

ORIGINAL ARTICLE

Physicochemical delivery of amphiphilic copolymers to specific organelles

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In this study, a novel polymer material was introduced into cells and was delivered to specific organelles. Various fluorescently labeled copolymers of 2-methacryloyloxyethylphosphorylcholine (MPC) and alkyl methacrylates, such as *n*-hexyl methacrylate (HMA), *n*-dodecyl methacrylate (DMA) and stearyl methacrylate (SMA), were synthesized. HeLa cells were treated with the copolymers and subsequently observed by confocal fluorescence microscopy. The results indicated that the localization behavior of the copolymers was dependent on their structure. For instance, poly(HMA-co-MPC) was non-cytotoxic and was localized in specific cellular organelles such as the endoplasmic reticulum. In contrast, poly(DMA-co-MPC) was cytotoxic and was partially transported to specific organelles. Poly(SMA-co-MPC) hardly entered the cells. The mechanism of delivery and structure–function relationships of the copolymers are also discussed.

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INTRODUCTION

Recently, drug delivery systems (DDSs) that control drug distribution quantitatively, locally and temporally have been extensively studied to enhance the beneficial effects and to reduce the adverse effects of drugs by controlling their disposition.¹ Most DDSs are based on targeting, controlled release or improved biomembrane permeability. In particular, drug targeting has progressed significantly, and many techniques based on the targeting of specific regions have been realized.^{2–8} In addition, many carriers that distribute drugs in the cell nucleus have been reported.^{9–25} However, reports on DDSs that target specific organelles other than the nucleus are scarce. As the function of organelles is elucidated, DDSs for organelles are being developed. For instance, Gomes *et al.*²⁶ prepared nanoparticle-containing drugs and verified that the nanoparticles were localized inside the mitochondria. Smith *et al.*²⁷ developed a strategy to target bioactive molecules to the mitochondria by attaching the active species to a lipophilic triphenylphosphonium cation via an alkyl linker. Meanwhile, Yoshikawa *et al.*²⁸ delivered peptide complexes in fusogenic liposomes to endoplasmic reticulum (ER) by adding an ER insertion signal sequence to the peptide complex.^{29,30} As stated above, DDSs that target the ER or the Golgi apparatus are rare. These organelles are important for cellular functions; the ER is involved in protein synthesis, storage, transport and modification, and the Golgi apparatus participates in glycoprotein processing. Therefore, DDS that target the Golgi apparatus and the ER must be developed.

Herein, various fluorescently labeled copolymers of 2-methacryloyloxyethylphosphorylcholine (MPC) and alkyl methacrylates, such as

n-hexyl methacrylate (HMA), *n*-dodecyl methacrylate (DMA) and stearyl methacrylate (SMA), were synthesized. The alkyl chains of alkyl methacrylates were expected to permeate the plasma membrane and interact with cellular membrane structures such as the ER and the Golgi apparatus. Moreover, MPC has a phosphorylcholine moiety, which should improve the biocompatibility and water solubility of the copolymers.³¹ The behavior of the copolymers in cells was observed by confocal fluorescence microscopy, and the results revealed that poly(HMA-co-MPC) became localized in specific organelles such as the ER and the Golgi apparatus. Moreover, despite its amphiphilic structure, poly(HMA-co-MPC) did not exhibit cytotoxicity. The mechanism of copolymer localization was evaluated, and the novel material is anticipated to have useful applications in biomaterials.

EXPERIMENTAL PROCEDURE

Materials

HMA, DMA, SMA and acryloyl chloride were purchased from Tokyo Chemical Industry (Tokyo, Japan). Ethylenediamine and ammonium persulfate were obtained from Wako Pure Chemical Industries (Osaka, Japan). Fluorescein-4-isothiocyanate was purchased from Dojindo Laboratories (Kumamoto, Japan). The reagents were used without further purification. MPC was synthesized according to a previously reported method.³² The structure of HMA, DMA, SMA and MPC is shown in Figure 1.

