Regular Article

Preparation and Evaluation of PEGylated Poly-L-ornithine Complex as a Novel Absorption Enhancer

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Polycationic compounds, such as poly-L-arginine and poly-L-ornithine (PLO), enhance the nasal absorption of hydrophilic macromolecular drugs. However, the bio availability corresponding to the dose of these enhancers has not been obtained in an open system study, where an administered solution is transferred to the pharynx because they do not exhibit mucoadhesion/retention in the nasal cavity. In this study, we prepared PEGylated-poly-L-ornithine (PEG-PLO) and investigated the effects of PEGylation on *in vitro* adhesion/retention properties, permeation enhancement efficiency, and cytotoxicity. PEG-PLO bearing 3–4 polyethylene glycol (PEG) chains per PLO molecule was more retentive than unmodified PLO on an inclined plate. The permeability of a model drug, FD-4, across Caco-2 cell sheets was enhanced by PEG-PLO as well as by PLO. PLO showed cytotoxicity at high concentrations, whereas PEG-PLO did not decrease cell viability, even above the concentration giving a sufficient enhancement effect. These findings suggest that PEGylation of polycationic absorption enhancers improves their adhesion/retention and decreases their cytotoxicity, which may lead to enhancers with greater utility.

Key words poly-L-ornithine (PLO); polyethylene glycol (PEG); permeation enhancer

Bioactive peptides, such as insulin, teriparatide, liraglutide, and somatropin, have been used widely as therapeutic drugs because of their intrinsic and effective activities, however, they have poor permeability across the epithelium because they are hydrophilic macromolecules. Orally administered peptides are often inactivated in the gastrointestinal tract, and thus peptides are usually administered as injectable formulations. However, injection can result in poor compliance because it is painful and burdensome.[1)] Therefore, alternative routes of administration are required.

The nasal mucosa has a large surface area for absorption because of its villus structure. Drugs absorbed across the nasal mucosa avoid the hepatic first pass effect because of the abundant vasculature under the nasal mucosa.[2)] The intranasal route is effective for absorbing high molecular weight drugs[3)] and is usually not painful. Therefore, intranasal administration is an important alternative route to injection for peptides and proteins.

Polycationic compounds, such as poly-L-arginine (PLA), poly-L-lysine, and poly-L-ornithine (PLO), enhance the transmucosal absorption of hydrophilic macromolecules.[4,5)] PLA enhances the paracellular permeability of rabbit nasal mucosa and Caco-2 cell sheets reversibly.[6,7)] The absorption of hydrophilic macromolecules across the nasal mucosa in rats is also increased by co-administration with PLA.[8)] PLA enhances the permeability by altering the localization of tight junction proteins from the cell–cell junction to the intracellular space without causing cytotoxicity.[9)] Therefore, polycationic absorption enhancers are useful in developing efficient *trans*-mucosal drug delivery systems.

Nevertheless, it is difficult to increase the bioavailability of drugs *via* intranasal administration by only increasing the absorption rate. There are two clearance mechanisms in the nasal cavity: mucociliary clearance by cilia and physical clearance *via* the structure of the nasal cavity.[3,10,11)] The substances trapped in the nasal mucus are transferred to the pharynx by these mechanisms, and drugs and absorption enhancers administered in the nasal cavity are removed from the absorption area. Polycationic absorption enhancers do not have mucoadhesive/retentive properties. Hence, the bioavailability corresponding to the dose of these absorption enhancers has not been obtained in an open system study, where an administered solution is transferred to the pharynx. Therefore, the development of functional polycations with mucoadhesive/retentive properties is important for the transmucosal delivery of hydrophilic macromolecules.

PLO is a homopolymer consisting of L-ornithine, which has a primary amine on its side chain and is positively charged under physiological conditions, similar to PLA. In a preliminary study, we confirmed that PLO also altered localization of tight junction proteins and enhanced the permeation of hydrophilic macromolecules (unpublished results).

