

Dextrans are known to present no toxicity and have been introduced into medicine in the early 1950's by Swedish scientists after a long search for a practical nontoxic plasma substitute (Gronwall, 1957; de Jonge *et al.*, 2001). Since then, dextrans are increasingly used as plasma substitutes (Atik, 1967). Besides their plasma expanding properties, they also exert an anticoagulant effect. They have shown to be effective in preventing postoperative venous thrombosis and pulmonary embolism (Ljungstrom, 1983; Clagett *et al.*, 1998; Bergqvist, 1998). Furthermore, dextrans are readily available and are relatively inexpensive.

7.3. HYDROXYPROPYLCELLULOSE (HPC)

HPC is a non-ionic water-soluble cellulose ether, formed by reaction of cellulose (containing β(1-4) glycosidic linkages) with propylene oxide at high temperature and pressure (Figure 7.1.B). It combines organic solvent and aqueous solubility (Alvarez-Lorenzo *et al.*, 2000).

HPC is used as excipient in many oral solid dosage forms, in which it acts as a binder in granulation (Skinner *et al.*, 1999). *In vivo*, HPC tends to undergo intimate contact

with the absorbing intestinal membrane, thus increasing residence time within the small intestine (Eiamtrakarn *et al.*, 2002). Moreover, HPC is known to present no cytotoxicity (Obara *et al.*, 1992). The World Health Organization (WHO) has not specified an acceptable daily intake for HPC, since the levels consumed are not considered to represent any hazard to health (FAO/WHO, 1990). Finally, HPC is readily available and relatively inexpensive.

DEX and HPC do not form micelles in water, and have very low affinity for CsA as shown in Figure 7.2. Therefore, a modification of their structure was required for them to form micellar assemblies in an aqueous environment. For this, a small number of polyoxyethylene alkyl groups were grafted on DEX and HPC chains.

7.4. POLYOXYETHYLENE ALKYL ETHERS

Polyoxyethylene alkyl ethers ($(POE)_y$-C_n, commercially available under the Brij® trade name) are nonionic surfactants consisting of a hydrophobic alkyl chain attached *via* an ether linkage to a hydrophilic POE chain of varying length (Figure 7.1.C).

To modify either DEX or HPC, $(POE)_y$-C_n residues were grafted by formation of an ether linkage, since this group is more resistant to temperature and pH changes, and is inert towards enzymatic activity in biological fluids, compared to other types of chemical bonds such as the ester linkage (Sovak *et al.*, 1980; Cavallaro *et al.*, 2001; Oishi *et al.*, 2003). This issue was important to consider for the modified polymers in order to ensure stability in the GI environment.

From a formulation point of view, $(POE)_y$-C_n surfactant micelles were shown to be able to incorporate high levels of CsA, varying between 13 – 21 % *w/w* (Figure 7.2. and Table 7.1.), confirming the high affinity of CsA for these molecules. The $(POE)_y$-C_n amphiphiles are readily available and relatively inexpensive. Yet, these surfactants exhibit significant toxicity towards epithelial cells, due to the alteration of cell membrane integrity.

HM-polysaccharides were synthesized *via* ether formation between a tosylated $(POE)_y$-C_n and hydroxyl groups of the corresponding polysaccharide. As the polymers and $(POE)_y$-C_n have similar solubility characteristics, the coupling could be carried out in homogeneous solution. Under these conditions, high levels of hydrophobic modification can be achieved and the distribution of alkyl chains along the polymer chain tends to be random rather than "blocky".

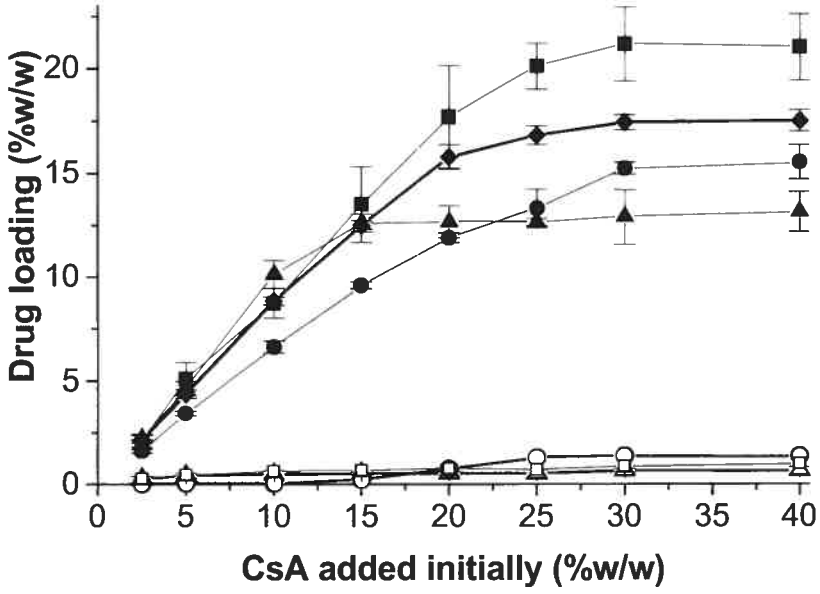

Figure 7.2. CsA loading (% *w*/*w*) in micelles of $(POE)_{10}$-C_{16} (♦), $(POE)_{10}$-C_{18} (▲), $(POE)_{20}$-C_{16} (■) and $(POE)_{20}$-C_{18} (●) surfactants as well as unmodified DEX (MW 10,000Da) (Δ), DEX (MW 40,000Da) (□) and HPC (O) hydrophilic polymers at 2.5 – 40 (*w*/*w* %) CsA initial loading. Mean ± S.D. ($n = 3$).

7.5. DIFFERENT COPOLYMER CANDIDATES

A series of HM copolymer candidates (Table 7.1.) was prepared by varying experimental parameters, such as (*i*) the composition of the hydrophilic polysaccharide backbone (either DEX or HPC), (*ii*) the molecular weight of the hydrophilic chain, for instance, DEX of different molecular weights (10,000 and 40,000 Da or ca 62 and 247 glucose units per chain, for DEX10 and DEX40, respectively) was used; (*iii*) the size of the $(POE)_y$ moiety consisting of 10 or 20 units, (*iv*) the size of the hydrophobic alkyl group (hexadecyl or octadecyl), and (*v*) the level of grafting, *i.e.* the number of hydrophobic substituents linked to the chain.

Table 7.1. Characteristics of different copolymer candidates.

