Multi-layered nanoball as high performance permselective membrane

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Abstract

Multi-layered structure of the core-polymerized polymeric micelle carrying aldehyde groups (aldehyde-nanoball) and polyallylamine (PAA) was prepared on a porous polymer membrane by the layer-by-layer (LBL) coating, followed by reductive amination. Aldehyde-nanoball with diameter of 40 nm was made from a core-shell type polymeric micelle of acetal-poly(ethylene glycol)/polylactide-methacryloyl block copolymer (acetal-PEG/PLA-methacryloyl) by a gentle acid treatment to convert acetal into aldehyde group, followed by the polymerization of the methacryloyl group in the core. Permselectivity of hydrophilic macromolecules (dextran, myoglobin, albumin) through the nanoball-coated membrane was evaluated by the use of two-chamber cell. With an increasing number of the coating layers, permeation of the macromolecules was reduced in the molecular weight-dependent manner. Especially, in the case of protein molecules as solutes, a definite cut-off molecular weight was justified by changing the number of the coating layer. The multi-layered coating of nanoballs developed here is expected to be useful as a high performance separation membrane for proteinous compounds.

Keywords: Block copolymer; Polymeric micelle; Surface modification; Layer-by-layer structure; Permeation control; Protein separation

1. Introduction

Poly(ethylene glycol)/polylactide (PEG/PLA) block copolymer, one of the typical amphiphilic A-B type block copolymers, spontaneously assemble into core-shell type stable polymeric micelle with a several tens nanometer size. This nanometric-scaled assembly is anticipated as one of the important devices in nanomedicine, especially as a carrier for drug delivery [1,2]. Recently, new synthetic approach was developed by our group to obtain PEG/PLA block copolymer possessing an acetal group at PEG chain end (acetal-PEG/PLA) [3,4]. The acetal-PEG/PLA block copolymer assembles into reactive polymeric micelle in an aqueous medium because the acetal groups located on the micelle periphery can be converted into the reactive aldehyde group by the gentle acid treatment [4]. Furthermore, a methacryloyl group was installed at the hydroxyl end of PLA chain in PEG/PLA block copolymer to obtain α-functionalized derivative (PEG/PLA-methacryloyl), from which the core polymerized polymeric micelle can be prepared [5]. Such micelle with a covalently polymerized core, so called “nanoball”, is fairly stable and can be utilized as a high performance DNA separation media in microchip electrophoresis [6]. By the use of heterotelechelic block copolymer, viz., aldehyde-PEG/PLA-methacryloyl, a nanoball with a reactive aldehyde group on its surface (aldehyde-nanoball) can be obtained [7]. This aldehyde-installed nanoball, possessing densely packed PEG brushes on the surface, was found to be useful as a unique agent for the surface modification of the amine-derivatized substrates via the reductive amination reaction [8–11]. The obtained nanoball-modified surface showed extremely high nonfouling character because of the densely packed PEG brushes [9]. Notably, this surface-modification procedure was further applied for the preparation of a layer-by-layer (LBL) structure of aldehyde-nanoball and polyallylamine (PAA) [9–11]. The surface thickness of LBL layer was readily controlled by changing the coated number of nanoball layer.

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As we reported previously, nanoball-packed micro-capillary provides an effective medium for the size-dependent separation of biomacromolecules, including DNA [6]. Unique sieving effect of tethered PEG layer of the packed nanoballs may play a substantial role in this highly sensitive size discrimination. It is reasonable to assume that similar effect of molecular sieving may occur for the cumulative layer of nanoballs constructed on porous membrane through LBL method as shown in Fig. 1. In this way, release control of biomacromolecules including proteins and nucleic acid derivatives from an appropriate reservoir is expected to be achieved, which is certainly an emerging topic in the field of drug delivery [12,13]. This paper deals with our first study devoted to reveal that LBL nanoball structure on porous membrane works as a sieving layer of biomacromolecules with varying molecular weight, providing a basis to use the cumulative nanoball layer in the controlled release formulations.

2. Experimental

2.1. Materials

Commercial tetrahydrofuran (THF, WAKO), 3,3-diethoxy-1-propanol (Aldrich), ethylene oxide (EO) (Sumitomo Gas Chemical), DL-lactide (LA) (Tokyo Kasei), and methacrylic anhydride (Aldrich) were purified conventionally. Potassium naphthalene was used as a THF solution, whose concentration was determined by titration. Sodium dodecyl sulfate (SDS) (WAKO), fluorescein isothiocyanate (FITC) (DOJINDO), tetramethylrhodamine isothiocyanate (TRITC) (Sigma), poly(2-hydroxyethyl methacrylate) (Aldrich), polyallylamine (Nittobo), FITC-dextran (Mw=4400, 19500, 37000) (Sigma), horse heart myoglobin (Sigma) and bovine serum albumin (Sigma) were used as received.

