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Encapsulation of hydrophobic drugs in polymeric micelles through co-solvent evaporation: The effect of solvent composition on micellar properties and drug loading

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Abstract

This study was designed to develop an optimized co-solvent evaporation procedure for the efficient encapsulation of hydrophobic drugs in polymeric micelles of methoxy poly(ethylene oxide)-*block*-poly(ε-caprolactone) (MePEO-*b*-PCL). MePEO-*b*-PCL block copolymers having varied MePEO and PCL molecular weights were synthesized, assembled to polymeric micelles, and used for the encapsulation of cyclosporine A (CyA) by a co-solvent evaporation method. The co-solvent composition was varied by changing the type of organic co-solvent (using acetone, acetonitrile and tetrahydrofuran), the ratio of organic to aqueous phase, and their order of addition. Carrier size, morphology and encapsulated CyA levels were defined by dynamic light scattering (DLS), transmission electron microscopy (TEM) and HPLC, respectively, and the effect of co-solvent composition on micellar properties and loaded CyA levels was evaluated. Application of acetone and acetonitrile as the selective co-solvent for the core-forming block led to a decrease in the average diameter of self-assembled structures. When acetone was added to water, a decrease in the ratio of organic to aqueous phase led to an increase in the loading efficiency of CyA in MePEO-*b*-PCL micelles. A similar trend in CyA loading was observed for MePEO-*b*-PCL micelles of varied MePEO and PCL block lengths. The ratio of organic to aqueous phase did not affect CyA loading when water was added to acetone. Irrespective of the order of addition, the decrease in the organic to aqueous phase ratio caused a reduction in the average diameter of the empty and CyA loaded micelles. We conclude that the co-solvent evaporation method may be optimized to improve the efficiency of drug encapsulation in polymeric micelles. For CyA encapsulation in MePEO-*b*-PCL micelles, addition of acetone to water at lower organic to aqueous phase ratio is shown to be the optimum procedure leading to higher drug encapsulation and smaller average diameter for the self-assembled structures.

Keywords: Polymeric micelles; Cyclosporine A; Poly(ethylene oxide); Poly(ε-caprolactone); Solubilization

1. Introduction

The problem of poor water solubility is one of the major obstacles for successful and effective therapy for many existing or emerging therapeutic agents. Conventional solubilizing agents currently in use are mostly toxic and add to the complications associated with drug administration (Ellis et al., 1996; Gelderblom et al., 2001; Theis et al., 1995; Thiel et al., 1986; Tibell et al., 1993). Nanoscopic core/shell structures formed from the assembly of amphiphilic polymers, namely polymeric micelles, have been the subject of several studies for the solubilization and targeted delivery of hydrophobic drugs in recent years (Allen et al., 1999; Kataoka et al., 2001; Kwon, 2003; Lavasanifar et al., 2002; Torchilin, 2004; Zhang et al., 1997a,b, 1996). Efforts for the development of optimal polymeric micellar carriers for individual drug has shown the chemical structure and molecular weight of the core-forming block as well as the self-assembly process to play an important role in determining the success of a given polymeric micellar system in drug solubilization, controlled release and targeted delivery (Aliabadi and Lavasanifar, 2006).

We have reported on the development of polymeric micelles based on block copolymers of poly(ethylene oxide)-*block*-poly(ε-caprolactone) (MePEO-*b*-PCL) for the solubilization

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and delivery of cyclosporine A (CyA) using a co-solvent evaporation method (Aliabadi et al., 2005). A review of individual data from separate research works points to the influence of solvent/co-solvent composition on the properties of MePEO-*b*-PCL micelles prepared by the co-solvent evaporation method (Jette et al., 2004; Shuai et al., 2004a,b). The aim of this research was to conduct a systematic investigation on the effect of solvent/co-solvent system in the self-assembly procedure on the morphology, size, polydispersity index, and CyA loading efficiency of MePEO-*b*-PCL nanocarrier and define the preferred condition for the preparation of optimum MePEO-*b*-PCL based carriers of CyA. The results show addition of concentrated polymeric solutions in organic phase to aqueous phase in the co-solvent evaporation method provides the optimum condition in terms of carrier diameter and CyA loading in MePEO-*b*-PCL micelles.

2. Materials and methods

2.1. Materials

Stannous octoate (96% pure) was obtained from Aldrich (Milwaukee, WI, USA). Methoxy polyethylene oxide (average molecular weight of 5000 g mol^{-1}), ε-caprolactone, amiodarone HCl (98%) were purchased from Sigma (St. Louis, MO). Methoxy polyethylene oxide (average molecular weight of 12,000 g mol^{-1}) was supplied by Polymer Source Inc. (Montreal, Quebec, Canada). Cyclosporine A (CyA) was purchased from Wuhan Zhongxin Company, China. Acetonitrile (MeCN), methanol, and chloroform were supplied by Fisher Scientific (Nepean, Ontario, Canada). Sucrose crystals were obtained from EM Science (Darmstadt, Germany). All other chemicals were reagent grade.

