Pharmaceutical Research, Vol. 22, No. 2, February 2005 (© 2005)
DOI: 10.1007/s11095-004-1188-0

Research Paper

Engineering Polysaccharide-Based Polymeric Micelles to Enhance Permeability of Cyclosporin A Across Caco-2 Cells

Mira F. Francis,[1] Mariana Cristea,[2] Yali Yang,[3] and Françoise M. Winnik[1,3,4]

Received September 6, 2004; accepted October 26, 2004

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 stability of micelles in simulated gastric and intestinal fluids was studied as a function of contact time, and their cytotoxicity toward Caco-2 cells was evaluated using the MTT colorimetric assay. The bidirectional transport across Caco-2 cell monolayers of CsA entrapped in HM-DEX and HM-HPC micelles 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 toward 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.

KEY WORDS: Caco-2; cyclosporin A; dextran; hydroxypropylcellulose; oral delivery; P-glycoprotein; polymeric micelles; transport.

INTRODUCTION

The cyclic undecapeptide cyclosporin A (CsA) is a potent immunosuppressive agent used to prevent allograft rejection after organ transplantation (1,2). It is also effective in the treatment of patients with selected autoimmune diseases, such as rheumatoid arthritis (3). CsA is currently available for oral administration in the form of a microemulsion containing, among other components, polyoxyethylated castor oil (Cremophor EL) (4,5). Cremophor EL is a nonionic surfactant that is present in various oral lipophilic drug formulations, due to its solubilizing properties and its ability to inhibit intestinal P-glycoprotein (P-gp) efflux (6,7). Cremophor EL, however, is known to induce undesirable side effects, such as nephrotoxicity (8,9) and induction of anaphylactic reactions in sensitized patients (10,11). 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 (12–16). Given the limited stability of liposomes *in vivo*, they have not been widely applied in clinical use (17–19). Other particulate vehicles, however, have been developed and show promising properties in terms of controlled drug release and distribution (20–23).

It is often observed that the gastrointestinal (GI) uptake of microparticles is affected significantly by particle size. For example, Desai and co-workers (24) reported that the uptake efficiency of particles ~100 nm in diameter by the GI tract is 15- to 250-fold higher than that of micrometer-sized particles.

[1] Faculty of Pharmacy, University of Montreal, Montreal, Quebec H3C 3J7, Canada.
[2] "Petru Poni" Institute of Macromolecular Chemistry, Iasi 700487, Romania.
[3] Department of Chemistry, University of Montreal, Montreal, Quebec H3C 3J7, Canada.
[4] To whom correspondence should be addressed. (e-mail: francoise.winnik@umontreal.ca)

ABREVIATIONS: AP, apical side; BL, basolateral side; CsA, cyclosporin A; DEX, dextran T10; HPC, hydroxypropylcellulose; P85, Pluronic P85; PGI, P-glycoprotein inhibitor; P-gp, P-glycoprotein; POE-C_{16}, polyoxyethylene (10) cetyl ether.

Thus, a major thrust of current research toward 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 during the past few years as nanoparticulate carriers for CsA include hydrophobic biodegradable polymers, such as poly(caprolactone) (25), poly(D,L-lactic acid) (26), positively charged chitosan hydrochloride (27), and hydroxypropyl methylcellulose phthalate (HPMCP) (28).

We reported recently the synthesis and physicochemical properties of amphiphilic polymers consisting of a polysaccharide main chain decorated with lipidic side-chains made up of *n*-hexadecyl or *n*-octadecyl chains (29,30). 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 (31,32), polymeric micelles are remarkably stable toward dilution and tend to exhibit minimal cytotoxicity (33–35). 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 nontoxic polysaccharides, either dextran (DEX-C_{16}) or hydroxypropylcellulose (HPC-C_{16}) (Fig. 1). Dextran, consists of β-D-glucose units, predominantly linked by 1-6 glycosidic linkages (36). It possesses excellent biocompatibility and has been used in medicine as plasma substitute, due to its known innocuousness both locally and systemically (37). More recently, dextran-based gels and prodrugs have been investigated as carriers for a variety of bioactive agents (38–41). Hydroxypropylcellulose is a common excipient in oral formulations, due to its excellent processability and disintegrant and bioadhesive properties. The latter feature is of interest in our application as well, as it may promote the contact of HPC-based micelles with the intestinal epithelial cells and their transport through the cell membranes.

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 (42): 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 (43). The Caco-2 cell line, which is derived from human colon adenocarcinoma, undergoes spontaneous differentiation in culture, forming monolayers of polarized enterocytes that possess morphologic and functional similarities to the small intestine (44–47). In particular, Caco-2 cells express on the apical (AP) surface the P-gp efflux pump (48), an absorption barrier limiting the oral bioavailability of hydrophobic drugs from the gastrointestinal tract (49). The permeability assay through Caco-2 monolayers has emerged as a leading method

