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Cyclosporin A-Loaded Poly(ethylene glycol)-*b*-poly(D,L-lactic acid) Micelles: Preparation, *in Vitro* and *in Vivo* Characterization and Transport Mechanism across the Intestinal Barrier

Yanhui Zhang,[†,‡] Xinru Li,[†,‡] Yanxia Zhou,[†] Yating Fan,[†] Xiaoning Wang,[†] Yanqing Huang,[§] and Yan Liu*[,†]

Department of Pharmaceutics and Pharmaceutical Teaching Experiment Center, School of Pharmaceutical Sciences, Peking University, Beijing 100191, China

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Abstract: To improve the oral bioavailability of poorly water-soluble cyclosporin A (CyA), polymeric micelles based on monomethoxy poly(ethylene glycol)-*b*-poly(D,L-lactic acid) (mPEG-PLA) were prepared. *In vitro* release test showed that the cumulative release percentage, about 85%, of CyA from polymeric micelles within 24 h was comparable to that from Sandimmun Neoral, the currently available oral formulation of CyA. A relative oral bioavailability of 137% in rats compared with Sandimmun Neoral was demonstrated for CyA-loaded polymeric micelles. The other aim of the current work was to study the transport mechanism of mPEG-PLA micelles across the intestinal barrier. It was found that polymeric micelles could significantly increase the permeability of CyA across Caco-2 monolayers without significantly affecting transepithelial electrical resistance values, and the apparent permeation coefficient (P_{app}) of CyA was significantly higher in the AP−BL direction compared to that in the BL−AP direction, suggesting that polymeric micelles might undergo an active AP to BL transport that probably involved endocytosis which was confirmed by confocal microscope observation. The permeation of CyA through Caco-2 monolayers showed that the P_{app} was significantly increased when CyA was formulated with the copolymer below its critical association concentration (CAC) and no significant difference was found above its CAC, implying that mPEG-PLA monomers affected the intestinal P-gp efflux pumps. Therefore, the mPEG-PLA micelles seemed to be a good candidate for oral delivery of poorly soluble drugs.

Keywords: Cyclosporin A; monomethoxy poly(ethylene glycol)-*b*-poly(D,L-lactic acid); polymeric micelles; oral administration; Caco-2 cells; transport mechanism; P-glycoprotein

Introduction

Cyclosporin A (CyA) is a highly effective immunosuppressive agent which is widely used in clinic for prevention of allograft rejection after organ transplantation and treatment of autoimmune disease.[1−3] However, it also has some drawbacks such as large molecular weight (1202 Da), low solubility in water (23 μg/mL at room temperature),[4] very

* Correspondence to Prof. Yan Liu, Department of Pharmaceutics, School of Pharmaceutical Sciences, Peking University, Xueyuan Road 38, Beijing 100191, China. Phone: 86-10-82801508. Fax: 86-10-62015584. E-mail: yanliu@bjmu.edu.cn.

[†] Department of Pharmaceutics.

[‡] These authors contributed equally to this work.

[§] Pharmaceutical Teaching Experiment Center.

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high lipophilicity (log $P = 2.92$),[5] being a substrate of P-glycoprotein (P-gp), and vulnerability to intestinal mucosa and liver P450 3A4. These factors lead to a low and irregular oral bioavailability, about 10% to 60%.[6] The currently available oral formulation of CyA in the form of a microemulsion, Sandimmun Neoral, has more stable drug metabolism, but its gastrointestinal absorption is still incomplete and variable due to its hydrophobic character.[7,8] Moreover, Cremophor RH 40 present in Sandimmun Neoral has been reported to induce undesirable side effects, such as anaphylactic reactions in sensitized patients.[9,10] In view of the clinical importance of CyA, various oral formulations of CyA without Cremophor RH 40 such as liposomes,[11] microspheres,[5] nanoparticles,[3,12−15] microemulsions[16,17] and polymeric micelles[18] have been developed to improve the oral bioavailability of CyA. Given the limited stability of liposomes *in vivo*, they have not been widely applied in clinical use.[19−21] Among other particulate vehicles, polymeric micelles have presented their great potential in oral delivery of poorly water-soluble drugs in recent years.

Generally, block copolymers with concentration above the critical association concentration (CAC) self-assemble into spherical polymeric micelles with a core−shell structure in water: the hydrophobic segments aggregate to form an inner core able to accommodate hydrophobic drugs with improved solubility by hydrophobic interactions; the hydrophilic shell consists of a brushlike protective corona that stabilizes the micelles in aqueous solution.[22−24] Polymeric micelles as novel drug vehicles present numerous advantages, such as reduced side effects of drugs, selective targeting, stable storage, stability toward dilution, and prolonged blood circulation time.[24,25] Furthermore, polymeric micelles possess a nanoscaled size with a narrow distribution. They can protect drugs against premature degradation *in vivo* owing to their core−shell architecture.[26,27] More importantly, polymeric micelles are fabricated according to the physicochemical properties of drugs and the compatibility between the core of micelles and drug molecules.[24,28] Since most of polymeric micelles are intended to be administered intravenously, the development of polymeric micelles via the oral route has

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attracted attention.[29−31] Nevertheless, the documented increased uptake using polymeric micelles may then be merely the result of an increase in membrane permeability[32,33] and/or inhibition of P-gp efflux, which has been established for several types of micelles (or its monomers),[34] including TPGS,[35] poly(ethylene glycol)-*block*-poly(alkyl acrylate-*co*-methacrylic acid),[36] and Pluronic P85,[37] the bioavailability enhancement of CyA loaded in polymeric micelles by oral delivery has been only predicated so far, and the mechanism through which oral absorption of polymeric micelles would occur remains elusive.

The purposes of the present work were (1) to fabricate polymeric micelles to improve the oral bioavailability of CyA and (2) to characterize transport mechanisms of polymeric micelles through the intestinal barrier. Therefore, monomethoxy poly(ethylene glycol)-*b*-poly(D,L-lactic acid) (mPEG-PLA) copolymers, which were selected as micelle-forming material based on our screening of a wider range of block copolymers according to the compatibility between the core of micelles and the drug,[38,39] were synthesized and characterized. The micelle preparation, CyA solubilization and micelle properties were investigated by size measurement, drug loading content (LC), encapsulation efficiency (EE) and *in vitro* drug release. Pharmacokinetic profiles of CyA-loaded polymeric micelles and CyA commercial formulation (Sandimmun Neoral) after oral administration were assessed in rats. Caco-2 cell monolayers representing intestinal cell lines were used in this study to better characterize the transport mechanism of polymeric micelles through the intestinal barrier.

Materials and Methods

Materials. Cyclosporin A (CyA) was received as a gift from Hangzhou Zhongmei Huadong Pharmaceutical Co., Ltd. (Hangzhou, China). Monomethoxy poly(ethylene glycol) (mPEG) with molecular weight of 5 kDa and stannous 2-ethylhexanoate were purchased from Sigma (St. Louis, MO). 3,6-Dimethyl-1,4-dioxane-2,5-dione (D,L-dilactide) was obtained from Daigang Biological Technology Co., Ltd. (Shandong, China). Sandimmun Neoral capsule was purchased from Novartis (Switzerland). Cremophor RH 40 was obtained from BASF Corp. (Parsippany, NJ). Dulbecco's modified Eagle medium (DMEM), penicillin−streptomycin (10,000 IU/mL penicillin-G and 10 mg/mL streptomycin), 0.25% trypsin−0.53 mmol/L EDTA and Hanks balance salt solution (HBSS) were obtained from M&C Gene Technology (Beijing, China). Fetal bovine serum (FBS) was obtained from Gibco, Invitrogen (Ontario, USA), and nonessential amino acid solution was obtained from Sigma-Aldrich (St. Louis, MO). 25 cm^2 and 75 cm^2 plastic culture flasks, 12-well and 96-well tissue culture plates and Transwell 12-well plate (12 mm, 3.0 μm pore size inserts) were obtained from Costar (Corning Incorporated, USA). Sulforhodamine B sodium salt (SRB) and coumarin-6 were obtained from Sigma-Aldrich (St. Louis, MO). Tris was obtained from Shenggong Biological Technology Co. Ltd. (Shanghai, China). Bis Benzimide Hoechst 33258 was purchased from Biodee Biotechnology Co. Ltd. (Beijing, China). TritonX-100 was obtained from Amresco Inc. (Solon, USA).

