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Dissolution of biomacromolecules in organic solvents by nano-complexing with poly(ethylene glycol)

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Abstract

Various biomacromolecules (BMs) such as proteins, DNA, and carbohydrates are extremely difficult to be dissolved in a single organic solvent phase for sustained release or targeted delivery formulation. In this study, three different BMs could be solubilized in selected organic solvents by forming poly(ethylene glycol) (PEG)-assisted nano-complexes while maintaining their structural integrity. Dynamic light scattering (DLS) and atomic force microscopy (AFM) analysis revealed that proteins, DNA, and carbohydrate polymers could be nano-complexed with PEG in various organic solvents. The diameter of nano-complexes decreased roughly from ~600 nm to ~100 nm with increasing weight ratio of PEG/BM. The present solubilization technique could be potentially applied for sustained release formulations of various therapeutic biological drugs.

Keywords: Biomacromolecules; Bovine serum albumin; DNA; Hyaluronic acid; Poly(ethylene glycol); Solubilization in organic solvents

1. Introduction

A wide range of biomacromolecules (BMs) such as proteins, nucleic acids, and carbohydrate polymers have emerged as new therapeutic agents for treating various diseases (Crommelin et al., 2003; Opalinska and Gewirtz, 2002; Purcell et al., 2007). However, many BMs, which have high molecular weights with charged structures, are extremely difficult to be dissolved in a single organic solvent phase for sustained release or targeted delivery formulation. For example, in order to fabricate sustained protein release formulations based on biodegradable polymer microparticles, protein drug molecules were dissolved within a small volume of aqueous phase for the subsequent microencapsulation within hydrophobic polymer matrices. Biodegradable poly(lactic-*co*-glycolic acid) microparticles microencapsulating hydrophilic BMs have been conventionally prepared by a water-in-oil-in-water ($W_1/O/W_2$) double emulsion method. This method, however, elicits undesirable protein stability problems at an oil/water interface, such as denaturation and aggregation, during the formulation process. To enhance solubility of proteins and peptides in organic solvents, they were often chemically conjugated with hydrophobic polymers, lipids, and fatty acids (Palumbo et al., 2006; Veronese, 2001). Protein–metal complexes, insoluble particles with several micron sizes, have been also used for the formulation of proteins in organic solvents (Johnson et al., 1997). Cationic or anionic amphiphiles such as lipids, polymers, and surfactants were used to form nanosized polyelectrolyte complexes with oppositely charged proteins and nucleic acids (Bromberg and Klibanov, 1994; Ganguli et al., 2004; Matsuura et al., 1993; Sergeyev et al., 1999). Negatively charged nucleic acids or proteins upon addition of positively charged amphiphile molecules can be condensed by electrostatic interactions, and the resultant hydrophobically ion-paired complexes could be solubilized or dispersed to some extent in various organic solvents. However, cationic polymers, lipids, and surfactants have limitations for clinical applications due to their severe cytotoxic effects (Brunot et al., 2007; Rajasekaran et al., 2006).

Poly(ethylene glycol) (PEG) is a well-known hydrophilic and nonionic polymer widely used for drug delivery systems and biomedical devices due to its proven biocompatibility (Allen and Cullis, 2004; Larsen et al., 2002). PEG has been used for separating many BMs in downstream processes, such as liquid–liquid phase separation and precipitation, due to its unique behavior

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in aqueous solution (Arakawa and Timasheff, 1985; Atha and Ingham, 1981; Hancock and Hsu, 1996; Makkar et al., 1995; Morita et al., 2000; Kim et al., 2002; Lee and Lee, 1987; Mok and Park, 2006; Sheth and Leckband, 1997; Stenekes et al., 1999). Indeed, the addition of PEG in aqueous solution at a higher concentration range above 8% (w/v) results in precipitating proteins by the volume exclusion effect of PEG (Atha and Ingham, 1981; Morita et al., 2000). However, at a lower concentration regime in aqueous solution, PEG can interact with proteins by hydrogen bonding and/or hydrophobic interactions (Arakawa and Timasheff, 1985; Hancock and Hsu, 1996; Makkar et al., 1995; Kim et al., 2002; Lee and Lee, 1987; Mok and Park, 2006; Sheth and Leckband, 1997). Thus PEG in the low concentration region has been used as an effective excipient to stabilize proteins in aqueous solution by assisting preferential hydration of proteins. PEGylation to BMs also enhances their solubility in organic solvents because PEG is soluble both in water and in organic solvents (Mine et al., 2001; Secundo et al., 1999).