2.2. Methods

2.2.1. Synthesis and characterization of MePEO-b-PCL block copolymers

MePEO-*b*-PCL block copolymers were synthesized by ring opening polymerization of ε-caprolactone using MePEO (molecular weight of 5000 or 12,000 g mol^{-1}) as initiator and stannous octoate as catalyst as described previously (Aliabadi et al., 2005). The molar feed ratio of monomer (ε-caprolactone) to initiator (MePEO) was varied to achieve MePEO-*b*-PCL block copolymers with PCL average molecular weights of 5000, 13,000 and 24,000 g mol^{-1}. A nomenclature of 5000–5000, 5000–13,000, 5000–24,000, and 12,000–5000 (in which the left and right numbers define MePEO and PCL molecular weights, respectively) is used throughout the manuscript to distinguish between different MePEO-*b*-PCL block copolymers used in this study. Prepared block copolymers were characterized for their average molecular weights and polydispersity by ^{1}H NMR and gel permeation chromatography (GPC) using MePEO standards as explained in a previous manuscript (Aliabadi et al., 2005).

2.2.2. Self-assembly and encapsulation process

A co-solvent evaporation method was used for the self-assembly of MePEO-*b*-PCL block copolymers and drug encapsulation (Aliabadi et al., 2005). The type of applied organic solvent, the ratio of organic to the aqueous phase, and the order of addition of the phases in the co-solvent evaporation method were changed to optimize the preparation method in terms of carrier size and encapsulation efficiency. MePEO-*b*-PCL (30 mg) was dissolved in acetone, tetrahydrofuran (THF) or acetonitrile. The volume of organic solvent was either 0.5 or 1.5 mL, corresponding to a final 1:6 or 1:2 organic:aqueous phase ratio, respectively. Either this solution was added drop-wise to water (3 mL) or water was added drop-wise to this solution. The mixture was then stirred at room temperature for 4 h. Vacuum was applied to remove the remainder of the organic solvent. Drug encapsulation was accomplished by dissolving 3 mg of CyA in the organic solvent and following an identical procedure to the self-assembly condition. At the end of encapsulation process, the colloidal solution was centrifuged at 11,600 × *g* for 5 min, to remove any CyA precipitate.

2.2.3. Characterization of empty and CyA loaded nanocarriers

Mean diameter and polydispersity of prepared polymeric micelles in an aqueous media were defined by light scattering (3000HS_A Zetasizer Malvern, Malven Instrument Ltd., UK) at a polymer concentration of 10 mg/mL. Transmission electron microscopy (TEM) (Hitachi-H7000, Tokyo, Japan) of a polymeric micellar solution negatively stained by 1% phosphotungstic acid (H_3PO_4 $12WO_3{\cdot}24H_2O$) was used to assess the morphology of prepared polymeric carriers (Lavasanifar et al., 2001).

2.2.4. Determining the encapsulated levels of CyA in MePEO-b-PCL micelles

An aliquot of the micellar solution in water was diluted with three times of acetonitrile to disrupt the self-assembled structures. Encapsulated levels of CyA were measured using reverse phase HPLC. The HPLC instrument consisted of a Chem Mate pump and auto-sampler. The HPLC system was equipped with an LC1 column (Supleco) with a mobile phase of KH_2PO_4 (0.01 M), methanol and acetonitrile (25:50:25). The flow rate and column temperature were set at 1 mL/min and 65 °C (Eppendorf CH-30 column heater), respectively. CyA concentrations were determined by UV detection at 205 nm (Waters 481) after injection of 100 μL samples, using amiodarone as the internal standard. The calibration samples were prepared at a concentration range of 0.1–10 μg/mL. Each experiment was conducted in triplicate. CyA loading and encapsulation efficiency were calculated from the following equations:

$$\text{CyA loading (w/w)} = \frac{\text{amount of loaded CyA in mg}}{\text{amount of polymer in mg}}$$

$$\text{CyA loading (M/M)} = \frac{\text{moles of loaded CyA}}{\text{moles of polymer}}$$

Table 1
Characteristics of MePEO-*b*-PCL block copolymers used in this study

MePEO molecular weight (g mol^{-1})	PCL molecular weight (g mol^{-1})[a]	[M]/[I][b]	MePEO-*b*-PCL molecular weight (g mol^{-1})[c]	MePEO-*b*-PCL molecular weight (g mol^{-1})[d]	MePEO-*b*-PCL polydispersity (M_w/M_n)[e]
5,000	5000	44	9.923×10^3	8.11×10^3	1.84
5,000	13,000	114	1.81×10^4	1.68×10^4	1.54
5,000	24,000	210	2.88×10^4	2.67×10^4	1.32
12,000	5000	44	1.34×10^4	1.52×10^4	1.52