Synthesis and characterization of mPEG-PLA Copolymers. mPEG-PLA copolymers were synthesized from D,L-dilactide and mPEG by the ring-opening polymerization method under continued supply of nitrogen, with stannous 2-ethylhexanoate used as a catalyst and methylbenzene as a solvent as previously described.[40] The synthesized diblock

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copolymers were referred to as mPEG*x*-PLA*y*. *x* and *y* represented the weight-averaged molecular weight of the mPEG and PLA block in kDa. mPEG5-PLA2.5, for example, consisted of a 5 kDa mPEG block connected to a 2.5 kDa PLA block.

The critical association concentration (CAC) of mPEG-PLA copolymer was determined by fluorescence spectroscopy using pyrene (Fluka, > 99%) as a hydrophobic probe as previously described.[40]

Preparation and Characterization of mPEG-PLA Polymeric Micelles. *Preparation of mPEG-PLA Polymeric Micelles.* The preparation process of mPEG-PLA micelles was optimized by using drug loading content, entrapment efficiency and the micelle size as indicators by orthogonal design. mPEG5-PLA2.5 and mPEG5-PLA5 polymeric micelles were prepared by rotary evaporation method.[41] Briefly, 25 mg of mPEG-PLA was dissolved in 10 mL of methanol followed by evaporation under vacuum at 60 °C to form a homogeneous film. The resulting film was dispersed in 10 mL of water at 60 °C and then vortexed for 3 min. The mixture was filtered through a 0.45 μm filter (Millex-GV, Millipore, USA) to obtain a clear and homogeneous micellar solution. The CyA-loaded micelles were prepared as described above except 25 mg of mPEG-PLA was replaced by a mixture of 5 mg of CyA and 25 mg of mPEG-PLA. Coumarin-6-loaded polymeric micelles were prepared as described above.

mPEG5-PLA10 and mPEG5-PLA15 polymeric micelles were prepared by a dialysis method.[42] Briefly, 25 mg of mPEG-PLA and 5 mg of CyA were dissolved in 3 mL of dimethyl sulfoxide (DMSO). The solution was then introduced into a dialysis bag (Spectrapor, MWCO = 3500), and dialyzed against 1 L of distilled water, which was replaced every 4 h in the course of 48 h. The micellar solution obtained in the dialysis bag was then filtered through a 0.45 μm filter to remove nonencapsulated CyA. CyA-free micelles were produced by the same method without adding CyA at the first stage of the preparation.

Particle Size Measurement. The average particle size and size distribution of polymeric micelles were determined by dynamic light scattering (DLS) (Zetasizer ZEN 3600, Malvern, U.K.). All DLS measurements were performed with a scattering angle of 90° at 25 °C after diluting the micellar solution to an appropriate volume with water. The results were the mean values of three samples.

Determination of Encapsulation Efficiency and Drug Loading Content. To determine drug loading content (LC, w/w %) and entrapment efficiency (EE, w/w %) of micelles, CyA-loaded polymeric micelles solution was freeze-dried and then dissolved in methanol, and CyA content in micelles was determined on a Shimadzu series HPLC system (Shimadzu LC-10AT, Kyoto, Japan) equipped with a UV detector (Shimadzu SPD-10A) and reversed phase column (ODS C18, 5 μm, 4.6 mm × 250 mm, Dikma, China). The mobile phase consisted of acetonitrile/water (90/10, v/v) and was pumped at a flow rate of 1.0 mL/min. The detection wavelength was 210 nm. The column temperature was set to 70 °C. The LC and EE of the micelles were then calculated based on the following formula:

$$\text{LC}\ (\%) = \frac{\text{mass of CyA extracted from freeze-dried micelles}}{\text{total mass of freeze-dried micelles}} \times 100\%$$

$$\text{EE}\ (\%) = \frac{\text{mass of CyA extracted from freeze-dried micelles}}{\text{mass of feed CyA}} \times 100\%$$

In Vitro Release of CyA from Micelles. One milliliter of micellar solution in simulated gastric fluid (SGF) or simulated intestinal fluid (SIF) was placed into a dialysis bag with molecular weight cutoff of 50,000. The dialysis bag (containing 0.25 mg of CyA loaded in polymeric micelles) was immersed into a flask with 30 mL of release medium (SGF or SIF) containing 30% (v/v) ethanol (under sink conditions) which was kept in a constant temperature shaking water bath at 37 °C and 100 rpm. At predetermined time intervals, aliquots (1 mL) of the release medium were taken and immediately replaced with the same volume of fresh release medium. The content of CyA in the medium was measured by HPLC method as described above. The cumulative release percentage of CyA was calculated. The *in vitro* release of CyA from Sandimmun Neoral (containing equivalent content of CyA to polymeric micelles) was also tested as control.

Pharmacokinetics Study. *In Vivo Experiment.* Male Sprague-Dawley (SD) rats were purchased from Peking University Health Science Center (weighing 200 ± 20 g). Animals were housed under standard conditions with free access to food and water. All of the animal experiments adhered to the principles of care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee of Peking University.

Two groups (6 rats per group) of male SD rats were used to examine the effect of solubilizing vehicle on the pharmacokinetics of CyA. The control group received CyA commercial formulation (Sandimmun Neoral), and test groups received CyA-loaded polymeric micelle formulation. All animals were provided with standard food and tap water ad libitum and exposed to alternating 12 h periods of light and darkness. Temperature and relative humidity were maintained at 25 °C and 50%, respectively. After an acclimatization period of two days, the rats were fasted for 12 h but allowed free access to water prior to the experiments. The animals were singly dosed by oral gavage at a dose of 10 mg/kg, and fasting continued for a further 6 h after oral administration. 0.5 mL of blood samples were collected and transferred into heparinized glass tubes at 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 10, 12, 24, 48, and 72 h postdose via ocular vein, and then stored at −20 °C until analysis.

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Blood Sample Analysis. A published method was used for analysis of blood with minor modification.[3] A 0.5 mL blood sample was incubated in a 37 °C water bath for 20 min, and then 50 μL of internal standard (cyclosporin D, Kerui Pharmaceutical Co., Ltd., China) solution with concentration of 60 μg/mL in methanol and 1.0 mL of hydrochloric acid (180 mmol/L) were added. The mixture was vortexed for 1 min, and then 5.0 mL of redistilled ether was added. After vortexing for 10 min, the organic layer was collected by centrifugation at 3500 rpm for 10 min, and then 1.0 mL of sodium hydroxide (95 mmol/L) was added. After vortexing and centrifugation as described above, the organic layer was collected and evaporated under a light stream of nitrogen at 40 °C. The residue was redissolved in 120 μL of mobile phase (acetonitrile/water, 80/20, v/v) and 1.0 mL of *n*-hexane. This solution was vortexed for 2 min and centrifuged at 3500 rpm for 8 min. The organic layer was removed, and 1.0 mL of *n*-hexane was added. Then the same vortexing, centrifugation and reconstitution procedure as described above was performed, and 20 μL samples were injected into the HPLC system as described above. The linearity of the calibration curves ranged from 0.200 to 25.0 μg/mL. The coefficient of variation of the interday and intraday precision of the quality control samples ranged from 0.78% to 1.48%, and accuracy ranged from 97.1% to 101.1%. The limit of detection (LOD) and the limit of quantification (LOQ) were 0.0625 μg/mL and 0.122 μg/mL, respectively. The extraction recovery ranged from 90% to 105%.