We intend to develop functional polycations by chemical modification of the functional compound. It has been suggested that both the absorption enhancement and the retentivity in nasal cavity were important to improve the transnasal delivery of unabsorbable drugs[12)] and PEGylation of proteins increased the viscosity of them.[13)] Polyethylene glycol (PEG) is widely used as a pharmaceutical additive and in pharmaceutical modifications.[14)] The chemical modification of pharmaceuticals, such as peptides, proteins, and antibodies, with PEG modifies their physiological and physicochemical properties.[15–17)] Furthermore, it has been reported that hydrogel having PEG chain on its surface shows the mucoadhesive ability by interaction with mucosa.[18)]

In this study, we investigated the effect of PEGylating polycations on their permeation enhancement to develop superior absorption enhancers. We prepared PEGylated-PLO and deter-

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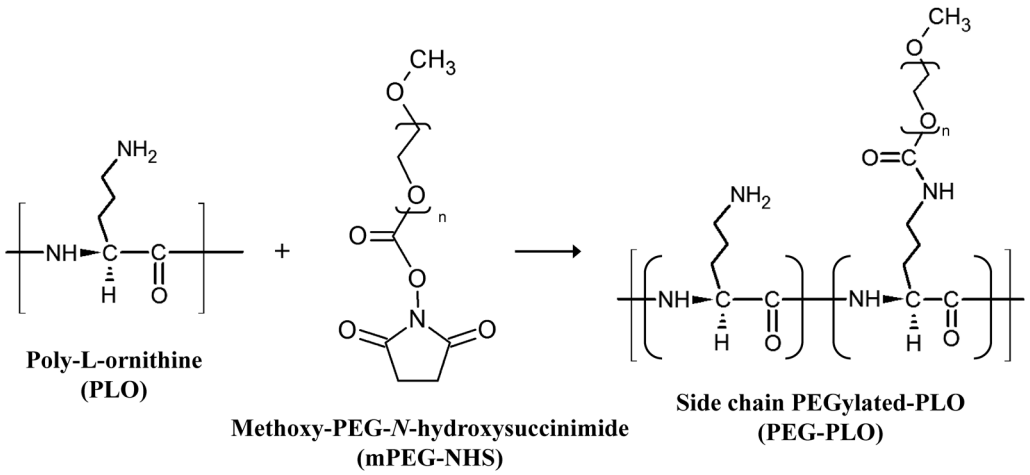

Fig. 1. Synthesis of the Side Chain PEGylated-PLO with mPEG-NHS

PLO and mPEG-NHS were mixed in a 1 : 5 M ratio. The mixture was stirred overnight at room temperature (r.t.).

mined its *in vitro* adhesion/retention, permeation enhancement efficiency, and cytotoxicity.

MATERIALS AND METHODS

Materials PLO hydrobromide (molecular weight (MW) 18.5 kDa) was purchased from Alamanda Polymers, Inc. (Huntsville, AL, U.S.A.). α-Succinimidyloxy carbonyl-ω-methoxy polyoxyethylene (mPEG-NHS; SUNBRIGHT ME-400TS, MW 40.2 kDa), α-methyl-ω-aminopropoxy polyoxyethylene (mPEG-NH_2; SUNBRIGHT MEPA-40T, MW 42.5 kDa), and α-mercaptoethyl-ω-methoxy polyoxyethylene (mPEG-SH; SUNBRIGHT ME-400SH; MW 39.4 kDa) were obtained from NOF Corporation (Tokyo, Japan). Fluorescein isothiocyanate dextran (FD-4, MW 3.7 kDa) was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). 2-Morpholinoethanesulfonic acid monohydrate (MES) and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) were obtained from Dojindo Laboratories (Kumamoto, Japan). The cell culture reagents and supplies were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, U.S.A.). 2,4,6-Trinitrobenzenesulfonic acid (TNBS) was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other reagents were of analytical grade.

Cell Culture Human colorectal adenocarcinoma Caco-2 cells were purchased from the American Type Culture Collection (Manassas, VA, U.S.A.) and used for all cell culture experiments. Caco-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% non-essential amino acids, 1% Gluta MAX-1, 1% antibiotic–antimycotic, 10% fetal bovine serum at 37°C, under a 5% CO_2 atmosphere. The cells were seeded on a polycarbonate membrane (Transwell, 12 well, Corning, Corning, NY, U.S.A.) at 1.0×10^5 cells/cm^2, and used for experiments 21–28 d after seeding.

