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Characterization of stable lysozyme-entrapped polyion complex (PIC) micelles with crosslinked core by glutaraldehyde

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Abstract

To gain an insight into the effect of core-crosslinking on polyion complex (PIC) micelles, the properties of PIC micelles prepared by mixing model protein, chicken egg white lysozyme with the synthesized α-methoxy-poly(ethylene glycol)–poly(α,β-aspartic acid) block copolymer (PEG–P(Asp)–NH_2) followed by glutaraldehyde addition, were investigated in detail. The added glutaraldehyde interacted with both of the lysine residues in lysozyme and P(Asp) ω-end amino groups in the micellar core, which elicited variations in the spectroscopic characteristics of the entrapped lysozyme, but no changes in its secondary structure. Micellar tolerability versus dilution, ionic strength increase, organic agent addition, and pH variation were all notably improved possibly due to strong core–shell structure-fixation resulting from this crosslinking. Such crosslinking may inhibit lysozyme denature in the presence of an organic agent, and also may prevent lysozyme escape from the micellar core usually observed due to weak association force between the lysozyme and P(Asp) segments. Additionally, the lysozyme reactivity in the micelles before and after the crosslinking seemed identical and even higher than that for free lysozyme. Such obtained stable protein-entrapped PIC micelles may find new applications in the fields of biotechnology and pharmaceutical sciences as a novel bioconjugating system.

Keywords: Polyion complex (PIC) micelles; Poly(ethylene glycol)–poly(α,β-aspartic acid) block copolymer; Lysozyme

1. Introduction

Taking advantage of advanced biotechnologies [1,2], and considering the increased awareness about bioactivities of various proteins, the therapeutic utilities of proteins as biopharmaceutical agents present more and more promising prospects. However, proteins are inherently both physically and chemically unstable [3], and biopharmaceutical utilities attempt to promote their stabilization. It has been noted that a matrix-encapsulation method may overcome this obstacle, and now the most widely studied protein encapsulating system, particularly useful in biomedical field, is based on the use of biodegradable polymer matrices, such as poly(lactide-*co*-glycolide) [4]. Nevertheless, such method requires complicated formulation procedures and the use of toxic organic solvents, both of which may perturb the protein structure and thus induce decreased bioactivities [5].

Our research group has recently established a very simple but appreciably stable protein nano-encapsulation system [6]: the core-crosslinked polyion complex (PIC) micelles, obtained by glutaraldehyde (a mild crosslinking agent for protein immobilization [7]) addition after the association of α-methoxy-poly(ethylene glycol)–poly(α,β-aspartic acid) block copolymer (PEG–P(Asp)–NH_2) as a block aniomer with a model cationic protein, trypsin. Higher apparent micellar enzymatic reactivity than free trypsin was observed because of the improved micellar stability together with the diminished trypsin self-digestion (autolysis). This system can be regarded as a novel bio-nanoreactor potentially useful in the bioengineering field.

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To get more detailed insight into the structure and the functions of this stabilized nanoreactor system containing an enzyme, thorough investigations were performed in this study on this type of core-crosslinked PIC micelles, prepared through electrostatic interactions between PEG–P(Asp)–NH_2 block copolymer with an cationic enzyme, chicken egg-white lysozyme. Characteristics for the micelles before crosslinking have been well specified by our group [8]. The variations in entrapped lysozyme conformations, ultraviolet absorption as well as fluorescence emission spectra, and apparent enzymatic reactivity were evaluated here in detail in relation to the core-crosslinking structure of PIC micelles.

2. Experimental section

2.1. Materials

α-Methoxy-poly(ethylene glycol)–poly(α,β-aspartic acid) (PEG–P(Asp)–NH_2, M_w of PEG: 12,000, degree of polymerization (DP) of (P(Asp): 34) and α-methoxy-poly(ethylene glycol)–poly(β-benzyl-L-aspartate) (PEG–PBLA–NH_2, M_w of PEG: 12,000, DP of PBLA: 36) block copolymers, were separately synthesized as previously reported [9]. Glutaraldehyde (25% aq. solution) and chicken egg-white lysozyme were obtained from Sigma (St Louis, MO, USA). 2,4,6-Trinitrobenzenesulfonic acid sodium salt dihydrate (TNBS), N^{α}-acetyl-L-lysine methyl ester hydrochloride (Ac-lysine-OMe·HCl), pyridinium chloride, and dioxane were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). *p*-Nitrophenol was purchased from ICN MP Biomedicals Inc. (Irvine, CA, USA). *p*-Nitrophenyl tetra-*N*-acetyl-β-chitotetraoside (NP-$(GlcNAc)_4$) used as a synthesized substrate for lysozyme was provided by Seikagakukogyo Co. Ltd (Osaka, Japan). These reagents were used without further purification.