2.2. Measurements

Dynamic light scattering (DLS) measurements were carried out using a Photal dynamic laser light-scattering spectrometer DLS-7000 (Otsuka Electronics, Tokyo, Japan) using an argon laser at 488 nm. All light-scattering experiments were carried out at 25 °C. Fluorescence intensities were measured using F-2500 spectrometer (HITACHI High Technologies, Tokyo, Japan). The excitation and emission bandwidths were both 2.5 nm.

2.3. Polymer synthesis

Details of the synthesis of acetal-PEG/PLA-methacryloyl block copolymer were reported previously [7]. Briefly, 1 mmol (0.16 mL) of 3,3-diethoxy-1-propanol and 1 mmol of potassium naphthalene were added to 30 mL of dry THF to form potassium 3,3-diethoxypropanolate (PDP). After stirring for 20 min, 130 mmol (6.5 mL) of EO was added via a cooled syringe to the formed PDP solution. The polymerization of the EO proceeded for 2 days at room temperature. Then, 35 mmol (35 mL) of an LA solution in THF (c=1.0 mmol) was introduced, and the polymerization proceeded for 120 min. After the polymerization, 20 mmol (3.10 mL) of a methacrylic anhydride was added to the reaction mixture, and stirred for further 2 days. The polymer was recovered by precipitation into a 20-fold excess of cold 2-propanol and centrifuged for 30 min at 6000 rpm. The polymer was then freeze-dried from benzene. The yield of the obtained polymer was ca. 88%. The number averaged molecular weight and MWD of the obtained polymer were 4800(PEG)/4400(PLA) (calculated from 1H-NMR and GPC data) and 1.09 (calculated from GPC data), respectively.

2.4. Preparation of core-polymerized micelle possessing aldehyde groups on the surface (aldehyde-nanoball)

According to our previous report [8], the core polymerized PEG/PLA micelle possessing aldehyde groups on the surface (aldehyde-nanoball) was prepared. Briefly, 280 mg of the acetal-PEG/PLA-methacryloyl was dissolved in 40 mL of dimethylacetamide. Then, the polymer solution was transferred into a preswollen dialysis membrane tube (MWCO=12000–14000), and was dialyzed against 2 L of water for 24 h with exchanging water at 2, 5 and 8 h passage. To convert the acetal groups on the micelle periphery into aldehyde groups, the polymer was adjusted to pH 2 with 1 mol/L hydrochloric acid. After stirring for 2 h, the mixture was neutralized with 0.1 mol/L sodium hydroxide, and the solution was dialyzed against water to remove the salt. After the preparation of the aldehyde-installed micelle, the methacryloyl end group in the core of the micelle was polymerized in the presence of radical initiator as follows: After potassium persulfate (2.0 wt % polymer) was added into the aqueous solution of the micelles in a 300 mL two-necked flask, the micelle solution
was degassed for 30 min under reduced pressure, and then, was purged with argon for 30 min to remove the oxygen. The polymerization reaction was carried out at 55 °C for 24 h. The size of the polymerized micelle (nanoball) determined by dynamic light scattering was ca. 40 nm. The nanoball solution was stored in a refrigerator until use.

2.5. Membrane preparation

The LBL surface was constructed on the dialysis membrane by our previously reported method [10] with some modifications. A commercial dialysis membrane (Spectra/Por molecular weight cut-off size 1,000,000) was soaked in methanol solution of poly(2-hydroxyethyl methacrylate) (PHEMA) (1 wt.%) for 1 h to prepare PHEMA coated membrane. The obtained PHEMA membrane was aminated by the plasma amination reaction with nitrogen/hydrogen mixed gas plasma (pressure=1 Torr, N2/H2=20 mL/min:40 mL/min, ICP power=50 W, treated time=10 min). Then, the alternate surface modification by the aldehyde-nanoball and polyallylamine (PAA) was carried out under reduced condition as follows: The aminated membrane was immersed in 1.73 mg/mL nanoball solution in 0.04 M HEPES (pH 6.7) containing 0.0032% (w/v) NaCNBH3 at room temperature for 2 h. After rinsing in pure water, the nanoball-coated membrane was immersed into 0.6 % (w/v) PAA in 0.04 M HEPES (pH 6.7) containing 0.25% NaCNBH3 at room temperature for 2 h. The above procedure was repeated until the desired number of coatings was obtained. Finally, the remained aldehyde group on the surface was converted to a hydroxyl group by the treatment with 0.25% NaCNBH3 in 0.04 M HEPES (pH 6.7) at room temperature for overnight. The nanoball-coated membrane was rinsed with pure-water repeatedly and stored in water until use.