Synthesis of PEGylated-Poly-L-ornithine PEGylated-poly-L-ornithine (PEG-PLO) was synthesized as shown in Fig. 1. PLO (37 mg) was dissolved in 0.1 M MES buffer (pH 7.0, 20 mL), and then mPEG-NHS (402 mg) was dissolved in the PLO solution (20 mL). The mixture was stirred overnight at room temperature to conjugate mPEG to PLO. Size-exclusion chromatography consisting of a PROTEIN KW-803 column (Shodex) and RI-101 refractive index detector (Shodex) was used to confirm that the reaction had finished. A 0.5 M sodium acetate–acetic acid buffer (pH 4.7) was used as the eluent. To elute the cationic compound from the reaction mixture, an ion exchange spin column (Strong Cation Exchange Spin Column Maxi, Thermo Fisher Scientific) was used according to the manufacturer's protocol. The reaction solution (18 mL) was added to the membrane and centrifuged at $500\times\boldsymbol{g}$ for 30 min. Purification buffer (10 mL, 25 mM sodium acetate buffer, pH 5.5) was added and centrifuged at $500\times\boldsymbol{g}$ for 10 min. This procedure was repeated twice. Elution buffer (5 mL, 25 mM sodium acetate buffer containing 1.0 M NaCl, pH 5.5) was added and centrifuged at $500\times\boldsymbol{g}$ for 5 min. This procedure was repeated twice. The elution solution was dialyzed (Spectra/Por 7, RC, MWCO=8 kDa) against distilled water (1 L) for 24 h (four changes of the outer distilled water). The dialysate was freeze-dried to obtain powdered PEG-PLO.

Characterization of PEGylation Ratio of PEG-PLO Complex Elemental analysis and a TNBS assay were used to determine the PEGylation ratio of PEG-PLO. Elemental analysis was carried out according to a reported method.[19] PLO and mPEG-SH were dissolved in distilled water and mixed completely at each ratio, and then these mixtures were freeze-dried. The dried samples were measured with a micro element analyzer (MT-6 CHN Corder, Yanaco, Tokyo, Japan). The carbon-to-nitrogen (C/N) ratios were calculated for known samples, and a standard curve was plotted. The C/N ratio in PEG-PLO was measured in the same manner, and the PEGylation ratio of PEG-PLO was determined from the standard curve. The TNBS assay was performed as previously reported with slight modifications.[20] Fifty micrograms per milliliter PEG-PLO (1 mL) solution and 0.1 M TNBS solution (75 μL) were added to 0.1 M sodium tetraborate solution (2 mL). The mixture was incubated at 37°C for 60 min. After incubation, the absorbance at a wavelength of 420 nm was determined by using a UV-Vis spectrophotometer (UVmini 1240, Shimadzu, Kyoto, Japan). A standard curve of the PEG-PLO/PLO absorbance ratio were calculated from the theoretical values of the remaining free primary amine groups. The PEGylation ratio was obtained by reading off the value corresponding to the experimental absorbance ratio from the standard curve.

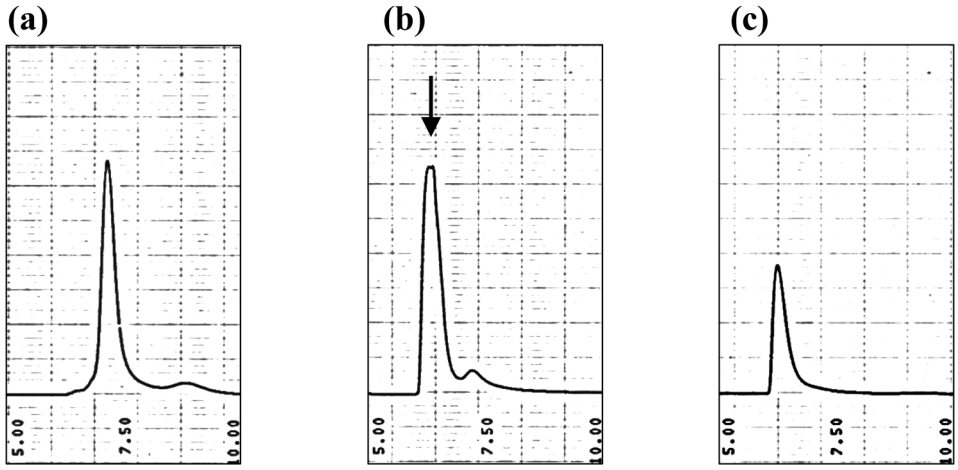

Fig. 2. Typical Chromatograms of Size Exclusion HPLC

(a) Before the reaction of PLO and mPEG-NHS. Peaks were observed at 7.2 and 9.0 min. (b) After the reaction of PLO and mPEG-NHS. Peaks were observed at 6.3 (arrow) and 7.3 min. (c) After extracting the cation compound on a spin column. Only one peak was observed at 6.3 min.