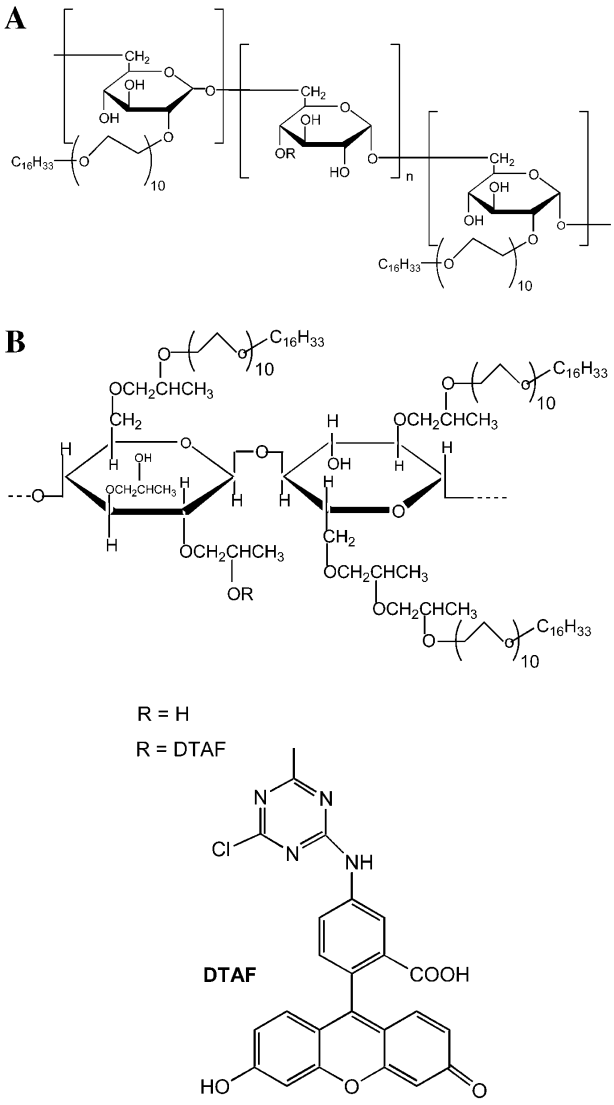

Fig. 1. Chemical structures of unlabeled and fluorescein-labeled (A) DEX-C_{16} (15 mol%) and (B) HPC-C_{16} (5.4 mol%) copolymers.

for determining the apparent permeability coefficient of drugs and to investigate their absorption mechanisms (46,50–53), as a strong correlation was observed between *in vitro* permeability across Caco-2 cells and *in vivo* human absorption for a variety of compounds (54–56).

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 radiolabeled 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.

MATERIALS AND METHODS

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 (57,58)], 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, USA). 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, USA). Dextran T10 (DEX; MW 10,000 Da) was supplied by Amersham Biosciences (Uppsala, Sweden). High performance liquid chromatography (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, USA) 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 nonessential 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. (Parsippany, NJ, USA). [^{3}H] cyclosporin A (8.00 Ci/mmol) was purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA). Ultrapure water, used for all aqueous solutions, was from a Milli-Q Filtration system (Millipore, Bedford, MA, USA).

Synthesis of Fluorescein-Labeled HM-Polysaccharides

The labeled polymers were prepared by modification of HPC-C_{16} and DEX-C_{16} (Fig. 1), which were synthesized following the general procedure reported elsewhere (59). The composition and molecular characteristics of these polymers are listed in Table I. The solubility characteristics of the two polymers are different, therefore the labeling procedure had to be adapted to each sample as follows.

Table I. Characteristics of the Polymers Used in the Current 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 (59).
[b] From UV-Vis spectra (see "Materials and Methods" section).

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 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 cutoff: 6000–8000 Da, Spectrum Laboratories Inc., Rancho Dominguez, CA, USA) and isolated by freeze-drying (yield: 107.2 mg). The degree of DTAF substitution was determined by quantitative UV-Vis spectrophotometry (Hewlett-Packard 8452A photodiode array spectrometer; Hewlett-Packard, Palo Alto, CA, USA). The labeled polymer was dissolved in an aqueous solution of pH 9. DTAF was used as reference (molar extinction coefficient ε_{492nm}: 70,000 $cm^{-1}\cdot mol^{-1}$ at λ = 492 nm) (60).

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, 5.6×10^{-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 cutoff: 3500 d). The dialyzed polymer solution was further purified by ultrafiltration through an Amicon YM3 ultrafiltration membrane (Amicon, Beverly, MA, USA). The labeled polymer was isolated by freeze-drying (yield: 132 mg), and its degree of labeling was determined by UV-Vis spectrophotometry.

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] = 7×10^{-7} M) and monitoring the changes in the ratio of the pyrene excitation spectra intensities (61) 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°.

Physical Loading of CsA in HM DEX and HPC Polymeric Micelles

A dialysis method was used 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 an 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 to 400 mg/L, with a first-order correlation coefficient (r^2) greater than 0.99. Drug loading (DL) was calculated using Eq. 1:

$$\mathrm{DL}\,(\%) = 100(W_c/W_M) \quad (1)$$

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

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 deionized water (250 ml) and added to 0.2 N NaOH (190 ml) in deionized water (400 ml). This solution was adjusted to a pH of 6.8 with 0.2 N NaOH and diluted to 1000 ml. Dialysis bags (MW cutoff: 6000–8000 Da) containing a solution of CsA-loaded micelles (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 triplicate; data are presented as the mean ± SD.

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) nonessential 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.

Cytotoxicity 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 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 (62,63). 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, USA) at 570 nm.

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, USA) at a density of approximately 8×10^4 cells/cm^2. Cells were incubated for 21–25 days to allow confluency, full maturation, including P-gp expression (48) and increased transepithelial electrical resistance (TEER) due to the formation of tight junctions in the cell monolayer (64). 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.). 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 triplicate. The amounts of CsA and polymer transported across Caco-2 cell monolayers were determined as depicted in Fig. 2.

Transport of CsA

CsA-loaded fluorescently labeled 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 (1 μM)-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} (Fig. 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

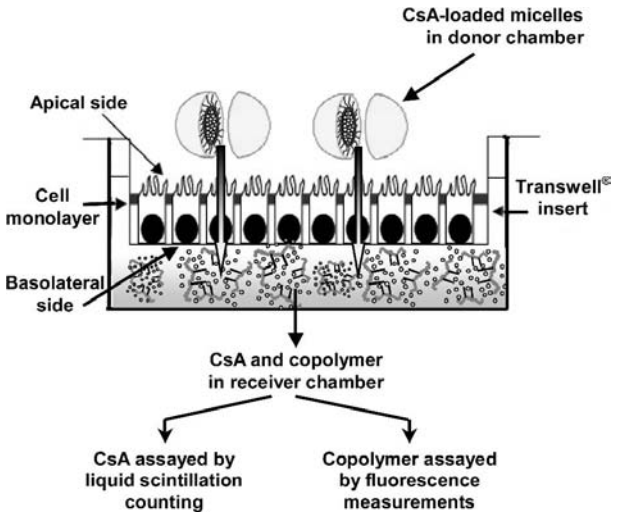

Fig. 2. Schematic representation of the procedure used to study the transport of CsA-loaded polymeric micelles across Caco-2 cell monolayer.

