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1 Hydrophobic Nature of Methacrylate-POSS in
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3 Phosphorylcholine for Enhanced Solubility and
4 Controlled Release of Paclitaxel

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10

Abstract

Amphiphilic copolymers consisting of 2-(methacryloyloxy)ethyl phosphorylcholine (MPC) and hydrophobic monomers are known as biomaterials for the administration of poorly water-soluble drugs such as paclitaxel (PTX). However, the hydrophobic monomers to be copolymerized with MPC have not been optimized for PTX solubilization and its dosage forms. Here, we show the enhanced PTX solubility by only an MPC-based amphiphilic copolymer using a polyhedral oligomeric silsesquioxane (POSS) methacrylate (MA) bearing ethyl (C_2H_5) group as a vertex group. MPC was copolymerized with POSS methacrylates bearing different vertex groups of ethyl (C_2H_5), hexyl (C_6H_{13}) and octyl (C_8H_{17}) via radical polymerization. We found that the strong interaction between the C_2H_5 -POSS and PTX contributed to the slow release of PTX without any burst release. The C_2H_5 -POSS-MA MPC copolymer was internalized into the cultured HeLa cells, which was confirmed by using a fluorescein-4-isothiocyanate (FITC)-labelled PTX, and the PTX-dissolved copolymer induced cell death. We anticipate that C_2H_5 -POSS-MA MPC copolymer is a good solubilizer bearing controlled release function for PTX.

Keywords

2-methacryloyloxyethyl phosphorylcholine; polyhedral oligomeric silsesquioxanes; copolymer; paclitaxel; solubility; controlled release

1 Introduction

2 Paclitaxel (PTX) is a hydrophobic anti-cancer drug for the treatment of breast, ovarian and lung
3 cancers. Because of its low water solubility, some special solvents are required to solubilize PTX
4 in water such as 50:50 v/v mixture of polyoxyethylated castor oil and dehydrated ethanol¹. These
5 solvents often cause a serious hypersensitivity reaction, and the injection sites are subject to some
6 reactions such as erythema and swelling. So, the alternative dosage form is needed to be developed
7 to reduce the undesirable side effects. The applications of liposomes^{2, 3}, polymeric assemblies⁴,
8 and cyclodextrin complexes⁵ for delivery of PTX have already been reported, but the good clinical
9 effect has not yet reported. Recently, low-dose metronomic (LDM) chemotherapy of PTX is
10 reported both *in vitro* and *in vivo*. Oral administration of PTX is a major route for successful
11 implantation of LDM chemotherapy. The studies revealed that the amorphous solid dispersion of
12 PTX, polyvinylpyrrolidone (PVP) K30 and sodium lauryl sulfate (SLS) showed a major increase
13 in the apparent solubility and release rate of PTX⁶. In this sense, the choice of water-soluble
14 polymers is likely to be an important factor to increase solubility and release rate of PTX⁷.

15 The water-soluble polymers have been extensively studied for effective therapeutic use.
16 Among them, a zwitterionic water-soluble polymer, 2-methacryloyloxyethyl phosphorylcholine
17 (MPC) polymer (poly MPC) has been used extensively in blood-contacting medical devices for a
18 high level of biocompatibility and resistance to protein adsorption^{8, 9}. The MPC monomer can
19 copolymerize with hydrophobic monomers, and the obtained amphiphilic copolymers have been
20 considered for solubilization and delivery of PTX^{10, 11, 12, 13, 14}. According to the report from K.
21 Ishihara *et al.*, the most effective polymer to dissolve the PTX was poly[MPC-*co*-*n*-butyl
22 methacrylate(BMA) (PMB30W)] with 70 mol % of the BMA unit¹¹. In addition, cellular uptake
23 of a poly MPC-based polymeric micelle was superior to that of poly(ethylene oxide) (PEO)- and

poly(*N*-(2-hydroxypropyl)methacrylamide) (PHPMA)-based polymeric micelle, indicating that poly MPC shall be advantageous to interact with cell membranes¹⁵. However, the hydrophobic part that is essential for interaction with the PTX molecule has been mainly constructed by BMA, and the other candidates of the hydrophobic monomers have not been investigated yet.

Polyhedral oligomeric silsesquioxane (POSS) is an organic/inorganic hybrid molecule. POSS is soluble in many solvents and highly compatible with various polymeric matrix due to the synergy between its inorganic core and organic side groups. Because of the hydrophobic in nature of POSS, amphiphilic characteristics can be achieved by incorporating POSS moiety into a hydrophilic polymer chain. The aggregation and order stacking nature of POSS cage are responsible for the generation of self-assemble POSS-based amphiphilic molecules. In recent years, POSS-containing hybrid polymers were synthesized and self-assembly behavior in selective solvents was studied. Their studies have achieved some novel interesting self-assemble morphologies, such as ellipsoidal aggregates, giant capsules, complex spheres, dendritic cylinders etc.. For example, POSS-based amphiphilic polymers have been studied by introducing the POSS moiety to the ends of hydrophilic chain such as PEO and poly (ethylene glycol) (PEG), resulting in the self-assembled micelle and vesicle in solutions and solvent-sensitive self-association behavior¹⁶. Kim *et al.* successfully encapsulated insulin by PEG-POSS for drug delivery, suggesting that the insulin was well protected inside PEG-POSS nanoparticles at gastric pH for 2 h. and start releasing at intestine pH 6-7¹⁷. Yuan *et al.* synthesized poly(L-glutamic acid) dendrimers bearing POSS nanocubic core, conjugated with doxorubicin via pH-sensitive hydrazine bond and biotin as a targeting ligand¹⁸. Thus, POSS moiety has been shown to possess good hydrophobic nature suitable for application as drug carriers as well as artificial molecular chaperones¹⁹ and polymeric capsule for photodynamic therapy²⁰.

We have synthesized R-POSS-based MPC random copolymers (R-groups are ethyl (C_2H_5), hexyl (C_6H_{13}), octyl (C_8H_{17})), and studied its mechanical, thermal and surface properties²¹. Our study showed that the incorporation of only the C_2H_5 -POSS in polyMPC matrix increased the hydrophobicity at water-polymer interface. This finding is the first report of biomaterial application of element block polymers that are based on a new concept of polymer hybrids: Incorporation of the inorganic component with domain size at the nano or molecular scale into the polymer matrix produces organic-inorganic polymer hybrids²². Therefore, we came up with an idea to expand the strategy of C_2H_5 -POSS-based random MPC copolymers towards the amphiphilic nature in solution, which can act as a solubilizer and a polymeric carrier for PTX.