In our previous study, we have shown that plasmid DNA could be solubilized in selected organic solvents by forming nano-complexes in the presence of PEG used as an effective complexing excipient (Mok and Park, 2006). It was found that PEG and plasmid DNA were complexed to form a few hundred nanoscale particles in selected organic solvents, probably due to the hydrogen bonding. In this study, we extended the previous finding to proteins and carbohydrate polymers. Three different BMs, proteins, nucleic acids, and carbohydrate polymers, were attempted to be solubilized in various organic solvents by PEG as an effective additive for the production of nano-complexes. Three model BMs, salmon sperm DNA (ssDNA) (nucleic acid), hyaluronic acid (HA) (carbohydrate polymer), and bovine serum albumin (BSA) (protein), were solubilized in various organic solvents by varying weight ratios of PEG/BM and by changing molecular weight of PEG. Size and morphology of PEG/BM nano-complexes in organic solvents were also characterized. The structural integrities of proteins and nucleic acids before and after solubilization in various organic solvents were evaluated.

2. Materials and methods

2.1. Materials

Salmon sperm DNA was obtained from Stratagene (La Jolla, CA). Hyaluronic acid (MW 17k) was purchased from Lifecore Biomedical (Chaska, MN). BSA, PEG, dimethyl sulfoxide (DMSO), and methanol (MeOH) were products of Sigma–Aldrich Chemical (St. Louis, MO). Chloroform, dimethyl formamide (DMF), and dichloromethane (DCM) were purchased from Junsei Chemical (Tokyo, Japan). Dialysis membrane (MW cut-off 10k) was purchased from Spectrum (Houston, TX). All other chemicals were of analytical grade.

2.2. Solubilization of BMs (BSA, ssDNA, and HA)

BMs, such as BSA (66k), ssDNA (0.5–2 kb), and HA (17k), were dialyzed in de-ionized water for more than 1 day for desalting. PEG (3.35 kDa) was used without further purification. BSA (1 mg), ssDNA (0.5 mg), or HA (0.5 mg) dissolved in 500 μl of de-ionized water was mixed with PEG in 500 μl of de-ionized water at various weight ratios of PEG/BM from 1 to 45, and then frozen in liquid nitrogen. The frozen samples were lyophilized at −50 °C under a pressure of 9 mTorr without further annealing step. The three PEG/BM mixtures in a dry powder state were solubilized in 1 ml of various organic solvents to adjust a final concentration of BSA, ssDNA, and HA to 1, 0.5, and 0.5 mg/ml, respectively. To determine the degree of solubilization in different organic solvents containing the PEG/BM complexes, transmittance values were measured at 400 nm using a spectrophotometer (UV-1601, Shimadzu, Japan). To evaluate the fractional soluble amount of BSA and ssDNA, the organic solvents containing dispersed PEG/BM nano-complexes were centrifuged at 14,000 rpm for 15 min. The supernatants were collected and re-solubilized in de-ionized water, and then the amounts of BSA and ssDNA were measured by BCA assay and absorbance value at 260 nm, respectively. To determine the effect of PEG molecular weight on the extent of solubilization, PEGs with various molecular weights (3.35k, 10k, and 35k) were used.

2.3. Differential scanning calorimetry (DSC)

To characterize the binding nature of PEG/BSA nano-complexes, differential scanning calorimetry (DSC 6100, Seiko Instruments, Japan) was used to trace thermal melting behaviors of PEG (MW 3.35k) and PEG/BSA complexes. After co-lyophilization of PEG and BSA at different weight ratios, each sample was scanned from 10 °C to 100 °C under a flow of nitrogen gas with a heating rate of 5 °C/min.

2.4. Particle size of PEG/BM (BSA, ssDNA, and HA) nano-complexes in organic solvents

The effective diameter of PEG (3.35 kDa)/BSA nano-complexes in various organic solvents (chloroform, DMF, and DMSO) was measured at different PEG/BSA weight ratios by a dynamic light scattering (DLS) instrument (Zeta plus, Brookhaven, NY) equipped with a He–Ne laser at a wavelength of 632.2 nm. For nano-complexes of PEG with ssDNA and HA in methanol, their diameters were determined at a PEG/BM weight ratio of 45.

2.5. Observation of PEG/BM nano-complexes in organic solvents

Morphological characters of BSA, ssDNA, and HA complexed with PEG (3.35 kDa) in dichloromethane were observed by atomic force microscopy (AFM). One drop of dichloromethane solution containing PEG/BM mixture at a weight ratio of 45 was loaded on a freshly cleaved mica surface and dried in air at room temperature. AFM measurements were done by using a 50 μm × 50 μm scanner of PSIA XE-100 AFM system (Santa Clara, CA, USA) in a non-contact mode. The scanned image was collected from a 5 μm × 5 μm area.