presence of P-glycoprotein inhibitor (PGI) were conducted using Pluronic P85 unimers (P85; 30 μM in transport medium) (65–67). P85 solution was added to the AP side of monolayers, and after sampling, equal volumes were replaced using P85-containing solution. At the end of the experiment, TEER values were measured in triplicate 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, USA). The amount of transported CsA was determined by liquid scintillation counting using a Tri-Carb liquid scintillation analyzer (Packard Instrument Co., Meriden, CT, USA) after correction for changes of volume and concentration 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, USA). Transport results were normalized for total protein content of the cells in each well. All experiments were performed in triplicate; the data presented are the mean ± SD.

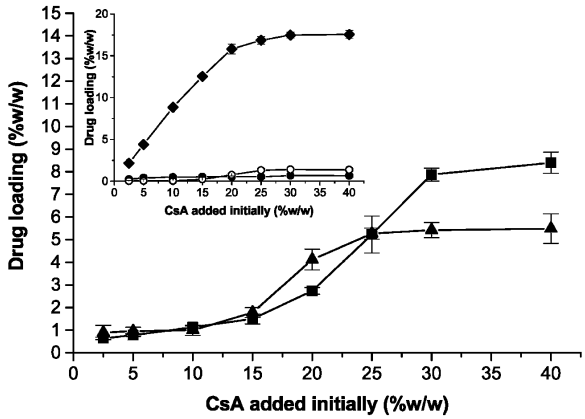

Fig. 3. CsA loading (% w/w) in micelles of (■) DEX-C_{16} (15 mol%) copolymer and (▲) HPC-C_{16} (5.4 mol%) copolymer at 2.5–40 (w/w%) CsA initial loading. Inset: CsA loading (% w/w) in presence of (♦) free POE-C_{16} surfactant as well as unmodified (●) DEX and (o) HPC polymers. Mean ± SD (n = 3).

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 Eq. 2:

$$P_{app}\,(\text{cm/s}) = \frac{1}{AC_0} \cdot \frac{dQ}{dt} \qquad (2)$$

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_0 is the CsA concentration (pM) on the donor side at $t = 0$.

Transport of HM Polysaccharides

The polymer permeability across Caco-2 cells was determined by a fluorescence assay using the intrinsic fluorescence of the labeled HM-polysaccharides. Solutions of CsA-loaded fluorescein-labeled polymeric micelles (CsA content of 1 μM) in HBSS transport medium (24×10^{-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 $\lambda_{ex} = 493$ nm, and the emission intensity was monitored at $\lambda_{em} = 519$ 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.

Statistical Analysis

All experiments were performed in triplicate; the data are presented as the mean ± SD, standardized on individual well protein concentrations. The differences between the mean values were analyzed for significance using analysis of variance (ANOVA) test. Results are considered statistically significant from the control when $p < 0.05$.

RESULTS AND DISCUSSION

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, toward 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 nontoxic toward 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 toward Caco-2 cells after incubation periods as long as 24 h and at concentrations as high as 10 g/L (Fig. 4). The cell viability dramatically decreased in the presence of POE-C_{16} even at concentrations

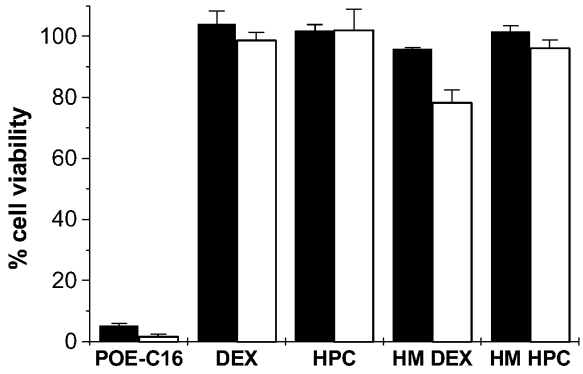

Fig. 4. Caco-2 cell viability determined by MTT colorimetric assay following 4-h (solid columns) and 24-h (open columns) incubation periods in presence of 10 g/L of free POE-C_{16} surfactant, unmodified DEX and HPC polysaccharides as well as HM copolymers of DEX-C_{16} and HPC-C_{16}. Mean ± SD (n =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

below 1 g/L (data not shown), confirming prior reports (29,30). Thus, linking POE-C_{16} to nontoxic 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.

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 II). 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 (~7×10^{-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 II). 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 I). 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 II).

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 (Fig. 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 (Fig. 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 used 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 toward 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 (Fig. 3, inset).

Table II. 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 ($\times 10^6$ 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_{336nm}/I_{333nm} 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.

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 (Fig. 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 toward pH changes and enzymatic degradations.

Transport Study

Experiments were designed that would allow detection of 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 (68–70), 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 (65). 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) (67,71) 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.