[a] Theoretical molecular weight.
[b] [ε-caprolactone]/[methoxy polyethylene oxide].
[c] Number average molecular weight determined by ^{1}H NMR.
[d] Number average molecular weight determined by GPC.
[e] Determined by GPC.

$$\text{Encapsulation efficiency (\%)} = \frac{\text{amount of loaded CyA in mg}}{\text{amount of CyA added in mg}} \times 100$$

3. Results and discussion

Encapsulation process has shown to affect loading efficiency of a number of hydrophobic drugs in polymeric micelles (Aliabadi and Lavasanifar, 2006). For instance, switching the encapsulation method from dialysis to solvent evaporation is shown to increase the encapsulated levels of amphotericin B in MePEO-*block*-poly(*N*-hexyl stearate L-aspartamide) (MePEO-*b*-PHSA) micelles (Lavasanifar et al., 2001). Similarly, an O/W emulsion method was proven to be more effective than a dialysis method for the encapsulation of doxorubicin in MePEO-*block*-poly(β-benzyl-L-aspartate) (MePEO-*b*-PBLA) micelles (Kataoka et al., 2000; Kwon et al., 1995).

Assembly of MePEO-*b*-PCL block copolymers and drug loading in the assembled structures has mostly been accomplished through either dialysis (Allen et al., 2000) or co-solvent evaporation methods (Jette et al., 2004; Shuai et al., 2004a,b). In the co-solvent evaporation method, the block copolymer and the drug are dissolved in a volatile, water miscible organic solvent (selective co-solvent for the core-forming block). Self-assembly and drug entrapment is then triggered by the addition of water (non-solvent for the core-forming block) to the organic phase (or vise versa) followed by the evaporation of the organic co-solvent. The co-solvent evaporation method bears several advantages over dialysis method including more feasibility for scale up and less chance for drug loss during dialysis in the encapsulation process. A comparison between individual studies by separate research groups on the self-assembly of MePEO-*b*-PCL by co-solvent evaporation method, implies that aside from block copolymer molecular weight, solvent composition in the self-association process may play a significant role in determining the final properties of the assembled structures (Jette et al., 2004; Kim et al., 1998; Shuai et al., 2004a,b). We have reported on the encapsulation of a relatively large hydrophobic drug, e.g., CyA in MePEO-*b*-PCL micelles by co-solvent evaporation method and showed an enhanced size and drug loading levels for self-assembled structures formed from block copolymers with larger PCLs (Aliabadi et al., 2005). In that study, acetone was used as the organic co-solvent at an organic:aqueous phase ratio of 1:2. To achieve an optimum polymeric micellar carrier for the delivery of CyA, a systematic study determining the effect of solvent/co-solvent composition on carrier size, morphology and CyA encapsulation has been conducted here. Three important factors involved in the co-solvent evaporation method were determined as: the type of organic co-solvent, the volume fraction of organic to aqueous phase during the assembly process and the order of addition of the two phases (aqueous to organic phase or organic to aqueous phase). Each factor was individually manipulated and the effect of each change on carrier size, morphology and/or CyA loaded levels was assessed.

Table 2
Average diameter and polydispersity index of 5000–13,000 MePEO-*b*-PCL carriers prepared by the addition of different organic solvents to water at organic:aqueous phase ratio of 1:2. The final polymer concentration was 10 mg/mL ($n = 3$)

Organic phase	Average diameter of particles ± S.D. (nm)	PI
Acetone	87.8 ± 9.4	0.111
Acetonitrile	82.9 ± 12.3	0.104
THF	109 ± 29.0[a]	0.523

[a] A secondary peak was observed at around 440 nm at an approximate 20% of the total population in 2 out of 3 experiments.

Characteristics of polymer batches used in this study are shown in Table 1. The first set of experiments was carried out using 5000–13,000 MePEO-*b*-PCL block copolymers. Acetone, THF, and acetonitrile were used as the organic co-solvent dissolving the polymer and added to the aqueous phase at a defined organic:aqueous phase ratio of 1:2. The selection of the organic solvent was made based on their miscibility with water, ability to dissolve both CyA and MePEO-*b*-PCL and low boiling point (to facilitate the evaporation and ensure the complete removal of the organic co-solvent). Analysis of the size distribution for 5000–13,000 MePEO-*b*-PCL colloidal dispersions prepared by the three different solvents showed a higher average diameter for carriers prepared by THF (110 nm) and existence of secondary peaks suggesting some degree of aggregation among the assembled structures (Table 2). The average diameter of 5000–13,000 MePEO-*b*-PCL micelles formed with acetonitrile and acetone were similar (83 and 88 nm, respectively) and showed a narrow polydispersity.