Polymer composition	Grafted $(POE)_y$-C_n (mol %)	CAC (mg/l)	Mean diameter (nm)	Maximum CsA loading (*w*/*w*%)
$(POE)_{10}$-C_{16}	100	2.5 ± 0.3	-	17.5 ± 0.5
$(POE)_{10}$-C_{18}	100	3.0 ± 0.1	-	13.1 ± 0.9
$(POE)_{20}$-C_{16}	100	4.6 ± 0.6	-	21.0 ± 1.5
$(POE)_{20}$-C_{18}	100	3.7 ± 0.5	-	15.5 ± 0.8
Dextran T10	0	-	-	0.6 ± 0.1
Dextran T40	0	-	-	1.0 ± 0.02
HPC	0	-	-	1.4 ± 0.1
DEX10-*g*-$(POE)_{10}$-C_{16}	3.0 ± 0.1	8 ± 1	18 ± 2	4.0 ± 0.1
DEX10-*g*-$(POE)_{10}$-C_{16}	7.0 ± 0.1	7 ± 2	9 ± 1	4.8 ± 0.4
DEX10-*g*-$(POE)_{10}$-C_{16}	15.0 ± 0.5	4 ± 0.2	11 ± 5	8.5 ± 0.6
DEX10-*g*-$(POE)_{10}$-C_{18}	3.9 ± 0.1	13 ± 1	21 ± 1	3.0 ± 0.2
DEX40-*g*-$(POE)_{10}$-C_{16}	2.3 ± 0.1	110.0 ± 5	23 ± 1	1.2 ± 0.1
DEX40-*g*-$(POE)_{10}$-C_{16}	3.5 ± 0.1	18.0 ± 2	30 ± 1	1.5 ± 0.1
HPC-*g*-$(POE)_{10}$-C_{16}	0.9 ± 0.1	75 ± 14	85 ± 2	2.3 ± 0.2
HPC-*g*-$(POE)_{10}$-C_{16}	4.7 ± 0.1	17 ± 3	80 ± 1	5.3 ± 0.3
HPC-*g*-$(POE)_{10}$-C_{16}	5.4 ± 0.5	17 ± 2	76 ± 2	5.5 ± 0.6
HPC-*g*-$(POE)_{20}$-C_{16}	1.1 ± 0.1	65 ± 12	90 ± 1	2.5 ± 0.3
HPC-*g*-$(POE)_{20}$-C_{16}	3.9 ± 0.1	15 ± 5	78 ± 1	6.7 ± 0.4
HPC-*g*-$(POE)_{20}$-C_{18}	1.1 ± 0.1	135 ± 10	85 ± 1	1.7 ± 0.3
HPC-*g*-$(POE)_{20}$-C_{18}	3.1 ± 0.1	22 ± 6	83 ± 2	4.8 ± 0.3

7.6. MICELLAR PROPERTIES OF HM-POLYSACCHARIDES IN WATER

In aqueous solution, HM DEX and HM HPC form polymeric micelles. The major driving force for the assembly of amphiphilic copolymers in water is the removal of hydrophobic fragments from the aqueous surroundings resulting in the formation of micelles consisting of a hydrophobic core stabilized by hydrated hydrophilic chains exposed into water (Gao *et al.*, 1993). A steady-state fluorescence spectroscopy assay was used to determine the polymer concentration at which micellization first takes place. The critical association concentration (CAC) values (Table 7.1.) range from ~ 3 mg/l to ~ 135 mg/l. They depend on (*i*) the nature of the polysaccharide main chain, where the CAC values for HPC-based micelles were generally higher than for DEX-based micelles, (*ii*) the molecular weight of the hydrophilic polymer, where the CAC values of grafted DEX increased with increasing the molecular weight of DEX, and (*iii*) the molar content of $(POE)_y$-C_n residues, where the CAC values decreased with increasing molar content of $(POE)_y$-C_n residues, for each hydrophobic group, and each polymer, reflecting the increase in hydrophobicity of the copolymer and consequent enhanced stability of the polymeric micelles. Similarly, Nagarajan *et al.* have reported that the increase in the length of a hydrophilic polymer chain causes noticeable decrease in CA value and increase in micelle stability in aqueous solution (Nagarajan *et al.*, 1989).

It is often observed that the gastrointestinal (GI) uptake of microparticles is affected significantly by particle size. For example, Desai and coworkers (Desai *et al.*, 1996)

reported that the uptake efficiency of particles ~ 100 nm in diameter by the GI tract is 15 to 250-fold higher than that of micron-sized particles.

The average size of the polysaccharide-based micelles was determined by dynamic laser light scattering (DLS) at 25°C, with a scattering angle of 90°. The hydrodynamic diameter of the polymeric micelles varied depending on the polymer structure, and ranged from 10 - 90 nm (Table 7.1.). HM-DEX and HM-HPC micelles in dilute solution showed a unimodal size distribution, indicative of the absence of free polymer chains and of large aggregates. In both cases, the size of micelles decreases with increasing level of grafting of $(POE)_y$-C_n substituent on the polysaccharide chain. We note that copolymers with longer polysaccharide chain (e.g dextran T40; MW 40 000 Da; 247 glucose units per chain) form micelles of larger mean diameter than those formed by dextran T10 (MW 10 000 Da; 62 glucose units per chain) copolymers, independently of the size of the hydrophobic substituent and of the level of modification. This observation can be taken as an indication of the steric hindrance induced by the carbohydrate chains, which are expected to take place over a larger volume for the polymer of higher molecular weight (Maksimenko *et al.*, 2001; Baldwin *et al.*, 1988).

It should be noted that CsA-free micelles of DEX and HPC showed mean diameters generally different (lower and higher, respectively) from those of the corresponding CsA-loaded micelles, independently of their content in hydrophobic substituent. Although this aspect of the work puzzles us as well, we do not have an explanation and prefer not to add

speculative explanation. Most importantly, the size of the micelles was not altered substantially and the difference was statistically non-significant.

Sass et al. (Sass *et al.*, 1990) showed that the uptake of nanoparticles within the intestine and the extent of drug absorption increase with decreasing particle size and increasing specific surface area. Moreover, Kriwet et al. (Kriwet *et al.*, 1998) showed that neutral nanopatricles have a high affinity to intestinal epitheli than charged nanoparticles, which may also enhance the transport process. Therefore, the small size exhibited by all the polymeric micelles studied here shows a favorable trend towards oral drug delivery.

7.7. MORPHOLOGY OF POLYMERIC MICELLES

It has been well established that the particle sizes and morphologies of micellar aggregates depend not only on the polymer structure (i.e. the polymer composition) but also on preparation conditions (i.e. polymer concentration, solvent, preparation method) (Jones *et al.*, 1999). In literature, a variety of morphologies such as spheres, rods, vesicles, lamellae, large compound micelles, tubules and hexagonally packed hollow hoops have been reported for amphiphilic block copolymer aggregates in dilute solutions, as observed by transmission electron microscopy (TEM) measurements (Zhang, L.F. *et al.*, 1996c; Zhang, L.F. *et al.*, 1996b; Zhang, L. *et al.*, 1996a; Liu *et al.*, 2003; Ouhib *et al.*, 2005).