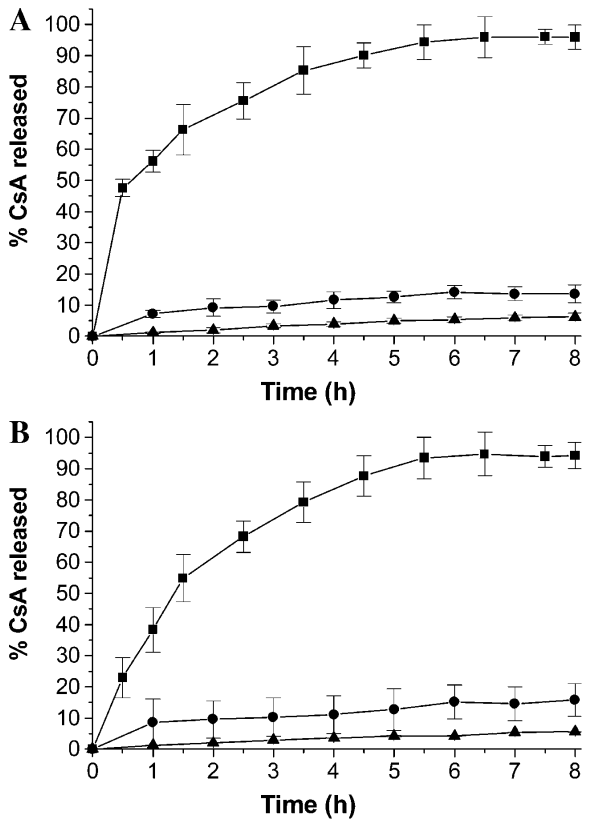

Fig. 5. Release profile of CsA at 37°C in (A) simulated gastric fluid at pH 1.2 and (B) simulated intestinal fluid at pH 6.8, from (■) free CsA solution (control); (●) CsA-loaded DEX-C_{16} polymeric micelles and (▲) CsA-loaded HPC-C_{16} polymeric micelles. Mean ± SD (n = 3).

Monitoring first the transport of CsA across Caco-2 cell monolayers (Fig. 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. Moreover, in the presence of P85, CsA transport was significantly enhanced in the AP-BL direction but nearly precluded in the BL-AP direction (Figs. 6B and 6D), in agreement with recent reports on the major role of the P-gp efflux mechanisms in determining CsA transport in Caco-2 cells (7,68,72,73). 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 (Fig. 7).

Next, we investigated the bidirectional permeability across the Caco-2 cell monolayers of the host polysaccharides, using fluorescein-labeled 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} (Fig. 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 (74–76).

Structure of the Polymeric Micelles and Its Effect on Their Transport Through Caco-2 Cell Monolayers

The polysaccharides used 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 (29,30). The molar content of (POE)-C_n residues is the determining factor, within a family of polymers, dextran or HPC, which affects the physicochemical 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

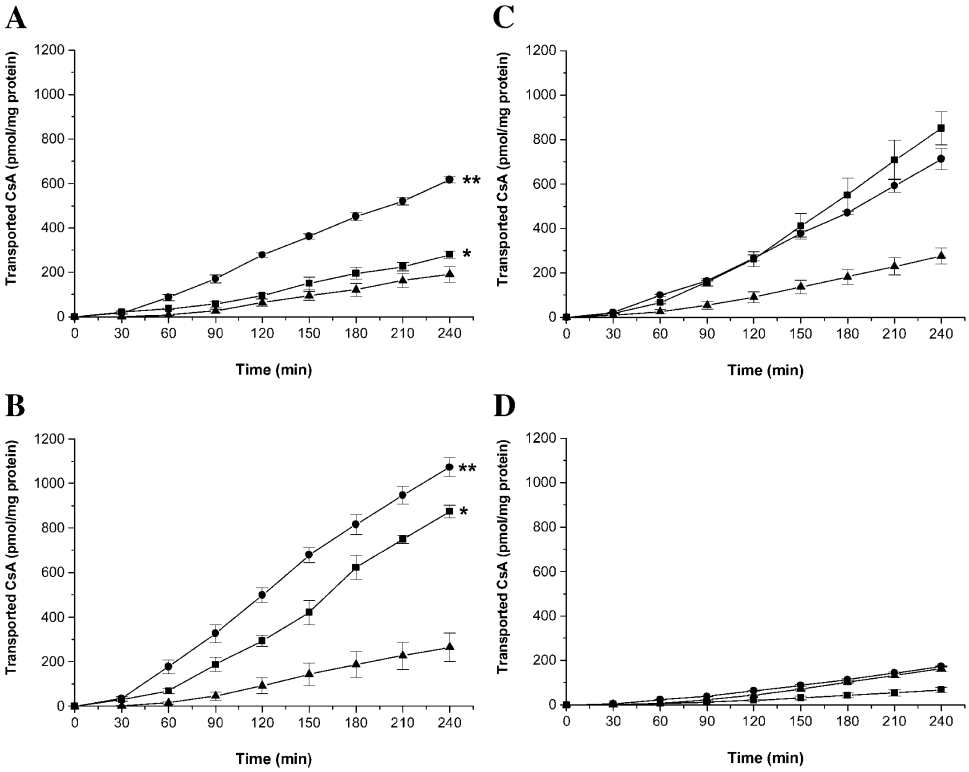

Fig. 6. CsA (pmol/mg protein) transported across Caco-2 monolayers after 240-min incubation in the AP-BL direction in absence (A) and presence (B) of P85, and in the BL-AP direction in absence (C) and presence (D) of P85 for free CsA (▲) and CsA loaded in DEX-C_{16} (■) and HPC-C_{16} (●) polymeric micelles. Mean ± SD (n = 3). *Statistically significant compared to free CsA; **statistically significant compared to both free CsA and CsA-loaded in DEX-C_{16} polymeric micelles.

of the polymer framework plays a role as well: micelles formed by DEX-C_{16} constructed from a dextran ~40,000 Da in size were less effective CsA carriers than those based on the shorter dextran (10,000 Da) (29).

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 nanoparticles 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 (77–80). 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. (81). The HPC-C_{16} and the DEX-C_{16} micelles investigated differ by a factor of nearly 10 in terms of diameter, but both types are substantially larger than dendrimers, and their size is within the range considered to be ideal for mucosal uptake.