Using THF as the organic solvent, Shuai et al. (2004a) have reported an average diameter of 41–86 nm for MePEO-*b*-PCL particles having 5000 g mol^{-1} of MePEO and 5000–24,000 g mol^{-1} of PCL. In that study, the ratio of organic:aqueous phase was set at 1:10. With this in mind, we have decreased the ratio of organic to aqueous phase from 1:2 to 1:6 for THF:water system and studied the effect of organic:aqueous phase ratio on the size of self-assembled structures. The reduction in the THF:water ratio from 1:2 to 1:6 resulted in a significant decrease in the average diameter of self-assembled structures from 110.0 to 58.0 nm. However, a higher polydispersity (0.52) for colloidal particles prepared at this condition was observed and secondary peaks were still present. In comparison, the polydispersity of colloidal particles prepared by acetone was lower (Table 2), and secondary peak was not seen. In further studies, acetone was chosen over THF, because it produced relatively monodispersed micelles. The selection of acetone over acetonitrile was due to a lower boiling point of acetone which makes its evaporation more efficient and rapid.

In the next step, the order of phase addition (acetone to water or water to acetone) as well as the acetone to water ratio was changed and its effect on the average diameter of self-assembled structures from 5000–13,000 MePEO-*b*-PCL was investigated. The volume fraction of the organic to aqueous phase was shown to be the most crucial factor determining the average diameter of self-associated colloids (Table 3). For both addition methods (acetone to water or water to acetone) application of lower acetone:water ratios led to the formation of smaller particles ($p < 0.05$, unpaired student's *t*-test). When water was added to acetone, colloidal particles with average diameters of 74.9 and 57.2 nm were formed using 1:2 and 1:6 acetone:water phase ratios, respectively. When acetone was added to water at 1:2 and 1:6 acetone:water phase ratios, the average carrier size was found to be 87.8 and 63.0 nm, respectively (Table 3). Statistical analysis comparing the size of MePEO-*b*-PCL micelles prepared through addition of water to acetone and those prepared by the addition of acetone to water at identical acetone to water ratios did not show any significant difference ($p > 0.05$, unpaired student's *t*-test). Addition of lower acetone to water ratios led to the assembly to smaller structures from 5000–5000 to 12,000–5000 MePEO-*b*-PCL, as well (Table 4). Compared to other block copolymers, 12,000–5000 MePEO-*b*-PCL micelles had a higher polydispersity and secondary peaks were present in the micellar population. This might be explained by a higher chance of intermicellar interaction, because of a longer chain in the shell of individual micelles. Interaction of MePEO chains in adjacent micellar shells could cause clumping and eventually, aggregation of micelles, creating a second peak in the size spectrum. The diameter of nanostructures formed from assembly of 5000–24,000 MePEO-*b*-PCL was not significantly different between the 1:2 and 1:6 organic:aqueous phase ratios, however ($p < 0.05$, unpaired student's *t*-test).

Table 3
The average diameter and polydispersity index (PI) of 5000–13,000 MePEO-*b*-PCL micelles prepared by different methods ($n = 3$)

Order of addition	Ratio (organic: aqueous)	Average diameter of particles ± S.D. (nm)	PI
Acetone to water	1:2	87.8 ± 9.4	0.111
	1:6	63.0 ± 4.0[‡]	0.142
Water to acetone	1:2	74.9 ± 1.9[a]	0.087
	1:6	57.2 ± 9.8[‡,a]	0.117

[‡] Significantly different from 1:2 ($p < 0.05$).
[a] Not significantly different from "acetone to water".

Table 4
The effect of loading process on the average diameter and polydispersity index of MePEO-*b*-PCL micelles of different PCL molecular weights prepared by the addition of acetone to water

MePEO-*b*-PCL	Ratio (organic: aqueous)	Average diameter of particles ± S.D. (nm)	PI
5000–5000	1:2	68.5 ± 2.0	0.136
	1:6	50.2 ± 3.8[‡]	0.169
5000–13,000	1:2	87.8 ± 9.4	0.111
	1:6	63.0 ± 4.0[‡]	0.142
5000–24,000	1:2	93.6 ± 1.7	0.112
	1:6	92.0 ± 3.1	0.223
12,000–5000	1:2	401 ± 47.7[a]	0.559
	1:6	92.3 ± 24.3[‡,b]	0.382

[a] Secondary peak was observed at approximately 60 nm (peak population <25% total population).
[b] Secondary peak was observed at about 300 nm (peak population <25% total population).
[‡] Significantly different from 1:2 ($p < 0.05$).