In aqueous environment, hydrophobically-modified polysaccharide-based copolymers are believed to associate into micelles consisting of a hydrophobic core made up of the alkyl chains surrounded by a hydrophilic corona of highly hydrated

polysaccharide chains. Although Lavasanifar *et al.* reported TEM microphotography for PEO-based micelles prepared by a dialysis method indicating the presence of spherical particles with nanoscopic dimensions (Lavasanifar *et al.*, 2001), at this point, we do not have enough strong data to put forward a structure for the micelles. We rather not base our description of micellar structure solely on our imagination. However, the dynamic light scattering measurements carried out on the CsA-loaded micelles confirmed that the integrity of the micelles was preserved during the loading process, since the size of the micelles was not significantly altered.

7.8. Drug Loading in HM-DEX and HM-HPC Polymeric Micelles

After selecting the best copolymer candidates, we evaluated their ability to solubilize poorly-water soluble drugs. The drug loading capacity of the copolymers was evaluated using Cyclosporin A (CsA), a highly lipophilic undecapeptide selected as model drug. The incorporation of CsA within the hydrophobic core of micelles was performed by a dialysis method. The amount of loaded CsA was quantitatively assayed by HPLC.

We assessed the incorporation of CsA within polymeric micelles in parallel to $(POE)_y$-C_n micelles as well as by unmodified DEX and HPC polymers as controls. The level of CsA incorporation within polymeric micelles, expressed in *w/w* % (CsA/polymer), ranged from ~ 1 – 8 % in the case of HM-DEX and ~ 2 – 7 % in the case of HM-HPC. The loading efficiency varied, depending on the initial CsA/polymer ratio, on the degree of grafting and on the chemical composition of the polymer, as evidenced in Figure 7.3. The

Figure presents CsA loading in the polymeric micelles as a function of the initial CsA/copolymer ratio, in the case of two DEX-based micelles and two HPC-based micelles with the same molar content of hydrophobic residues (~ 3 mol % of either $(POE)_{10}$-C_{16} or $(POE)_{10}$-C_{18} for each polysaccharide) as well as unmodified DEX and HPC as controls. In the case of polymers prepared with DEX10, the amount of CsA loaded within the micelles was 4 % for DEX10-*g*-$(POE)_{10}$-C_{16} (3 mol%), while DEX10-*g*-$(POE)_{10}$-C_{18} (3.9 mol%) resulted in a maximum CsA loading of 3%. CsA displays a higher affinity for HPC than for DEX, where HPC-*g*-$(POE)_y$-C_n micelles incorporated higher amounts of CsA compared to DEX-*g*-$(POE)_y$-C_n micelles, for the same molar content of $(POE)_y$-C_n. This trend is consistent with the inherent hydrophobicity of HPC due to the presence of isopropoxy substituents, known as "the hydrophobic pockets" within the HPC structure, as reported by Klug (Klug, 1971). This heterogenous structure of HPC explains the high affinity of CsA for unmodified HPC, showing a level of CsA incorporation of 1.4 % w/w. The micelles formed by the dextrans of higher molecular weights (DEX40) were not as effective CsA carriers (Table 7.1.). Nonetheless, in all cases the amount of incorporated CsA was larger in the case of polymeric micelles than in the case of unmodified dextrans and HPC (Figure 7.3), which have a much lower affinity for CsA.

By comparing the highest CsA loading percentage in the various polymers, it is apparent that the CsA loading increases with increasing molar content of POE_{10}-C_{16} grafted on the dextran backbone. Other structural features of the copolymers also affect the micellar loading capacity towards CsA. For example, micelles formed by polymers of higher molecular weights (e.g. DEX40) are not as effective CsA carriers as those based on

DEX10. Also, for a constant number of POE_{10}-C_n units grafted per dextran chain, POE_{10}-C_{16}-based micelles seem more effective in solubilizing CsA than POE_{10}-C_{18}-based micelles, indicating that the micropolarity of a hydrophobic core made up of hexadecyl-POE chains might present a better solubilizing microenvironment than that offered by the octadecyl-POE moieties.

It is interesting to note also that, at low CsA initial concentrations (< 15 % *w*/*w*), HPC-*g*-$(POE)_y$-C_n polymeric micelles exhibit a solubilizing/loading trend different from that displayed by DEX-based micelles (Figure 7.3). While the degree of CsA incorporation within DEX-based micelles increases steadily with increasing initial CsA concentration, the level of CsA incorporation in polymeric micelles remains low and nearly constant with initial CsA concentration < 15 %, then increases rapidly as the initial CsA concentration exceeds 15 %.

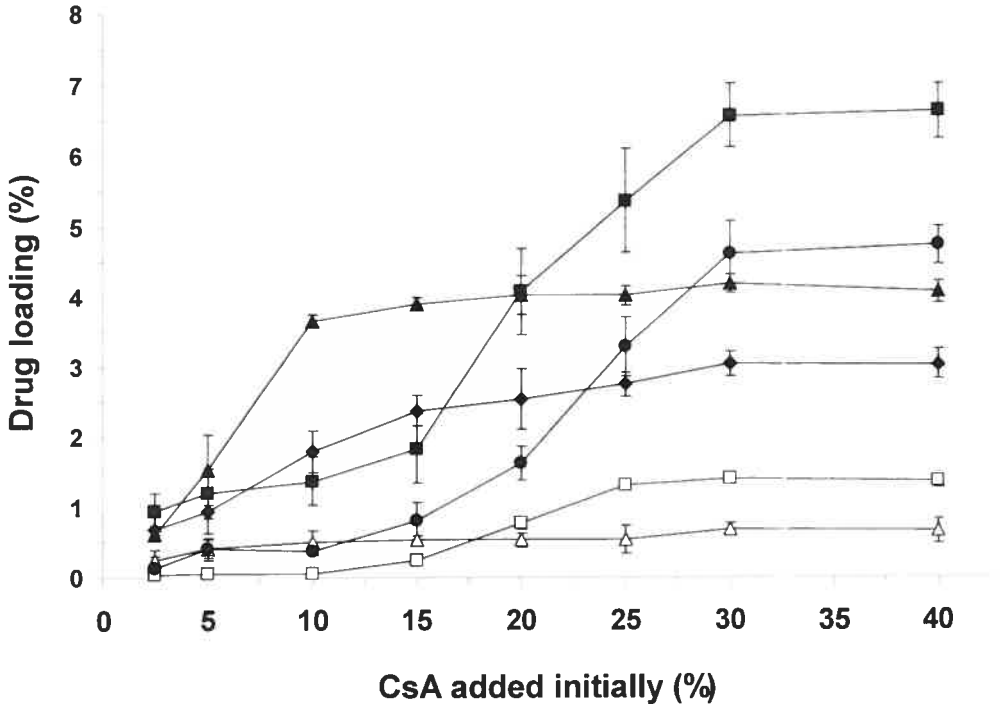

Figure 7.3. CsA final loading (*w/w* %) in micelles of (▲) DEX10-g-$(POE)_{10}$-C_{16} (3 mol%), (♦) DEX10-g-$(POE)_{10}$-C_{18} (3.9 mol%), (■) HPC-g-$(POE)_{10}$-C_{16} (3.9 mol%) and (●) HPC-g-$(POE)_{10}$-C_{18} (3.1 mol%) copolymers at 2.5 – 40 (*w/w* %) of initially added CsA. For comparison, CsA was incorporated in (Δ) unmodified dextran T10 and (□) unmodified HPC polymers. Mean ± SD (n = 3).