2.6. Co-localization of PEG and BMs in organic solvents

To show that BSA and PEG (3.35 kDa) were co-localized (nano-complexed) together in dichloromethane, rhodamine-labeled PEG was prepared. Amine-modified methoxy-PEG (MW 5k, 50 mg) was reacted with rhodamine isothiocyanate (21.4 mg) in sodium carbonate buffer (pH 9.0) for 3 h at room temperature. After purification with de-ionized water using desalting column (D-Salt^{TM} dextran desalting column, Pierce, Rockford, IL), the solution was freeze-dried. FITC-labeled BSA (Sigma, St. Louis, MO) was mixed with rhodamine-labeled PEG at the PEG/BSA weight ratio of 5 and co-lyophilized according to the previous method. After solubilizing the PEG/BSA mixture in dichloromethane, the solution was diluted and loaded onto a slide glass. After air-drying, the sample was visualized by confocal microscopy (Carl Zeiss, Germany).

2.7. Circular dichroism (CD) spectroscopy

The secondary structure of BSA and ssDNA was analyzed by circular dichroism spectroscopy (JASCO J710, Japan) spectra at 25 °C before and after solubilization in organic solvents. After the organic solvents containing PEG/BM mixtures were evaporated, samples were re-solubilized with de-ionized water.

3. Results and discussion

To eliminate salt effects, the three BMs were completely desalted before lyophilization with PEG. BSA (MW 66k), ssDNA (MW 165–660k), and HA (MW 17k) were used as representative model BMs for proteins, nucleic acids, and carbohydrate polymers, respectively. To evaluate the solubility of BMs in organic solvents, transmittance values of the solvents containing PEG/BM complexes were first measured at various weight ratios of PEG/BM. Diverse organic solvents with different polarity index values (dichloromethane: 3.1; chloroform: 4.1; methanol: 5.1; DMF: 6.4; DMSO: 7.2) were used for testing the solubilization. As shown in Fig. 1A–C, three BMs (BSA, ssDNA, and HA) exhibited increased transmittance values in all organic solvents with increasing weight ratio of PEG/BM, although the transmittance values at a fixed PEG/BM weight ratio were largely dependent on the polarity of organic solvents. In general, BMs dissolved in polar organic solvents such as DMSO showed greater transmittance values than those in less polar ones. In particular, BSA, ssDNA, and HA in DMSO showed over 90% transmittance above the PEG/BM weight ratio of 15, while those in other solvents exhibited different extents of solubilization. The PEG/BSA mixture had poor solubility in methanol, while the PEG/ssDNA and PEG/HA mixtures showed poorer solubility in chloroform. It is likely that different hydrophilic/hydrophobic natures of the three BMs might contribute to varying extents to the PEG-assisted BM solubilization in organic solvents that have also different polarities. The inset pictures in Fig. 1A–C clearly reveal that the three BMs in the presence of PEG (PEG/BM ratio = 45) are completely transparent and solubilized in dichloromethane, whereas those in the absence of PEG are turbid or precipitated.