In further studies, the effect of acetone to water volume fraction and order of phase addition on the level of CyA loading in MePEO-*b*-PCL micelles and the diameter of loaded particles was assessed (Table 5). For 5000–13,000 MePEO-*b*-PCL (similar to unloaded particles) the average diameter of CyA loaded micelles was significantly smaller when organic:aqueous phase ratio was 1:6 (unpaired student's *t*-test, $p < 0.05$). When acetone was added to water at 1:2 and 1:6 organic to aqueous phase ratios, CyA-loaded 5000–13,000 MePEO-*b*-PCL particles with average diameters of 118 and 89.3 nm were formed, respectively (Table 5). When water was added to acetone, the average diameter of assembled 5000–13,000 MePEO-*b*-PCL structures containing CyA were found to be 94.4 and 66.9 nm for 1:2 and 1:6 organic to aqueous phase ratios, respectively (Table 5). Application of 1:6 organic to aqueous phase ratio also led to a higher polydispersity for the assembled structures (Table 5). TEM images provided further evidence for the smaller average diameter and higher polydispersity of polymeric carriers prepared at 1:6 acetone to water ratio (Fig. 1A) compared to those prepared at an acetone:water ratio of 1:2 (Fig. 1B). Micelles prepared at the ratio of 1:6 consist of a population of smaller structures (the majority of the micelles) accompanied with some larger ones. The diameter of CyA loaded 5000–5000 and 12,000–5000 MePEO-*b*-PCL micelles were also significantly smaller when 1:6 acetone to water ratios were used in the encapsulation process (Table 6). Similar to unloaded carrier, CyA loaded 12,000–5000 MePEO-*b*-PCL micelles have shown higher polydispersity in the micellar population. The average

Table 5
The effect of loading process on the encapsulation of CyA in 5000–13,000 MePEO-*b*-PCL micelles ($n = 3$)

Order of addition	Ratio (organic: aqueous)	CyA initial concentration (mg/mL)	CyA loading ± S.D. (w/w)	Encapsulation efficiency ± S.D. (%)	Average diameter of micelles ± S.D. (nm)	PI
Acetone to water	1:2	3	0.1071 ± 0.0072	35.7 ± 2.4	118.0 ± 16.73	0.121
	1:6	3	0.2286 ± 0.215[‡]	75.9 ± 7.5	89.3 ± 15.3[‡]	0.207
Water to acetone	1:2	3	0.1483 ± 0.0132[†]	49.4 ± 4.4	94.4 ± 11.0	0.180
	1:6	3	0.1496 ± 0.0147[†]	50.0 ± 4.9	66.9 ± 6.0[‡,†]	0.210

[†] Significantly different from "acetone to water' ($p < 0.05$)'.
[‡] Significantly different from 1:2 ($p < 0.05$).

diameter of loaded 5000–24,000 MePEO-*b*-PCL micelles was not significantly different for 1:2 and 1:6 acetone to water ratios (Table 6).

The results also showed organic to aqueous phase ratio and the order of phase addition, both, to affect the final CyA loading in MePEO-*b*-PCL micelles (Tables 5 and 6). CyA reached a final aqueous concentration of 2.3 mg/mL in the presence of 5000–13,000 MePEO-*b*-PCL micelles when acetone was added to water at an initial organic to aqueous phase ratio of 1:6. With the same order of addition, a ratio of 1:2 led to a final aqueous CyA concentration of 1.07 mg/mL on average. A similar trend in CyA loading was observed for 5000–5000, 5000–24,000 and 12,000–5000 MePEO-*b*-PCL micelles (Table 6). Addition of acetone to water at 1:6 phase ratio led to 1.7, 2.1, 1.5 and 4.3-fold increase in CyA encapsulated levels in 5000–5000, 5000–13,000, 5000–24,000 and 12,000–5000 MePEO-*b*-PCL micelles, respectively (Table 6, Fig. 2). When water was added to acetone, there was no significant difference in the CyA loading efficiency in 5000–13,000 MePEO-*b*-PCL micelles for the two different organic to aqueous phase ratios ($p > 0.05$, unpaired student's *t*-test) (Table 5).

An increase in the PCL molecular weight from 5000 to 13,000 and 24,000 led to an increase the molar loading of CyA in polymeric micelles for the acetone:water ratio of 1:6 ($p < 0.05$, student's *t*-test) (Fig. 2). Identical results were observed when an acetone:water ratio of 1:2 was applied ($p < 0.05$, student's *t*-test). A comparison between 5000–5000 and 12,000–5000 MePEO-*b*-PCL micelles showed no significant difference in molar CyA loading between these two structures (irrespective of the organic:aqueous phase ratio) which may imply the association of CyA with the PCL block.

Assembly of MePEO-*b*-PCL block copolymers having a MePEO molecular weight of 5000 g mol^{-1} and varied PCL molecular weights of 1000–4000 g mol^{-1} by adding water to acetonitrile have been studied by Jette et al. (2004). The authors reported on the formation of large structures with hydrodynamic diameters of 200–800 nm at 10–40% water contents. This observation was attributed to the formation of swollen MePEO-*b*-PCL micelles or alternative conformations at water contents around critical water content of micellization (CWC), i.e., the water content needed to induce assembly of block copolymers. With further addition of water (>50% water content) the PCL core was shown to collapse resulting in the formation of nanostructures of 20–30 nm. The results of that study showed an increase in the concentration of MePEO-*b*-PCL to reduce CWC. In 1999, Zhang et al. showed the importance of the water content on the morphology of crew-cut micelles prepared by a co-solvent evaporation method (Zhang and Eisenberg, 1999). They suggested that in low water contents, the micelle formation process might be thermodynamically controlled; however, as the water content increases, kinetic aspects are expected to become more important. Vangeyte et al. (2004) have suggested the same hypothesis by showing that the size of the MePEO-PCL micelles increases when the polymer concentration in the organic solvent is decreased. Johnson and Prud'homme (2003a,b) showed that a higher polymer concentration in the organic phase can decrease the aggregation time of micellization and lead to the formation of more compact nanoparticles.