7.9. SOLID STATE OF CSA IN MICELLES

Free Cyclosporin A is completely crystalline. It consists of white prismatic needles prepared from acetone at –15°, showing definite specific peaks in its X-ray diffractogram and a melting point at 148 – 151°C (The Merck Index, 13th edition, 2003). Craig et al. (Passerini *et al.*, 2002) showed that, following incorporation into poly(D,L-lactide-co-glycolide) microspheres using the solvent evaporation method, CsA is transformed into a completely amorphous state. Similar behavior has been detected for progesterone within poly(D,L-lactide) microspheres, although at high progesterone loading, a crystalline state was detected resulting in microspheres showing rough surface (Hill *et al.*, 1998).

On the other hand, Zhao et al. reported the presence of an amorphous CsA within CsA-poloxamer solid dispersion prepared using the melt solvent method (Zhao *et al.*, 1997).

In order to determine the solid state of CsA solubilized in the hydrophobic core of polymeric micelles, we carried out X-ray powder diffraction and calorimetric studies on free CsA (as received) and freeze-dried CsA-loaded polymeric micelles in the powder form. Both powder X-ray diffractograms and differential scanning calorimetry thermograms correlated to show the absence of an amorphous form of CsA within polymeric micelles. However, further investigation will be aimed at confirming that CsA crystals preserved the same molecular conformation inside the micelles as in the free form.

7.10. STABILITY OF POLYMERIC MICELLES IN SIMULATED GI FLUIDS

The design of an oral drug delivery system should include stability testing in simulated gastric fluid (pH 1.2) and intestinal fluid (pH 6.8). In order to evaluate the stability of the polymeric micelles in the simulated GI fluids, the release of CsA was evaluated both from HM-DEX and HM-HPC micelles loaded with the drug at saturation. Micellar solutions were placed in a releasing bag separated by a dialysis membrane (6000 - 8000 Da MW cutoff) from a receiving compartment containing gastric or intestinal fluids, which were replaced with fresh fluid following sampling to assure sink conditions. The membrane allowed permeation only to the free drug present in equilibrium with the complex in the releasing cell and not to the CsA-loaded micelles.

Compared to various nanocarrier systems, such as niosomes, liposomes and surfactant micelles (Hu *et al.*, 1999; Ozpolat *et al.*, 2003), HM-polysaccharide based micelles represent a significant improvement by eliminating physical stability problems, and may therefore offer improved bioavailability of poorly soluble drugs. We have demonstrated that polysaccharide-based micelles were highly stable at varying pH values during 8 h of incubation at 37 °C. The high stability of micelles is due to the known stability of the ether linkages linking the $(POE)_{10}$-C_{16} residues to the polysaccharide backbone in the micelle structure. Ether linkage is known to be highly stable towards pH changes and enzymatic reactions, compared to ester linkage. Therefore, the micellar structure of these polymeric carriers is maintained. The release of encapsulated CsA, in each type of fluid, reached a maximum of 12 % and 4 % in the case of DEX- and HPC-

micelles, respectively, indicating that these systems are quite stable towards drug release during the average residence time period of the macromolecular carriers in the GIT before reaching the systemic circulation. In this period the stability of the drug carrier towards release is very important in order to prevent the enzymatic degradation of CsA in the stomach, as well as the metabolizing enzymes cytochrome P-450 3A4 and the multidrug transporter P-glycoprotein in the intestinal barrier.

The drug release from the micelles, however, takes place slowly over time, due to the drug partition between the micelles and the aqueous solution which becomes a dynamic process, promoting drug release from the micelles, when the free drug present in solution is continuously removed by the absorption *in vivo* (Zuccari *et al.*, 2005).

7.11. CYTOTOXICITY ASSAY

The cytotoxicity of new polymers always needs to be performed on the cellular level in order to evaluate the cytotoxicity of the micelle components towards the cell line used, before performing further specific studies. From previous reports, we knew that while both HPC and DEX present no toxicity (Couch, 1965; Obara *et al.*, 1992), free $(POE)_v$-C_n inhibit cell growth by affecting the integrity of cell membranes (Dimitrijevic *et al.*, 2000). Hofland *et al* showed that, the toxicity of alkyl polyoxyethylene surfactants was related to their hydrophilic/lipophilic balance: an increase in alkyl chain length is accompanied by a decrease in toxicity, while an increase in the polyoxyethylene chain length causes an increase in toxicity (Hofland *et al.*, 1992).

Therefore, in the present study, it was important to assess if linking $(POE)_y$-C_n chains to a polysaccharide framework would alleviate their toxicity. The evaluation of polymers cytotoxicity was carried out using the colorimetric MTT assay. The test is based on mitochondrial dehydrogenase cell activity as an indicator of cell viability. We carried out toxicity tests for DEX, HPC, various $(POE)_y$-C_n, and the different copolymers towards human intestinal epithelial cells (Caco-2 cells), which are widely used to investigate the intestinal absorption mechanisms of drugs (Krishna *et al.*, 2001). The effect of polymer composition, concentration and incubation time on intestinal cell viability was examined. As expected, DEX and HPC show no toxicity at high concentrations, whereas all studied $(POE)_y$-C_n inhibit cell growth, when added to cells at concentrations as low as 0.5 g/l. Like DEX and HPC, the modified polysaccharides DEX-*g*-$(POE)_y$-C_n and HPC-*g*-$(POE)_y$-C_n exhibit no significant cytotoxicity at a concentration as high as 10 g/l, independently of the grafting level. Even in the case of DEX- and HPC-based copolymers with the highest levels of $(POE)_{10}$-C_{16} grafting (15 mol%, and 5.4 mol%, respectively), the cytotoxicity was negligeable compared to equivalent concentrations of free $(POE)_{10}$-C_{16}. These results confirm 1) that upon linking to a polymer chain, $(POE)_y$-C_n loses its cytotoxicity, and 2) that the polymer purification method efficiently removed any free $(POE)_y$-C_n from the polymer. This effect may be due to the fact that the non toxic hydrophilic polysaccharide chains forming the polymeric micelle outer shell stay in contact with the cells and effectively insulate them from the surfactant residues assembled in the inner core of the micelle. For these to escape from the micellar assembly, it is necessary to break an ether bond, known to be stable against hydrolysis over a wide range of pH values.