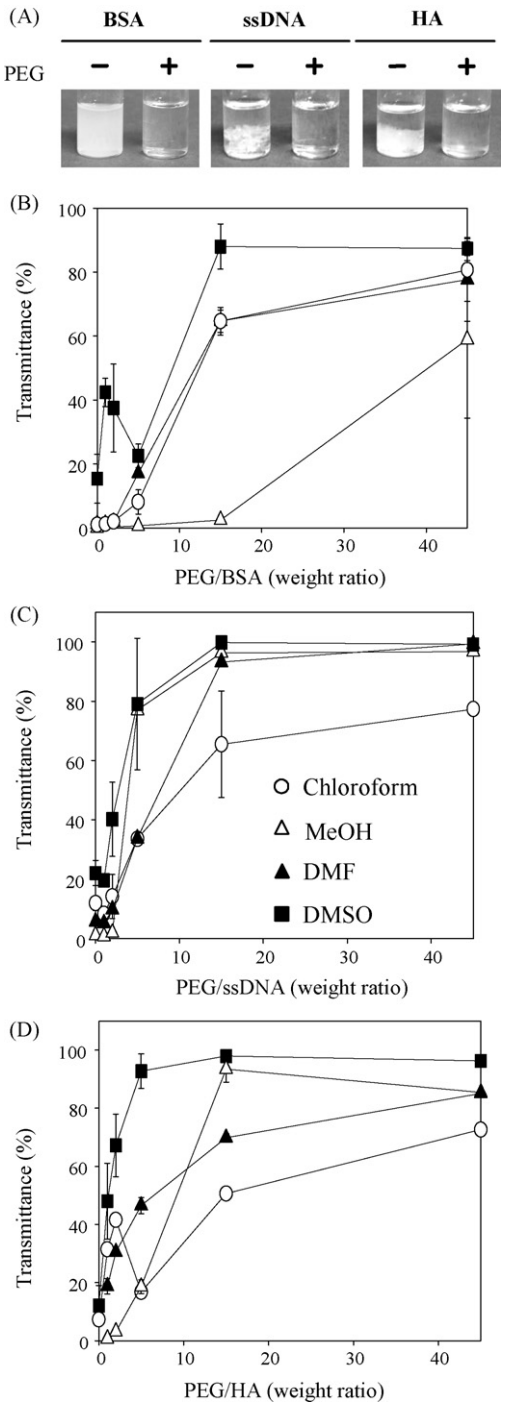

Fig. 1. (A) Photographs of dichloromethane solutions containing BSA, ssDNA, and HA with or without PEG. (B–D) Transmittance values of organic solvents containing (B) BSA, (C) ssDNA, and (D) HA at various PEG/BM weight ratios. Chloroform (open circle), methanol (open triangle), dimethyl formamide (filled triangle), and dimethyl sulfoxide (filled square). Final concentration (100%) in organic solvent was 0.5 mg/ml for ssDNA and HA, and 1 mg/ml for BSA.

It should be noted that the term "solubilization" of BMs in organic solvents is defined here as a transparent state without showing any turbidity originated from aggregates and precipitates in the solution (Bromberg and Klibanov, 1994; Ganguli et al., 2004). The solubilization means that BMs are collapsed and complexed in an organic phase to form sufficiently

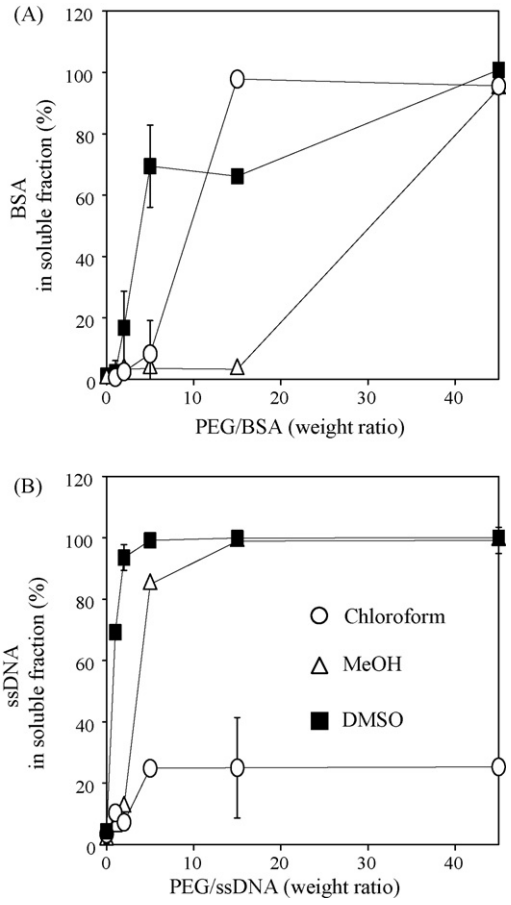

Fig. 2. Relative soluble amounts of (A) BSA and (B) ssDNA in organic solvents at various PEG/BM weight ratios. Chloroform (open circle), methanol (open triangle), and dimethyl sulfoxide (filled square).

small nanoscale particulates that cannot scatter a visible light irradiated with a wavelength of 400 nm. Strictly speaking, BMs in organic solvents are not solubilized in a molecularly dissolved state, but they are present as dispersed BM/PEG nano-complexes. The non-covalent nano-complexation between PEG and BMs resulted in increased transmittance values with visual transparency in organic solvents.

To measure the soluble amount of BSA and ssDNA in organic solvents, the supernatant of the organic solvent phase containing PEG/BM mixtures was collected after centrifugation. The amount of BSA in soluble fraction increased as raising weight ratio of PEG/BSA in all selected organic solvents, as shown in Fig. 2A. In particular, BSA could be solubilized almost 100% in chloroform at a PEG/BSA ratio of 15, but was unable to be solubilized in methanol at the same condition. The solubility data are consistent with the transmittance results in Fig. 1B. The amount of ssDNA soluble in the three organic solvents is shown in Fig. 2B. ssDNA can be solubilized very well in DMSO and methanol, but not in chloroform. While the transmittance value of chloroform containing the PEG/DNA mixture increased as raising weight ratio of PEG/DNA, the soluble amount of DNA was not noticeably increased to the same extent under the similar condition. It is probably because the size of PEG/ssDNA nano-complexes in chloroform was

