# Preparation of Polypseudorotaxanes Composed of Cyclodextrin and Polymers in Microspheres

Katsunori Shinohara,[a] Miki Yamashita,[b] Wataru Uchida,[b] Chie Okabe,[b] Shinji Oshima,[b] Masahiro Sugino,[b] Yuya Egawa,*[,b] Ryotaro Miki,[b] Osamu Hosoya,[b] Takashi Fujihara,[c] Yoshihiro Ishimaru,[d] Tohru Kishino,[a] Toshinobu Seki,[b] and Kazuhiko Juni[b]

[a] *Department of Pharmacy Services, Saitama Medical Center, Saitama Medical University; 1981 Kamoda, Kawagoe, Saitama 350–8550, Japan:* [b] *Faculty of Pharmaceutical Sciences, Josai University; 1–1 Keyakidai, Sakado, Saitama 350–0295, Japan:* [c] *Research and Development Bureau, Comprehensive Analysis Center for Science, Saitama University; and* [d] *Division of Material Science, Graduate School of Science and Engineering, Saitama University; 255 Shimo-ohkubo, Sakura-ku, Saitama 338–8570, Japan.*
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**We prepared polypseudorotaxanes (PPRXs) composed of cyclodextrin (CyD) and polyethylene glycol (PEG) inside microspheres (MSs) by an emulsifying process using polypropylene glycol (PPG) that shows temperature-dependent hydrophilicity changes; PPG is hydrophobic at high temperatures but hydrophilic at low temperatures. An aqueous solution of CyD and PEG was dispersed as droplets in PPG at 60°C then cooled to 0°C to allow water of droplets to transfer into PPG. On removal of water in the droplets, CyD and PEG were left behind as a CyD/PEG PPRX inside the solid-state MSs. Examination of *α*-, *β*-, and *γ*-CyD revealed that *α*-CyD was suitable for the formation of PPRX containing PEG in this MS preparation procedure. Interestingly, a new PPRX composed of *α*-CyD and PPG was formed in the *α*-CyD MSs when they were prepared in the absence of PEG from the aqueous solution of *α*-CyD. This MS fabrication procedure can control the size and shape of PPRX particles, and will contribute to the production of new types of CyD inclusion complexes.**

**Key words** cyclodextrin (CyD); microsphere (MS); polyethylene glycol (PEG); polypropylene glycol (PPG); polypseudorotaxane (PPRX)

Cyclodextrins (CyDs) are cyclic oligosaccharides composed of 6, 7, or 8 glucopyranoside units, which are named *α*-, *β*-, and *γ*-CyD, respectively. CyDs consist of a hydrophobic cavity in which hydrophobic molecules are enclosed to form an inclusion complex. CyDs are widely used as a pharmaceutical additive for their ability to improve the solubility and stability of drugs *via* CyD complexation.[1,2] Recently, CyDs have attracted much attention as components of supramolecular nanostructures because of their unique structures and functions.[3–6] CyDs include not only low-molecular-weight molecules but also polymers[7–10]; the latter complexes, in which many CyDs are threaded by a polymeric molecule, are referred to as polypseudorotaxanes (PPRXs). CyDs show the selectivity for guests depending on the size of the cavity and sectional area of the polymers; specifically, *α*-CyD includes polyethylene glycol (PEG),[8] *β*-CyD includes polypropylene glycol (PPG),[10] and *γ*-CyD includes PPG or double-stranded PEG.[9,10] When the both end groups of polymer of PPRX are modified with bulky stoppers, the structures are called as a polyrotaxane (PRX).