7.12. CACO-2 PERMEABILITY STUDIES

Absorption of orally administered drugs, a major determinant of bioavailability, is mainly controlled by two key factors; drug solubility in the intestinal lumen and its permeability across the intestinal barrier. Recently, the use of Caco-2, human intestinal epithelial cells, has emerged as a leading method to investigate absorption mechanisms of several classes of potential drugs in the early development stages (Delie *et al.*, 1997; Artursson *et al.*, 1997; Krishna *et al.*, 2001; Kamm *et al.*, 2000; Faassen *et al.*, 2003). In order to gain insight into the absorption of solubilized CsA from the GI tract into the blood stream, we investigated the permeability of CsA entrapped in polymeric micelles across Caco-2 cell monolayers. Caco-2 cells retain many features of small intestinal cells (Delie *et al.*, 1997; Hidalgo *et al.*, 1989; Artursson *et al.*, 2001), and a strong correlation was observed between *in vivo* human absorption and *in vitro* permeability across Caco-2 cells for a variety of compounds (Artursson *et al.*, 1991; Yee, 1997; Grès *et al.*, 1998).

The efflux transporter protein, P-gp, is expressed at the apical side of the Caco-2 monolayer and is not expressed at the basolateral side (Hosoya *et al.*, 1996). The intestinal efflux caused by P-gp acts as an absorption barrier to limit the oral bioavailability of hydrophobic drugs from the gastrointestinal tract (Hunter *et al.*, 1997). In the present study, the bi-directional transport across Caco-2 cells was assayed by liquid scintillation counting to determine the amount of CsA that crossed the cell monolayer. Following 240 min of incubation with Caco-2 cells, the apical to basal permeability of CsA loaded in polymeric micelles was generally higher than that of free CsA. Moreover, the bidirectional transport

of CsA solubilized in HPC-*g*-POE-C_{16} polymeric micelles was higher than that in DEX-*g*-POE-C_{16} micelles, suggesting that HPC micelles adhered to the cell monolayer, allowing a slow diffusion of solubilized CsA to the basal side, while DEX micelles remained suspended in the apical side. A fluorescence spectroscopy technique was used to determine the amount of host polymeric micelles that crossed the cell monolayer. Using fluorescein-labelled micelles, we demonstrated also that the amount of transported HM-HPC is greater than that of HM-DEX copolymer. This property may be attributed to the bioadhesive characteristic of HPC polymers.

It has been demonstrated that, for compounds that are substrates of P-gp, the use of a P–gp inhibitor resulted in a better estimate of absorption in humans (Yee, 1997). Natural or synthetic fatty acid ester based surfactants, such as polysorbates and solutol, have been investigated and were found to inhibit P-gp mediated drug efflux (Woodcock *et al.*, 1992; Nerurkar *et al.*, 1996). Recently, amphiphilic triblock copolymers of poly(ethylene oxide)-*b*-poly(propylene oxide)-*b*-poly(ethylene oxide), also known as Pluronic block copolymers, have been shown to enhance cellular accumulation, membrane permeability, and to modulate multidrug resistance of numerous P-gp substrates (Seeballuck *et al.*, 2003; Batrakova, E.V. *et al.*, 1998; Batrakova, E. *et al.*, 1999a). In the present study, we chose to use Pluronic P85 as P-gp inhibitor since it has been recently approved by the US Food and Drug Administration (FDA) (BASF, 1993). Pluronic P85 has been observed to block P-gp mediated efflux in Caco-2 and bovine brain microvessel endothelial cells (Batrakova *et al.*, 1999) which suggested that this agent may be useful for formulations to enhance oral and brain absorption. On the other hand, several previous studies examining the effects of non-

ionic surfactants on transport in cell monolayers have clearly indicated that the AP-BL transport of drugs that are P-gp substrates increased at surfactant concentrations at or below the CMC (Nerurkar *et al.*, 1996; Batrakova *et al.*, 1998). In contrast, at concentrations above the CMC, the Pluronic block copolymers were found to cause an increased efflux of P-gp substrate from Caco-2 cells (Batrakova *et al.*, 1998). Nerurkar et al. concluded that the free unimers were responsible for inhibiting P-gp efflux transport (Nerurkar *et al.*, 1996). The CMC of Pluronic P85 has been reported to be 67 μM (Miller *et al.*, 1997). In this study, we used a PGI solution containing 30 μM Pluronic P85, to ensure that the copolymer concentration is below the CMC, i.e. P85 copolymer is in the form of unimers. It should be mentioned that the BL-AP transport of CsA in presence of PGI on the basal side was higher than that shown when PGI was added on the apical side, in the case of free as well as micelle-loaded CsA (data not shown), due to the presence of P-gp transporter on the apical side of Caco-2 cells.

7.13. VB_{12}-TARGETED POLYMERIC MICELLES

At this point in our understanding and in an attempt to override such inherent intestinal barrier, we exploited the use of the cobalamin absorption pathway to further enhance the permeability of CsA solubilized in DEX-based micelles, following a receptor-mediated endocytosis mechanism. VB_{12} residues were covalently linked to the surface of polymeric micelles. The permeability of CsA across Caco-2 monolayers increased significantly when loaded in VB_{12}-modified DEX-micelles compared to unmodified DEX-micelles as well as free CsA. Meanwhile, a further increase was observed in intrinsic factor

(IF)-mediated permeability, meaning that, in presence of IF, VB_{12}-modified polymeric micelles were specifically trancytosed across Caco-2 cell monolayers through IF receptors on the apical side. These results appear to be consistent with previous findings on a major role of increased permeability of VB_{12}-conjugates across Caco-2 cells through receptor-mediated endocytosis mechanism (Russell-Jones *et al.*, 1995; Alsenz *et al.*, 2000). However, Moestrup *et al.* proposed that, in absence of IF, the VB_{12}-conjugate binds to intracellular TCII, secreted unidirectionally from AP to BL side in Caco-2 cultures (Ramanujam *et al.*, 1991), and the complex is internalized by the cells and transported to the BL side of the monolayer (Moestrup *et al.*, 1996).

In order to localize internalized CsA within Caco-2 cells, its presence was investigated in cell debris as well as in the insert filter membrane, where CsA may be trapped and could not move into the BL chamber of the Transwell. The results demonstrated that; following 4-h of incubation, although the amount of CsA transported to the BL chamber was not negligible, the amount of CsA internalized by the cells was higher. However, the amount of CsA trapped in the filter was negligible and remained constant in all cases. In the case of 24-h incubation period, the amount of transported CsA was higher than that internalized by the cells, while the entrapment of CsA in filter membranes remained negligible. However, it should be noted that the amount of CsA internalized within the cells after 24 h remained comparable to that after 4 h of incubation with Caco-2 cells.