Synthesis of fluorescent monomers

Specifically, 100 mg (0.257 mmol) of fluorescein-4-isothiocyanate, 172 μ l (2.57 mmol) of ethylenediamine and 7 ml of methanol were added into a flask and the mixture was stirred at room temperature for 2 h. The flask was

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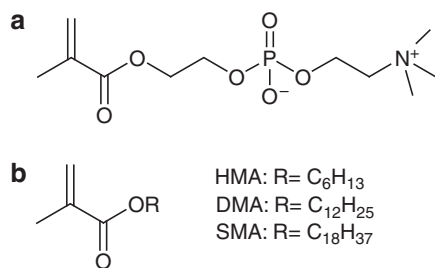


Figure 1 Structure of (a) 2-methacryloyloxyethylphosphorylcholine and (b) alkyl methacrylates. DMA, *n*-dodecyl methacrylate; HMA, *n*-hexyl methacrylate; SMA, stearyl methacrylate.

protected from light. Subsequently, the mixture was added to a large amount of acetonitrile and the supernatant was removed after centrifugation. Reprecipitation was conducted in a solution of water/acetonitrile. Finally, the precipitate was dissolved in water and freeze dried to yield 95.6 mg (83.1%) of a red-orange compound. ¹H nuclear magnetic resonance (600 MHz, D₂O) spectra supported the structure of **1** (FITC-NHCH₂CH₂NH₂): δ 7.59 (s, 1H), δ 7.40 (d, *J*=8.2 Hz, 1H), δ 7.21 (d, *J*=7.7 Hz, 1H), δ 7.13 (d, *J*=9.4 Hz, 2H), δ 6.57–6.54 (m, 4H), δ 3.82 (br t, 2H) and δ 3.13 (t, *J*=5.8 Hz, 2H).

In total, 90.6 mg (0.202 mmol) of **1**, 64.1 mg (0.605 mmol) of sodium carbonate and 10 ml of methanol were added into a flask. After the flask was cooled to 0 °C, 24.4 μl (0.302 mmol) of acryloyl chloride was slowly added. The reaction was carried out in the dark at room temperature for 3 h, and the product was purified by silica gel chromatography (eluent: chloroform/methanol=7/1 containing 1% of acetic acid). Subsequently, the eluted substance was adsorbed to Diaion Sepabeads HP20 (Mitsubishi Chemical Corporation, Tokyo, Japan) and washed with water and 10% methanol to remove the acetic acid. Finally, the product was extracted from HP20 with methanol and dried under reduced pressure to yield 19.3 mg (19.0%) of a yellow compound. ¹H nuclear magnetic resonance spectra (600 MHz, d₃-MeOH) supported the structure of fluorescent monomer δ 8.10 (s, 1H), δ 7.73 (d, *J*=7.1 Hz, 1H), δ 7.16 (d, *J*=8.2 Hz, 1H), δ 6.69–6.67 (m, 4H), δ 6.53 (dd, *J*=2.2, 8.8 Hz, 2H), δ 6.27–6.19 (m, 2H), δ 5.66 (dd, *J*=2.7, 9.3 Hz, 1H), δ 3.78 (br t, 2H) and δ 3.54 (t, *J*=6.1 Hz, 2H).

Synthesis of poly (alkyl methacrylate-co-MPC)

Polymerization was carried out according to a previously reported method.³³ In short, 2.7 mg (0.0054 mmol) of fluorescent monomer, 296 mg (1.00 mmol) of MPC and 22.9 mg (0.100 mmol) of ammonium persulfate were weighed in a flask. Subsequently, 20 ml of solvent (dimethylsulfoxide/water=19/1, 20/0 and 18/2 for HMA, DMA and SMA, respectively) and 1.00 mmol of alkyl methacrylate (HMA, DMA or SMA) were added to the mixture. The mixture was degassed, filled with Ar and polymerized at 60 °C for 20 h. Polymerization was terminated by adding the mixture to a large amount of acetone. The resulting precipitate was filtered, and an aqueous solution of the precipitate was dialyzed for 3 days using a 3500 cutoff membrane. The chemical composition of poly(HMA-co-MPC), poly(DMA-co-MPC) and poly(SMA-co-MPC) was 51:49, 50:50 and 40:60 alkylmethacrylate:MPC, respectively. The aforementioned values were determined by elemental analysis, and the molecular weight of copolymers synthesized without a fluorescent monomer was determined by gel permeation chromatography.³³ The Mn of poly(HMA-co-MPC), poly(DMA-co-MPC) and poly(SMA-co-MPC) was 1.0×10⁴, 2.4×10⁴ and 0.8×10⁴, respectively.