The maximal blood concentration of drug (C_{max}) and the time to reach maximum blood concentration (T_{max}) were directly obtained from blood data. The area under the drug concentration−time curve (AUC) of CyA for each formulation was assessed by the WinNonlin program (version 5.2, Pharsight Corp., Mountain View, CA) using the noncompartment method.

Cell Culture. The human colon adenocarcinoma cells, Caco-2 cells, at passage 25 were obtained from Cell Culture Center of Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences. The Caco-2 cells were routinely maintained in DMEM with 4.5 g/L D-glucose, supplemented with 10% of heat inactivated FBS, 1% of nonessential amino acids, 100 U/mL penicillin, 100 μg/mL streptomycin and 2 mmol/L L-glutamine. The cells were cultured in an incubator at 37 °C with a controlled atmosphere containing 5% CO_2 and 90% relative humidity.[43,44] The cells were subcultivated every 5 days at 80−90% confluence. The Caco-2 cells used for experiments were between passages 35 and 50.

Cytotoxicity Assay. The cytotoxicity of test samples was evaluated using the sulforhodamine B (SRB) assay.[45,46] Briefly, Caco-2 cells were seeded in sextuplicate in 96-well plates at a density of approximately 1.2×10^4 cells per well. The cells were cultured for 48 h at 37 °C in a humidified atmosphere with 5% CO_2.[47] Subsequently, the culture medium was removed and then 200 μL of sample solutions (mPEG5-PLA5 copolymer solution in PBS, CyA-loaded polymeric micelles, CyA solution in ethanol, Sandimmun Neoral in PBS and Cremophor RH 40 in PBS), negative control (20 μL of PBS with 180 μL of culture medium) and positive control (1%, v/v, Triton X-100, a known toxin for cells) were added to the wells. The cells were further cultured for 24 h. Thereafter the medium was removed and the cells were washed once by cold PBS. The cells were fixed with 200 μL of 10% trichloracetic acid (TCA) at 4 °C for 1 h. The cells were then washed five times with deionized water after removal of TCA and dried at 37 °C, and then stained with 200 μL of 0.4% (w/v) SRB in 1% (v/v) acetate solution for 30 min at room temperature. Thereafter the cells were washed five times with deionized water after removal of SRB and dried at 37 °C, and then 96-well plates were shaken for 30 min at 37 °C after 200 μL of 10 mmol/L Tris was added. The absorbance was measured at 540 nm with a Microplate Reader (Bio-Rad model 550, USA). Relative cell viability % was determined as follows:

$$\text{relative cell viability (\%)} = \frac{\text{absorbance}_{\text{test}}}{\text{absorbance}_{\text{control}}} \times 100$$

Transport Studies of Drug-Loaded Polymeric Micelles. *Assessment of Cell Monolayer Integrity.* For the transport experiments, Caco-2 monolayers were obtained by seeding Caco-2 cells with a density of approximately 1.5×10^5 cells/cm^2 on Transwell polycarbonate inserts and culturing them for 21 days to allow confluency, full maturation, including P-gp expression and increased transepithelial electrical resistance (TEER) due to the formation of tight junctions in the cell monolayer.[33,43,47] The culture medium was replaced every other day for the first week and daily thereafter as the apical (AP) and basolateral (BL) compartments received 0.5 and 1.5 mL of the culture medium, respectively. The integrity of the cell monolayers was evaluated both before and after transport studies using an EVOM resistance volt-ohm-meter (WPI Inc., FL). Monolayers presenting with TEER values below 200 Ω/cm^2 were

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excluded from the experiments. The resistance of HBSS alone (12 Ω/cm^2) was considered as background resistance and subtracted from each TEER value.

Transport Experiments. For all transport experiments, the culture medium was removed and the monolayers were washed twice with 0.5 and 1.5 mL of HBSS for AP and BL side, respectively, and incubated for 30 min with HBSS at 37 °C. Thereafter HBSS was removed and 0.5 mL of test formulation was added to the AP side of inserts while the BL compartments were filled with 1.5 mL of prewarmed fresh HBSS for investigating the AP to BL transport. For the BL to AP transport, 1.5 mL of test formulation was added to the BL compartments while the AP side of inserts was filled with 0.5 mL of prewarmed fresh HBSS. 100 μL of solution was sampled from the receiver compartments and replaced with prewarmed fresh HBSS at 30, 60, 90, 120, 180 min after incubation at 37 °C. The amount of drug crossing the monolayer was assayed by HPLC method as described above for CyA. The amount of coumarin-6 was measured by using a Shimadzu RF-5301 PC fluorescence spectrometer. The excitation wavelength was adjusted to 521 nm, and the detection of fluorescence was performed at 488 nm. Calibration curves were prepared in the concentration range from 0.625 to 20 μg/mL for CyA and 1.56 to 50 ng/mL for coumarin-6, respectively. The coefficients of variation (CV) for intra- and interassay were all within 3%. At the end of the experiment, TEER values were measured in triplicate to assess the integrity of the cell monolayers.

The cumulative amount of CyA permeated was plotted as a function of time. The apparent permeability coefficient (P_{app}, cm/s) was determined from the linear slope of the plot using the following equation: $P_{app} = (dQ/dt)(1/C_0A)$, where, P_{app} is the apparent permeability coefficient (cm/s), dQ/dt is the steady state flux (mg/s), C_0 is the initial concentration of CyA in AP (for AP to BL transport) or BL (for BL to AP transport), and A is the surface area of the membrane filter (1.13 cm^2).[48−49]

The transport studies were conducted for seven treatment groups as follows:

1. 1 μg/mL of coumarin-6 dissolved in HBSS with 1% (v/v) DMSO
2. 1 μg/mL of coumarin-6 loaded in polymeric micelles
3. 1 μg/mL of coumarin-6 dissolved in HBSS with 1% (v/v) DMSO along with 10 μg/mL of blank polymeric micelles
4. 25 μg/mL of CyA dissolved in HBSS with 10% (v/v) ethanol
5. 25 μg/mL of CyA loaded in polymeric micelles
6. 25 μg/mL of CyA dissolved in HBSS with 10% (v/v) ethanol along with blank polymeric micelles
7. 25 μg/mL of CyA dissolved in HBSS with 10% (v/v) ethanol and copolymer.

Confocal Laser Scanning Microscopy (CLSM) Study. CLSM was used to determine the pathway by which the drug-loaded polymeric micelles crossed the cell monolayers. Briefly, 0.5 mL of coumarin-6 solutions (group 1), coumarin-6-loaded polymeric micelles (group 2) or coumarin-6 solutions along with blank polymeric micelles (group 3), each with concentration of 1 μg/mL of coumarin-6, were added to the AP side of inserts while the BL compartments were filled with 1.5 mL of prewarmed fresh HBSS and then incubated for 2 h at 37 °C. Then monolayers were washed three times with PBS and fixed for 30 min at room temperature using freshly prepared 3.7% (v/v) formaldehyde solution and then replaced. Thereafter, the cells were washed three times with PBS and stained for 30 min at room temperature using 1 μg/mL of Hoechst 33258 and then replaced. The cells were washed three times with PBS, and then the Transwell polycarbonate inserts were cut and preserved in blocking solution (glycerol-PBS, 9:1, v/v) for CLSM observation by confocal laser scanning microscope (Leica SP2, Heidelberg, Germany).