It has been shown that, after the binding of IF-Cbl, there is a delay where the complex is internalized and Cbl is released inside the enterocyte. The TCII-Cbl complex is then formed intracellularly (Quadros *et al.*, 1999; Pons *et al.*, 2000). It has been suggested that the transfer of Cbl from IF to TCII occurs within a delay of 3 - 4 h (Rothenberg *et al.*, 1978; Ramanujam *et al.*, 1991). Further more, Alsenz *et al.* (Alsenz *et al.*, 2000) showed that the pharmacokinetics of orally administered Cbl and of Cbl conjugates were very similar after 4h, where the highest tissue concentration in the small intestine with almost no urinary excretion of Cbl were observed after 4h. On the other hand, between 4 h and 24 h, most of the Cbl conjugates disappeared from the small intestine and high concentrations were localized in the kidney and liver with little urinary excretion.

7.14. CONCLUSION

In this study, optimized polysaccharide-based polymeric micelle formulations with improved chemical structures and physicochemical characteristics are proposed for the oral delivery of poorly soluble therapeutic agents. We succeeded in enhancing the aqueous solubility of CsA by reaching relatively high solubilization capacity in the inner core of polysaccharide-based polymeric micelles, and consequently increasing its permeability across model intestinal epithelium, compared to free CsA. Therefore, the polysaccharide-based polymeric micelles offer unique opportunities for the oral delivery of poorly-water soluble drugs, namely:

1) The small size of polymeric micelles and their very low onset of micellization.

2) The high encapsulation capacity
3) The stability of the micelle system in simulated biological fluids.
4) The absence of cytotoxicity towards intestinal epithelial cells.
5) The ability to enhance the permeability of solubilized drug across the intestinal barrier *versus* free drug.

Consequently, it would be expected that:

1) The nanoscopic size of polymeric micelles would result in an enhanced intracellular drug concentration, since the uptake is size-dependent; where smaller particles are taken up to higher degree than larger particles.
2) The stability of the drug in biological fluids would increase through micelle incorporation, by decreasing contacts with inactivating species (enzymes) in the gastrointestinal fluid (Yokoyama *et al.*, 1990).
3) This may lead to decrease CsA administered dose.
4) Consequently, the undesirable systemic side effects of CsA are expected to decrease.

To the best of our knowledge, this is the first time that the permeability of a drug and its carrier across Caco-2 monolayers is studied.

Collectively, the results of this research will aid in understanding the relationship between structural features of polysaccharide-based carriers in solubilizing lipophilic drugs and their intestinal permeability, with the prospect of designing novel polymeric carriers for oral drug delivery. The use of this delivery approach can, in principle, modulate both the

pharmacokinetic behaviour and bioavailability of the drug, resulting in an overall increase in the drug therapeutic index.

7.15. IMPACT ON THE BIOPHARMACEUTICAL INDUSTRY

In recent years, Canada has experienced a tremendous development of the pharmaceutical industry. In the area of drug formulation, research on polymeric micelles is growing in Canada. At least two companies, Supratek Pharma Inc. (Montreal, QC) and Angiotech Pharmaceuticals Inc. (Vancouver, BC) have patented formulations based on polymeric micelles for parenteral delivery. Since oral dosage forms represent more than 90% of all drug formulations, the development and long term commercialization of an efficient oral vehicle for poorly-water soluble drugs could have tremendous economical benefits for Canada. For example, the sales of CsA oral microemulsion (Neoral®) have generated 54 million dollars in Canada in 2003 (IMS Health Canada). The present project offers new options for oral delivery of poorly-water soluble drugs, thus warranting further developments in pharmaceutical industry.

7.16. REFERENCES

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Zhang, L. F., Yu, K. and Eisenberg, A. (1996c) *Ion-Induced Morphological Changes in "Crew-Cut" Aggregates of Amphiphilic Block Copolymers.* Science, 272, 1777-1779.

Zhao, H., Zheng, J. M. and Zhang, H. H. (1997) *Studies on some physical characteristics of cyclosporine A (CyA) solid dispersions.* Yao Xue Xue Bao, 32, 777-781.

Zuccari, G., Carosio, R., Fini, A., Montaldo, P. G. and Orienti, I. (2005) *Modified polyvinylalcohol for encapsulation of all-trans-retinoic acid in polymeric micelles.* J. Control. Release, 103, 369-380.

CHAPTER EIGHT

SUMMARY AND PERSPECTIVE

There is a great need to increase the ability to deliver the broad range of poorly-water soluble drugs efficiently by the peroral route of administration. This thesis describes polysaccharide-based delivery approaches and targeting schemes for manipulating such therapeutic agents, and enhancing their transport across the transcellular pathway in the GI tract.

This study has increased our understanding of the effect of the chemical structure of amphiphilic copolymers on their efficiency to encapsulate lipophilic drugs, a crucial property to allow their transcellular transport from the small intestine to the systemic circulation following oral administration. Initial work on targeting *via* VB_{12}-absorption pathway neds to be continued to take full advantage of the flexibility of a polymeric micelle structure, in order to enhance the oral absorption of drugs *via* specific uptake pathways across the intestinal epithelium.

To achieve the final goal of producing polymeric micelles acting as effective oral drug carriers, further aspects must be examined before starting the maturation process from *in vitro* investigations to *in vivo* studies. For instance, the solid state of CsA solubilized within the micelle hydrophobic core needs to be investigated on the molecular level. Quantitative studies aimed at enhancing the drug loading in the micellar system are also needed. Three specific areas appear to be very important for the production of polymeric micelle vehicles able to overcome the limited success in this area: that is, increasing the grafting level of the hydrophobic residues in the micelle structure without losing their solubility, using different hydrophobic moieties and polysaccharide of various MW, and by

using other methods of drug loading such as the direct dissolution method and the oil-in-water emulsion method. The bioadhesive properties of HPC-based micelles need to be evaluated more in depth, towards the intestinal mucin layer. Finally, the biological fate (metabolism/elimination) of the entrapped CsA and of the polymeric carriers needs to be investigated. These basic studies will put efficient tools at the pharmaceutical scientist's disposal, bringing new means for enabling lipophilic drugs to overcome the GI tract absorption barrier.

The information gathered through this research is a step towards the realization of the full potential of polymeric micelle-based oral dosage forms with economically viable commercial-scale production, so as to bridge the gap between a research concept and market products. Although a recipe for immediate success is beyond the scope of this work is certainly difficult to offer, it would seem that the use of polymeric micelle system as a chemical passport for the oral delivery of therapeutics remains a field rich in opportunities, with many interesting aspects to explore.

Appendix I. Publications and Honors

Academic History

2001 –	Ph.D. candidate, Faculty of Pharmacy "Pharmaceutical Technology", University of Montreal, Montreal (Quebec) Canada.
1999 – 2001	Master degree, Faculty of Pharmacy "Pharmaceutical Technology", University of Montreal, Montreal (Quebec) Canada
1993 – 1998	B. Pharm., Faculty of Pharmacy, University of Alexandria, Alexandria, Egypt

Publications

I- Scientific Papers

9. "Exploiting The Vitamin B_{12} Pathway To Enhance Oral Drug Delivery *via* Polymeric Micelles"
Mira F. Francis, Mariana Cristea and Françoise M. Winnik.
Biomacromolecules, ***2005***, submitted.