Table 6
The effect of loading process on the encapsulation of CyA in MePEO-*b*-PCL micelles of different MePEO and PCL molecular weights prepared by the addition of acetone to water

MePEO-PCL	Ratio (organic: aqueous)	CyA initial concentration (mg/mL)	CyA loading ± S.D. (w/w)	Encapsulation efficiency ± S.D. (%)	Average diameter of particles ± S.D. (nm)	PI
5000–5000	1:2	3	0.0969 ± 0.0270	32.3 ± 12.5	74.1 ± 4.8	0.206
	1:6	3	0.1678 ± 0.0036[‡]	55.9 ± 8.5[‡]	59.9 ± 6.3[‡]	0.231
5000–13,000	1:2	3	0.1071 ± 0.0072	35.7 ± 2.4	118 ± 16.7	0.121
	1:6	3	0.2286 ± 0.215[‡]	75.9 ± 7.5[‡]	89.3 ± 15.3[‡]	0.207
5000–24,000	1:2	3	0.1176 ± 0.0163	39.2 ± 8.5	105 ± 3.6	0.072
	1:6	3	0.1827 ± 0.0097[‡]	60.9 ± 3.2[‡]	101 ± 4.8	0.245
12,000–5000	1:2	3	0.0351 ± 0.0247	11.7 ± 8.2	550 ± 175[a]	0.724
	1:6	3	0.1512 ± 0.0247[‡]	50.4 ± 8.2[‡]	384 ± 11.4[‡]	0.174

[a] Secondary peak observed at approximately 60 nm (peak population <25% total population).
[‡] Significantly different from 1:2 ($p < 0.05$).

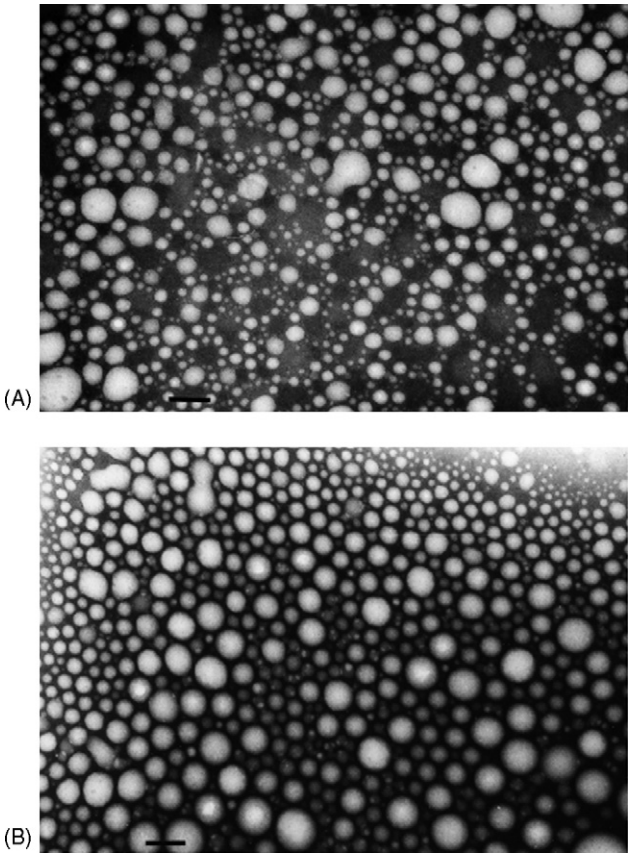

Fig. 1. TEM images of CyA loaded 5000–13,000 MePEO-*b*-PCL micelles prepared by the addition of acetone to water at an acetone:water ratio of (A) 1:6 and (B) 1:2. The bar represents 50 nm (magnification of 18,000 × 6.1, for enlargement of samples in the electron microscope and the magnification from negative to the picture, respectively).