The unique structures of PPRXs and PRXs have been applied toward a new generation of drug delivery systems.[11–19] Seo *et al.* have prepared a PRX using a ligand-modified CyD.[15] Both the rotation of CyD rings around the polymer axis and free arrangement of ligands were favorable in promoting multiple interactions between ligands and receptors on particular cell membranes. Ohya *et al.* have prepared a stimuli-sensitive PRX composed of a drug-modified CyD and polymer capped with hydrolyzable ester groups.[16] Hydrolysis of the end-capped linkages by an esterase induced disintegration of the PRX, ultimately resulting in the release of drug-modified CyDs. Higashi *et al.* reported PPRXs composed of CyDs and PEG-modified insulin.[17–19] These PPRXs showed a slow release of PEGylated insulin, which would correspond to a novel sustained-release system of PEGylated proteins.

Although PPRXs have great potential as a new material for drug delivery systems, the repertoire of PPRX preparation methods is limited. In general, PPRXs are obtained by spontaneous precipitation from a mixed solution of CyD and polymer. For example, a small amount of PEG solution is added to a saturated CyD solution, resulting in the precipitation of crystalline PPRXs.[7–10] With this spontaneous precipitation method, it seems difficult to expand upon variations in the combination of CyD and polymer for PPRXs; furthermore, this method makes it difficult to control the size and shape of PPRX particles.

We have developed a new PPRX preparation method to extend their applicability as carrier systems. This method has been previously reported as a microsphere (MS) preparation method for water-soluble polymers[20] (Fig. 1). This MS preparation method utilizes PPG as a dispersion medium. PPG is both hydrophobic at high temperatures and hydrophilic and freely soluble in water at low temperatures.[21,22] Therefore, the PPG/water system forms two liquid phases of a water-in-oil (W/O) emulsion at a high temperature and transforms to one liquid phase on cooling. This temperature-dependent property of PPG can be used to remove water from aqueous polymer solutions dispersed in PPG to form solid polymer particles. In the previous report, water-soluble starch, gelatin, whey protein, and dextran were used for the MS preparations.

In this study, we have attempted to produce PPRXs composed of CyD and PEG within MS systems. PEG is impor-

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* To whom correspondence should be addressed. e-mail: yegawa@josai.ac.jp
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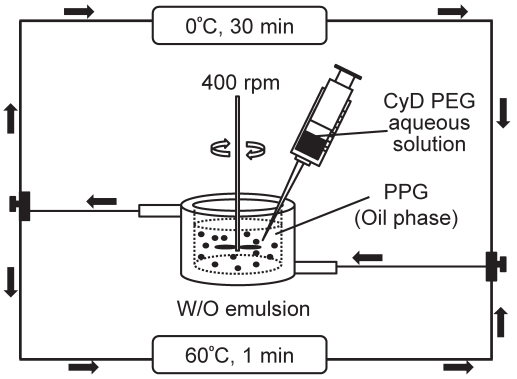


Fig. 1. Schematic Illustration of MS Preparation Procedure

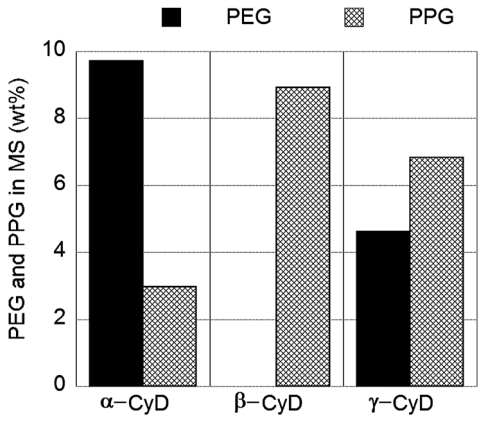


Fig. 2. The Amount of PEG and PPG in MSs after a Single Acetone Washing

MSs were prepared in the presence of PEG.

tant for drug delivery systems owing to its biocompatibility and approval as a pharmaceutical additive. Furthermore, PEGylation technologies have been widely used to improve therapeutic efficacies of proteins, and some PEGylated proteins are available in the market.[23,24] CyDs were used as a water-soluble material, and PEG was added into an aqueous solution of CyD. The aqueous CyD and PEG solution was dispersed in PPG as droplets, and water was removed from the droplets by inducing a change in temperature. We found that $\alpha$-CyD was suitable for the preparation of PPRXs containing PEG. This new preparation method for CyD/PEG PPRXs is anticipated to be useful in pharmaceutical applications. Furthermore, we observed a new type of PPRX composed of $\alpha$-CyD and PPG when preparing $\alpha$-CyD MSs in the absence of PEG. In this paper, we present the details of the preparation procedure and structural analyses of CyD MSs, and discuss the PPRX formation mechanism.