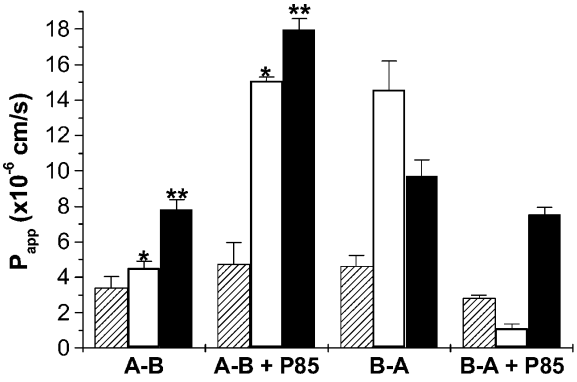

Fig. 7. Permeability coefficient (P_{app}, cm/s) of free CsA (hatched columns) and CsA-loaded in polymeric micelles of DEX-C_{16} (open columns) and HPC-C_{16} (solid columns). Mean ± SD (n = 3). *Statistically significant compared to free CsA; **statistically significant compared to both free CsA and CsA-loaded in DEX-C_{16} polymeric micelles.

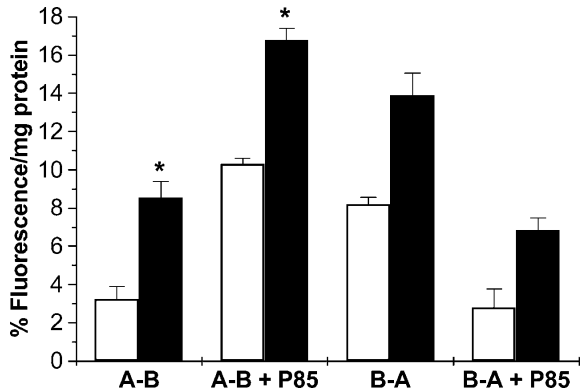

Fig. 8. Permeability of fluorescein-labeled polysaccharides for CsA-loaded micelles of DEX-C_{16} (open columns) and HPC-C_{16} (solid columns) across Caco-2 monolayers following 4-h incubation with Caco-2 cells. Mean ± SD (n = 3). *Statistically significant compared to DEX-C_{16} polymeric micelles.

Table III. 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

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 III) points to the enhanced performance of HPC-C_{16} micelles as judged by the Caco-2 permeability assay used here. Unmodified dextran dissolves rapidly in water and has a low affinity for the mucous layer (82). 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 (83,84). HPC-C_{16} micelles readily adhere to the Caco-2 cell monolayers, allowing the slow diffusion of the encapsulated drug to the basal side, whereas DEX-C_{16} micelles tend to remain suspended in the apical side.

CONCLUSIONS

The coupling of hydrophobic groups to water-soluble polysaccharides significantly promotes the solubilizing power of either dextran or hydroxypropylcellulose toward CsA. Moreover, the bioadhesive characteristics of HPC enhance the association of polymer micelles toward 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 nontoxic 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.

ACKNOWLEDGMENTS

This work was financially supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) under its strategic grants program. M. F. Francis acknowledges a scholarship from the Rx&D Health Research Foundation (HRF)/Canadian Institutes of Health Research (CIHR). We would like to thank Dr. Patrice Hildgen and Dr. Huy Ong for providing some useful pieces of equipment.

REFERENCES

1. R. M. Merion, D. J. White, S. Thiru, D. D. Evans, and R. Y. Calne. Cyclosporine: five years experience in cadaveric renal transplantation. *N. Engl. J. Med.* **310**:148–154 (1984).
2. I. J. Klompmaker, J. M. Wierda, W. J. Sluiter, D. R. Uges, E. B. Haagsma, R. Verwer, and M. J. Slooff. Pharmacokinetics of cyclosporine A after intravenous and oral administration in liver transplant patients measured with high-performance liquid chromatography. *Ther. Drug Monit.* **15**:60–64 (1993).
3. F. Galla, V. Marzocchi, L. Croattino, D. Poz, M. Baraldo, and M. Furlanut. Oral and intravenous disposition of cyclosporine in psoriatic patients. *Ther. Drug Monit.* **17**:302–304 (1995).
4. A. C. Foradori, L. Martinez, A. Vacarezza, L. Elberg, A. Loveluck, and C. Pinto. Pharmacokinetics of a new galenical formulation of oral cyclosporine A in stable kidney transplanted patients. *Transplant. Proc.* **26**:2969–2972 (1994).
5. I. S. Sketris, J. G. Lawen, L. Beauregard-Zollinger, P. Belitsky, D. Landsberg, M. L. Givner, and P. Keown. Comparison of the pharmacokinetics of cyclosporine sandimmune with sandimmune neoral in stable renal transplant patients. *Transplant. Proc.* **26**: 2961–2963 (1994).
6. D. M. Woodcock, S. Jefferson, M. E. Linsenmeyer, P. J. Crowther, G. M. Chojnowski, B. Williams, and I. Bertoncello. Reversal of the multidrug resistance phenotype with cremophor EL, a common vehicle for water-insoluble vitamins and drugs. *Cancer Res.* **50**:4199–4203 (1990).
7. F. Seeballuck, M. B. Ashford, and C. M. O'Driscoll. The effects of Pluronics block copolymers and Cremophor EL on intestinal lipoprotein processing and the potential link with P-glycoprotein in Caco-2 cells. *Pharm. Res.* **20**:1085–1092 (2003).
8. J. Mason. Renal side-effects of cyclosporin A. *Br. J. Dermatol.* **122**:71–77 (1990).
9. K. L. Skorecki, W. P. Rutledge, and R. W. Schrier. Acute cyclosporine nephrotoxicity-prototype for a renal membrane signalling disorder. *Kidney Int.* **42**:1–10 (1992).
10. L. S. Friedman, J. L. Dienstag, P. W. Nelson, P. S. Russell, and A. B. Cosimi. Anaphylactic reaction and cardiopulmonary arrest following intravenous cyclosporine. *Am. J. Med.* **78**:343–345 (1985).
11. D. L. Howrie, R. J. Ptachcinski, B. P. Griffith, R. J. Hardesty, J. T. Rosenthal, G. J. Burckart, and R. Venkataramanan. Anaphylactoid reactions associated with parenteral cyclosporine use: possible role of Cremophor EL. *Drug Intell. Clin. Pharm.* **19**:425–427 (1985).
12. C. Allen, D. Maysinger, and A. Eisenberg. Nano-engineering block copolymer aggregates for drug delivery. *Colloids Surf. B Biointerfaces* **16**:3–27 (1999).
13. X. Chen, T. Young, M. Sarkari, R. Williams, and K. Johnston. Preparation of cyclosporine A nanoparticles by evaporative precipitation into aqueous solution. *Int. J. Pharm.* **242**:3–14 (2002).
14. A. Sanchez and M. J. Alonso. Poly(D,L-lactide-co-glycolide) micro and nanospheres as a way to prolong blood/plasma levels of subcutaneously injected cyclosporin A. *Eur. J. Pharm. Biopharm.* **41**:31–37 (1995).
15. K. Itoh, A. Pongpeerapat, Y. Tozuka, T. Oguchi, and K. Yamamoto. Nanoparticle formation of poorly water-soluble drugs from ternary ground mixtures with PVP and SDS. *Chem. Pharm. Bull. (Tokyo)* **51**:171–174 (2003).
16. M. Al-Meshal, S. H. Khidr, M. A. Bayomi, and A. A. Al-Angary. Oral administration of liposomes containing cyclosporine: a pharmacokinetic study. *Int. J. Pharm.* **168**:163–168 (1998).
17. B. Ozpolat, G. Lopez-Berestein, P. Adamson, C. J. Fu, and A. H. Williams. Pharmacokinetics of intravenously administered liposomal all-trans-retinoic acid (ATRA) and orally administered ATRA in healthy volunteers. *J. Pharm. Pharm. Sci.* **6**:292–301 (2003).
18. F. Nacka, M. Cansell, P. Meleard, and N. Combe. Incorporation of alpha-tocopherol in marine lipid-based liposomes: in vitro and in vivo studies. *Lipids* **36**:1313–1320 (2001).
19. M. C. Taira, N. S. Chiaramoni, K. M. Pecuch, and S. Alonso-