Inhibition of P-gp Efflux Pumps. Studies of CyA transport in presence of P-gp inhibitor, Pluronic P85 unimers (BASF Corp, Parsippany, NJ) with a concentration of 30 μM in transport medium,[37,50] were conducted. HBSS with P85 was added to the AP side of monolayers to preincubate for 30 min and then replaced by sample solutions containing 30 μM P85 (groups 4−7). At the end of the experiment, TEER values were measured in triplicate to assess the integrity of the cell monolayers. The amount of transported CyA was determined by the HPLC method as described above.

Statistical Analysis. All data were expressed as mean ± standard deviation (SD) unless particularly outlined. The statistical significance of differences was determined by one-way ANOVA using SPSS for Windows versions 13.0 (SPSS Inc., Chicago, IL). Values of $p < 0.05$ and $p < 0.01$ were considered statistically significant and highly significant, respectively.

Results

Characterization of mPEG-PLA Copolymers and Micelles. Polymeric micelles can be formed only when the block copolymer concentration is higher than CAC which characterizes the micelle stability. Table 1 summarizes the CAC values of the various synthesized mPEG-PLA diblock copolymers ranging from 1.08 to 3.07 × 10^{-7} mol/L. Compared with low molar mass surfactants, the copolymers exhibited a remarkably lower CAC. These data indicate that micelles formed from mPEG-PLA copolymers as drug

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Table 1. Characterization of mPEG5-PLA*y* Copolymers and Polymeric Micelles[a]

copolymers	mPEG5-PLA2.5	mPEG5-PLA5	mPEG5-PLA10	mPEG5-PLA15
M_n (PLA)	2269	4802	9247	15825
CAC (10^{-7} mol/L)	3.07	1.99	1.57	1.08
CyA-free				
d (nm)	43.8 ± 2.4	54.9 ± 3.1	75.0 ± 3.0	79.0 ± 5.8
PDI	0.21 ± 0.01	0.23 ± 0.02	0.21 ± 0.01	0.25 ± 0.01
CyA-loaded				
d (nm)	60.1 ± 8.2	71.9 ± 0.42	89.0 ± 0.48	95.2 ± 1.2
PDI	0.23 ± 0.01	0.19 ± 0.01	0.20 ± 0.01	0.23 ± 0.10
LC (%)	12.1 ± 0.8*	15.2 ± 1.1	10.7 ± 1.5*	9.7 ± 0.2*
EE (%)	44.0 ± 3.0**	77.9 ± 4.1	33.1 ± 2.1**	10.9 ± 0.2*

[a] *d* and PDI represent the average diameter and the polydispersity index of micelles, respectively. CyA-free: blank polymeric micelles; CyA-loaded: CyA-loaded polymeric micelles. *$p < 0.05$ vs mPEG5-PLA5 micelles. **$p < 0.01$ vs mPEG5-PLA5 micelles.

carriers could preserve stability without dissociation after dilution,[51] which was of major interest for oral administration where considerable dilution will take place. Notably, the CAC values gradually decreased with increasing of the length of PLA chains, which was in agreement with a previous report.[52]

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. The mean size and size distribution of CyA-free and CyA-loaded polymeric micelles were therefore determined by DLS. The mean diameter ranged from 43 to 79 nm for CyA-free micelles and from 60 to 96 nm for CyA-loaded micelles with narrow distributions (Table 1), and no significant change was observed within at least 24 h ($p > 0.05$, data not shown). It appeared that the micelle size, which varied depending on the polymer constitution, gradually increased with increasing of the length of PLA chains. This result was in agreement with the characteristic of amphiphilic copolymeric micelles, i.e., the shorter the hydrophobic block length, the smaller the micelles. This might be accounted for by the fact that it is difficult to form compact polymeric micelles for amphiphilic copolymers with longer hydrophobic chain length. In addition, similar to drug-loaded micelles reported earlier,[52,53] the size of drug-loaded micelles was about 15 nm bigger than that of drug-free micelles, suggesting that CyA molecules were trapped in the hydrophobic inner cores and that these entrapped CyA molecules increased the average size of CyA-loaded polymeric micelles.

The encapsulation efficiency (EE) and loading content (LC) of micelles were also presented in Table 1. It could be found that the LC and EE of CyA-loaded mPEG5-PLA5 polymeric micelles were significantly higher than those of others ($p < 0.05$ for LC and $p < 0.01$ for EE, respectively). Notably, the results were not the general trend that LC and EE of polymeric micelles increased with increasing hydrophobic chain length. This finding may be assigned to the factors contributing to LC and EE. In general, LC and EE depend on the composition of the copolymers,[51,54,55] initial diblock copolymeric concentration or the feed weight ratio of the drug to the copolymer, solvent used in formulation process[55,56] and micelle preparation method,[57,58] and so on. In the case of our experiment, mPEG5-PLA2.5 and mPEG5-PLA5 polymeric micelles were prepared by the rotary evaporation method, whereas mPEG5-PLA10 and mPEG5-PLA15 polymeric micelles were prepared by the dialysis method, which were attributed to the fact that the preparation method of micelles depended on the hydrophilic property of micelle-forming copolymers. In general, in order to obtain micelles with higher LC and EE, the rotary evaporation method is usually used for the copolymers with better hydrophilic property (or with shorter hydrophobic segment), whereas the dialysis method is used for the copolymers with

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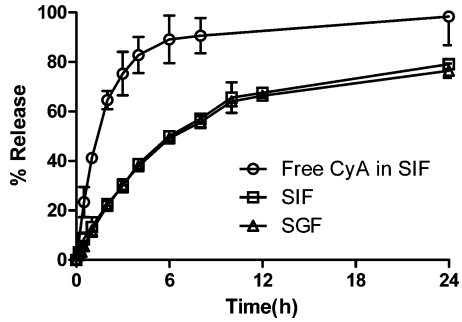

Figure 1. Release profiles of CyA from mPEG5-PLA5 polymeric micelles in SGF and SIF at 37 °C, respectively ($n = 3$).

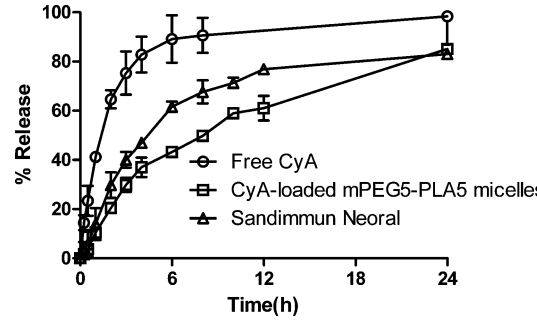

Figure 2. Release profiles of CyA from polymeric micelles and Sandimmun Neoral in SIF at 37 °C, respectively ($n = 3$).

poor hydrophilic property (or with longer hydrophobic segment). In addition, the LC and EE of polymeric micelles prepared by the rotary evaporation method are higher than those of polymeric micelles prepared by the dialysis method.[58−60] The results in current study were in agreement with this trend. Moreover, for mPEG5-PLA2.5 and mPEG5-PLA5 polymeric micelles, the results of LC and EE of polymeric micelles followed the general trend, i.e., increased with increasing hydrophobic chain length, however, the results were opposite for mPEG5-PLA10 and mPEG5-PLA15 polymeric micelles, as reported that LC and EE decreased with increasing hydrophobic chain length when polymeric micelles were prepared by the dialysis method due to enhanced water insolubility of copolymers.[61] Based on the result of CyA-loading experiment, mPEG5-PLA5 polymeric micelles were therefore selected for the subsequent studies.