## Experimental

**Materials** $\alpha$-CyD, $\beta$-CyD, and $\gamma$-CyD were obtained from Junsei Chemical Co., Ltd. (Tokyo, Japan). PPG (diol type, molecular weight (MW) 400) and PEG (diol type, MW 2000) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals were reagent grade and used as received.

**Apparatus** Hydrogen-1 NMR spectra were measured using a JNM GX-270 FT NMR spectrometer at 270 MHz (JEOL, Tokyo, Japan). Solid-state $^{13}C$ cross polarization/magic-angle spinning (CP/MAS) NMR was recorded at 125.7 MHz on a Bruker AVANCE500T (Bruker BioSpin K.K., Kanagawa, Japan) with a sample spinning rate *ca.* 8 KHz. Chemical shifts were referenced carbonyl carbon of glycine as an external standard. Differential scanning calorimetry (DSC) was carried out using a Thermo Plus2 series (Rigaku Corporation, Tokyo, Japan). A sample was heated in an aluminum pan at a heating rate of 5K/min under nitrogen atmosphere. Powder X-ray diffraction (XRD) patterns were measured using a Mini Flex II (Rigaku Corporation, Tokyo, Japan) using Cu$K\alpha$ radiation under the following conditions: Diffraction was performed at 30 kV and 15 mA with a scanning speed of 4°/min and measurement range of $2\theta$=5–40°. The volume particle-size distributions of powdered products were measured using a laser diffraction method (Mastersizer 2000, Malvern Instruments Ltd., Worcestershire, U.K.). Acetone was used as a dispersant with a wet sample dispersion unit (Hydro 2000SM). Particle surface morphologies were examined using a scanning electron microscope (SEM; model S3000N, Hitachi Ltd., Tokyo, Japan). An acceleration voltage of 15 kV was used for all samples. Prior to microscopy, the samples were mounted onto aluminum stubs 12 mm in diameter and coated with gold using an SEM Coating Unit E-1010 ion-sputter coater (Hitachi Ltd., Tokyo, Japan) at 50 mA for 70 s under an atmosphere of argon.

**Procedure for Microsphere Preparation** PPG (MW 400, 15 mL) was added to a jacketed beaker, and the temperature of PPG was kept at 60°C. CyD (150 mg) and PEG (14 mg) were dissolved in water (3 mL) at 60°C. This aqueous CyD and PEG solution was added to PPG and mixed using an agitating propeller with triple wings ($d$=22 mm) at 400 rpm for 1 min at 60°C. The mixture was cooled to 0°C and kept at this temperature for 30 min. For the first washing step, the mixture was added to 350 mL of acetone, and MSs were collected by filtration under reduced pressure with a membrane filter (0.5 $\mu$m, PTFE, Advantec, Tokyo, Japan). The MSs were dried at room temperature under reduced pressure overnight. For repeated washing steps, MSs were suspended in 50 mL of acetone. After stirring well, the MSs were collected by filtration using the membrane filter. This washing process was repeated several times.

$\alpha$-CyD MSs were prepared in a similar manner as above in the absence of PEG.