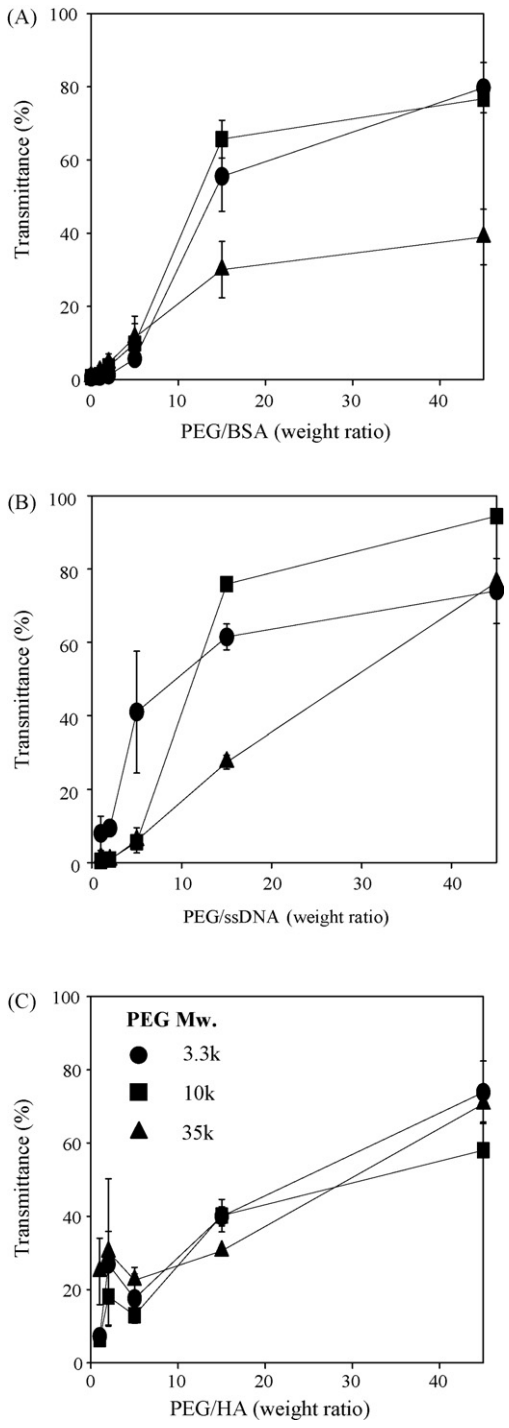

Fig. 3. Transmittance of the organic solvents containing (A) BSA, (B) ssDNA, and (C) HA nano-complexed with three kinds of PEG with different molecular weights (circle: 3.35k, square: 10k, and triangle: 35k).

large enough so that they were precipitated by centrifugation.

The effect of PEG molecular weight on the solubilization of BMs in organic solvents was examined in Fig. 3. Three kinds of PEG with different molecular weights of 3.3k, 10k, and 35k were co-lyophilized with BSA, ssDNA, and HA, respectively, at various weight ratios, and they were solubilized in dichloromethane. PEG 3.3k and 10k were more effective in the solubilization of

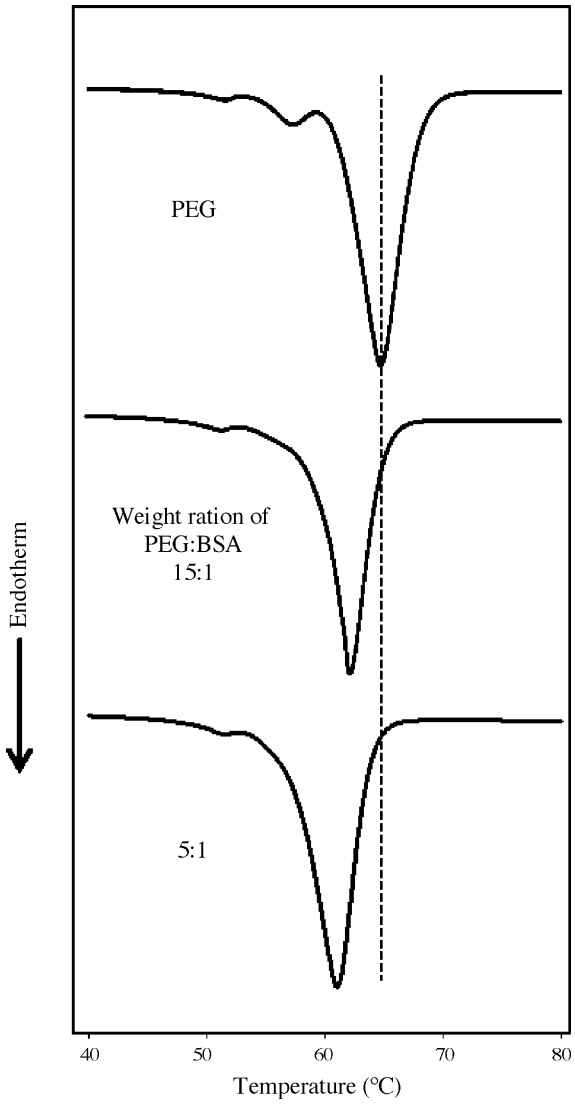

Fig. 4. Differential scanning calorimetry thermograms of co-lyophilized PEG/BSA mixture as a function of PEG/BSA weight ratio.