When developing oral colloidal delivery systems for highly hydrophobic drugs such as CyA, it is important to adequately control the release rate in order to avoid precipitation upon dilution in the stomach and maximize the absorption in the intestine. The *in vitro* release of CyA from polymeric micelles at different release medium was therefore investigated. Prior to conducting these release assays, it was verified that CyA could freely diffuse through the dialysis membrane when the molecular weight cutoff of the membrane was 50 kDa, which is much larger than the molecular weight of CyA (Figure 1), which was assigned to the fact that CyA is a neutral cyclic undecapeptide, consisting of hydrophobic amino acids linked via 11 amide bonds, seven of which are N-methylated and four intramolecular hydrogen bonds contribute to the rigidity of its skeleton. Moreover, the sink condition was respected by addition of 30% (v/v) ethanol in the release medium. More importantly, no significant change in micelle size upon incubation of the micelle sample with release medium at 37 °C with time from 0 to 24 h was observed (data not shown), indicating that the CyA-loaded polymeric micelles were stable in release medium for a long enough time. As shown in Figure 1, the release profile of CyA from polymeric micelles (i.e., mPEG5-PLA5) in SGF did not differ from that in SIF, indicating that the release medium had no effect on the release of CyA. Consequently, the SIF was chosen as the release medium for the subsequent release studies.

As shown in Figure 2, no burst effect could be seen in the release profile, indicating that the drug was efficiently entrapped within the core of micelles. The comparison of the profiles of CyA release from the micelles and the aqueous solution showed that the entrapment of CyA in the nanoparticles could significantly retard its *in vitro* release.[30,62] In addition, the cumulative release percentage, about 85%, of CyA from polymeric micelles within 24 h was comparable to that from commercial formulation. Nevertheless, the release rate of polymeric micelles was slower than that of commercial formulation before the first 6 h, the release rate of the two formulations was comparable during 6−12 h and the former was faster thereafter. In addition, it was observed that the release profile of CyA from polymeric micelles was biphasic, with an abrupt decrease of release rate occurring after 4 h from the beginning. During the first phase (0−4 h) 42% of the drug was released. Moreover, the cumulative release of mPEG5-PLA5 polymeric micelles was significantly lower than that of the commercial formulation at almost all the time points, which might be accounted for the stronger interaction between CyA and PLA (the difference of partial solubility parameter between them was 4.03 $(J \cdot cm^{-3})^{1/2}$) compared with that between CyA and corn oil-monoditriglycerides (the difference of partial solubility

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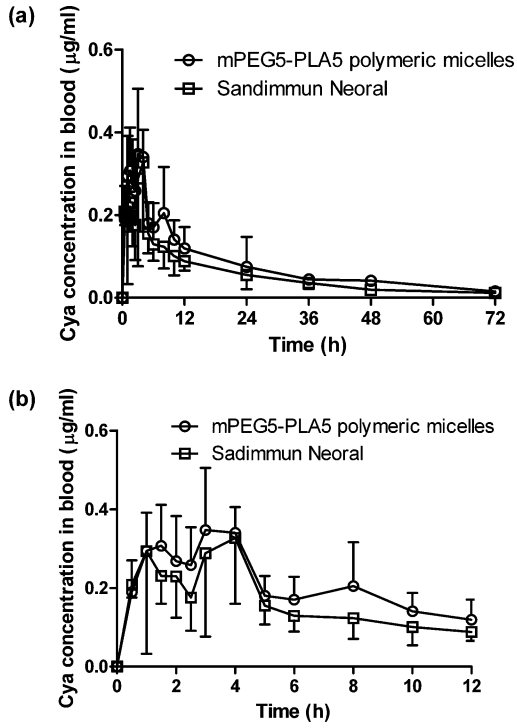

Figure 3. Blood concentration vs time curve of CyA after oral administration of Sandimmun Neoral and CyA-loaded mPEG5-PLA5 micelles to fasted SD rats (a) within 72 h and (b) within 12 h at a dose of 10 mg/kg ($n = 6$).

Table 2. Pharmacokinetic Parameters of CyA after Oral Administration of Sandimmun Neoral and CyA-Loaded mPEG5-PLA5 Micelles to Rats at a Dose of 10 mg/kg ($n = 6$)

parameter	Sandimmun Neoral	polymeric micelles[a]
C_{max} (μg/mL)	0.43 ± 0.09	0.40 ± 0.12
T_{max} (h)	3.50 ± 0.50	3.30 ± 0.90
AUC_{0-72h} (μg·h/mL)	4.06 ± 1.81	5.57 ± 0.94

[a] $p > 0.05$ vs Sandimmun Neoral.

parameter between them was 6.72 to 7.86 $(J \cdot cm^{-3})^{1/2}$) presented in microemulsion.[63]

Bioavailability and Pharmacokinetics Study. The oral bioavailability of CyA incorporated in mPEG5-PLA5 micelles was evaluated and compared with Sandimmun Neoral. The results are shown in Figure 3, and the relevant pharmacokinetic parameters are listed in Table 2. As shown in Figure 3, the high variability of the blood concentration indicated the high intersubject absorption variability of CyA, which was in agreement with previous reports.[7,8] CyA from polymeric micelles was absorbed a little fast and exhibited slightly short time ($p > 0.05$) to reach maximum blood drug concentration (C_{max}). CyA incorporated in polymeric micelles gave lower C_{max} (0.40 ± 0.12 μg/mL) and higher AUC_{0-72h} (5.57 ± 0.94 μg·h/mL) compared to Sandimmun Neoral ($p > 0.05$). The relative bioavailability of CyA from self-assemblies increased to 137% in comparison to Sandimmun Neoral, indicating that the CyA-loaded polymeric micelles displayed higher bioavailability compared to Sandimmun Neoral although the difference was not statistically significant ($p > 0.05$). Apart from this, the advantage of polymeric micelles is the simplicity of the system and the absence of formulation additives, such as low molecular weight surfactants. Indeed, the commercial formulation Sandimmun Neoral contains Cremophor RH 40 as an emulsifier.

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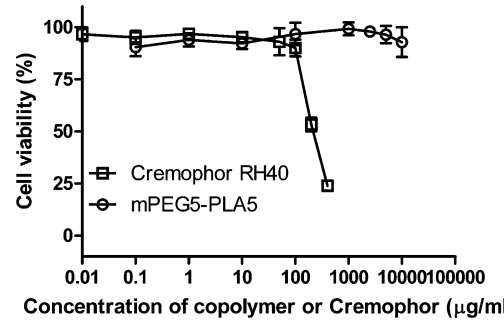

Figure 4. Cytotoxicity of mPEG5-PLA5 and Cremophor RH 40 to Caco-2 cells ($n = 6$).

In addition, the percentage of the cumulative release of CyA from micelles was less than 30% in the first 3 h. Based on the results of pharmacokinetics and *in vitro* release, it was inferred that CyA might be absorbed in intact polymeric micelles, which would be validated by the subsequent transport studies.

Cytotoxicity of Interests. The successful pharmaceutical exploitation of self-assembling polymeric micelles will depend on several factors including their safety, loading efficiency and ability to delivery poorly water-soluble drugs after administration. In order to gauge toxicity, a number of *in vitro* assessments were completed using SRB assay. The cytotoxicity of the drug-free polymeric micelles was evaluated using a microtiter well protocol in which Caco-2 cells were exposed to various concentrations (0.0001−10 mg/mL) of the copolymer over a 24 h time course. The dose-dependent viability of Caco-2 cells treated with the various materials is presented in Figure 4. At these concentrations a relative cell viability of at least 90% was observed for mPEG5-PLA5 copolymer, whereas the cells incubated with 1% Triton X-100 exhibited a relative cell viability of less than 15%. No significant change was detected in cell viability with the increasing copolymer concentration ($p > 0.05$). By comparison, the cytotoxicity of Cremophor RH 40 used in Sandimmun Neoral was also determined for 24 h (Figure 4). It could be seen that the cell viability was in the range of 100% to 89% at concentrations below 100 μg/mL while the cell viability dramatically decreased thereafter. As anticipated, free Cremophor RH 40 inhibited cell growth even at concentrations below 200 μg/mL. Also, mPEG5-PLA5 copolymer exhibited no significant toxicity toward Caco-2 cells, up to a concentration of 10 mg/mL. When the cell viability was 80%, the concentration (i.e., IC_{20}) of mPEG5-PLA5 copolymer was at least 50-fold higher than that of

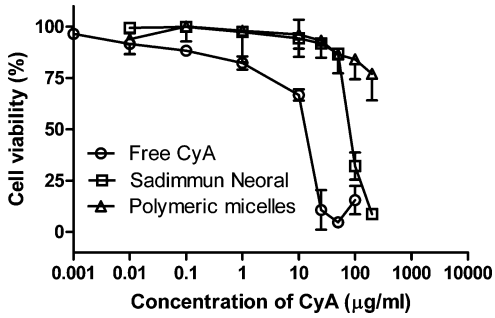

Figure 5. Cytotoxicity of CyA-loaded polymeric micelles, Sandimmun Neoral and free CyA to Caco-2 cells ($n = 6$).