## Results and Discussion

We prepared MSs using $\alpha$-, $\beta$-, and $\gamma$-CyD in the same procedure for the purposes of comparison. An aqueous solution of CyD and PEG was dispersed in PPG to prepare a W/O emulsion. Lowering temperature induced the increased hydrophilicity of PPG, and water in the droplets was transferred into the PPG phase, resulting in the formation of a solid-state MSs. The obtained MSs were dissolved into DMSO-$d_6$ and examined by $^1H$-NMR to estimate the amounts of components, *i.e.*, CyD, PEG, and PPG. The amounts of PEG and PPG were calculated by the integration of peaks around $\delta$=3 and 1 ppm, respectively.[8,10] Figure 2 shows the amount (wt%) of polymers entrapped by a series of CyD MSs after a single acetone washing. The MSs using $\alpha$-CyD showed the highest amount of PEG. In the case of $\beta$-CyD, the MSs did not contain PEG, but rather contained the highest amount of PPG. The MSs using $\gamma$-CyD contained certain amounts of PEG and

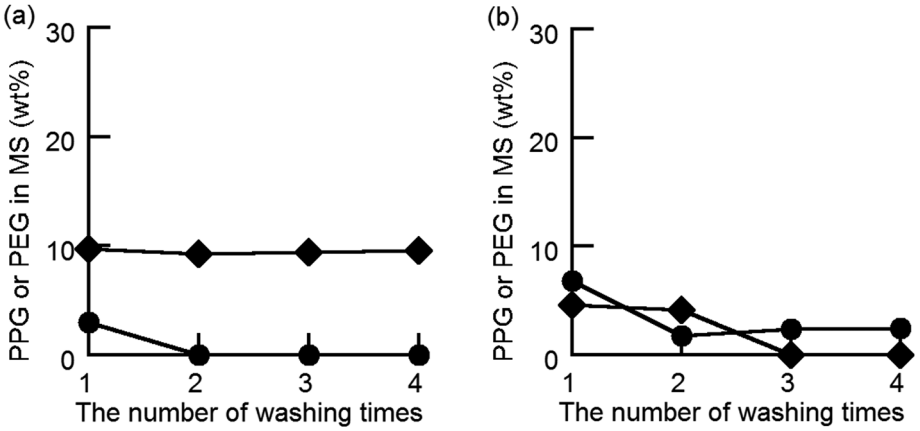


Fig. 3. The Change of Polymer Amount in CyD MSs through Repeated Acetone Washing

(a) $\alpha$-CyD MSs prepared in the presence of PEG. (b) $\gamma$-CyD MSs in the presence of PEG. PEG (diamond), PPG (circle).

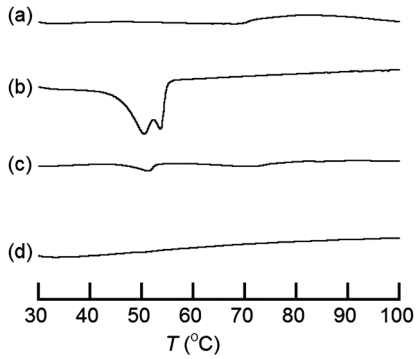


Fig. 4. DSC Thermograms

(a) $\alpha$-CyD, (b) PEG, (c) physical mixture of $\alpha$-CyD, PEG, and PPG in a weight ratio of 87 : 10 : 3, (d) $\alpha$-CyD MSs prepared in the presence of PEG.

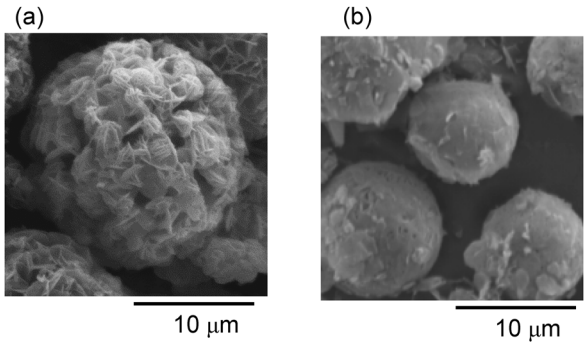


Fig. 5. SEM Images of $\alpha$-CyD MSs

(a) $\alpha$-CyD MSs prepared in the presence of PEG. (b) $\alpha$-CyD MSs prepared in the absence of PEG.