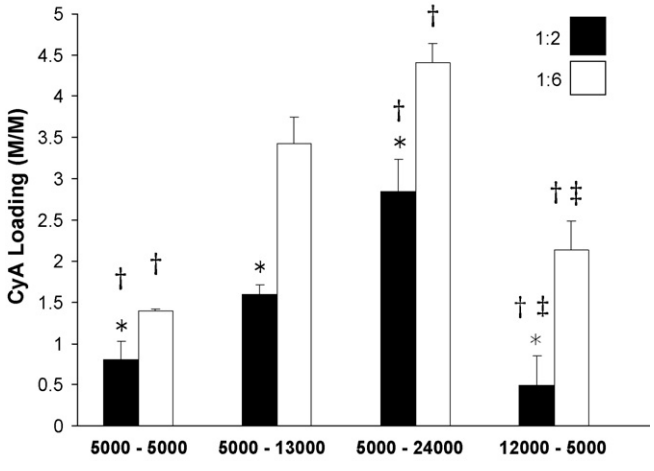

Fig. 2. The effect of organic:aqueous phase ratio on molar CyA loading in MePEO-*b*-PCL micelles of different MePEO/PCL molecular weights. *Significantly different from 1:6 ($p < 0.05$). †Significantly different from 5000–13,000 ($p < 0.05$). ‡Not significantly different from 5000–5000 ($p > 0.05$).

In an attempt to explain the reason for the effect of solvent composition on micellar size and CyA loading, we have calculated the water content of the solvent/co-solvent mixture during drop-wise addition of water to acetone or acetone to water assuming a 50 μL volume for each drop of water or acetone added (Fig. 3A and B). We have also made the same assumption and calculated the change in the concentration of block copolymer during drop-wise addition of water to acetone or acetone to water (Fig. 3C and D). As demonstrated in Fig. 3A, in this study by drop-wise addition of water to acetone, the water content of system is gradually raised from 0 to 66.7 or 85.7% for 1:2 and 1:6 organic to aqueous phase ratios, respectively, passing CWC at some stage during this process. In this case, transition from polymeric unimers to swollen micelles (or other possible assembled conformations) is probable (model shown on the left side of Fig. 3A and B). Lower levels of drug loading by the addition of water to acetone may reflect the release and/or precipitation of the hydrophobic CyA from flexible and liquid like core of these transitional conformations. On the other hand, when acetone is added to water, water content in the system remains above CWC values at all times during the process (Fig. 3B). In this case, the system may not go through the transitional stages and polymeric micelles with collapsed and rigid cores will be formed instantly after acetone droplets (containing both drug and polymer) hit water, minimizing the chance of drug loss from the carrier. This may explain the higher level of drug loading through addition of acetone to water compared to addition of water to acetone in the self-assembly process.

With the addition of acetone to water, we have also observed a raise in the level of encapsulated CyA and a reduction in micellar size when the volume fraction of acetone to water was decreased (Table 5). The trend in CyA encapsulation was found to be consistent for all MePEO-*b*-PCL molecular weights and compositions under this study (Table 6, Fig. 2). The higher level of CyA loading in polymeric micelles prepared through the addition of one part acetone to six part water is most probably a result of high polymer concentrations in acetone added to water in this case (Fig. 3D). During the polymer assembly process, 30 mg of MePEO-*b*-PCL is dissolved in 0.5 mL of acetone and added to 3 mL of water in a drop-wise manner when 1:6 organic:aqueous phase ratio is used. For 1:2 organic:aqueous phase ratio 30 mg of MePEO-*b*-PCL is dissolved in 1.5 mL of acetone and added to 3 mL of water. Therefore, at an acetone to water ratio of 1:6, the concentration of polymeric solution added to water is three-fold higher than the polymer concentration in acetone solutions added to water at 1:2 ratio.

By adding an organic solvent to a non-solvent micellarization may kinetically be favored when concentrated polymeric solutions in selective co-solvent (i.e., acetone) are added to the non-selective solvent (i.e., water). This may lead to the association of block copolymers to more compact micelles, which can explain the smaller average diameter of loaded and unloaded micelles prepared under this condition. When water is added to the polymer and drug solution in acetone, the significant difference in drug loading between the 1:6 and 1:2 organic:aqueous phase ratios are not observed anymore (Table 5). This may be due to drug loss from transitional conformation formed through