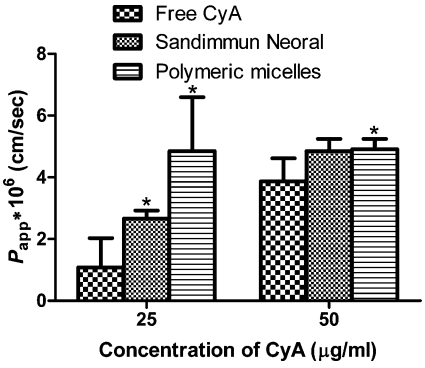

Figure 6. Apparent permeability coefficients (P_{app}) of polymeric micelles, Sandimmun Neoral and free CyA ($n = 3$). *$p < 0.05$ vs free CyA.

Cremophor RH 40. The oral dose of copolymer used in micelles and Cremophor RH 40 in Sandimmun Neoral was about 50 mg/kg and 15 mg/kg, respectively. Thus the higher safety of the micelle-forming copolymer was shown compared with Cremophor RH 40.

The cytotoxicity of CyA-loaded polymeric micelles and Sandimmun Neoral against Caco-2 cells was also determined for 24 h, respectively. As shown in Figure 5, the cell viability decreased gradually to 86% when the concentration of CyA in Sandimmun Neoral was lower than 50 μg/mL while the cell growth was inhibited at concentrations up to 200 μg/mL. Overall, Sandimmun Neoral showed evident cell cytotoxicity when the concentration of CyA was higher than 50 μg/mL. By contrast, the cell viability was higher than 85% when the concentration of CyA loaded in polymeric micelles was up to 100 μg/mL. The IC_{20} of CyA-loaded mPEG-PLA polymeric micelles was 4-fold higher than that of Sandimmun Neoral, indicating that the cytotoxicity of CyA-loaded mPEG-PLA polymeric micelles was significantly decreased compared with Sandimmun Neoral.

Effect of Polymeric Micelles on Transport of Drug through Caco-2 Cell Monolayers. To assess whether drug transport through cell monolayers was affected by the applied copolymeric micelles, apparent permeability (P_{app}) values of CyA from AP to BL were also determined, and the results are shown in Figure 6. At higher concentration of CyA (50 μg/mL), there was no significant difference in P_{app} between polymeric micelles and Sandimmun Neoral and between free

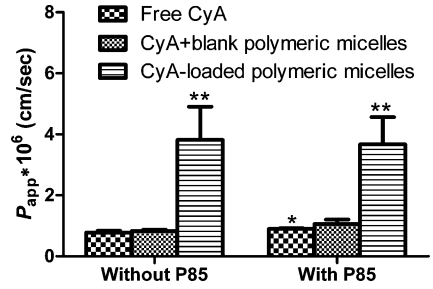

Figure 7. P_{app} of free CyA, free CyA along with blank polymeric micelles and CyA-loaded polymeric micelles from apical to basolateral compartment (AP to BL) in the presence or absence of P85 ($n = 3$). **$p < 0.01$ vs free CyA, *$p < 0.05$ vs free CyA without P85.

CyA and Sandimmun Neoral ($p > 0.05$), respectively, whereas a significant difference ($p < 0.05$) in P_{app} was found between polymeric micelles and Sandimmun Neoral at lower concentration of 25 μg/mL. More importantly, the P_{app} of polymeric micelles and Sandimmun Neoral was significantly higher than that of free CyA ($p < 0.05$), respectively, which was in accordance with the earlier report.[32] Based on the results of permeation tests and bioavailability study, it was deduced that incorporation of CyA in polymeric micelles and microemulsion vehicle improved the oral absorption of CyA mainly through solubilization of CyA other than enhancement of the permeability of CyA across intestinal cells. These results also demonstrated that, for low solubility−high permeability (i.e., BCS Class II) drugs, such as CyA, whose oral bioavailability is solubility or dissolution rate-limited, any factor that improves solubility will improve bioavailability.[44]

Moreover, as shown in Figure 7, the P_{app} of free CyA along with blank polymeric micelles was significantly enhanced in the presence of P85 as an inhibitor of P-gp ($p < 0.05$). However, the P_{app} of CyA loaded in polymeric micelles was not significantly different in the absence ($(3.82 \pm 1.09) \times 10^{-6}$ cm/s) and presence ($(3.67 \pm 0.90) \times 10^{-6}$ cm/s) of P85, which was about 3-fold higher than that of free CyA ($(0.78 \pm 0.06) \times 10^{-6}$ cm/s without P85 and $(0.90 \pm 0.03) \times 10^{-6}$ cm/s with P85). These results indicated that it might be more important for CyA to be solubilized by polymeric micelles to cross Caco-2 monolayers compared with inhibition of P-gp activity. A detailed discussion concerning inhibition of the copolymer to P-gp activity is presented below.

Transport Route of Drug-Loaded Polymeric Micelles through Caco-2 Cell Monolayers. In order to investigate the possible transepithelial transport route of polymeric micelles, the permeation of CyA-loaded polymeric micelles through enterocytes was compared in Caco-2 cell monolayers treated with free CyA and free CyA along with blank micelles while the concentration of CyA for all test samples was 25 μg/mL. As shown in Figure 7, the P_{app} of CyA was not significantly influenced by the presence of blank micelles ($p > 0.05$), however, the P_{app} of CyA-loaded polymeric micelles was about 3-fold higher than that of free CyA in the absence or presence of blank polymeric micelles after