PPG. These results match the selectivity of CyDs for guests. Previous reports have shown that $\alpha$-CyD includes PEG but not PPG, and $\gamma$-CyD includes both PEG and PPG; on the other hand, $\beta$-CyD includes PPG but not PEG.[7–10]

To clarify that PEG was included in the CyD cavities in the MSs, repeated acetone washings were conducted for $\alpha$-CyD MSs and $\gamma$-CyD MSs (Fig. 3). In the case of $\alpha$-CyD MSs (Fig. 3(a)), PPG was completely removed after the second wash, which suggests that PPG was adsorbed on the surface of the $\alpha$-CyD MSs. On the contrary, the amount of PEG remained unchanged over four washings at 9.6 wt%, corresponding to an $\alpha$-CyD : ethylene glycol unit stoichiometry of 1 : 2.4. This value is similar to the reported $\alpha$-CyD : ethylene glycol unit value of 1 : 2 in an $\alpha$-CyD/PEG PPRX prepared by spontaneous precipitation.[8] This result supports the formation of $\alpha$-CyD/PEG PPRX within the MS interior. In contrast, in the case of $\gamma$-CyD MSs (Fig. 3(b)), PEG was removed after the third wash with acetone. In order to evaluate the effect of repeated acetone washing on PPRXs, we prepared PPRXs by using spontaneous precipitation,[8–10] and evaluated the polymer amount (Fig. S1). In the case of $\alpha$-CyD/PEG PPRX, there is little change in the amount of PEG by acetone washing. On the contrary, other PPRXs using $\beta$-CyD and $\gamma$-CyD show drastic decline. From this result, acetone washing is suitable for evaluating the amount of polymer included by $\alpha$-CyD: however, it is difficult to discuss the amount of polymer included by $\beta$-CyD or $\gamma$-CyD.

Next, DSC thermograms were recorded to confirm the formation of $\alpha$-CyD/PEG PPRX in the MSs. PEG showed an endothermic peak around 50°C as the melting point (Fig. 4(b)). A physical mixture of $\alpha$-CyD, PPG, and PEG showed a small peak around 50°C (Fig. 4(c)); however, $\alpha$-CyD MSs prepared with PEG did not show an endothermic peak (Fig. 4(d)), demonstrating that PEG inside of the MSs was covered by $\alpha$-CyD.[25] From these results, we conclude that $\alpha$-CyD is the most suitable of the examined complexes to prepare PPRX in the MS preparation method.

The morphologies and sizes of the as-prepared PEG-containing $\alpha$-CyD MSs were evaluated. Size-distribution measurements of the MSs revealed that 10%, 50%, and 90% diameters by volume were 2.4 $\mu$m, 10.2 $\mu$m, and 22.3 $\mu$m, respectively (Fig. S2(a)). Figure 5(a) shows an SEM image of the surface of the MSs, which appears as an aggregation of a crystalline material, suggesting that crystalline $\alpha$-CyD/PEG PPRX forms before water transfer from droplets into PPG phase is completed. When the dispersed droplets in PPG were cooled to 0°C, crystalline $\alpha$-CyD/PEG PPRXs could form easily. In fact, we prepare the aqueous solution of $\alpha$-CyD and PEG at 60°C for MS preparation to avoid formation of $\alpha$-CyD/PEG PPRXs.

We propose a mechanism for the formation of $\alpha$-CyD/PEG PPRX in the MSs as follows. At first, the aqueous solution formed by dissolution of PEG and $\alpha$-CyD is heated to 60°C. At higher temperatures, PEG is relatively hydrophobic; how-

ever, α-CyD/PEG PPRX does not form because high temperature is disadvantaged for PPRX formation. As the aqueous solution is added to PPG at 60°C, it is dispersed as aqueous droplets by the hydrophobic PPG. The hydrophilicity of PPG increases when the two-phase system is cooled to 0°C, causing the transfer of water from the aqueous droplets into the PPG phase. The aqueous droplets shrink and the concentrations of α-CyD and PEG increase, which enhances the complexation based on Le Chatelier's principle. The α-CyD/PEG PPRX forms in the concentrated droplets, and the crystalline α-CyD/PEG PPRX particles then assemble as a solid-state MSs in the final stage. The temperature control from 60°C to 0°C is needed to obtain spherical PPRX. In fact, we obtained angular PPRX when we used PPG at 0°C from the beginning.