Cell culture conditions

Dulbecco's modified Eagle's medium nutrient mixture F-12 and Hanks' balanced salt solution were purchased from Gibco, Life Technologies Japan Ltd. (Tokyo, Japan). Fetal bovine serum was obtained from JRH Biosciences, Nichirei Bioscience (Tokyo, Japan). Antibiotic–antimycotic mixed stock solution was purchased from Nacalai Tesque (Kyoto, Japan). All other reagents used in the cell experiments were purchased from Invitrogen, Life Technologies Japan Ltd. HeLa cells were incubated in Dulbecco's modified Eagle's medium F-12

containing 10% (v/v) fetal bovine serum and 1% (v/v) antibiotic–antimycotic mixed stock solution. Cells were detached through the application of 0.25% trypsin–EDTA, passaged every 3 or 4 days and maintained in a humidified atmosphere of 5% CO₂ at 37 °C.

Confocal fluorescence microscopy

In total, 3.0×10⁵ HeLa cells were seeded on the glass coverslip in a six-well plate. After 20 h of incubation, the medium was changed to Hanks' balanced salt solution containing the copolymer (1 mg ml⁻¹, 2 ml per well), and the cells were incubated for 1 or 3 h. Subsequently, the cells were fixed by adding cold methanol and were stained with wheat germ agglutinin-Alexa 647 (5 μg ml⁻¹, 2 ml per well, 10 min at room temperature) and propidium iodide (0.25 μg ml⁻¹, 1 ml per well, 5 min of incubation). Finally, the glass coverslip was fixed on a glass slide and was observed by confocal fluorescence microscopy. The cells were washed twice with 2 ml per well of Hanks' balanced salt solution after each step in the aforementioned process.

The localization of poly(HMA-co-MPC) was also determined using ER-Tracker (Molecular Probes, Life Technologies Japan Ltd.). After the cells were treated with the copolymer incubated for 3 h, and washed, the cells were treated with 1 μM of ER-Tracker Hanks' balanced salt solution (1 ml per well, 20 min incubation) without cell fixation. Other procedures and conditions used to determine the localization behavior of the polymers were identical to those described above.

Cytotoxicity evaluation

To determine the cytotoxicity of the copolymers, HeLa cells were incubated in culture medium containing the copolymer. Specifically, 2.0×10⁵ HeLa cells were seeded in a 6 cm dish. After 24 h of incubation, the medium was changed to copolymer-containing medium (1 mg ml⁻¹) and the cells were incubated for 24 or 48 h. Subsequently, the cells were collected and dead cells were stained with Trypan blue. Finally, the number of living and dead cells was determined.

RESULTS AND DISCUSSION

Figure 2 shows a fluorescent image of the HeLa cells. The incubation time after copolymer treatment was varied, and the fluorescence of the copolymers was compared with the fluorescence of propidium iodide and wheat germ agglutinin. Propidium iodide is a nuclear staining reagent, and wheat germ agglutinin recognizes *N*-acetyl-D-glucosamine and localizes in membrane structures, primarily in the Golgi apparatus.^{34,35} The quantity of copolymer taken up by the cell and localized in specific organelles was dependent on the copolymer species and the incubation time. Poly(HMA-co-MPC) localized primarily in the plasma membrane after 1 h of incubation. However, the copolymer colocalized with wheat germ agglutinin after 3 h. These results suggest that the copolymers interacted with the plasma membrane, entered the cells and localized in the membrane. Poly (DMA-co-MPC) also localized in the plasma membrane after 1 h of incubation. However, even after 3 h of incubation, the copolymer was only partially transported to membrane structures, and a portion of the copolymer remained at the plasma membrane. In contrast, poly(SMA-co-MPC) hardly entered the cells after 1 h and 3 h of incubation. Moreover, interactions between the copolymer and the plasma membrane were not observed. As a reference, HeLa cells were treated with fluorescently labeled MPC homopolymer. After 1 h of incubation, fluorescence was not observed in any of the cells. Moreover, after 3 h of incubation, the polymer diffused into the cells and did not localize into specific organelles.