Evaluation of *in Vitro* Retentivity of PEG-PLO The retentivity of PEG-PLO was evaluated by the inclined plate test as previously reported with modifications.[21] A stainless steel plate (SSP) was washed with methanol and dried for 5 min, the plate was inclined at 45°. A 0.1% (w/v) PLO or PEG-PLO solution (35 μL) was dropped on the plate, and the time it took to travel 4 cm was measured.

Determination of Membrane Conductance The transepithelial electrical resistance (TEER) was measured using a voltohmmeter (Millicell ERS-2, Merck, Darmstadt, Germany) and was converted to units of ohm square centimeter by multiplying the resistance by the surface area of the membrane. Caco-2 cell sheets were washed with Hank's balanced salt solution (HBSS), and DMEM culture medium was replaced with HBSS. The polycation solutions (PLO and PEG-PLO at various concentrations) were applied to the apical side of the cell sheets. The TEER values were measured at predetermined times after the polycation was applied to the Caco-2 cell sheet. TEER was converted to the membrane conductance (G_t=1/TEER, mS/cm^2), and the ratio of $G_{t\ 120\,min/0\,min}$ in the treatment group to that in the control group (G_t ratio) was calculated.

FD-4 Permeation Experiment The FD-4 permeation experiment was performed as reported previously.[22] Caco-2 cell sheets were washed with HBSS, and the DMEM culture medium was replaced with HBSS. The test solutions containing polycations (PLO and PEG-PLO at various concentrations) and FD-4 (1.0 mg/mL) were applied to the apical side of the cell sheet. Samples were collected from the basolateral side at predetermined times up to 120 min. The cumulative amount of FD-4 that crossed the cell sheet per unit surface area (μg/cm^2) was calculated. The fluorescence was determined with a spectrofluorophotometer (RF-5300PC, Shimadzu) with an excitation wavelength of 495 nm and an emission wavelength of 515 nm. The apparent permeability coefficient (cm/s, P_{app}) of FD-4 was calculated using P_{app} (cm/s)=dQ/dt/($A\times C_0$), where dQ/dt is the steady-state permeation rate (μg/s), A is the surface area of the Transwell membrane (cm^2), and C_0 is the initial concentration of FD-4 on the apical side (μg/mL).

Cytotoxicity The MTT assay was performed as previously reported.[6,23] In brief, PLO or PEG-PLO solutions were applied to the apical side of Caco-2 cell sheets at various concentrations for 120 min. The solution was replaced with DMEM containing MTT (0.5 mg/mL). After incubation at 37°C for 180 min, the formazan product was released by lysing the cells with dimethyl sulfoxide (DMSO). The absorbance at 540 nm was measured with a microplate reader (Multiskan Ascent, MTX Lab Systems, Bradenton, FL, U.S.A.). The cell viability was expressed as a percentage of the control.

Statistical Analysis The results are presented as the mean±standard errors (S.E.). The two groups were compared using Student's t-test. A p value of less than 0.05 was regarded as significant.

RESULTS

Synthesis of PEG-PLO Complex The side chain PEGylated-PLO was obtained from the amine NHS-ester reaction (Fig. 1), and the product was confirmed by size exclusion HPLC. Peaks derived from PLO and mPEG were observed at 9.0 and 7.2 min, respectively, before the reaction (Fig. 2a). After the reaction, a new peak at 6.3 min appeared, suggesting conjugation of PEG to PLO (arrow, Fig. 2b). The PLO peak was not observed, indicating that there was no unreacted PLO in the reaction solution. Therefore, the cation exchange spin column extracted only PEG-PLO (Fig. 2c).