The above-described MS preparation method consists of a stage involving water removal from the droplets. We discovered an unexpected phenomenon that seems to be related to the forced removal of water. When MSs were prepared in the absence of PEG, the obtained MSs contained α-CyD/PPG PPRX, representing a new PPRX combination.

α-CyD/PPG PPRX cannot be obtained in the conventional method.[10] The amount of PPG in the MSs after a single acetone washing was calculated to be 21 wt%, corresponding to an α-CyD : propylene glycol unit stoichiometry of 1 : 4.6. Figure 6 shows the change in the amount of PPG after repeated acetone washing. After the second wash, the amount of PPG decreased to 9 wt%, which suggests that some amount of PPG was adsorbed on the surface of the MSs and removed by repeated acetone washing. However, the value remained unchanged after the second washing, showing that PPG is strongly entrapped in the MSs. After four washings, the α-CyD : propylene glycol unit stoichiometry was 1 : 1.6, similar to the reported value of 1 : 2 for α-CyD : ethylene glycol in α-CyD/PEG PPRX prepared by spontaneous precipitation.[8] One possible explanation for this result is that PPG was included into the α-CyD cavity to form α-CyD/PPG PPRX, and the included PPG could not be washed out by acetone.

We next measured the XRD pattern of the MSs prepared in the absence of PEG, and compared its diffractogram with those of the parent α-CyD (Fig. 7(a)) and α-CyD/PEG PPRX prepared by spontaneous precipitation (Fig. 7(b)) to ascertain whether α-CyD/PPG PPRX was formed. The pattern of α-CyD MSs prepared in the absence of PEG (Fig. 7(c)) was very similar to α-CyD/PEG PPRX, exhibiting a sharp peak around 20°. The patterns of α-CyD MSs prepared without PEG corresponded to the α-CyD channel-type crystal structure, in which cyclodextrin molecules are stacked along an axis to form a cylinder and provide space for a long-chain molecule guest.[18,19,26] The XRD pattern of the channel structure also supported the formation of α-CyD/PPG PPRX in the MSs. At this point, it is difficult to explain the detailed structure α-CyD/PPG PPRX by using the XRD pattern because this is the first report for preparation of α-CyD/PPG PPRX, and further experimental work is needed in this respect.

In order to make doubly sure the formation of α-CyD/PPG PPRX inside of the MSs, we measured the solid-state $^{13}C$ CP/MAS NMR spectra (Fig. S3). As previously reported,[8] α-CyD shows finely split signals of C-1, C-4, and C-6 because all glucose units are not in the same environment, which means α-CyD has a less symmetrical conformation. On the contrary, the MSs show non-split signals of C-1, C-4, and C-6, indicating all glucose units of CyD are in the similar environment by including PPG. From these results of XRD and solid-state NMR, we conclude that α-CyD/PPG PPRX forms in the in the MS preparation procedure.

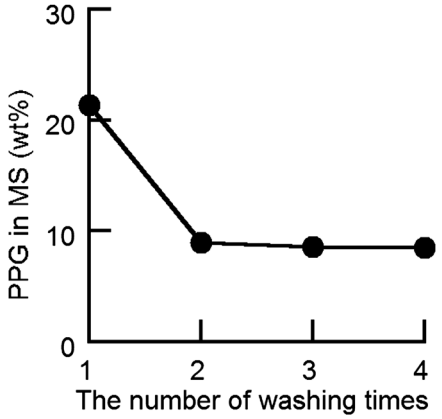


Fig. 6. The Change of PPG Amount in the MSs through Repeated Acetone Washing

The MSs were prepared in the absence of PEG.