BSA and ssDNA, respectively, compared to PEG 35k, while there was no noticeable difference in PEG molecular weight for HA solubilization. It was likely that lower molecular weight PEG was more easily complexed with BSA and ssDNA than higher molecular weight one. An optimal molecular weight of PEG was required for the solubilization of BMs, because very low molecular weight of PEG (MW 400) did not show any solubilization effect in dichloromethane (data not shown). Previously, it was also reported that PEG with molecular weights above about 1000 stabilized a duplex structure of DNA while low molecular weight PEG (PEG 200) caused no stabilization (Spink et al., 2007). As shown in Fig. 3, PEG with molecular weight 3.3–10k might be good enough to solubilize BMs in organic solvents.

It is well known that proteins are phase separated and precipitated in aqueous solution when an excess amount of PEG is added (Bhat and Timasheff, 1992; Bromberg et al., 2005; Heller et al., 1997; Randolph, 1997; Gustafsson et al., 1986). According to previous report (Bromberg et al., 2005), the presence of high concentration of PEG (16.6 wt%) in aqueous solution nucleated and aggregated protein molecules, resulting in the formation of protein microparticles that increased their sizes with time. It was also reported that sub-micron protein particles could be readily prepared by phase separation from an aqueous solution by adding PEG at the concentration range of 5–8% (w/v) during annealing and subsequent co-lyophilization process (Morita et al., 2000). For the aqueous phase separation, high molecular weight PEG was more favorable for the production of smaller dry protein powders than low molecular weight PEG (Diamond and Hsu, 1989; Morita et al., 2001). However, in this study, much lower amount of PEG (0.1–4.5% (w/v) for BSA and 0.05–2.25% for DNA and HA) was mixed with BMs, and then frozen under rapid liquid nitrogen quenching condition without further annealing steps. Since low molecular weight PEG was more effective for the solubilization of BMs in the organic solvent, it can be concluded that the formation of individual PEG/BM nano-complexes was caused primarily by non-covalent interactions between BM and PEG in an organic phase, not like the reported phase separation mechanism in the aqueous phase.

In an attempt to characterize the binding nature between PEG and BSA in the dry-state nano-complexes, differential scanning calorimetric study was performed. As shown in Fig. 4, thermal melting temperature of pure PEG is observed at 64.8 °C. When PEG was co-lyophilized with BSA at PEG/BSA weight ratios of 15:1 and 5:1, melting temperatures of PEG appeared at 62.1 °C and 61.0 °C, respectively. The melting temperature of PEG was slightly reduced, but clearly shifted in the presence of BSA. The DSC data suggest that the PEG crystalline structure was influenced obviously by non-covalent interactions between PEG and BSA (Mosharraf et al., 2007). To further elucidate the interaction nature between BMs and PEG in the organic solvents, a more detailed study such as employing 2D-NOESY NMR would be necessary to identify what types of specific interactions played a role in forming the nano-complexes.

The size of PEG/BSA nano-complexes in organic solvents was measured using DLS as shown in Fig. 5A. As increasing the PEG/BSA weight ratio, the size of the nano-complexes decreased in all three solvents. The effective diameter of PEG/BSA complexes at a weight ratio of 15 was 122.5 ± 0.8 nm in DMSO, 174.0 ± 3.6 nm in DMF, 254.8 ± 9.21 nm in chloroform. The sizes of PEG/ssDNA and PEG/HA at molar ratio of 45 in methanol were 194.8 ± 9.2 nm and 263.2 ± 3.9 nm, respectively (data not shown). BMs in the absence of PEG were aggregated into large precipitated particles. However, BMs could be stabilized and dispersed to nanoscale particles by an amphiphilic function of PEG when PEG was present in a sufficient amount in an organic phase. The size of PEG/BM nano-complexes in organic solvents was mainly dependent on the strength of non-covalent interactions between BM and PEG. Presumably, hydrogen bonding between the two species might play a role in stabilizing the nano-complexes. It was likely that PEG/BM nano-complexes formed in organic solvent were in equilibrium with their individual components, PEG and BM. The reduced sizes of nano-complexes with increasing PEG/BM ratios can be attributed to the thermodynamic equilibrium, PEG + BM ↔ PEG/BM