The copolymers synthesized in this study do not have a bioactive domain, such as a nucleic acid, peptide or carbohydrate. Hence, the copolymers localize in specific organelles because of physicochemical interactions. Hydrophobic alkyl chains of HMA, DMA and SMA probably interact with membrane structures. Moreover, the phosphorylcholine moiety of MPC is not repelled by the lipid bilayer because

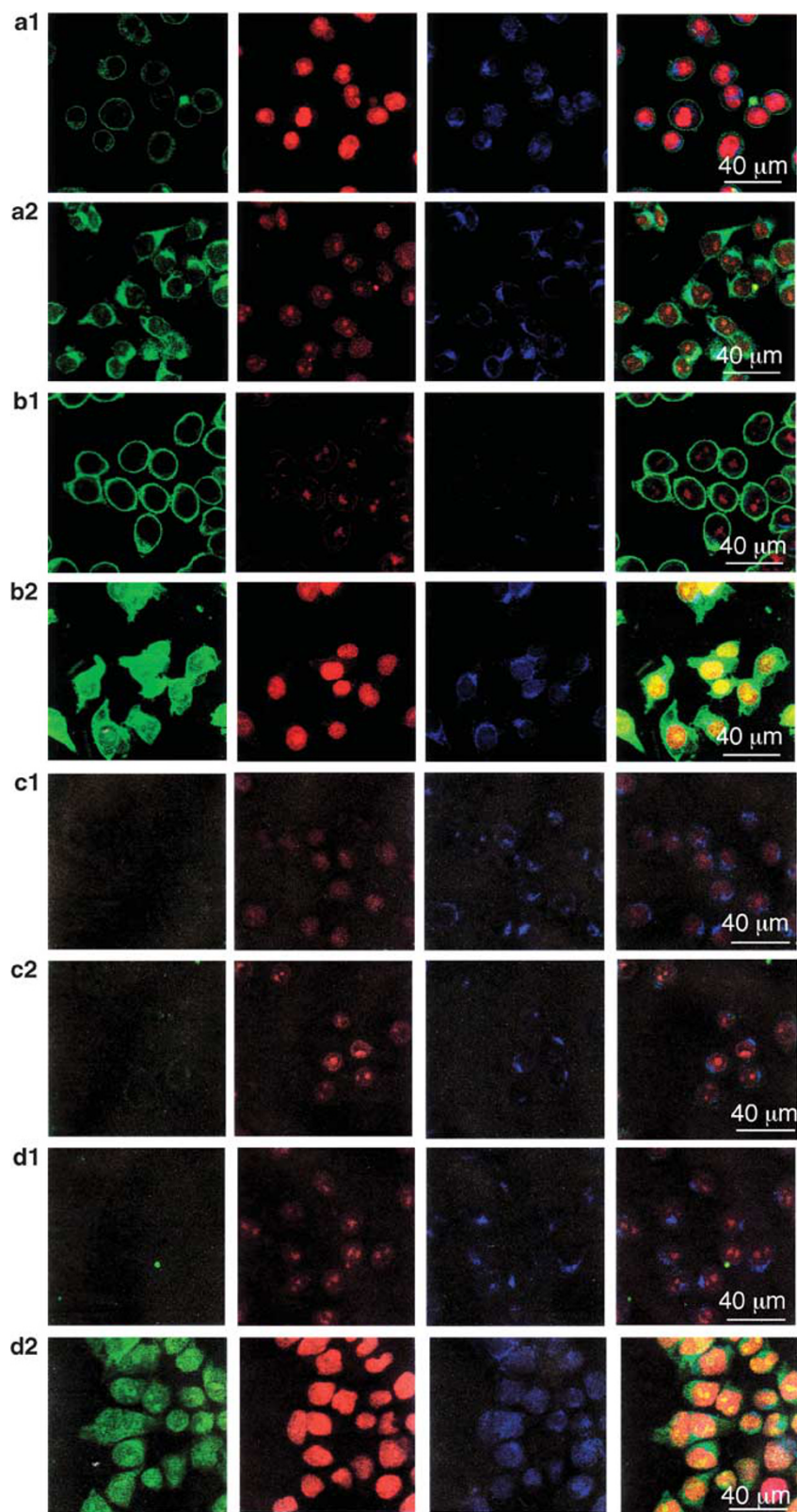


Figure 2 Fluorescence image of HeLa cells treated with poly(HMA-co-MPC) (panels **a1** and **a2**), poly(DMA-co-MPC) (panels **b1** and **b2**), poly(SMA-co-MPC) (panels **c1** and **c2**) and MPC homopolymer (panels **d1** and **d2**). The incubation time after treatment with the copolymer was 1 h for a1, b1, c1 and d1, and 3 h for a2, b2, c2 and d2. The fluorescence of green, red and blue represents the copolymers, cell nucleus (propidium iodide) and membrane structures in the cells (wheat germ agglutinin-Alexa 647), respectively. DMA, *n*-dodecyl methacrylate; HMA, *n*-hexyl methacrylate; MPC, 2-methacryloyloxyethylphosphorylcholine; SMA, stearyl methacrylate.