Characterization of PEGylation Ratio of PEG-PLO Complex The elemental analysis and TNBS assay were used to determine the PEGylated ratio of PEG-PLO. The elemental analysis showed that the C/N ratio and correcting value of PEG-PLO were 38.76 and 33.62, respectively, and that the PEGylation ratio of PEG-PLO was 3.83 (Table 1, Fig. 3a). The TNBS assay showed that absorbance ratio of PEG-PLO/PLO was 0.1234 and that the PEGylation ratio of PEG-PLO was 3.22 (Fig. 3b). Because the values obtained by the elemental analysis and TNBS assay were similar, it was concluded that PEG-PLO bears an average of 3–4 PEG chains per PLO molecule.

***In Vitro* Retentivity of PEG-PLO** The inclined plate test was used to evaluate the retentivity of PEG-PLO (Fig. 4). A 0.1% (w/v) PLO solution took 6.88 s to flow down the SSP, similar to water (7.23 s). However, 0.1% (w/v) PEG-PLO solution took 15.86 s. These results suggested that the adhe-

Table 1. The Measurement Values and C/N Ratio of PEG-PLO by the Elemental Analysis

	Measurements (%)			C/N (Measurement)	C/N (Theoretical)	Measurement/Theoretical	Measurement deviation
	C	H	N				
PLO+PEG=1:3	50.52	8.62	1.65	30.62	26.42	1.16	1.15
PLO+PEG=1:4	51.12	8.74	1.30	39.32	34.52	1.14	
PLO+PEG=1:5	51.43	8.79	1.04	49.45	42.61	1.16	
	Measurements (%)			C/N from measurement	Correcting value	PEG/PLO ratio	
	C	H	N				
PEG-PLO	51.94	8.67	1.34	38.76	33.62	3.83	

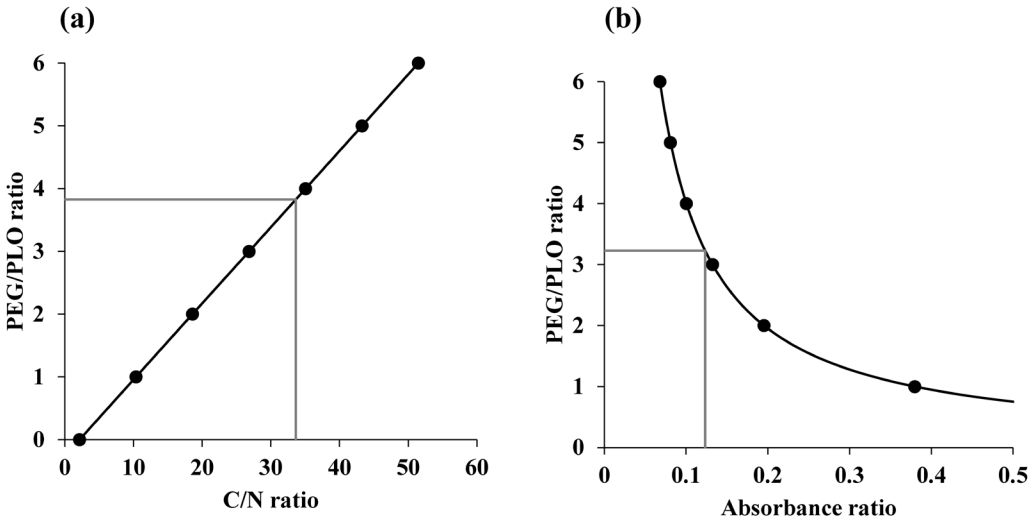

Fig. 3. Determination of PEGylated Ratio of PEG-PLO

(a) Elemental analysis results. Black circles are the C/N ratios obtained from each known sample. (b) TNBS assay results. Black circles are the theoretical absorbance ratios of PEG-PLO/PLO.

sion/retention properties of polycations could be improved by PEGylation.

Membrane Conductance and FD-4 Permeation To determine the effects of PLO and PEG-PLO on membrane conductance (G_t) and permeability of macromolecules across the Caco-2 cell sheets, an FD-4 permeation study and TEER measurements were performed after exposure to these polycations. Each G_t was expressed in the ratio of 120 to 0 min values because the effects of polycations were reached a steady state (Fig. 5A). G_t and P_{app} of FD-4 in Caco-2 cell sheets were increased dose-dependently by exposure to PEG-PLO and to PLO (Fig. 5B). However, the permeation profiles and cumulative absorption of FD-4 after application of PLO 1 μM and PEG-PLO 20 μM were almost same (Fig. 5A).