Romanowski. Stability of liposomal formulations in physiological conditions for oral drug delivery. *Drug Deliv.* **11**:123–128 (2004).
20. S. Bonduelle, M. Carrier, C. Pimienta, J. P. Benoit, and V. Lenaerts. Tissue concentration of nanoencapsulated radiolabelled cyclosporin following peroral delivery in mice or ophthalmic application in rabbits. *Eur. J. Pharm. Biopharm.* **42**:313–319 (1996).
21. J. Molpeceres, M. R. Aberturas, and M. Guzman. Biodegradable nanoparticles as a delivery system for cyclosporine: preparation and characterization. *J. Microencapsul.* **17**:599–614 (2000).
22. E. Ugazio, R. Cavalli, and M. R. Gasco. Incorporation of cyclosporin A in solid lipid nanoparticles (SLN). *Int. J. Pharm.* **241**: 341–344 (2002).
23. J. Daia, T. Nagaib, X. Wanga, T. Zhangc, M. Menga, and Q. Zhang. pH-sensitive nanoparticles for improving the oral bioavailability of cyclosporine A. *Int. J. Pharm.* **280**:229–240 (2004).
24. M. P. Desai, V. Labhasetwar, G. L. Amidon, and R. J. Levy. Gastrointestinal uptake of biodegradable microparticles: effect of particle size. *Pharm. Res.* **13**:1838–1845 (1996).
25. M. C. Varela, M. Guzman, J. Molpeceres, M. D. R. Aberturas, D. Rodriguez-Puyol, and M. Rodriguez-Puyol. Cyclosporine-loaded polycaprolactone nanoparticles: immunosuppression and nephrotoxicity in rats. *Eur. J. Pharm. Sci.* **12**:471–478 (2001).
26. R. Gref, P. Quellec, A. Sanchez, P. Calvo, E. Dellacherie, and M. J. Alonso. Development and characterization of CyA-loaded poly(lactic acid)-poly(ethylene glycol)PEG micro- and nanoparticles. Comparison with conventional PLA particulate carriers. *Eur. J. Pharm. Biopharm.* **51**:111–118 (2001).
27. A. M. De Campos, A. Sanchez, and M. J. Alonso. Chitosan nanoparticles: a new vehicle for the improvement of the delivery of drugs to the ocular surface. Application to cyclosporin A. *Int. J. Pharm.* **224**:159–168 (2001).
28. G. Sertsou, J. Butler, J. Hempenstall, and T. Rades. Solvent change co-precipitation with hydroxypropyl methylcellulose phthalate to improve dissolution characteristics of a poorly water-soluble drug. *J. Pharm. Pharmacol.* **54**:1041–1047 (2002).
29. M. F. Francis, L. Lavoie, F. M. Winnik, and J. C. Leroux. Solubilization of cyclosporin A in dextran-*g*-polyethyleneglycolalkyl ether polymeric micelles. *Eur. J. Pharm. Biopharm.* **56**:337–346 (2003).
30. M. F. Francis, M. Piredda, and F. M. Winnik. Solubilization of poorly water soluble drugs in micelles of hydrophobically modified hydroxypropylcellulose copolymers. *J. Control. Rel.* **93**: 59–68 (2003).
31. D. D. Lasic. Mixed micelles in drug delivery. *Nature* **355**:279–280 (1992).
32. H. E. J. Hofland, J. A. Bouwstra, J. C. Verhoef, G. Buckton, B. Z. Chowdry, M. Ponec, and H. E. Junginger. Safety aspects of non-ionic surfactant vesicles-a toxicity study related to the physicochemical characteristics of non-ionic surfactants. *J. Pharm. Pharmacol.* **44**:287–294 (1992).
33. B. G. Yu, T. Okano, K. Kataoka, S. Sardari, and G. S. Kwon. In vitro dissociation of antifungal efficacy and toxicity for amphotericin B-loaded poly(ethylene oxide)-block-poly(beta benzyl L-aspartate) micelles. *J. Control. Rel.* **56**:285–291 (1998).
34. S. C. Kim, D. W. Kim, Y. H. Shim, J. S. Bang, H. S. Oh, S. W. Kim, and M. H. Seo. In vivo evaluation of polymeric micellar paclitaxel formulation: toxicity and efficacy. *J. Control. Rel.* **72**:191–202 (2001).
35. R. Jevprasesphant, J. Penny, D. Attwood, N. B. McKeown, and A. D'Emanuele. Engineering of dendrimer surfaces to enhance transepithelial transport and reduce cytotoxicity. *Pharm. Res.* **20**: 1543–1550 (2003).
36. C. Larsen. Dextran prodrugs-structure and stability in relation to therapeutic activity. *Adv. Drug Deliv. Rev.* **3**:103–154 (1989).
37. N. P. Couch. The clinical status of low molecular weight dextran: a critical review. *Clin. Pharmacol. Ther.* **6**:656–665 (1965).
38. J. P. Draye, B. Delaey, A. Van de Voorde, A. Van Den Bulcke, B. Bogdanov, and E. Schacht. In vitro release characteristics of bioactive molecules from dextran dialdehyde cross-linked gelatin hydrogel films. *Biomaterials* **19**:99–107 (1998).
39. I. S. Kim, Y. I. Jeong, and S. H. Kim. Self-assembled hydrogel nanoparticles composed of dextran and poly(ethylene glycol) macromer. *Int. J. Pharm.* **205**:109–116 (2000).