In this study, we evaluated the random copolymers prepared by copolymerization with POSS methacrylate bearing different vertex (R-) groups (ethyl, hexyl and octyl) and MPC as a solubilizer of PTX. The self-assembled nature was characterized by fluorescence spectroscopy using pyrene as a fluorescence probe to determine critical association concentration (CAC), dynamic light scattering (DLS) measurements, and transmission electron microscopy (TEM) observation. Among the series, we found that PTX solubility in water increased greatly by employing only the ethyl ($R = C_2H_5$)-POSS in the copolymers, which was related to the formation of assembled structure. The relationship between the C_2H_5 -POSS-MA MPC copolymer PTX solubility and cytotoxicity was discussed with cellular uptake of the C_2H_5 -POSS-MA MPC copolymer using HeLa cell line using a fluorescein-4-isothiocyanate (FITC)-labelled PTX. Furthermore, we demonstrated PTX release from the assembly of PTX-solubilized C_2H_5 -POSS-MA MPC copolymer to discuss the role of C_2H_5 -POSS-MA as a hydrophobic monomer.

Materials and Methods

2.1 Materials

The R-POSS-MA MPC copolymers ($R = C_2H_5$, C_6H_{13} , and C_8H_{17}) were synthesized by our previously described method (**Figure 1**)²¹. The mol. % of R-POSS in the copolymers were found to be 1-2 mol. %. PTX was purchased from FUJIFILM Wako Pure Chemical Corporation, Japan. Dulbecco's Modified Eagle Medium (DMEM), Dulbecco's phosphate-buffered saline (DPBS), 0.25 %-Trypsin/1mM-EDTA solution, penicillin-streptomycin mixed solution and sodium carbonate was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Fetal Bovine Serum (FBS) was purchased from Sigma-Aldrich Co. LLC. (St. Louis, U.S.A). The other reagents and solvents were used without further purification. As the C_2H_5 -POSS MA MPC is partially soluble in water, the copolymer water solution was prepared by first dissolving the copolymer in methanol, followed by addition of water with agitation. Then, the flask was attached to a rotary evaporator to remove the methanol. Also, it was kept inside vacuum dryer for 1 h at 40°C for complete removal of methanol. The solution was then diluted with water to get the required concentration. HeLa cells (Health Science Research Resources Bank, Osaka, Japan) were cultured in DMEM supplemented with 10% FBS and 100 U/mL penicillin-streptomycin. Cells were grown in a humidified incubator at 37°C under 5% CO₂.

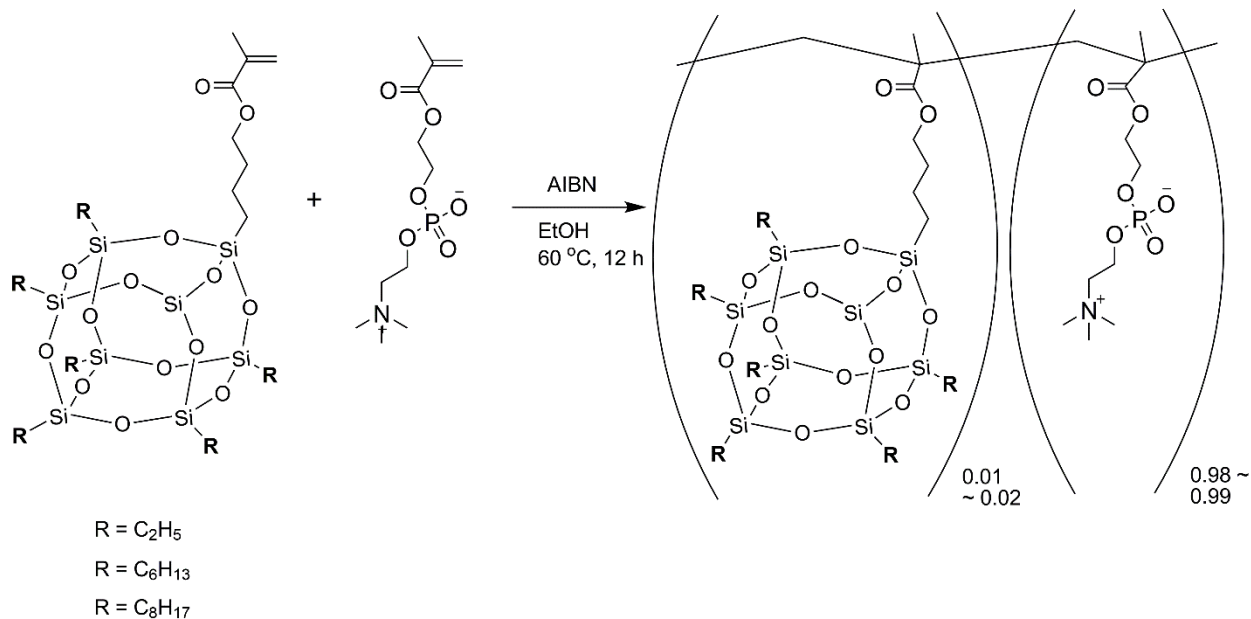


Figure 1. Synthesis of R-POSS methacrylate-based MPC copolymers (R-POSS-MA MPC)

2.2 Measurements

Critical Association concentration (CAC) was determined by fluorescent spectroscopy using pyrene as a hydrophobic fluorescent probe^{23 24}. Pyrene stock solution in acetone was prepared (15 μ M) and added to the aqueous polymers solution of different concentrations ranging from 0.0001 to 50 mg/ml to obtain samples containing pyrene (6.0×10^{-7} M). It was kept overnight in dark at room temperature for complete evaporation of acetone from the solution. Fluorescence spectra were recorded on a fluorescence spectrophotometer (JASCO Spectrofluorometer FP-8200) at room temperature. To determine CAC, excitation spectra of pyrene were measured by varying the concentration of the copolymer solutions. The emission wavelength was 390 nm.

Dynamic light scattering (DLS) measurements were carried out using a light scattering instrument (Malvern, Zetasizer Nano ZS) with 90° as scattering angle. The measurement was performed at 25 °C in water with the copolymer concentration of 1 and 100 mg/ml.