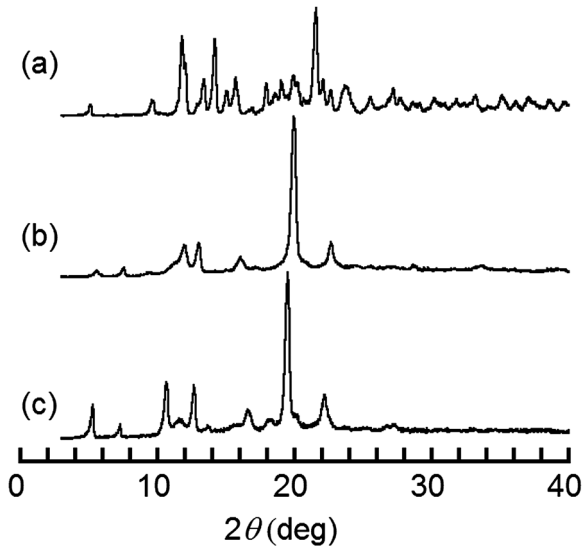


Fig. 7. XRD Patterns of α-CyDs

(a) α-CyD, (b) α-CyD/PEG PPRX prepared in a spontaneous precipitation, (c) α-CyD MSs prepared in the absence of PEG.

The morphologies and sizes of the MSs prepared in the absence of PEG were evaluated. MS size-distribution measurements showed that 10%, 50%, and 90% diameters by volume were 3.0 μm, 9.7 μm, and 31.8 μm, respectively (Fig. S2 (b)). SEM images showed that the MS surface was relatively smooth (Fig. 5(b)) compared to that of the MS containing α-CyD/PEG PPRX, implying that α-CyD/PPG PPRX forms when the amount of water was small. If α-CyD/PPG PPRX formed when the plenty of water exists in the droplets, the surface of MSs would be rough due to the crystalline PPRX. The previous report shows that α-CyD/PPG PPRX does not form in the spontaneous precipitation.[10] We assume that α-CyD/PPG PPRX forms in the special condition where water disappears gradually.

A mechanism of formation of α-CyD/PPG PPRX in the absence of PEG is proposed as follows. The α-CyD aqueous

solution is dispersed as droplets into hydrophobic PPG at 60°C so that the aqueous droplets contain a certain concentration of PPG. The hydrophilicity of the outer phase of PPG is increased when the system is cooled to 0°C, inducing the transfer of water from the aqueous droplets into the outer phase of PPG. The aqueous droplets shrink and the concentrations of α-CyD and PPG are increased to saturation. This increase in α-CyD and PPG concentrations enhances complexation based on Le Chatelier's principle. The α-CyD/PPG complex remains inside the shrinking droplets, which do not precipitate spontaneously. The α-CyD/PPG PPRX is left in the solid-state MSs by the forced transfer of water from droplets. Although PPG is not a favorable guest for α-CyD, NMR and XRD analyses demonstrated the formation of α-CyD/PPG PPRX in the MSs. We believe that this MS preparation procedure may be applicable to the formation of host–guest complexes that might otherwise be difficult to obtain by spontaneous precipitation.

In the same MS fabrication procedure, we tried to prepare α-CyD/PPG PPRX with PPG (MW 1000). We obtained MSs and evaluated the amount of PPG. The amount of PPG was 27% after the first acetone washing; however, the value dropped to 0.4% after four washing. Currently, we do not have a reasonable answer for the effect of molecular weight of PPG on the formation of α-CyD/PPG PPRX. This will be the subject of further study.

In conclusion, we have successfully prepared PPRXs contained inside MSs; α-CyD/PEG PPRX was formed in the obtained MSs when PEG was used together with α-CyD. In the absence of PEG, a new combination of α-CyD/PPG PPRX was observed to form in the MSs. This new procedure has the potential to produce new types of CyD/polymer PPRX, which may not be obtained through common precipitation methods. In addition, the MS preparation method can control the size and shape of PPRX particles, which will contribute to the development of new drug delivery systems using PPRXs.

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