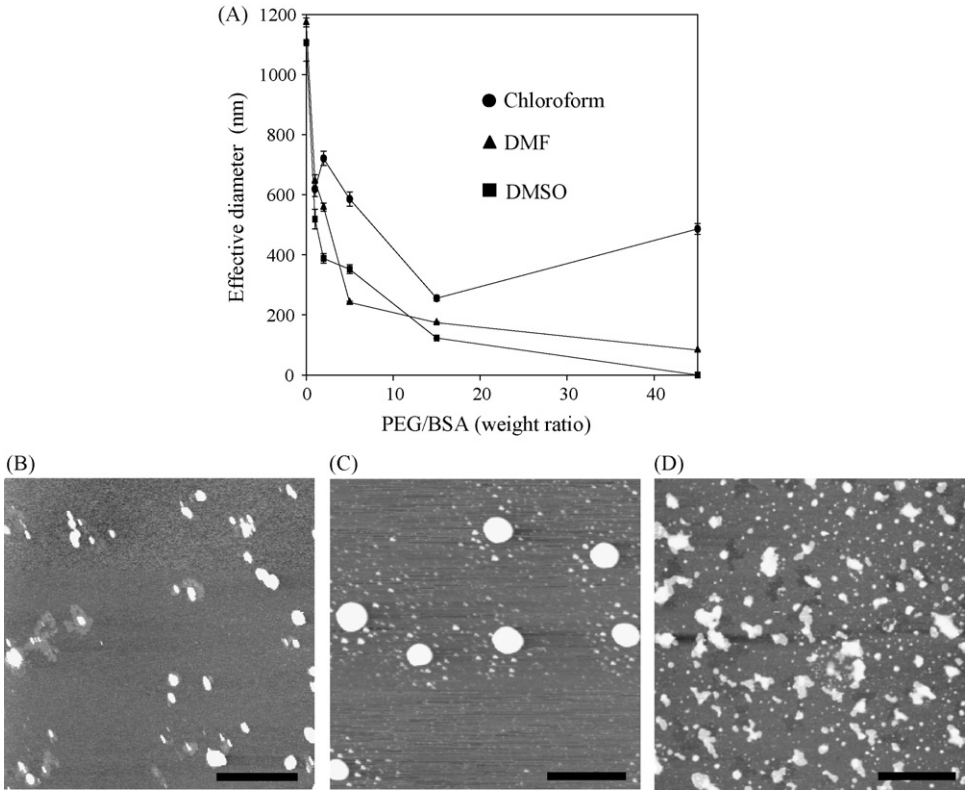

Fig. 5. (A) Effective diameters of PEG/BSA complexes at various PEG amount in various organic solvents. Chloroform (filled circle), dimethyl formamide (filled triangle), and dimethyl sulfoxide (filled square). (B–D) AFM images of (B) BSA, (C) DNA, and (D) HA which were complexed with PEG (PEG/DNA weight ratio = 45) in dichloromethane (scale bar = 1 μm).

in the organic phase. The size of PEG/BSA complexes in DMSO at PEG/BSA weight ratio of 45 was not detectable. It is probably because BSA was partially in a molecularly dissolved state in DMSO assisted with PEG under that condition.

The morphology of PEG/BM complexes at a weight ratio of 45 in dichloromethane was visualized using AFM in Fig. 5B–D. The three PEG/BM complexes appear as nanoscale particles with 100–500 nm in diameter. Average size of PEG/BSA at the molar ratio of 45, determined by measuring the diameter of at least 50 complexes in the AFM image, was 163.2 ± 49.2 nm in dichloromethane, while the effective diameter determined by DLS was 239.6 ± 4.5 nm (data not shown). Polydispersed sizes observed in the AFM image might be attributed to the deformation of PEG/BMs complexes dried onto a mica film. The PEG/ssDNA nano-complexes were more spherical and larger than the others, probably due to the higher molecular weight of ssDNA than those of BSA and HA.

The formation of nano-complexes between PEG and BSA in the dichloromethane phase was visualized by confocal microscopy as shown in Fig. 6. The size of PEG/BSA complexes was larger than that shown in DLS or AFM, which was probably caused by the air-drying process on the slide. Most BSA molecules were co-localized with PEG at the PEG/BSA weight ratio of 5. This directly reveals that BSA was not excluded and phase separated from the enriched PEG phase in

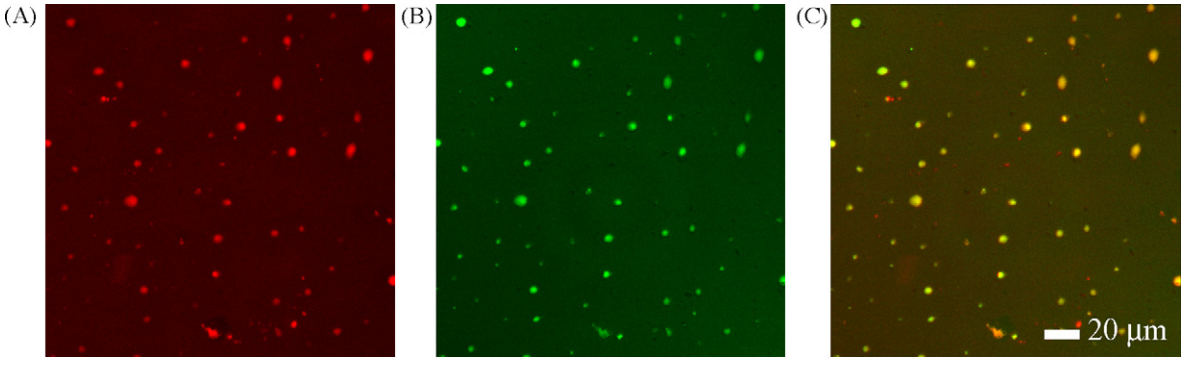

Fig. 6. Confocal microscopy image of (A) rhodamine-labeled PEG, (B) FITC-BSA, and (C) a merged image in dichloromethane after PEG-assisted solubilization.