2.6. Fluorescence labeling of proteins

To detect the permeated proteins through the membrane, each of proteins was labeled with fluorescence dye as follows: One gram of Bovine serum albumin and 10 mg of fluorescein isothiocyanate were dissolved into 100 mL boronate buffer (pH 7.8, 100 mM). Then the solution was stirred at 22 °C for overnight, followed by the purification by gel filtration. Purity of the fluorescein-labeled bovine serum albumin was checked by HPLC, followed by the lyophilization. Tetramethylrhodamine-labeled horse heart myoglobin was prepared in the similar way.

2.7. Permeation experiments

Permeation experiment was carried out with a two-chamber cell system as shown in Fig. 2. The system was kept at 25 °C by circulating thermostated water. The nanoball-coated membrane was fixed in between two chambers, and then, the chambers were filled with 33 mL of phosphate buffered saline (PBS) for overnight to reach equilibrium swelling of the membrane. After the buffer...
solution was removed, PBS buffer with and without 1 mg/mL fluorescence labeled solutes were filled in left and right chambers, respectively. The solute permeation was estimated from a change in the fluorescent intensities of fluorescein ($\lambda_{\text{ex}}=495$ nm, $\lambda_{\text{em}}=520$) or tetramethylrhodamine ($\lambda_{\text{ex}}=550$ nm, $\lambda_{\text{em}}=578$ nm).

3. Results and discussion

3.1. Characterization of the nanoball

PEG/PLA block copolymers possessing a suitable balance of both segment lengths are known to form spherical core-shell type polymeric micelle with several tens nanometer size [2]. The DLS analysis of the micelle obtained in this study revealed that the size and the distribution ($\mu I^2$) to be 40 nm and 0.10, respectively as typically seen in Fig. 3a. No remarkable difference in these values was observed before and after the core polymerization.

As we reported previously, core polymerization of the micelles is essential to maintain the core-shell structure even after their immobilization to the substrate surface [8]. The core polymerization was confirmed by the sodium dodecyl sulfate (SDS) addition to the micelle solution [5]. After the addition of 1 mL of SDS solution (20 mg/mL in water) to 2 mL of the micelle solution, the DLS analysis was carried out. Fig. 3 shows the size distribution of the micelle solution with SDS before and after the core polymerization. When SDS solution was added to the non-polymerized micelle solution, there occurred a significant decrease in the signal intensity due to the dissociation of the micelles (Fig. 3b). On the other hand, there was no change in the signal intensity as well as the size of the micelles when the core was polymerized even after SDS addition (Fig. 3c and d). This result clearly indicates the formation of stable core-polymerized polymeric micelles (aldehyde-nanoball).

3.2. Nanoball-coating of the porous membrane

Since the obtained nanoball possesses the aldehyde groups on the surface, the surface modification of the aminated porous membrane with nanoball was carried out via the reductive amination reaction. To construct the model surface, a dialysis membrane having molecular weight cutoff of 1000000 was used as a support material. The dialysis membrane was coated with poly(2-hydroxyethyl methacrylate) (PHEMA) followed by plasma treatment. The introduced primary amino groups on the surface form Schiff base with the aldehyde group possessed on the nanoball periphery. Nanoball was then covalently immobilized onto the surface via reduction of Schiff base with the reduced

Fig. 3. DLS histograms of micelles; (a) polymeric micelles without core-polymerization (control), (b) polymeric micelles without core-polymerization + SDS, (c) core-polymerized micelles (nanoballs), and (d) nanoballs + SDS.
agent, NaCNBH3 (reductive amination). Alternate coatings of nanoball and polyallylamine (PAA) via reductive amination gave the multi-layered nanoball structure on the membrane.

It is important that the concentration of NaCNBH3 should be optimized in the case of multi-layer nanoball-coating. Although NaCNBH3 is a mild reducing reagent, all the aldehyde groups on the nanoball should be reduced with 0.25% NaCNBH3 solution [9]. This is desirable for the treatment of the final nanoball layer to convert all of the remaining aldehyde groups into OH groups, but it is not suitable for the intermediate coating of the nanoball with aldehyde functionality to react with amino groups of PAA. Thus, highly diluted solution of NaCNBH3 (0.0032% (v/w)) was used to selectively reduce Schiff base with most of aldehyde groups to be remained intact.