8. "Engineering Polysaccharide-Based Polymeric Micelles To Enhance Permeability of Cyclosporin A Across Caco-2 Cells"
Mira F. Francis, Mariana Cristea, Yali Yang and Françoise M. Winnik.
Pharmaceutical Research, **2005**,22, 209-219.

7. "Polymeric Micelles For Oral Drug Delivery: Why and How"
Mira F. Francis, Mariana Cristea and Françoise M. Winnik.
Pure and Applied Chemistry (the official Journal of the International Union of Pure and Applied Chemistry (IUPAC), **2004**, 76, 1321-1335.

6. "Solubilization of Poorly-Water Soluble Drugs in Micelles of Hydrophobically-Modified Hydroxypropylcellulose copolymers"
Mira F. Francis, Mariella Piredda and Françoise M. Winnik.
Journal of Controlled Release, **2003**, 93; 59-68.

5. "Synthesis and Evaluation of Hydrophobically-modified polysaccharides as Oral Delivery Vehicles of Poorly-Water Soluble Drugs"
Mira F. Francis, Mariella Piredda, Mariana Cristea and Françoise M. Winnik.
Polymeric Materials: Science & Engineering (ACS Polymer Preprint), **2003**; 89; 55-56.

4. "Solubilization of Cyclosporin A in Dextran-g-Polyethyleneglycolalkyl Ether Polymeric Micelles"
Mira F. Francis, Luc Lavoie, Françoise M. Winnik and Jean-Christophe Leroux.
European Journal of Pharmaceutics and Biopharmaceutics, **2003**, 56; 337-346.

3. "Polymer based pH-sensitive carriers as a means to improve the cytoplasmic delivery of drugs"
Emmanuelle Roux, **Mira Francis**, Françoise M. Winnik and Jean-Christophe Leroux
International Journal of Pharmaceutics, **2002**; 242; 25-36.

2. "Preparation and Tumor Cell Uptake of Poly-(N-isopropylacrylamide) Folate Conjugates"
Denis Dubé, **Mira Francis**, Jean-Christophe Leroux and Françoise M. Winnik.
Bioconjugate Chemistry, **2002**; 13; 685-692.

1. "Characterization of pH-Sensitive Polymer/Niosome Complexes"
Mira F. Francis, Ganga Dhara, Françoise M. Winnik and Jean-Christophe Leroux.
Biomacromolecules, **2001**; 2; 741-749.

II- Book Chapters

3. "Hydroxypropylcellulose In Oral Drug Delivery"
Mira F. Francis, Mariella Piredda and Françoise M. Winnik
In: Marchessault R. (Ed), *Polysaccharides in Drug Delivery and Pharmaceutical Applications*, American Chemical Society, Washington (DC); **2005**, submitted.

2. "Synthesis and Evaluation of Hydrophobically-modified polysaccharides as Oral Delivery Vehicles For Poorly-Water Soluble Drugs"
Mira F. Francis, Mariella Piredda, Mariana Cristea and Françoise M. Winnik
In: Svenson S. (Ed), *Polymeric Drug Delivery: Science & Application*, American Chemical Society, Washington (DC); **2005**, in press.

1. "Stimuli-responsive liposome-polymer complexes: Towards the Design of Intelligent Drug Carriers"
Emmanuelle Roux, **Mira Francis**, Françoise M. Winnik and Jean-Christophe Leroux.
In: Svenson S. (Ed), *Carrier-Based Drug Delivery*, American Chemical Society, Washington (DC); **2004**; pp. 26-39.

III- Abstracts

16. **"Improving Cyclosporin A Permeability Across Caco-2 Cell Monolayers By Using Vitamin B_{12}-Targeted Polymeric Micelles"**
Mira F. Francis, Mariana Cristea and Françoise M. Winnik.
AAPS Pharm. Sci. Supplement, 2004; 6(4), Abstract W4018.
AAPS (American Association of Pharmaceutical Scientists) Annual Meeting and Exposition, 2004, November 7-11, Baltimore (MD), U.S.A.

15. **"Hydrophobically-Modified Hydroxypropylcellulose Based Polymeric Micelles For Oral Delivery of Poorly-Water Soluble Drugs"**
Mira F. Francis, Mariella Piredda and Françoise M. Winnik.
Journal of Pharmacy and Pharmaceutical Sciences, 2004; 7(2), 71-72.
Canadian Society for Pharmaceutical Sciences (CSPS) 7th Annual Symposium on Pharmaceutical Sciences, 2004, June 9-12, Vancouver (BC), Canada

14. **"Solubilization of Poorly-Water Soluble Drugs Using Hydroxypropylcellulose-g-Poly (Ethylene Glycol) Cetyl Ether Polymeric Micelles For Oral Delivery"**
Mira F. Francis*, Mariella Piredda and Françoise M. Winnik.
AAPS Pharm. Sci. Supplement, 2003; 5(1) 6061.
AAPS (American Association of Pharmaceutical Scientists) Annual Meeting and Exposition, 2003, October 26 -30, Salt Lake City (Utah), U.S.A.
***AAPS 2003 Award of the Pharmaceutics and Drug Delivery (PDD) section**

13. **"Dextran-g-Polyethyleneglycolcetyl Ether Polymeric Micelles For Oral Delivery of Cyclosporin A"**
Mira F. Francis*, Mariana Cristea, Françoise M. Winnik and Jean-Christophe Leroux.
BioContact Quebec, 2003, October 1-3, Quebec city (Qc), Canada
***CIHR/BioContact-Quebec 2003 Next Generation Award**

12. "Synthesis and Evaluation of Hydrophobically-modified polysaccharides as Oral Delivery Vehicles of Poorly-Water Soluble Drugs"
Mira F. Francis, Mariella Piredda, Mariana Cristea and Françoise M. Winnik.
226th ACS National Meeting, 2003, September 7 – 11, New York (NY), U.S.A.

11. "Dextran-g-Polyethyleneglycolcetyl Ether Polymeric Micelles For Oral Delivery of Cyclosporin A"
Mira F. Francis*, Mariana Cristea, Françoise M. Winnik and Jean-Christophe Leroux.
Proceedings International Symposium on Controlled Release of Bioactive Materials, 2003; 30
30th Annual Meeting & Exposition of the Controlled Release Society (CRS), 2003, July 19-23, Glasgow, Scotland, United Kingdom [Oral Communication].
***2003 Controlled Release Society (CRS) – Capsugel Graduate/Postdoc Award for Innovative Aspects of Gastrointestinal Drug Absorption and Delivery,** sponsored by the CRS and Capsugel, a division of Pfizer, Glasgow, Scotland, United Kingdom.