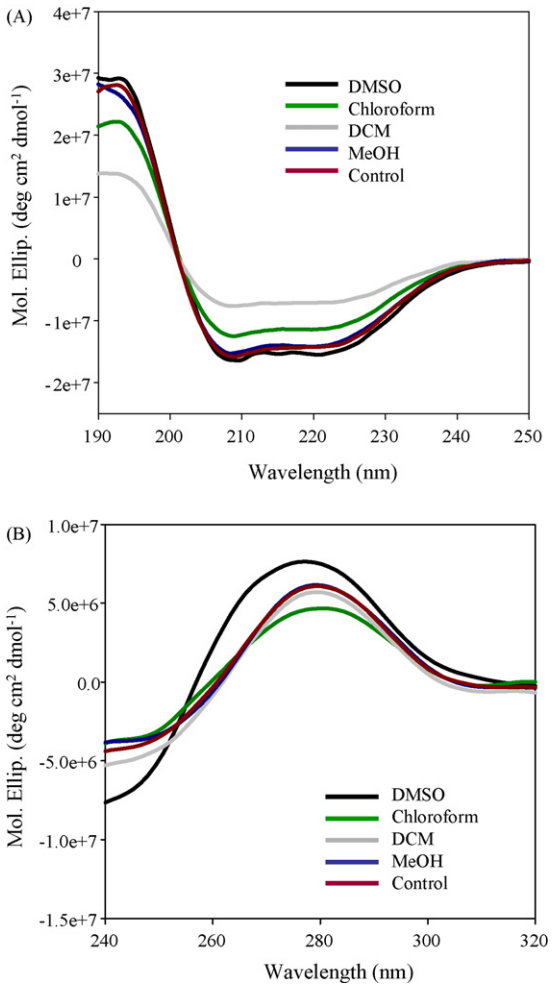

Fig. 7. CD spectra of reconstituted (A) BSA and (B) ssDNA in de-ionized water after PEG-assisted solubilization in organic solvents. (For interpretation of the references to colour in this artwork, the reader is referred to the web version of the article.)

the organic phase, but it indeed interacted with PEG to form nano-complexes.

The effect of BM solubilization in various organic solvents on their conformational structure after reconstitution in aqueous phase was investigated using CD spectroscopy. The organic phase containing PEG/BM nano-complexes was diluted in de-ionized water and extensively dialyzed to remove the organic solvent and PEG. The recovered BMs in de-ionized water were used to take CD spectra. CD spectra for naked BSA exhibit double minimum peaks at 222 nm and 208 nm, indicative of a typical α-helical structure (Fig. 7A). The relative ratio of molar ellipticity value at $[\theta]_{222}/[\theta]_{208}$ was around 0.9. Those of BSA nano-complexed in different organic solvents showed similar values: 0.93 (DMSO), 0.91 (chloroform), 0.92 (dichloromethane), and 0.91 (methanol), suggesting that the α-helical structure was maintained after the decomplexation in de-ionized water (Andreola et al., 2003). This shows a slight change in the secondary structure of samples compared to the native one. CD spectra of ssDNA nano-complexed in the four organic solvents also indicate that a typical B-helical conformation was preserved after the decomplexation in aqueous solution, since no detectable decrease in $[\theta]_{275}$ value was observed (Fig. 7B).

In conclusion, various BMs, such as proteins, nucleic acids, and carbohydrates could be solubilized in various organic solvents by forming well-dispersed and stable nano-complexes that did not show aggregation and precipitation behaviors. The PEG/BM nano-complexes in organic solvents were formed primarily by non-covalent interactions, perhaps hydrogen bonding in a low dielectric constant medium. It is well known that both backbone ether groups and terminal hydroxyl groups of PEG could interact with various hydrogen bond donors and acceptors in BMs via hydrogen bonding (Lacoulonche et al., 1998; Pochylski et al., 2006). The present solubilization method for three different BMs in organic solvents using a biocompatible polymer, PEG, could have potential applications for fabricating polymeric delivery systems to release therapeutic proteins, genes, carbohydrates in a sustained manner. It is conceivable that PEG/BM nano-complexes could be directly loaded within the polymer matrices employing a single organic phase. In addition, it would be also possible to conjugate hydrophobic polymers and lipids to hydrophilic BMs in the single organic phase. Based on the BM solubilization technique presented in this study, sustained release formulations for therapeutic protein drugs are currently under investigation, and will be reported in the near future.

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