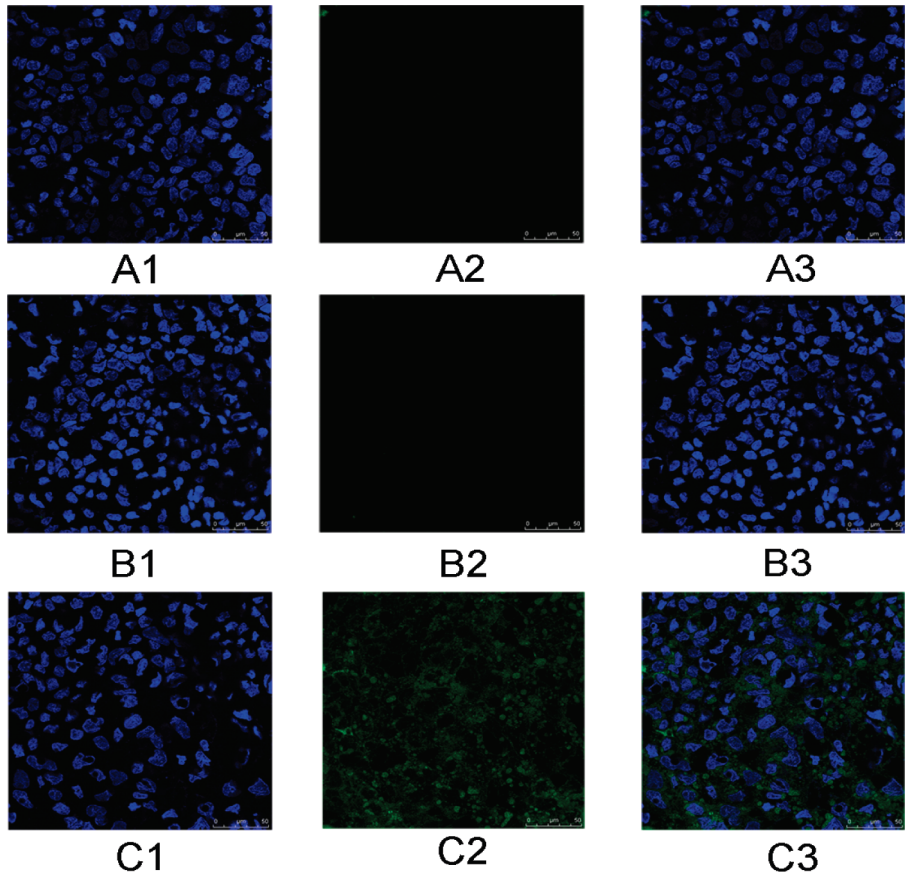

Figure 8. Confocal images of the Caco-2 cells after incubation with 1 μg/mL of free coumarin-6 (A), free coumarin-6 along with blank polymeric micelles (B) and coumarin-6-loaded polymeric micelles (C) for 2 h at 37 °C. A1−C1: Blue color indicates the nucleus of Caco-2 cells stained with Hoechst 33258. A2−C2: Green color shows coumarin-6 in Caco-2 cells after applying free coumarin-6 (A2), free coumarin-6 along with blank polymeric micelles (B2), and coumarin-6-loaded polymeric micelles (C2), respectively. A3−C3: Overlapping images exhibited drug in the nucleus of Caco-2 cells after applying free coumarin-6 (A3), free coumarin-6 along with blank polymeric micelles (B3), and coumarin-6-loaded polymeric micelles (C3), respectively.

incubation for 3 h. In addition, in all cases, TEER values were monitored throughout the experiments: the addition of micelles to Caco-2 monolayers for up to 3 h did not affect TEER values significantly (only about 10% reduction), confirming that the integrity of the cell monolayers was preserved. Based on the permeation experiments, it was suggested that the polymeric micelles were transported by routes other than the paracellular route. Further, CyA was transported across the Caco-2 cells in intact micelles other than in free form.

In order to further demonstrate and intuitively observe the effect of polymeric micelles in the transport of CyA, coumarin-6 with a green fluorescence, for which it was reported that it cannot be directly internalized by Caco-2 cell monolayers in free form,[56,64] was used as a fluorescence marker. Consequently, the fluorescence observed from the uptake samples reflected the fluorescent nanoparticles taken up by the cells but not the free fluorescence.[65] Confocal images of the Caco-2 cells after incubation with 1 μg/mL of free coumarin-6 solution, free coumarin-6 along with blank polymeric micelle solution and coumarin-6 loaded in polymeric micelles for 2 h at 37 °C, respectively, are shown in Figure 8. For free coumarin-6 in 1% (v/v) DMSO, there was no green fluorescence existing around the nucleus (Figure 8A), which showed that no coumarin-6 transported into Caco-2 cells. The same results were obtained for free coumarin-6 along with blank polymeric micelles (Figure 8B). For coumarin-6 loaded in polymeric micelles, in the cell cytoplasm, around the blue nucleus were aggregates of the green coumarin-6 (Figure 8C), which suggested that coumarin-6-loaded intact polymeric micelles had been internalized by the cells. In addition, cells exposed to micelles showed no decrement in resistances. Based on these results, it was similarly concluded that (1) polymeric micelles with a concentration of 5 mg/mL of copolymer could not disrupt

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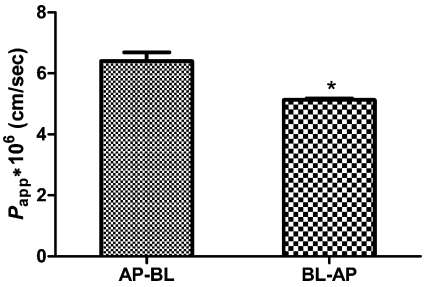

Figure 9. P_{app} of CyA loaded in polymeric micelles from apical to basolateral compartment (A to B) and vice verse (B to A) ($n = 3$). *$p < 0.01$.

the Caco-2 cell monolayers and (2) the paracellular pathway was not involved in the transepithelial transport of polymeric micelles.

In addition, bidirectional permeability of CyA loaded in polymeric micelles was investigated. As shown in Figure 9, the P_{app} of CyA was still significantly higher in the AP−BL direction ((6.40 ± 0.29) $\times$ 10^{-6} cm/s) compared to that in the BL−AP direction ((5.12 ± 0.05) $\times$ 10^{-6} cm/s) ($p < 0.01$), which was consistent with the recent report.[66] Based on the results in the current study, this difference in the P_{app} of CyA indicated that the absorption of CyA loaded in polymeric micelles might result from an active process such as endocytosis.[66]

Copolymer Effect on P-gp Efflux Pumps. In order to evaluate a possible inhibitory effect of mPEG-PLA on efflux pumps, which has been reported for other polymeric surfactants,[50,67,68] the influence of the polymer on CyA efflux, a good P-gp substrate,[69] was examined in the presence and absence of P85. It was reported that the P85 free unimers were responsible for inhibiting P-gp efflux pumps.[70] In the case of our experiments, P85 was added to the AP compartment at a concentration of 30 μM, a value lower than the P85 CAC (67 μM)[50] to ensure that P85 copolymer was in the form of unimers in all measurements. In all cases, TEER values were monitored throughout the experiments: the addition of polymers and/or P85 to the AP side of Caco-2 monolayers for up to 2 h did not affect TEER values significantly, confirming that the integrity of the cell monolayers was preserved.

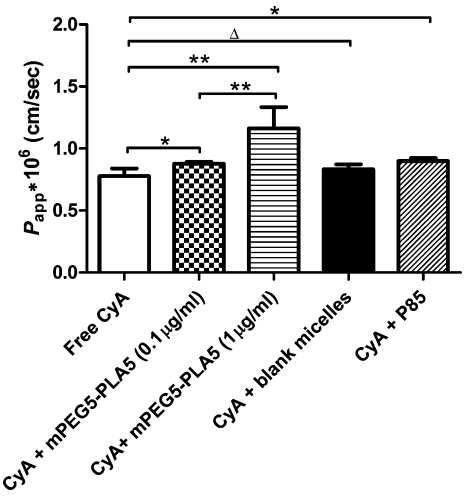

Figure 10. P_{app} of CyA across Caco-2 monolayers from apical to basolateral compartment (AP to BL) in the presence of P85 or mPEG5-PLA5 copolymer ($n = 3$). *$p < 0.05$, **$p < 0.01$, $^{\Delta}p > 0.05$.