Transmission electron microscopy (TEM) images were collected using a TEM apparatus (JEOL JEM-1230) at an accelerating voltage of 100 kV. Samples were prepared on 200 mesh copper grids. Before the measurements, the samples were vacuum dried and kept overnight *in vacuo*. An energy-dispersive X-ray analysis (EDS) attached to the scanning TEM-high angle annular dark field (STEM-HAADF) was performed with JEOL JEM-ARM200F at 200 kV.

2.3 Cytotoxicity of the copolymers

The cytotoxicity of the copolymers was determined using a Cell Counting Kit-8. Each copolymer (1 mg) was dissolved in 1 ml of 0.1 M phosphate buffer saline (PBS, pH 7.4). Additionally, regarding the C₂H₅-POSS-MA MPC copolymer, high concentration range (5, 10, 50, and 100 mg/ml) was subjected to evaluate the concentration-dependent cytotoxicity. Prior to the experiments, the copolymers were further purified against ultra-purified DI water using a dialysis tube (MWCO: 1,000). During the dialysis, no visible precipitation was observed in the dialysis tube of all the copolymers. HeLa cells were seeded into a 96-well plate in volumes of 90 µl (5000 cells/ well in DMEM media supplemented with 10% FBS) and incubated overnight at 37 °C. The copolymer-contained solutions were sterilized using UV light for 30 min. The 10 µl of the sample solution were added to each well and incubated for 24 hrs. at 37 °C. Only media and only cell in the well were subjected as blank (BL) and positive control (C), respectively. The 10 µl of CCK-8 reagent was added to each well and incubated for 2 hrs. at 37 °C. The absorbance of each well was

measured using a Corona Grating Microplate Reader SH-9000 Series (Corona Electric Co., Ltd, Japan) at 450 nm. The cell viability (%) was calculated by the following formula:

$$\text{Cell viability (\%)} = (I_{450}^{\text{S}} - I_{450}^{\text{BL}}) / (I_{450}^{\text{C}} - I_{450}^{\text{BL}}) \times 100\ldots$$

where I_{450}^{S} , I_{450}^{BL} and I_{450}^{C} represent the absorbance of the copolymer samples (S), blank (BL) and positive control (C), respectively.

2.4 Determination of PTX solubility in various POSS-MPC copolymers in aqueous solution

PTX (1 mg) was dissolved in 1 ml of methanol. Three different concentrations of the copolymer solutions were prepared in water at 5, 10 and 50 mg/ml. The 100 μ l of PTX stock solution prepared in methanol (1 mg/ml) was added to 900 μ l of each copolymer aqueous solution, followed by vortex mixing. Methanol was removed by keeping it under reduced pressure. PTX crystal appeared in all concentration of poly MPC solution, but for other copolymers, it appeared at only 5 mg/ ml. The solid phase was separated by centrifugation (3000 rpm) for 15 min. All the samples were filtered using 0.45 μ m pore size filter before HPLC measurement²⁵ using a GILSON HPLC system (UV-vis detector : Gilson 119 UV/VIS Detector, Autosampler : GILSON 231XL, Pump : GILSON 805 Manometric Module, GILSON 811c dynamic mixer and GILSON 306 Pump, Injector : GILSON DILTOR401) with a COSMOSIL 5C₁₈-MS-II (Φ 4.6 mm \times 150 mm) column (Nacalai Tesque Inc., Japan) as stationary phase at r.t.. The detection was performed at a detection wavelength of 227 nm. The mixture of acetonitrile (60 %) and water (40 %) was used as the mobile phase at the flow rate of 1 ml/min. The PTX concentration was calculated by comparing the peak area with the standard curve. Since the PTX solubility in C₂H₅-POSS-MA MPC copolymer

1 solution was found to be higher than that of the other polymers, the solubility was further measured
2 by increasing the copolymer concentration to 100 mg/ml.

3 4 2.5 Preparation of PTX-solubilized POSS-based MPC random copolymers assembly

5 The POSS-based MPC copolymers (20 mg) and PTX (1 mg) were dissolved in methanol
6 (1 ml), and the solution was added drop by drop into 10 ml water with vigorous stirring for
7 overnight. The solution was dialyzed against water using a dialysis tube (MWCO = 6,000-8,000)
8 for 24 h to eliminate the methanol. The inner phase was collected after centrifugation (4000 r.p.m.
9 for 30 min), and then the supernatant was filtered using a 0.45 μ m filter. The assembly solution
10 was used for characterization and stability study. The POSS-based MPC copolymer assemblies
11 without PTX was also prepared following the same procedure.

12 13 2.6 Characterization of the POSS-based MPC copolymer assemblies

14 The morphology of the POSS-based MPC copolymer assemblies without PTX in the dry
15 state was observed in day 1 and day 4 by transmission electron microscopy (TEM) measurements
16 using JEOL JEM-1230 at an accelerating voltage of 100 kV. Samples were prepared on 200 mesh
17 copper grids. Before the measurements, the samples were vacuum dried and kept overnight *in*
18 *vacuo*. In addition, an energy-dispersive X-ray analysis (EDS) attached to the scanning TEM-high
19 angle annular dark field (STEM-HAADF) was performed with JEOL JEM-ARM200F at 200 kV.

Dynamic light scattering (DLS) measurements were carried out to determine the average hydrodynamic diameter and size distribution of the assemblies both with or without PTX-loaded. The measurement was performed using a light scattering instrument (Malvern, EXSTER DSC600) with 90° as scattering angle. Stability of the assemblies was assessed after standing the sample at room temperature for 4 days. The DLS measurements were performed at 25 °C in water at the concentration of 1 mg/ml. Before the measurements, each sample was filtered using a 0.45 µm-pore size filter, and every sample was recorded three times to analyse the mean diameter.