Cytotoxicity Cytotoxicities of PLO and PEG-PLO in Caco-2 cells were determined by an MTT assay (Fig. 6). PLO produced a decrease in cell viability at 1 μM, a concentration that enhances adsorption. Furthermore, the viability was decreased significantly at the higher concentration (3 μM). In contrast, PEG-PLO had no effect on the cell viability even at the highest concentration in this study. The physical mixture of PLO and mPEG-NH_2 containing equimolecular amounts of the PEG-PLO group decreased cell viability at 1 μM. These results suggest that the cytotoxicity of PLO was decreased by PEGylation.

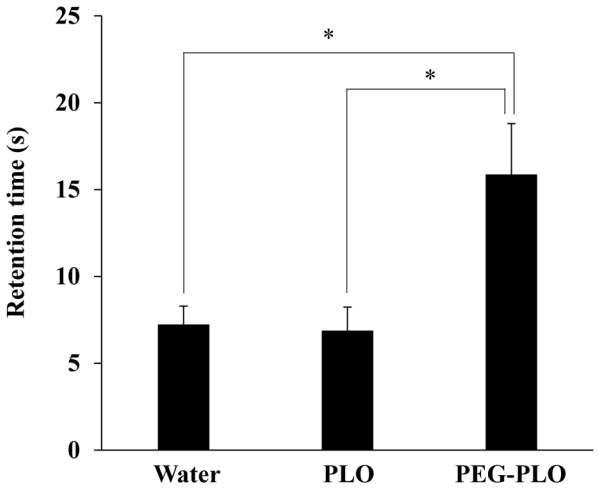

Fig. 4. Retention Time of the PEG-PLO Solution on the Inclined SSP

Retention time was defined as the time required for the solution to flow 4 cm. All measurements were performed at r.t. on the same day. Each data column represents the mean±S.E. (n=6), *p<0.05.

DISCUSSION

PLO was PEGylated by an amine-NHS coupling reaction. We had attempted to preparation of PEG-PLA, but it was hard to introduce PEG to PLA. We had confirmed that the permeation stimulatory effect of PLO was higher than PLA