3.3. Permeation profiles of FITC-dextran

Permeation profiles of polymer solutes were investigated by the use of two-chamber cell. The membranes coated with nanoballs were installed in the cell as depicted in Fig. 2. FITC-dextran with varying molecular weight were used as model polymers. Amount of the penetrated FITC-dextran through the nanoball-coated membrane was determined from fluorescence intensity of donor chamber. Fig. 4 shows the permeation profiles of FITC-dextran with varying molecular weight through the membrane modified with the different number of aldehyde-nanoball layers. Obviously, with an increasing layer of nanoballs, the permeation rate of FITC-dextran decreased successively. As seen in Fig. 4c, permeation of FITC-dextran with Mw of 37,000 was essentially prohibited by the membrane having more than four layers of nanoballs, indicating nanoball layer on the membrane effectively function as a regulating barrier of solute transport across the membrane. Fig. 5 shows the relationship between the number of nanoball layer and the amount of permeated FITC-dextran at 8 h. The figure clearly shows that the increase in the number of layers retarded the dextran permeation, and there seems to be a clear cut-off molecular weight in terms of permeation between 2 and 4×10^4 dalton for the membrane with more than four layers of nanoballs.

3.4. Permeation profiles of proteins

In order to confirm the clear molecular weight cut-off of the high molecular weight solutes, permeation of proteins having different sizes (horse heart myoglobin, Mw=17,000 and bovine serum albumin, Mw=66,000) was investigated for the membrane coated with the cumulative layer of nanoballs. Fig. 6a and b show permeation profiles of myoglobin and albumin, respectively. The profiles of the protein permeation were fairly different from that of the FITC-dextrans. For example, the permeation rate of myoglobin is much higher than that of FITC-dextran having the similar molecular weight (Mw=19,500). No clear effect of the number of the nanoball layer was observed. In the case of albumin permeation, on the other hand, it is clearly observed the effect of the number of the nanoball layers on the permeation rate as shown in Fig. 6b, and the membrane
with seven layers of nanoballs revealed an appreciable sieving effect of albumin. Fig. 7 shows cumulative amount of permeated myoglobin and albumin for 8 h as a function of the number of the nanoball layers. Obviously, permselectivity of myoglobin to albumin became significant with increasing the number of nanoball coating layer. Especially, the membrane with seven layers of nanoballs showed extremely high permselectivity as can be seen in Fig. 8. It is likely that the size of myoglobin may be compact enough to permeate the cumulative layer of nanoballs prepared in this study.

3.5. Comparison of permeation mechanism based on the hydrodynamic radii of molecules

To make clear the effect of cumulative layer of nanoballs on molecular sieving, permeation profiles of dextran and protein were compared based on the hydrodynamic radii of each molecule. Since protein molecule has a definite tertiary structure maintained by a spatially regulated intramolecular association force, hydrodynamic radius is appreciably smaller than that of random-coiled polymers having the same order of the molecular weight with the protein.
Therefore, the permeation behavior should be compared based on the actual size in aqueous medium, viz. hydrodynamic radius rather than on the molecular weight. Cumulative permeation of solute was shown in Fig. 9 as a function of their cross-sectional area. The cross-sectional area was estimated from the hydrodynamic radius referred to previous literatures\(^{[14–16]}\), with an assumption that each molecule is in the spherical form. Interestingly, dextrans showed a different permeation profile from proteins in regard to the cross-sectional area of each molecule. Although both dextran and protein revealed a decreased permeation with an increase in the cross-sectional area, the former has a higher permeation than the latter at the same cross-sectional area. Presumably, a difference in the partition coefficient may take a role in this distinctive permeation behavior of dextrans and proteins. Permeability coefficient, \(P\), is defined as the product of diffusion coefficient, \(D\), and partition coefficient, \(K\):

\[ P = KD \]

If the all solutes have the same partition coefficient, permeability only depends on the diffusion coefficient, which is inversely correlated with hydrodynamic radii of each solute molecule. As seen in Fig. 9, this is not the case for present experiments. It is likely that partition coefficient to the nanoball layer may be different between dextran and proteins. Since PEG and dextran are well known to be immiscible to cause a liquid-liquid phase separation\(^{[17]}\), the partition of dextran into the membrane coated with PEGylated nanoball may be substantially restricted. Consequently, this may lead to a decreased permeation of dextran compared to that of protein molecules having the same cross-sectional area.

4. Conclusion

In conclusion, core-polymerized block copolymer micelle (nanoball) possessing aldehyde groups on its surface (aldehyde-nanoball) was successfully prepared. Based on the LBL method, multi-layered nanoball-coated membrane was constructed in the presence of appropriate concentration of reductive reagent. By changing the number of the coating layers, permeation of macromolecular solutes was controlled in the molecular-weight dependent manner. Notably, a clear cut-off molecular weight was justified by changing the number of the coating layer. This unique membrane with multi-layered nanoball coating is applicable to a high performance protein separator and controlled-releasing device of bioactive compounds.

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References

[14] Hydrodynamic radii of each FITC-dextran were cited from manufacturer’s data sheet.