2.7 Measurements of cell viability of PTX-solubilized C₂H₅-POSS- MPC copolymer

PTX (1 mg/ml in ethanol) in 100 mg/ml C₂H₅-POSS-MA MPC copolymer aqueous solution was prepared following the same process as solubility testing. This copolymer-PTX solution was divided into three parts: one for solubility measurement using HPLC, second, to prepare neutral copolymer solution (pH 7) and third to prepare the copolymer solution at pH 5 using 0.5 M HCl. PTX (1 mg) was dissolved in 1 ml of ethanol to use as a control. HeLa cells were seeded into 96-well plate in volumes of 90 µl (5000 cells/ well in DMEM media supplemented with 10% FBS) and incubated overnight at 37 °C. All the solutions were sterilized using UV light for 30 min before the test. Each of the solutions (10 µl) were added to the cell seeded well plate and incubated for 24 and 48 h at 37 °C to measure the cell viability (Final concentration of PTX in the copolymer solution and control was 7.6 and 100 µg/ml, respectively). Only media and only cell in the well were considered as blank and positive control respectively in this experiment. 10 µl of CCK-8 reagent was added after 24 and 48 h to each well and incubated for 2 h at 37 °C. The

absorbance of each well was measured using microplate reader at wavelength of 450 nm. The cell viability (%) was calculated using the formula as mentioned above (see: section 2.3).

2.8 Cellular uptake of FITC-PTX solubilized C₂H₅-POSS-MA MPC copolymer

To visualize the cellular uptake of PTX solubilized C₂H₅-POSS-MA MPC copolymer, FITC-labelled PTX (FITC-PTX; **Figure 2**) was synthesized according to the literature^{26 27} with some modifications (**See Supporting Information**). The FITC-PTX was encapsulated inside the copolymer by dialysis approach. HeLa Cells were seeded into a 4-well slide and chamber plate (Watson Bio Lab, Kobe, Japan) with a cell density of 12,750 cells/ well in 0.8 ml DMEM media supplemented with 10% FBS and incubated overnight at 37 °C before the experiment. After 24 h incubation, the growth media removed, and the cells were treated with serum free medium containing FITC-PTX solubilized C₂H₅-POSS-MA MPC copolymer (concentration of FITC-PTX: 2 µg/ml). As a control, FITC-PTX (2 µg/ml) was used. At the incubation time for 2, 5 and 24 h, the media were removed and washed with D-PBS for 3 times. Then, the cells were fixed with cold acetone, washed with D-PBS for 3 times. The obtained cells on the glass plate were imaged by confocal laser scanning electron microscope (CLSM) measurements using a Fluo View™ FV1000 Confocal Microscope (Olympus Co., Tokyo, Japan).

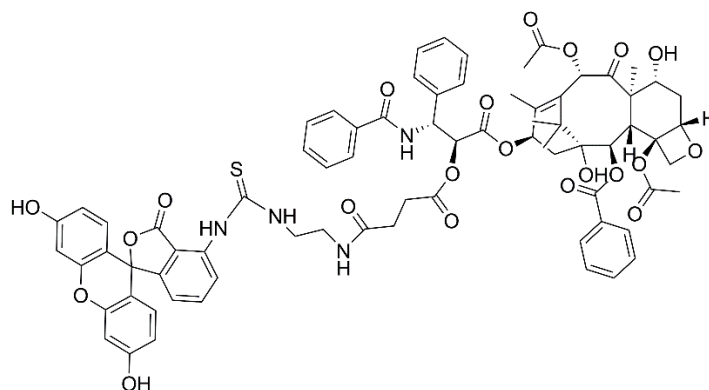


Figure 2. Chemical structure of FIT-PTX

2.9 *In vitro* PTX release from the copolymer assemblies

The PTX-solubilized POSS-based MPC copolymer assemblies (2.4 mg of lyophilized solid powder) were dispersed in 1 ml of D-PBS (pH 7.4). It was then poured into a dialysis membrane tube with a molecular cut-off of 6000 – 8000 Da, followed by introduced in a vial containing 25 ml of D-PBS (pH 7.4) containing 1 % Tween 80. The vials were introduced in a shaking bed maintaining the temperature 37 °C with the shaking speed of 100 r.p.m. One ml of the release medium was withdrawn at predetermined time interval for concentration measurement and equal volume of D-PBS (pH 7.4) plus 1 % Tween 80 mixed solution was added to keep the volume of the release medium constant. The concentration of PTX release in the releasing medium was monitored by HPLC measurement (GLISON HPLC system, see section 2.4) at a detection wavelength of 228 nm. Before measurement, the sample was diluted with equal volume of acetonitrile and filtered with 0.45 µm pore-size filter. Mixture of acetonitrile (60%) and water (40%) were used as the mobile phase at a flow rate of 1 ml/min. The concentration of PTX was determined by comparing the peak area with the standard curve of PTX prepared considering the

same measurement conditions. The amount of PTX remaining in the assemblies was also measured after collecting the inner phase of the dialysis membrane tube by the HPLC.

2.10 Statistical analysis

Statistical analysis was performed applying the one-way analysis of variance (ANOVA) followed by Fischer's test as post-hoc comparison at a significance level set at $p < 0.05$.

Results and Discussions

3.1 Characterization of POSS-based MPC random copolymers

As we reported before, number-average molecular weight (M_n) of all the POSS-MA MPC polymers was in the range of 10^4 , and the POSS-based MPC copolymers were soluble in water²¹. Characterization of POSS-based MPC copolymers was performed by ¹H NMR and FT-IR measurements. Both the spectra were provided in the supporting information (**Figures S1 and S2, Supporting Information**). In order to evaluate the hydrophobicity of POSS domain in the copolymers, the association formation of the copolymers in water was determined by fluorescence technique using pyrene as a hydrophobic probe. Critical association concentration (CAC) was calculated from the fluorescence excitation spectra of pyrene, participate between aqueous and micellar environment. With the increase in the concentration of the copolymer in the aqueous solution, a shift in excitation spectra of pyrene was observed. The intensity ratio (I_{339}/I_{334}) vs. logarithmic value of concentration (Log C) of the copolymers in the pyrene excitation spectra was plotted (**Figure S3, Supporting Information**). It was observed that the curves were almost flat at