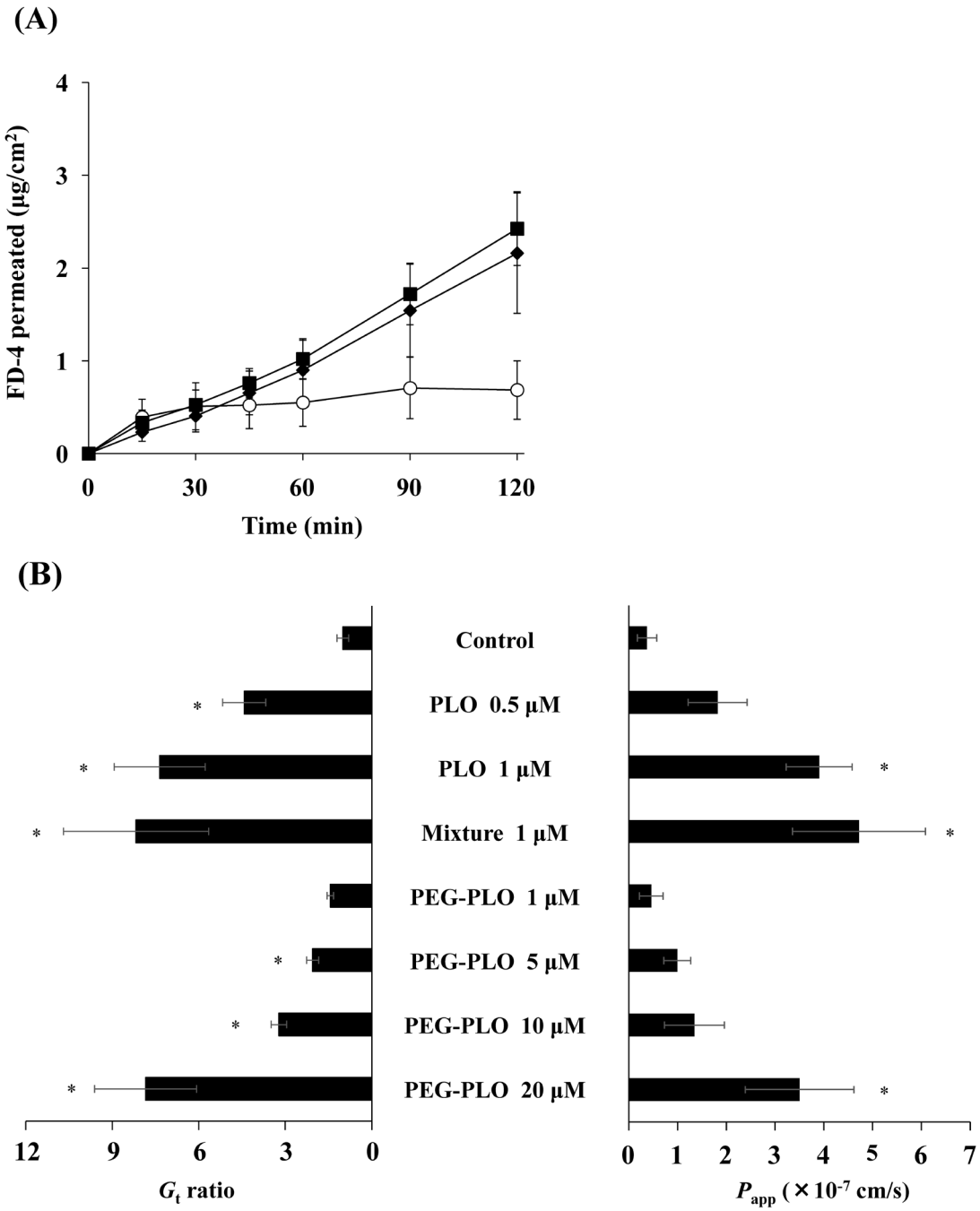

Fig. 5. Effects of Polycations on FD-4 Permeation (P_{app}) and Membrane Conductance (G_t) in Caco-2 Cell Sheets

Permeation profiles of FD-4 after application of PLO and PEG-PLO (A). ○: Control, ■: PLO 1 μM, ◆: PEG-PLO 20 μM. The relationships of P_{app} and G_t after application of PLO and PEG-PLO (B). Control group was treated with vehicle (HBSS). Mixture was a physical mixture of 1 μM PLO and 4 μM mPEG-NH_2. Each data column represents the mean±S.E. (n=3). *p<0.05 *vs.* control.

and PEGylation of PLO was relatively easy because of its primary amine on the side chain in preliminary study. Therefore, this paper has been described with a focus on the PEG-PLO. Furthermore, we had measured the viscosities of 40 kDa PEG and 10 kDa PEG in preliminary study, the viscosity tended to be increased with the increase in molecular weight of them. We inferred that the viscosity of PEG would become too high if the molecular weight was greater than 40 kDa, therefore, we used the 40 kDa PEG in this study. To simplify the purification, PLO was completely reacted by adding excess PEG. In our preliminary study, no unreacted PLO was observed when 5 eq of PEG were used. Hence, PEG-PLO could be purified by only cation extraction. The ion exchange spin column exhibited high selectivity for cationic compounds (Fig. 2). Although the column is suitable for small-scale studies, the purification method would need to be optimized for a large-scale experiment.

Elemental analysis and a TNBS assay were used to evaluate the PEGylation ratio of the PEG-PLO complex. Ideally, the PEGylated site and the number of PEG chains in the polymer are determined. However, modification of a specific site is impossible when using mPEG-NHS in the reaction. Moreover, the degree of polymerization of PLO is not controlled precisely. Therefore, the number of primary amine groups and