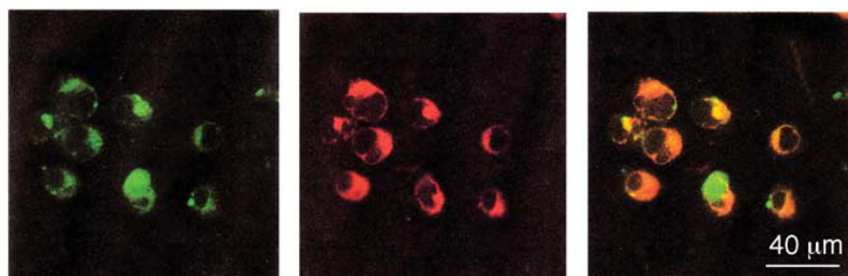


Figure 3 The fluorescence image of HeLa cells after treatment with poly(HMA-co-MPC). The cells were incubated for 3 h after copolymer treatment. The fluorescence of green and red represents the copolymer and endoplasmic reticulum (ER-Tracker), respectively. HMA, *n*-hexyl methacrylate; MPC, 2-methacryloyloxyethylphosphorylcholine.

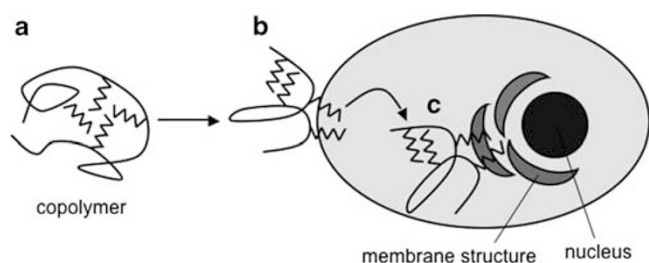


Figure 4 The anticipated mechanism of copolymer localization. Copolymers form micelle-like structures in aqueous solution (state **a**). Alkyl chains of copolymers interact with the plasma membrane of cell (state **b**). Copolymers that enter the cells interact with membrane structures such as the ER and Golgi apparatus (state **c**).

the surface of the lipid bilayer is composed of a phosphorylcholine moiety. The copolymers localize by physicochemical interactions in specific organelles; thus, the copolymers localize in various membrane structures such as the ER and the Golgi apparatus.

The relative amount of various types of membranes in the cell has been determined in previous studies.³⁶ The amount of membrane in each organelle is somewhat dependent on the species; however, among organelles, the ER contains the largest amount of membrane. Hence, the copolymers are anticipated to localize primarily in the ER. Therefore, the localization of the copolymers was compared with that of ER-Tracker (Figure 3). The results indicated that the localization of poly(HMA-co-MPC) was similar to that of ER-Tracker and the copolymer localized primarily in the ER.

Figure 4 shows the anticipated mechanism of copolymer localizations. First, the copolymers form micelle-like structures in aqueous solution because the alkyl chains of the alkyl methacrylate moiety are repelled from the aqueous solution (state **a** of Figure 4). Our previously reported data suggested that poly(HMA-co-MPC) polymerized according to the proposed method form micelle-like structures in aqueous solution.³² Second, the alkyl chains of the copolymers, which form loosened and deformed micelle-like structures, interact with the plasma membrane (state **b** of Figure 4). At this time, the local and dense interaction between alkyl chains and the plasma membrane induces the incorporation of copolymers into the cells. As mentioned above, the copolymers do not have a bioactive domain; thus, the entrance of the copolymers cannot be attributed to receptor-mediated endocytosis. Rather, the copolymers are incorporated because of physicochemical interactions. Third, copolymers that enter the cells interact with membrane structures such as the ER and Golgi apparatus (shown in state **c** of Figure 4) in a similar manner in which they proceed from state **a** to state **b**.