the low concentration of the copolymers, but those started decreasing at high concentration except for poly MPC. This phenomenon indicated the association formation of the copolymers in water. From the results, CAC values of each copolymer were determined from the intersection of two straight lines. The calculated CAC values of C₂H₅-POSS-MA MPC, C₆H₁₃-POSS-MA MPC and C₈H₁₇-POSS-MA MPC were 4.8×10^{-2} mg/ml, 6.3×10^{-2} mg/ml and 8.1×10^{-2} mg/ml, respectively. It was observed that the CAC value of the copolymers increased with the R groups (C₂H₅, C₆H₁₃, C₈H₁₇) in the POSS moiety. Our previous study showed that C₂H₅-POSS moiety in the Poly MPC matrix increased the hydrophobicity at water-polymer interface²¹. Due to the increased hydrophobicity of C₂H₅-POSS-MPC, CAC value was found to be lower than the other copolymers. Other factors to affect the CAC values were the difference of molecular weight. The M_n of the copolymers were found to be in the following order: C₂H₅-POSS-MA MPC ($M_n = 9.8 \times 10^4$) < C₈H₁₇-POSS-MA MPC ($M_n = 4.1 \times 10^5$) < C₆H₁₃-POSS-MA MPC ($M_n = 5.9 \times 10^5$). Since the hydrophobic part of each of the copolymer was the same (1-2 mol. %), the hydrophilic chain length increased with the M_n . Increase in hydrophilic chain length of C₂H₅-POSS-MA MPC decreases the association tendency of the hydrophobic POSS segment, as compared with C₆H₁₃-POSS-MA MPC and C₈H₁₇-POSS-MA MPC. Additionally, the appropriate molecular weight of hydrophilic MPC segment needs to have some balance to achieve lower CAC value^{28, 29}.

The z-average sizes of POSS-based MPC copolymers in water above the CAC were estimated by DLS measurements (**Figure 3 (A)**). The z-average diameter of the copolymers was found in the range 65 – 80 nm at the concentration of 1 mg/ml, and they showed a wide range of size distribution (**Figure S4 (a) in Supporting Information**). In the case of C₂H₅-POSS-MA MPC, two peaks were observed around 10 and 100 nm. The peak around 10 nm seems to be unimolecular assembly. Since the incorporated POSS moiety in the MPC polymeric matrix was only 1-2 mol. %,

the randomly incorporated R-POSS moieties induced the association states. After 4 days, the z-average diameter was almost the same in the case of C₂H₅-POSS-MA MPC, indicating that the assemblies are stable even after standing in water (**Figure 3 (A) and Figure S4 (b) in Supporting Information**). The stability of C₆H₁₃-POSS-MA MPC and C₈H₁₇-POSS-MA MPC showed the same tendency. ζ -Potentials of the copolymers were also measured in water, and the values were ranging from - 0.5 to - 4 mV (**Figure 3 (A)**). The lightly anionic charge would satisfy one of the requirements for obtaining good blood compatibility as seen in the MPC-based nanoparticles³⁰.

Morphology of the POSS-based MPC random copolymers (1 mg/ml) was investigated by TEM observation (**Figure 3 (B) (a)-(c)**) after 4 days. The shapes of the POSS-based MPC random copolymers were observed to be spherical, and the size of them was consistent with the size obtained from DLS results. After 4 days standing in water, TEM images of C₂H₅-POSS-MA MPC and C₆H₁₃-POSS-MA MPC were obtained: any noticeable change in the morphology and size was not observed (**Figure 3 (B) (d) and (e)**). Distribution of the POSS moiety in the assemblies (day 1) was determined by high-resolution dark field STEM/EDS observation³¹ (**Figures S5-S7 in Supporting Information**). Dark phase corresponds to the region with lower electron density, so that the copolymer assemblies are likely to be located in the bright phase. From the elemental map of nitrogen (N), oxygen (O), phosphorous (P) and silicon (Si), the POSS and the MPC moieties are likely to be located in the assemblies, and all the elements were homogeneously distributed. These results suggest that the formed assemblies were attributed to the randomly incorporated POSS moieties, and the random copolymerization results in the stable assemblies in water after 4 days.

The cytotoxicity of all the copolymers solutions (1 mg/ml) was determined by CCK-8 assay after 24 h incubation. The cell viability of each copolymer solution was as high as 100 % and some of them were even over 100 % (**Figure S8 in supporting information**).

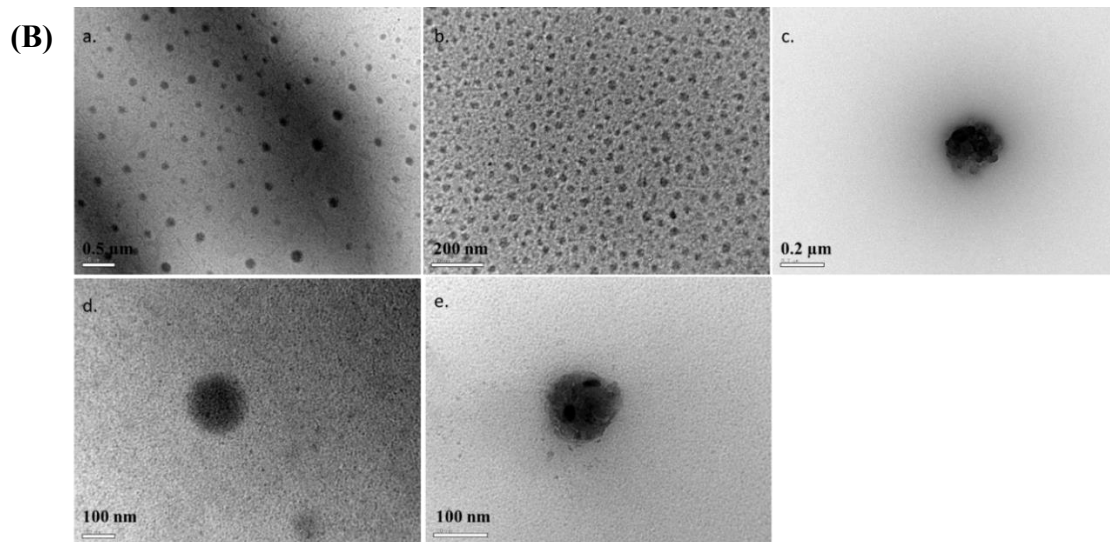
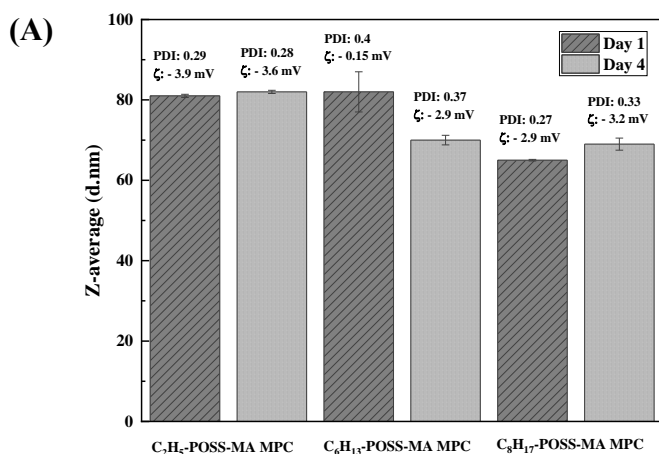


Figure 3. (A) Particle size (Z-average diameter) and ζ -potential of C_2H_5 -POSS-MA MPC, C_6H_{13} -POSS-MA MPC and C_8H_{17} -POSS-MA MPC (B) TEM images of a.) C_2H_5 -POSS-MA MPC (30K) b.) C_6H_{13} -POSS-MA MPC (120K) c.) C_8H_{17} -POSS-MA MPC (100K) d.) C_2H_5 -POSS-MA MPC (150K) and e.) C_6H_{13} -POSS-MA MPC (250K) after 4 days (Stability study).