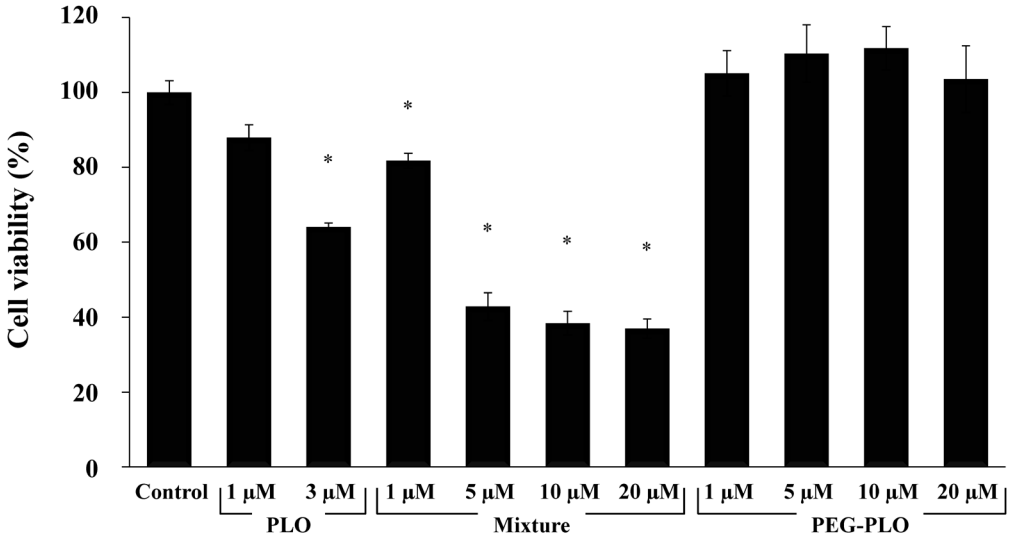

Fig. 6. Effects of Polycations on Cell Viability in Caco-2 Cell Sheets

Control group was treated with vehicle (HBSS). Mixtures: 1 μM: 1 μM PLO+4 μM mPEG-NH_2; 5 μM: 5 μM PLO+20 μM mPEG-NH_2; 10 μM: 10 μM PLO+40 μM mPEG-NH_2; 20 μM: 20 μM PLO +80 μM mPEG-NH_2. Each data column represents the mean±S.E. (n=3). *p<0.05 *vs.* control.

the elemental composition were calculated from the degree of polymerization according to the manufacturer's datasheet. The average PEGylated ratio per PEG-PLO molecule was determined by the change in these parameters after PEGylation.

Polycationic absorption enhancers, such as PLA and PLO, do not have mucoadhesive/retentive properties. The retention time of PEG-PLO on an SSP was significantly improved compared with that of PLO (p<0.05), suggesting that PEGylation can increase or impart mucoadhesive/retentive properties. However, the difference between the SSP and real mucosa must be considered. *In vivo* studies are required to investigate mucoadhesive/retentive properties.

Although PEG-PLO enhanced the permeation of FD-4, the effect was smaller than that of PLO because the steric hindrance of the PEG chain likely affects permeation. The permeation enhancement effect of polycations arises from the interaction between their positive charge and the cell membrane. The molecular weight of PEG used in this study was about 40 kDa, and its large chain may have inhibited contact between polycations and the membrane. This attenuation was caused by conjugation of PEG to PLO, but not by the presence of PEG, because there was little difference in the G_t ratio and P_{app} of FD-4 between PLO and the physical mixture of mPEG-NH_2 and PLO.

The cell viability was 100% in PEG-PLO at a concentration that showed a sufficient enhancement effect. Previous reports have shown that PLA reversibly enhances the absorption of hydrophilic macromolecules across the epithelium without causing epithelial damage.[7] PLO decreased cell viability at a concentration that showed a notable enhancement effect, although the difference was not significant. The cytotoxicity of cationic compounds is caused by the electrostatic interactions with the negative charges on the cell surface. The cell viability was decreased in the physical mixture of PLO and mPEG-NH_2, suggesting that the cytotoxicity was reduced by the conjugated PEG chains shielding the positively charged amine groups of PLO.

CONCLUSION

We investigated the effects of chemical modification with PEG on the permeation enhancement effect of PLO. The mucoadhesive/retentive properties of PEG-PLO were improved compared with those of PLO *in vitro*. PEGylation attenuated the permeation enhancement effect and decreased the cytotoxicity of PLO. These findings provide important information for the development of polycationic absorption enhancers with greater utility.

Conflict of Interest The authors declare no conflict of interest.

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