10. "Solubilization of Poorly-Water Soluble Drugs Using Dextran-g-Poly(Ethylene Glycol) Alkyl Ether Polymeric Micelles"
Mira F. Francis, Mariana Cristea, Françoise M. Winnik and Jean-Christophe Leroux.
Journal of Pharmacy and Pharmaceutical Sciences, 2003; 6 (2); 71-72.
Canadian Society for Pharmaceutical Sciences (CSPS) 6th Annual Symposium on Pharmaceutical Sciences, 2003, May 28-31, Montreal (Qc), Canada

9. "Efficient and controlled preparation of modified dextrans as vehicles in oral drug delivery of poorly-water soluble drugs"
Mariana Cristea, **Mira F. Francis** and Françoise M. Winnik
Journal of Pharmacy and Pharmaceutical Sciences, 2003; 6 (2); 67-68.
Canadian Society for Pharmaceutical Sciences (CSPS) 6th Annual Symposium on Pharmaceutical Sciences, 2003, May 28-31, Montreal (Qc), Canada

8. "Evaluation of Dextran-g-Poly(Ethylene Glycol Alkyl Ether) Polymeric Micelles As Vehicles In Oral Drug Delivery of Poorly-Water Soluble Drugs"
Mira F. Francis, Luc Lavoie, Françoise M. Winnik and Jean-Christophe Leroux
23rd Annual Meeting of the Canadian Biomaterials Society, 2003, May 29-31, Montreal (Qc), Canada

7. "Stimuli-responsive liposome-polymer complexes: Toward the design of intelligent drug carriers"
J.C. Leroux, F.M. Winnik, E.Roux and **M. Francis**.
223rd ACS (American Chemical Society) National Meeting, 2002, April 7-11, Orlando (Florida), U.S.A.

6. "In vitro Folate-mediated Tumor Cell Targeting Using Folate Conjugates of N-isopropylacrylamide Copolymer"
M. Francis, D. Dubé, F.M. Winnik and J.C. Leroux.
AAPS Pharm. Sci. Supplement, 2001; 3(3) : 2001.
AAPS (American Association of Pharmaceutical Scientists) Annual Meeting and Exposition, 2001, October 21-25, Denver (Colorado), U.S.A.

5. "Polymer-based pH-sensitive carriers as a means to improve the cytoplasmic delivery of drugs"
J.C. Leroux, **M. Francis**, E. Roux, F.M. Winnik.
13th International Symposium on Microencapsulation 2001, Anger, France.

4. "In vitro Evaluation of pH-Sensitive Niosome/Polymer Complexes"
M.F. Francis*, G. Dhara, F.M. Winnik and J.C. Leroux.
84th CSC (Canadian Society for Chemistry) Conference and Exhibition 2001, Montreal (PQ), Canada. [Oral communication].
***MSED Best Student Oral Presentation Award 2001**

3. "Synthesis and Characterization of Folate Derivatives of Hydrophobically-modified poly(N-isopropylacrylamides)"
D. Dubé, **M. Francis**, F.M. Winnik and J.C. Leroux.
84th CSC (Canadian Society for Chemistry) Conference and Exhibition 2001, Montreal (PQ), Canada.

2. "Study of the Mechanisms of Destabilization of pH-Sensitive Niosomes by N-isopropylacrylamide Copolymer Under Weakly Acidic Conditions"
M.F. Francis, G. Dhara, F.M. Winnik and J.C. Leroux.
10ème Forum des Sciences Pharmaceutiques 2001, Montreal (PQ), Canada [Oral Communication].

1. "Destabilization of Niosomes and Liposomes by pH-Sensitive N-isopropylacrylamide Copolymers Under Weakly Acidic Conditions"
M. Francis, G. Dhara, F.M.Winnik, J.C. Leroux.
AAPS Pharm. Sci. Supplement, 2000; 2 (4) : 2000.
AAPS (American Association of Pharmaceutical Scientists) Annual Meeting and Exposition, 2000, October 29 - November 2, Indianapolis (Indiana), U.S.A.

Volunteer Activities

2001 – 2004 Representative of Graduate Students in "Research Committee" of the Faculty of Pharmacy, University of Montreal

Scholarships, Distinctions and Honors

2004 Manulife Financial Corporation – Ph.D. Student Distinction Prize, Faculty of Graduate Studies, University of Montreal, Montreal (Qc), Canada.

2003 American Association of Pharmaceutical Scientists (AAPS) Award of the Pharmaceutics and Drug Delivery (PDD) section, October 28th, Salt Lake City (Utah), U.S.A.

2003 CIHR/BioContact-Quebec Next Generation Award, BioContact Quebec, a symposium on the biopharmaceutical partnership between North America, Europe and Asia, October 3rd, Quebec city (Qc), Canada.

2003 Banque de Montreal – Ph.D. Student Distinction Prize, Faculty of Graduate Studies, University of Montreal, Montreal (Qc), Canada.

2003 Controlled Release Society (CRS) - Capsugel Graduate/Postdoc Award for Innovative Aspects of Gastrointestinal Drug Absorption and Delivery, sponsored by the CRS and Capsugel, a division of Pfizer, July 21st, Glasgow, Scotland, United Kingdom.

2002 – 2003 Novartis Pharma Canada Excellence Scholarship, Montreal (Qc) Canada

2002 McKesson Canada Prize for academic distinction (Ph.D. graduate studies in Pharmaceutical sciences), Montreal (Qc), Canada.

2001 and 2002 Ph.D. Student Distinction Prize in Pharmaceutical sciences, Faculty of Pharmacy, University of Montreal, Montreal (Qc), Canada

2001 MSED Best Student Oral Presentation Award, 86th Canadian Society of Chemistry (CSC) Conference and Exhibition, Montreal (Qc), Canada.

2001 and 2002 J.A. De Sève Distinction scholarship, Montreal (Qc), Canada.

2001 – 2005 Rx & D Health Research Foundation (HRF)/Canadian Institutes of Health Research (CIHR), Ph.D. graduate studies scholarship in Pharmacy, Ottawa (ON), Canada.

2001 – 2003 Exemption of Differential University Fees for International Students, Faculty of Graduate Studies, University of Montreal, Montreal (Qc), Canada.

2001 Aventis Class Prize, M.Sc. graduate studies in Pharmaceutical Sciences, Montreal (Qc), Canada.

1999 – 2001 Canadian International Development Agency (CIDA) Distinction scholarship for Master studies, Ottawa (ON), Canada.

1998 Distinction and Honor degree, Faculty of pharmacy, University of Alexandria, Alexandria, Egypt.

Memberships

- Controlled Release Society **(CRS)**
- American Association of Pharmaceutical Scientists **(AAPS)**.
- Canadian Society for Pharmaceutical Sciences **(CSPS)**.
- Canadian Society for Chemistry **(CSC)**.