3.2 PTX-solubilized POSS-based MPC copolymer assemblies

Since the POSS-based MPC copolymers could form a kind of assembled structure, PTX solubilization was performed. After the PTX solubilization, the z-average diameter of the POSS-based MPC copolymer assemblies was calculated by DLS measurements (**Figure S9 in Supporting Information**). The diameter of both C₂H₅ POSS- MA MPC and C₆H₁₃ POSS- MA MPC decreased after the PTX solubilization (see **Figures S9 in Supporting Information**). This can be attributed to the PTX solubilization: strong hydrophobic interaction between PTX and the POSS moiety leads to the shrinkage in hydrodynamic diameter. After standing 4 days, PTX-solubilized C₂H₅ POSS- MA MPC and C₈H₁₇ POSS- MA MPC maintained its original hydrodynamic size, while PTX-solubilized C₆H₁₃ POSS - MA MPC showed an increase in size due to aggregation phenomena. These results suggest that the assembled structure, especially for the C₂H₅ POSS- MA MPC, was stably maintained even after 4 days.

3.3 PTX solubility in the presence of various POSS-based MPC copolymers

PTX solubility in three kinds of POSS-based MPC copolymers and poly MPC solutions at 5, 10 and 50 mg/ml were evaluated by using HPLC (**Figure 4 (a)**). The PTX solubility in poly MPC aqueous solution was less than detectable range (considered to be around 0 µg/ml). On the other hand, incorporation of R-POSS-MA moieties significantly contributed to increasing little PTX solubility, especially at the copolymer concentrations of 50 mg/ml. It is noted that the order of R-groups of POSS moiety towards PTX solubility enhancement was found to be C₂H₅ > C₆H₁₃ > C₈H₁₇, indicating that shorter alkyl chain attached to the POSS moiety enhanced the hydrophobic interaction with PTX, which was consistent with the lowest value of CAC value. The solubility

measurement was performed at 100 mg/ml using C₂H₅-POSS-MA MPC solution in order to check the copolymer concentration-dependent PTX solubility in aqueous solution. (**Figure 4 (b)**). The PTX solubility at 100 mg/mL was found to be 75.5 ± 8.8 µg/ml. According to those results, we selected C₂H₅-POSS-MA MPC copolymer for the following cell viability tests.

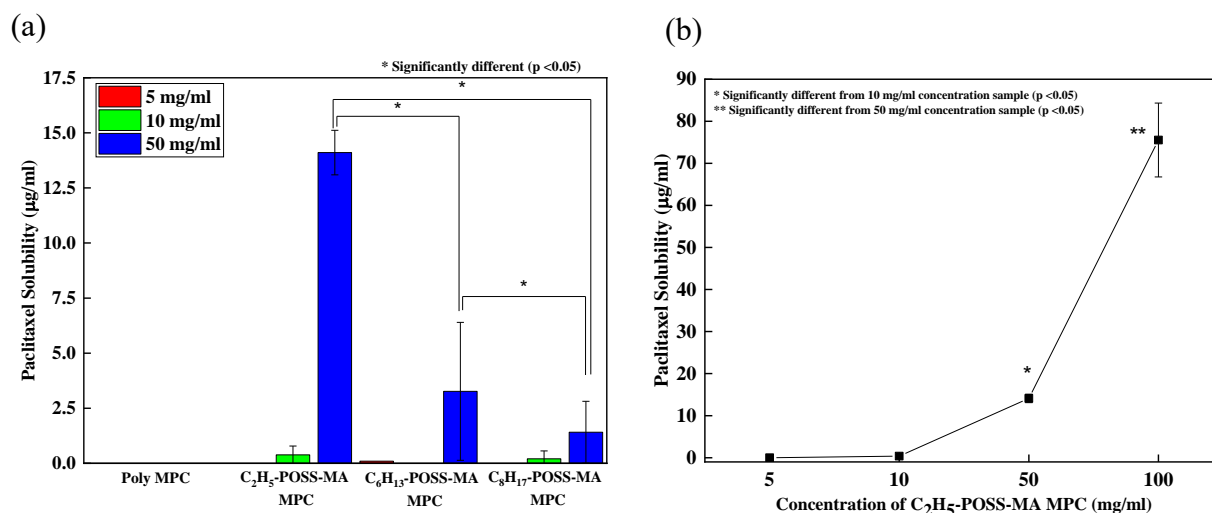


Figure 4 (a) PTX solubility of in the presence of C₂H₅-POSS-MA MPC, C₆H₁₃-POSS-MA MPC, C₈H₁₇-POSS-MA MPC and poly MPC at 5, 10, and 50 mg/ml. (b) C₂H₅-POSS-MA MPC concentration-dependent PTX solubility (5, 10, 50 and 100 mg/ml). Initial PTX concentration 1 mg/ml. Values are mean \pm S.D. (n = 3).