40. Y. Zhang and C. C. Chu. Biodegradable dextran-polylactide hydrogel networks: their swelling, morphology and the controlled release of indomethacin. *J. Biomed. Mater. Res.* **59**:318–328 (2002).
41. R. Mehvar. Dextrans for targeted and sustained delivery of therapeutic and imaging agents. *J. Control. Rel.* **69**:1–25 (2000).
42. V. Pade and S. Stavchansky. Estimation of the relative contribution of the transcellular and paracellular pathway to the transport of passively absorbed drugs in the Caco-2 cell culture model. *Pharm. Res.* **14**:1210–1215 (1997).
43. K. L. Audus, R. L. Bartel, I. J. Hidalgo, and R. T. Borchardt. The use of cultured epithelial and endothelial cells for drug transport and metabolism studies. *Pharm. Res.* **7**:435–451 (1990).
44. I. J. Hidalgo, T. J. Raub, and R. T. Borchardt. Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. *Gastroenterology* **96**:736–749 (1989).
45. J. N. Cogburn, M. G. Donovan, and C. S. Schasteen. A model of human small intestinal absorptive cells. 1. Transport barrier. *Pharm. Res.* **8**:210–216 (1991).
46. F. Delie and R. Werner. A human colonic cell line sharing similarities with enterocytes as a model to examine oral absorption: advantages and limitations of the Caco-2 model. *Crit. Rev. Ther. Drug Carrier Syst.* **14**:221–286 (1997).
47. P. Artursson, K. Palm, and K. Luthman. Caco-2 monolayers in experimental and theoretical predictions of drug transport. *Adv. Drug Deliv. Rev.* **46**:27–43 (2001).
48. K. I. Hosoya, K. J. Kim, and V. H. Lee. Age-dependent expression of P-glycoprotein gp170 in Caco-2 cell monolayers. *Pharm. Res.* **13**:885–890 (1996).
49. J. Hunter and B. H. Hirst. Intestinal secretion of drugs. The role of P-glycoprotein and related drug efflux systems in limiting oral drug absorption. *Adv. Drug Deliv. Rev.* **25**:129–157 (1997).
50. P. Artursson and R. T. Borchardt. Intestinal drug absorption and metabolism in cell cultures: Caco-2 and beyond. *Pharm. Res.* **14**: 1655–1658 (1997).
51. G. krishna. K. J. Chen, C. C. Lin and A. A. Nomeir. Permeability of lipophilic compounds in drug discovery using in-vitro human absorption model, Caco-2. *Int. J. Pharm.* **222**:77–89 (2001).
52. W. Kamm, A. Jonczyk, T. Jung, G. Luckenbach, P. Raddatz, and T. Kissel. Evaluation of absorption enhancement for a potent cyclopeptidic alpha(nu)beta(3)-antagonist in a human intestinal cell line (Caco-2). *Eur. J. Pharm. Sci.* **10**:205–214 (2000).
53. F. Faassen, J. Kelder, J. Lenders, R. Onderwater, and H. Vromans. Physicochemical properties and transport of steroids across Caco-2 cells. *Pharm. Res.* **20**:177–186 (2003).
54. P. Artursson, and J. Karlsson. Correlation between oral drug absorption in humans and apparent drug permeability coefficients in human intestinal epithelial (Caco-2) cells. *Biochem. Biophys. Res. Commun.* **175**:880–885 (1991).
55. S. Yee. In vitro permeability across Caco-2 cells (colonic) can predict in vivo (small intestinal) absorption in man: fact or myth. *Pharm. Res.* **14**:763–766 (1997).
56. S. Yamashita, Y. Tanaka, Y. Endoh, Y. Taki, T. Sakane, T. Nadai, and H. Sezaki. Analysis of drug permeation across Caco-2 monolayer: implication for predicting in vivo drug absorption. *Pharm. Res.* **14**:486–491 (1997).
57. Y. Tezuka, K. Imai, M. Oshima, and T. Chiba. Determination of substituent distribution in cellulose ethers by 13C- and 1H-NMR studies of their acetylated derivatives: *O*-(2-hydroxypropyl)cellulose. *Carbohydr. Res.* **196**:1–10 (1990).
58. M. G. Wirick and M. H. Waldman. Some solution properties of fractionated water-soluble hydroxypropylcellulose. *J. Appl. Polym. Sci.* **14**:579–597 (1970).
59. M. F. Francis, M. Cristea, and F. M. Winnik. Polymeric micelles for oral drug delivery: why and how. *Pure Appl. Chem.* **76**:1321–1335 (2004).
60. D. Blakeslee. Immunofluorescence using dichlorotriazinylaminofluorescein (DTAF). II. Preparation, purity and stability of the compound. *J. Immunol. Methods* **17**:361–364 (1977).
61. C. L. Zhao, M. A. Winnik, G. Riess, and M. D. Croucher. Fluorescence probe techniques used to study micelle formation in water-soluble block copolymers. *Langmuir* **6**:514–516 (1990).
62. T. Mosmann. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **65**:55–63 (1983).
63. M. B. Hansen, S. E. Nielsen, and K. Berg. Re-examination and