As the CAC of mPEG5-PLA5 was 1.95 μg/mL converted from 1.99×10^{-7} mol/L (Table 1), the concentration of the copolymer was selected to be 0.1 and 1.0 μg/mL. As shown in Figure 10, the P_{app} of free CyA was significantly enhanced in the AP to BL direction in the presence of the copolymer at 0.1 or 1.0 μg/mL ($p < 0.01$), whereas the P_{app} of free CyA was not significantly different in the presence or absence of blank micelles ($p > 0.05$). These data indicated that P-gp activity was inhibited by mPEG5-PLA5 unimers other than polymeric micelles, which was similar to the positive control P85. Besides, the effect of the copolymer unimers on P-gp activity exhibited concentration-dependence. These results were consistent with the previous report.[71]

Discussion

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 recent years, polymeric micelles have drawn much attention as drug delivery carriers due to their good pharmacological characteristics. mPEG-PLA micelles were therefore designed and prepared to encapsulate CyA, a highly lipophilic drug. As anticipated, the oral bioavailability of CyA was significantly improved and was comparable to Sandimmun Neoral (Table

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2). The enhancement in bioavailability of CyA by polymeric micelles could be attributed to the following facts: (1) Theoretically it is known that undissolved drugs cannot pass through intestine. The solubility of CyA was enhanced by polymeric micelles and CyA existed in the molecular level. (2) The size of micelles was less than 100 nm, which might promote the micelles to overcome unstirred water layer in the lumen of small intestine and adhere into the intestinal wall and thus could ensure sufficient time and concentration gradient for absorption of drugs.[72] It is known that prolonged residence time due to increased duration of contact with mucosal surface in the GI system can improve drug bioavailability. (3) The inhibition of P-gp activity by mPEG-PLA unimers might be an important factor due to the fact that CyA is also an inhibitor of P-gp. Overall, 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. Except for several surfactants of the Pluronics class, most of the existing amphiphilic polymers forming micelles have been tested after intravenous administration. The fact that mPEG-PLA could be delivered in a capsule to form, upon dilution in the gastrointestinal tract, the drug containing micelles with a low CAC is of particular interest.[73] These amphiphilic polymers forming micelles could open new opportunities for the oral delivery of poorly soluble drugs.

There are two routes of transepithelial drug transport by passive diffusion:[55] 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. Earlier investigation demonstrated that polymer unimers forming polymeric micelles transported through Caco-2 cell monolayers neither by the paracellular route nor by M-cells,[43] whereas the mechanism by which polymeric micelles cross the intestinal barrier has remained uncertain. The aim of the current work was therefore to explore the transport mechanism of polymeric micelles across enterocyte-like Caco-2 cell monolayers.

The gauge of cytotoxicity of the mPEG5-PLA5 copolymer used in preparation of polymeric micelles was first taken. The results suggested that the polymer up to a concentration of at least 10 mg/mL was not acutely toxic to Caco-2 cells. The concentration of mPEG-PLA copolymer was at least 50-fold higher than that of Cremophor RH 40 at 80% of the cell viability, suggesting the higher safety of the copolymer compared with Cremophor RH 40. Further, the cytotoxicity of CyA could be remarkably decreased by mPEG-PLA polymeric micelles compared with Sandimmun Neoral. This important result indicated that we succeeded in minimizing the cytotoxicity of Cremophor RH 40-based formulation on cells by using the mPEG-PLA polymeric micelle formulation.

Next, the transport route of polymeric micelles across Caco-2 monolayer was deeply investigated by permeation tests and confocal microscope observation. Before performing transport studies, TEER values, which controlled the quality of tight junctions, were measured in empty and cell seeded polycarbonate Transwell inserts. The results were 502 Ω and >400 Ω, respectively, which indicated that the tight junctions were formed in cells.[47] In the permeation test of free CyA through Caco-2 cell monolayers, 10% of ethanol was added to the transport medium due to the fact that the solubility of CyA was too low in transport medium to be determined in the received side. Significant increase in transport of CyA loaded in polymeric micelles was found whereas the blank polymeric micelles did not affect the transport of CyA (Figure 7), implying that CyA loaded in polymeric micelles was transported across the Caco-2 cells in intact micelles other than in free form, which was further confirmed by CLSM observation (Figure 8). Previous document reported that up to 80% of TEER value reduction could affect the tight junctions.[48,63] In the case of our experiment, only about 10% reduction in the TEER values of Caco-2 monolayers was observed (data not shown) throughout the experiments, suggesting that polymeric micelles did not significantly alter the integrity of the Caco-2 cellular tight junctions, that is to say, polymeric micelles did not open the tight junctions. In addition, the paracellular spaces represent less than 1% of the mucosal surface area and the pore diameter of the tight junctions does not exceed 10 Å, whereas mPEG5-PLA5 micelles exhibited a diameter of about 72 nm and unimers have an average molecular weight of 14200 Da. As the drug was entrapped in the micelles, it could become unavailable for passive diffusion through the monolayers. Further, paracellular transport is the passive diffusion through intercellular spaces. Based on these results, it was suggested that the polymeric micelles were transported by routes other than the paracellular route. To further elucidate the transport process of CyA in the presence of the polymeric micelles through the Caco-2 cell monolayers, bidirectional permeation experiments were performed. The significant difference in P_{app} in AP to BL and BL to AP directions through the Caco-2 cell monolayers showed that the transport of CyA loaded in polymeric micelles exhibited transport directionality, suggesting that polymeric micelles might undergo an active AP to BL transport that probably involved endocytosis. This was in accordance with the recent report in which the cell model used was FAE cells which mimic M-cells and are specialized in the uptake of macromolecules and particles via transcytosis.[43] This was further confirmed by intuitive observation of fluorescent imaging of coumarin-6-loaded polymeric micelles with confocal microscope which showed punctuated fluorescence in the

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Caco-2 cytoplasm (Figure 8). The possible endocytosis of polymeric micelles was first investigated by evaluating the P_{app} of CyA loaded in mPEG5-PLA5 in an *in vitro* Caco-2 cell model which is considered to be a representative model for assessing intestinal uptake of drugs. Further experiments on permeation of CyA loaded in polymeric micelles at 4 °C will be then conducted using Caco-2 cell model to explore whether temperature-dependent transport exists.

It is well-known that CyA is a P-gp substrate and the oral absorption is in part affected by P-gp efflux activity.[69,74] A lot of research had been done on investigation of the P-gp inhibition effect of surfactants such as Cremophor RH 40.[70,72] Cremophor RH 40 could specifically bind to the hydrophobic domain of the P-gp that may change its secondary and/or tertiary structure and reduce its function, which may be the reason that the P_{app} of Sandimmun Neoral was higher than that of free CyA (Figure 7). In addition, it has been demonstrated that several amphiphilic polymers can inhibit the P-gp efflux pumps.[67,68,75] Conversely, mmePEG$_{750}$-*p*(CL-*co*-TMC) was reported not to inhibit P-gp activity.[43] In the case of our experiment, the effect of the copolymer on P-gp efflux pump was therefore evaluated. Fortunately, the mPEG5-PLA5 also appeared to inhibit the intestinal P-gp efflux pumps below its CAC and not above its CAC without significantly affecting TEER values (Figure 10), which was similar to the positive control P85 whose efflux pump inhibitory mechanism is believed to be due to (a) polymer mediated inhibition of P-gp ATPase activity and ATP depletion[67] and (b) effects of polymer unimers on membrane fluidization, and it has been proved that the effect of polymers on energy conservation is mediated by single chain units (unimers) and the inhibitory effect decreases when reaching the critical association concentration.[76]

Conclusion

The mPEG5-PLA5 polymeric micelles were able to significantly enhance the oral absorption of CyA with a particle size below 100 nm and high encapsulation efficiency (about 78%) and had a higher AUC compared to the marketed CyA formulation Sandimmun Neoral. The data collected in the current study demonstrated that polymeric micelles could significantly increase the permeability of CyA (BCS class II drug) across Caco-2 cell monolayers and might undergo an active AP to BL transport that probably involved endocytosis. Furthermore, the mPEG5-PLA5 also appeared to inhibit the intestinal P-gp efflux pumps below its CAC and not above its CAC without significantly affecting TEER values. Overall, these amphiphilic polymers forming micelles could open new opportunities for the oral delivery of poorly soluble drugs.

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Supporting Information Available: Characterization of mPEG-PLA copolymers. This material is available free of charge via the Internet at http://pubs.acs.org.

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