3.4 Cell viability of PTX-solubilized C₂H₅-POSS-MA MPC copolymer

Cell viability in the PTX-solubilized C₂H₅-POSS-MA MPC copolymer at pH 7 and pH 5 was measured in comparison with free PTX (**Figure 5**). The final concentration of PTX dissolved in C₂H₅-POSS-MA MPC copolymer was around 7.6 µg/ml, whereas that of free PTX was 100 µg/ml. The cell viability of PTX itself was 38 ± 18 and 17 ± 10 %, for 24 and 48 h incubation, respectively. Xu *et al* reported that HeLa cell viability of free PTX at 10 µg/ml for 24 and 48 h

incubation was about 50 and 30 %, respectively³². From this report, the cell viability of free PTX at 100 µg/ml in this study was identical. When PTX was dissolved in C₂H₅-POSS-MA MPC (100 mg) copolymer solution at pH 7, cell viability was found around 87 ± 24 % after 24 h, while free PTX showed significantly lower level of cell viability. This means that the solubilized PTX was strongly incorporated inside the copolymer association till 24 h. After 48 h of incubation, cell viability was significantly decreased to 21 ± 5 %. This result suggests that PTX incorporated into the copolymer association was released inside of cells during that incubation time. The similar phenomena were observed when the sample solution pH was adjusted to 5 (**Figure 5**), suggesting PTX is likely to be released from the copolymer association under the intracellular pH condition. However, cellular uptake of the copolymer was still unknown under the condition, so we performed the cellular uptake study.

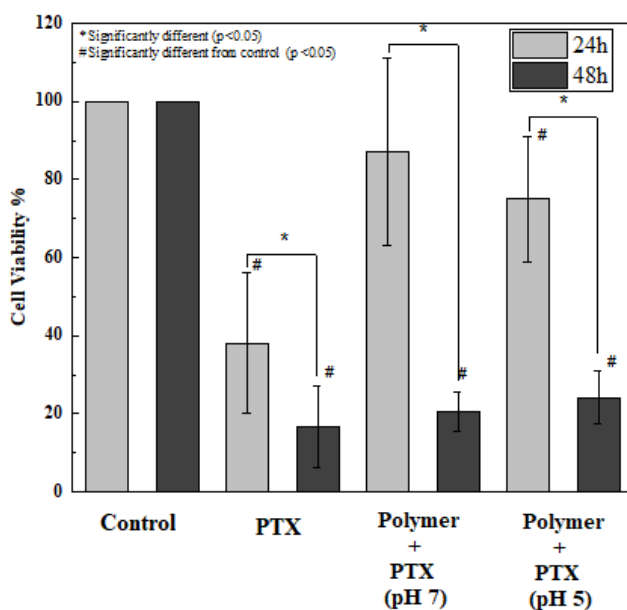


Figure 5. The cell cytotoxicity of PTX (initial conc.: 1mg/ml) and PTX dissolved in C₂H₅-POSS-MA MPC (100 mg/ml) copolymer solution at pH 7 and pH 5. Each of the solutions (10 µl) were added to HeLa cells seeded well plate (90 µl) and incubated for 24 and 48 h at 37°C to measure the cell viability. Final concentration of PTX in the copolymer solution and control was 7.6 and

100 $\mu\text{g/ml}$, respectively. Values are Mean \pm S.D. ($n = 6$) (*: $P < 0.05$ #: $P < 0.05$ vs control, one-way ANOVA, Fischer's test). Cell viability was determined using the CCK-8 Kits and the absorbance was detected at 450 nm.

3.5 Cellular uptake of FITC-PTX-solubilized $\text{C}_2\text{H}_5\text{-POSS-MA MPC}$ copolymer

In order to observe the cellular uptake of PTX-solubilized $\text{C}_2\text{H}_5\text{-POSS-MA MPC}$ copolymer, FITC-labelled PTX (FITC-PTX; see **Figure 2**) was prepared. The obtained FITC-PTX was confirmed by ^1H NMR and ESI-MS spectroscopy (**Figure S10 in supporting information**). FITC-PTX was solubilized in the $\text{C}_2\text{H}_5\text{-POSS-MA MPC}$ copolymer solution in the same manner, as PTX. As shown in **Figure 6**, the green fluorescence intensity inside the cells was observed after 2 h incubation with FITC-PTX-solubilized $\text{C}_2\text{H}_5\text{-POSS-MA MPC}$ copolymer. On the other hand, the cells treated with FITC-PTX did not show any fluorescence colour at the same time. After 24 h incubation, the fluorescence colour was observed in the cells treated with both FITC-PTX and FITC-PTX-solubilized $\text{C}_2\text{H}_5\text{-POSS-MA MPC}$ copolymer, although most of the cells were died due to the cytotoxicity of PTX (**Figure S11 in supporting information**). These results suggest that FITC-PTX-solubilized $\text{C}_2\text{H}_5\text{-POSS-MA MPC}$ copolymer was taken up by the HeLa cells through cellular uptake process within 2 h. Therefore, the $\text{C}_2\text{H}_5\text{-POSS-MA MPC}$ copolymer assembly is likely to play a role for rapid cellular uptake, followed by release of PTX in the target cells. .