further development of a precise and rapid dye method for measuring cell growth/cell kill. *J. Immunol. Methods* **119**:203–210 (1989).
64. C. A. Bailey, P. Bryla, and A. W. Malick. The use of the intestinal epithelial cell culture model, Caco-2, in pharmaceutical development. *Adv. Drug Deliv. Rev.* **22**:85–103 (1996).
65. M. M. Nerurkar, P. S. Burton, and R. T. Borchardt. The use of surfactants to enhance the permeability of peptides through Caco-2 cells by inhibition of an apically polarized efflux system. *Pharm. Res.* **13**:528–534 (1996).
66. E. V. Batrakova, H. Y. Han, V. Y. Alakhov, D. W. Miller, and A. V. Kabanov. Effects of pluronic block copolymers on drug absorption in Caco-2 cell monolayers. *Pharm. Res.* **15**:850–855 (1998).
67. E. V. Batrakova, H. Y. Han, D. W. Miller, and A. V. Kabanov. Effects of pluronic P85 unimers and micelles on drug permeability in polarized BBMEC and Caco-2 cells. *Pharm. Res.* **15**:1525–1532 (1998).
68. P. Augustijns, T. P. Bradshaw, L. S. Gan, R. W. Hendren, and D. R. Thakker. Evidence for a polarized efflux system in Caco-2 cells capable of modulating cyclosporin A transport. *Biochem. Biophys. Res. Commun.* **197**:360–365 (1993).
69. G. Fricker, J. F. Drewe, J. Huwyler, H. Gutmann, and C. Beglinger. Relevance of P-glycoprotein for the enteral absorption of cyclosporin A: in vitro-in vivo correlation. *Br. J. Pharmacol.* **118**: 1841–1847 (1996).
70. Y. Y. Chiu, K. Higaki, B. L. Neudeck, J. L. Barnett, L. S. Welage, and G. L. Amidon. Human jejunal permeability of cyclosporin A: influence of surfactants on P-glycoprotein efflux in Caco-2 cells. *Pharm. Res.* **20**:749–756 (2003).
71. D. W. Miller, E. V. Batrakova, T. O. Waltner, V. Y. Alakhov, and A. V. Kabanov. Interactions of pluronic block copolymers with brain microvessel endothelial cells: evidence of two potential pathways for drug absorption. *Bioconjugate Chem.* **8**:649–657 (1997).
72. V. J. Wacher, J. A. Silverman, Y. Zhang, and L. Z. Benet. Role of P-glycoprotein and cytochrome P450 3A in limiting oral absorption of peptides and peptidomimetics. *J. Pharm. Sci.* **87**:1322–1330 (1998).
73. E. V. Batrakova, S. Li, D. W. Miller, and A. Kabanov. Pluronic P85 increases permeability of a broad spectrum of drugs in polarized BBMEC and Caco-2 cell monolayers. *Pharm. Res.* **16**: 1366–1372 (1999).
74. R. Wiwattanapatapee, B. Carreno-Gomez, N. Malik, and R. Duncan. Anionic PAMAM dendrimers rapidly cross adult rat intestine in vitro: a potential oral delivery system? *Pharm. Res.* **17**:991–998 (2000).
75. M. El-Sayed, M. Ginski, C. Rhodes, and H. Ghandehari. Transepithelial transport of poly(amidoamine) dendrimers across Caco-2 cell monolayers. *J. Control. Rel.* **81**:355–365 (2002).
76. M. El-Sayed, C. A. Rhodes, M. Ginski, and H. Ghandehari. Transport mechanism(s) of poly (amidoamine) dendrimers across Caco-2 cell monolayers. *Int. J. Pharm.* **265**:151–157 (2003).
77. P. Jani, G. W. Halbert, J. Langridge, and A. T. Florence. Nanoparticle uptake by the rat gastrointestinal mucosa: quantitation and particle size dependency. *J. Pharm. Pharmacol.* **42**:821–826 (1990).
78. J. P. Ebel. A method for quantifying particle absorption from the small intestine of the mouse. *Pharm. Res.* **7**:848–851 (1990).
79. L. Simon, G. Shine, and A. D. Dayan. Translocation of particulates across the gut wall - a quantitative approach. *J. Drug Target.* **3**:217–219 (1995).
80. K. E. Carr, R. A. Hazzard, S. Reid, and G. M. Hodges. The effect of size on uptake of orally administered latex microparticles in the small intestine and transport to mesenteric lymph nodes. *Pharm. Res.* **13**:1205–1209 (1996).
81. A. T. Florence and N. Hussain. Trancytosis of nanoparticle and dendrimer delivery systems: evolving vistas. *Adv. Drug Deliv. Rev.* **50**:S69–S89 (2001).
82. Y. Miyazaki, S. Yakou, T. Nagai, and K. Takayama. Release profiles of theophylline from microspheres consisting of dextran derivatives and cellulose acetate butyrate: effect of polyion complex formation. *Drug Dev. Ind. Pharm.* **29**:795–804 (2003).
83. G. Ponchel. and J. Irache. Specific and non-specific bioadhesive particulate systems for oral delivery to the gastrointestinal tract. *Adv. Drug Deliv. Rev.* **34**:191–219 (1998).
84. J. K. Vasir, K. Tambwekar, and S. Garg. Bioadhesive microspheres as a controlled drug delivery system. *Int. J. Pharm.* **255**: 13–32 (2003).