Compared with the three kinds of copolymers evaluated in the present study, poly(HMA-co-MPC) smoothly follows the aforementioned processes and is the most likely to localize into specific organelles. Alternatively, poly(DMA-co-MPC) is anticipated to interact more strongly with the plasma membrane than poly(HMA-co-MPC) because the alkyl chains of DMA are longer than those of HMA. As shown in Figure 4, it is difficult for poly(DMA-co-MPC) to transit from state **b** to state **c** because of the strong interaction between the alkyl chains of DMA and the plasma membrane. In fact, poly(DMA-co-MPC) remained at the plasma membrane for a long period of time (**b1** and **b2** of Figure 2). Meanwhile, poly(SMA-co-MPC) likely forms rigid micelle-like structures because the hydrophobic interactions of SMA are the strongest among the evaluated alkyl methacrylates. Thus, poly(SMA-co-MPC) was hardly transmitted from state **a** to state **b**, as shown in Figure 4. As a result, poly(SMA-co-MPC) did not localize in the plasma membrane or in the membrane structures of the cells. Alternatively, the MPC homopolymer does not have alkyl chains and hardly interacts with the plasma membrane and membrane structures of cells. Hence, MPC homopolymer is not likely to enter the cells after 1 h of incubation. After 3 h of incubation, the MPC homopolymer did not localize in specific organelles but diffused into the cells.

The cytotoxicity of the copolymers was investigated because surface-active molecules, such as the proposed amphiphilic copolymers, generally exhibit cytotoxicity by destroying the plasma membrane.^{37–39} The viability of the cells after treatment with each of the copolymers is shown in Table 1. As shown in the table, the cell viability of poly(HMA-co-MPC), poly(SMA-co-MPC) and MPC homopolymer was almost identical to that of the negative control, which suggested that the polymers did not exhibit cytotoxicity. There are two possible reasons for the observed lack of cytotoxicity. First, the copolymers have many alkyl chains, which can locally interact with the plasma membrane. Alternatively, surface-active low-molecular-weight compounds interact with the plasma membrane in a non-local manner. Therefore, compared with surface-active low-molecular-weight compounds, it is more difficult for copolymers to destroy the plasma membrane. Second, MPC contains a biocompatible phosphorylcholine moiety, which likely diminishes the cytotoxicity of the copolymers. In contrast, poly(DMA-co-MPC) exhibited cytotoxicity. After treatment with the copolymer, 15 and 9% of cells died within 24 and 48 h, respectively. As shown in Figure 2, poly(DMA-co-MPC) remained at the plasma membrane for a long period of time and may destroy the plasma membrane.

To apply the proposed copolymers to DDS, bioactive molecules must be able to interact with the copolymer. One method of promoting such interactions is to conjugate bioactive molecules to the copolymer. Although the bioactivity of the molecule could be lost because of conjugation, the molecule will likely be delivered to the

Table 1 Cell viability after treatment with poly(HMA-co-MPC), poly(DMA-co-MPC), poly(SMA-co-MPC), MPC homopolymer and the negative control

	Poly(HMA-co-MPC)	Poly(DMA-co-MPC)	Poly(SMA-co-MPC)	MPC homopolymer	Negative control
24 h	98%	85%	97%	98%	97%
48 h	99%	91%	99%	99%	99%

Abbreviations: DMA, *n*-dodecyl methacrylate; HMA, *n*-hexyl methacrylate; MPC, 2-methacryloyloxyethylphosphorylcholine; SMA, stearyl methacrylate.

ER and Golgi apparatus. Second, a complex such as a polymer micelle could be formed between the copolymer and a bioactive molecule. In this case, a loss of bioactivity is not a concern; however, the stability of the complex and its localization in cells must be studied.

CONCLUSION

The localization of copolymers based on various alkylmethacrylates and MPC was examined in cells, and the results suggested that the behavior of the copolymers was dependent on the alkylmethacrylate species. Poly(HMA-co-MPC) localized primarily in specific organelles, such as the ER, and did not exhibit cytotoxicity. Alternatively, poly(DMA-co-MPC) was cytotoxic and poly(SMA-co-MPC) hardly entered the cells. On the basis of the localization behavior and biocompatibility of the copolymers, poly(HMA-co-MPC) is expected to be a novel biomaterial and may be used as a drug carrier or a precursor for the biosynthesis of valuable biomolecules.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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