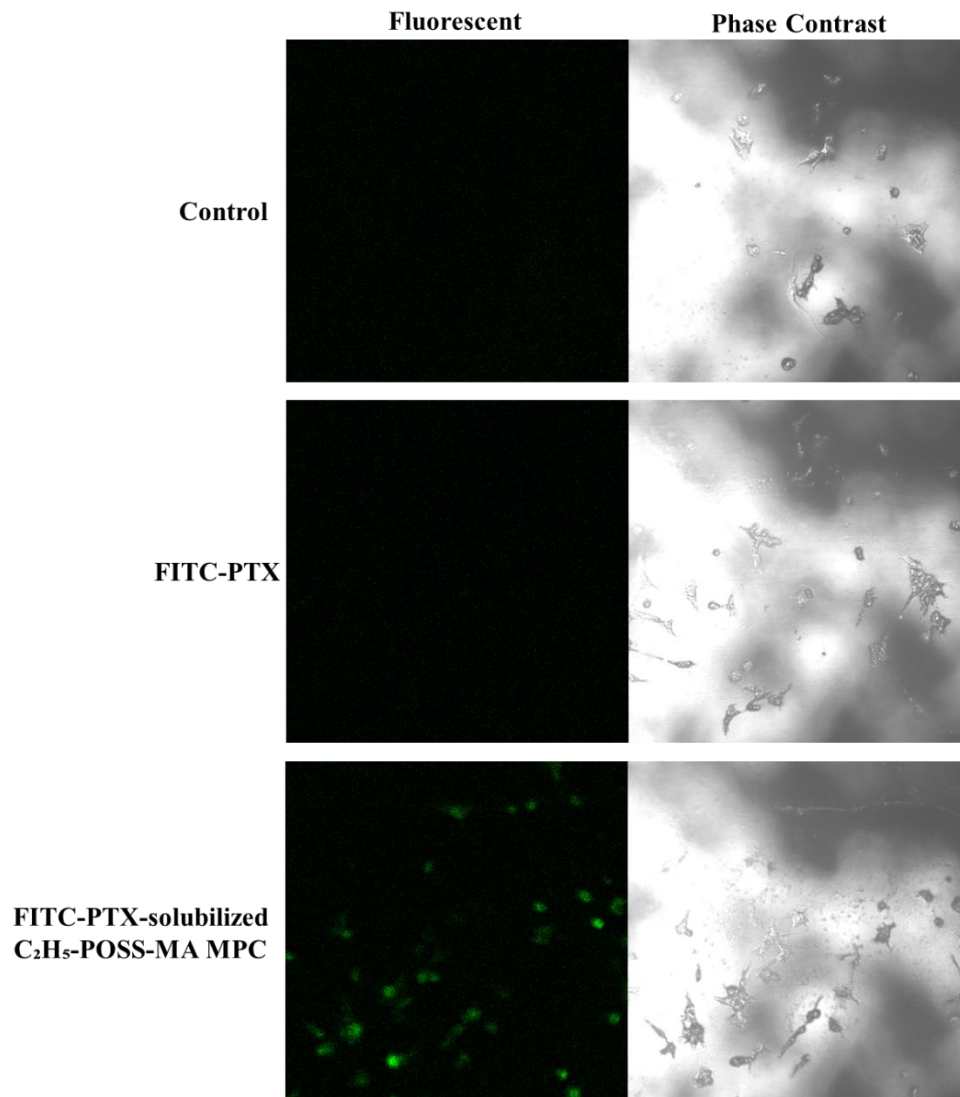


Figure 6. The CLSM images of HeLa cells treated with medium without FITC-PTX (upper), with FITC-PTX (middle) and FITC-PTX-solubilized C₂H₅-POSS-MA MPC copolymer (bottom) after 2 h incubation.

3.6 *In vitro* paclitaxel release from the assemblies

In vitro, PTX release profile from the PTX-solubilized C₂H₅-POSS-MA MPC copolymer was studied in D-PBS (pH 7.4) containing 1 % Tween 80. The release behaviour from the PTX-solubilized C₆H₁₃-POSS-MA- and C₈H₁₇-POSS-MA MPC copolymers was also performed. As

1 shown in **Figure 7**, three kinds of PTX-solubilized copolymers have shown different release
 2 pattern. Initial burst release was not observed in all the POSS-based MPC assemblies, which
 3 indicates that no residual PTX was located on the surface area. Cumulative release of PTX from
 4 the PTX-solubilized C₂H₅ POSS-MA MPC assembly was reached to 30 % till 47 h, while that
 5 from the other assemblies was over 40 %. According to the STEM/EDS experimental results
 6 (**Figures S5-S7 in Supporting Information**), the POSS moieties of the all the copolymers seem
 7 to be homogeneously distributed in the assemblies. Taking the morphology into account, the
 8 suppression of the PTX release is considered to be the strength of the hydrophobic interaction
 9 hydrophobic region in the assembled structure. Thus, C₂H₅-POSS moiety effectively contributed
 10 to the slow release of PTX. This is supported by the results of DLS (**Figure 3 (A) and Figure S9**
 11 **in Supporting Information**): the size of C₂H₅-POSS-MA MPC assembly (~ 80 nm) decreased
 12 after the PTX solubilization (~ 62 nm), and the structure was stable for 4 days. Therefore, strongly
 13 interacted PTX with C₂H₅ POSS-MA moiety was kept around the poly MPC matrix in a solution
 14 state.

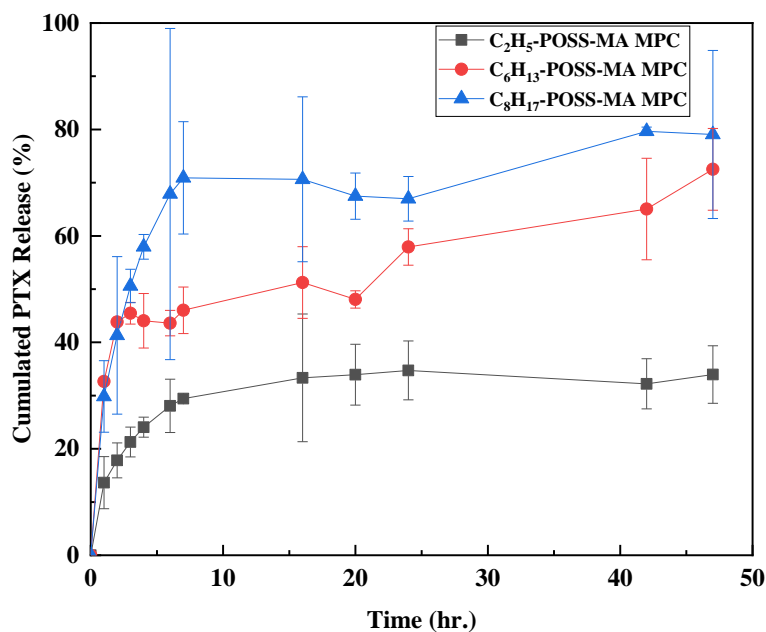


Figure 7. Released of Paclitaxel from PTX-solubilized POSS-based MPC copolymer vs. time curves of (square) PTX-solubilized C₂H₅-POSS-MA MPC, (circle) PTX-solubilized C₆H₁₃-POSS-MA MPC and (triangle) PTX-solubilized C₈H₁₇-POSS-MA MPC assemblies. Values are mean \pm S.D. (n = 3).

Conclusions

The R-POSS-based MPC random copolymers (R-groups are ethyl (C₂H₅), hexyl (C₆H₁₃), octyl (C₈H₁₇)) were evaluated as a solubilizer of PTX. Among them, the C₂H₅-POSS-MA MPC contributed to the enhanced solubilization due to assemble formation, leading to cellular uptake and intracellular PTX release. The PTX release rate and the amount could be controlled not only in solution state but also in the solid dosage form. Based on the findings of the current investigation, it can be inferred that the copolymer based on C₂H₅-POSS-MA MPC shows promising characteristics to be considered as polymeric biomaterials for enhancing poorly soluble compounds.

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1 Table of Contents Graphic

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