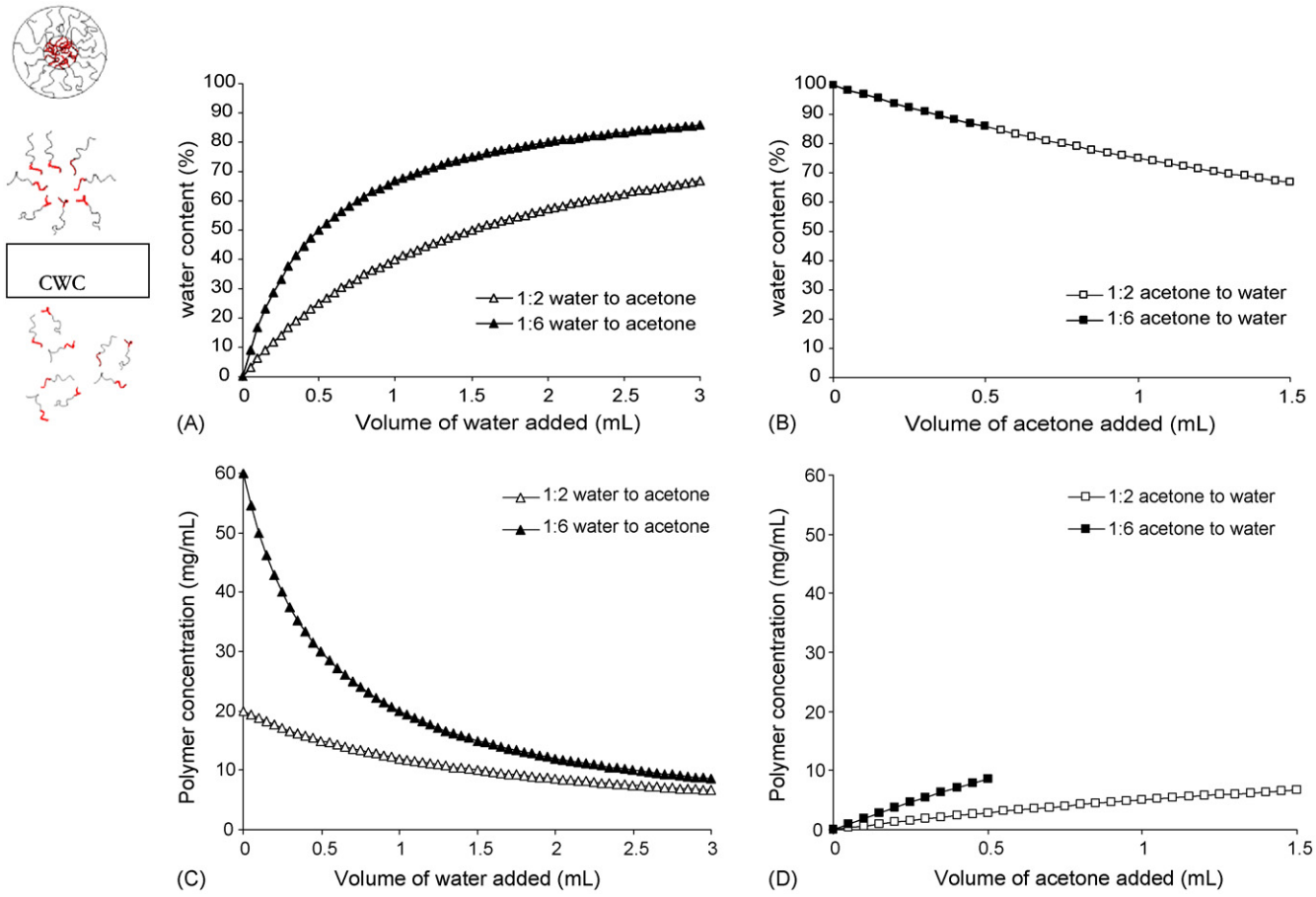

Fig. 3. (A and B) Changes in water content (in %) during drop-wise addition of (A) water to acetone and (B) acetone to water at 1:2 and 1:6 acetone:water phase ratios. The accumulative volume (in mL) of water (*X* axis for graph A) or acetone (*X* axis for graph B) was calculated assuming a 50 μL volume for each drop of water and acetone added to solvent mixture, respectively, during polymer assembly. Proposed model for changes in the micellar conformation at different water contents is also shown on the left side of graphs. (C and D) Changes in the polymer concentration during self-assembly of block copolymer through addition of (C) water to acetone and (D) acetone to water at 1:2 and 1:6 acetone:water phase ratios.

gradual addition of non-solvent (i.e., water) to selective co-solvent (i.e., acetone) for both organic:aqueous phase ratios. Formation or co-existence of alternative conformations (e.g., micellar aggregates, polymer vesicles or nano-emulsions) under this condition is possible, as well.

We hypothesize a slower rate of drug release for CyA from polymeric micelles prepared through addition of acetone to water at lower acetone:water ratio based on our assumption for the formation of compact micelles under this condition. Unfortunately, the *in vitro* release procedure used in our previous study does not provide sufficient sink to reveal the differences between CyA release from different polymeric micellar formulations (Aliabadi et al., 2005). Investigations on the development of an *in vitro* release experiment that can provide an appropriate sink and assess the impact of preparation method on the release of CyA from MePEO-*b*-PCL micelles are currently underway.

4. Conclusion

Manipulation of the self-assembly conditions such as organic to aqueous phase ratio and order of phase addition in the co-solvent evaporation method may be used to improve the efficiency of hydrophobic drug encapsulation in polymeric nanocarriers and average diameter of assembled structures. Addition of acetone to water at low organic to aqueous phase ratio leads to a smaller average diameter for the self-assembled structures and is shown to be more efficient for CyA encapsulation. The higher encapsulation capacity for CyA despite smaller size of the prepared nanocarriers may be attributed to the formation of compact micelles under this condition. Additional studies are underway to determine whether encapsulation of CyA under such condition (addition of concentrated polymeric solutions in acetone to water) may lead to a slower rate of drug release from the polymeric nanocarrier.

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