2.2. Synthesis of PEG–P(Asp)–NH–$COCH_3$ block copolymer

α-Methoxy-ω-acetyl-poly(ethylene glycol)–poly(α,β-aspartic acid) block copolymer (PEG–P(Asp)–NH–$COCH_3$) was obtained by eliminating the β-benzyl groups in the side chain of α-methoxy-ω-acetyl-poly(ethylene glycol)–poly(β-benzyl-L-aspartate) block copolymer (PEG–PBLA–NH–$COCH_3$) using alkali hydrolysis method [9]. This PEG–PBLA–NH–$COCH_3$ was prepared by ω-end acetylation of PEG–PBLA–NH_2, which was carried out in anhydrous DMF solvent at 40 °C for 2 h using acetic anhydride as acetylation reagent [6]. ^{1}H NMR measurement (270 MHz) was performed at 80 °C for the PEG–PBLA–NH–$COCH_3$ in DMSO-d_6 to determine the acetylation degree of the terminal primary amino groups that was found to be 90% by calculating the peak intensity ratio of the introduced acetyl protons ($COCH_3$: $\delta = 1.8$ ppm) to the PEG methylene protons (OCH_2CH_2: $\delta = 3.7$ ppm).

2.3. Formulation of crosslinked PIC micelles

The PEG–P(Asp)–NH_2 or PEG–P(Asp)–NH–$COCH_3$ block copolymer, and chicken egg white lysozyme were separately dissolved in a phosphate buffer (PBS, 10 mM, pH 7.4; $Na_2HPO_4 \cdot 12H_2O$: 2.865 g/L, $NaH_2PO_4 \cdot 2H_2O$: 0.312 g/L). After a filtration through a 0.1 μm filter to remove any dust, the block copolymer solution was mixed with the lysozyme solution to form PIC micelle followed by incubation at 25 °C over 24 h. The mixing ratio (r) of Asp residues in the P(Asp) segments to Lys and Arg residues in the lysozyme was set as unity. A given amount of glutaraldehyde (5.1 μL) was added into the PIC micelle solution (10 mL, total concentration = 1.0 mg/mL) to crosslink the micellar core through a reaction with the lysine residues on the surface of lysozyme for 30 h at 25 °C. By a dialysis against PBS to remove excess glutaraldehyde using a semi-permeable regenerated cellulose membrane with a molecular weight cut off (MWCO) = 3500, core-crosslinked PIC micelles was obtained after a further incubation at 25 °C over 24 h. Such obtained micelles was abbreviated as g-GR, where GR stands for the ratio of [aldehyde groups in glutaraldehyde] to [Lys residues in lysozyme] in the reaction mixture.

2.4. Free amino group content

Un-crosslinked lysine residues in the micellar core, expressed as the number of free amino groups per lysozyme molecule (N_{lysine}), were determined using TNBS [10a]. Briefly, a micelle solution and PBS (control solution) in identical volume were separately mixed and reacted with a given amount of TNBS solution (around 51-fold excess per lysine residue [10b]) in a dark place at 25 °C. The product of this reaction gives an absorbance around 340 nm after mixing with 10 w/v% sodium lauryl sulfate (SDS) solution and 0.2 M HCl [10a,c]. Therefore, the reacted lysine residues with TNBS was calculated from the difference in the absorption between the micelle and control PBS solutions. The molar absorption coefficient at 340 nm, $\Delta\varepsilon_{340}$, was estimated from the same procedure using Ac-lysine-OMe·HCl as a model of lysine residues, and was calculated to be 1.32×10^4 M^{-1}/cm, which is consistent with the reported values [10c].

2.5. Light scattering measurements

Light scattering measurements were carried out using a DLS-7000 instrument (Otsuka Electronic Co., Ltd, Japan). Vertically polarized light of 488 nm wavelength from an Ar ion laser (75 mW) was used as the incident beam.

In the dynamic light scattering (DLS) measurement, the photoelectron-count time correlation function, $g(\tau)$, was

analyzed using the cumulant method. It is expressed by the following equation:

$$g(\tau) = \exp[-\bar{\Gamma}\tau + (\mu_2/2)\tau^2 - (\mu_3/3!)\tau^3 \cdots] \quad (1)$$

where τ is the delay time, and $\bar{\Gamma}$ is the average characteristic line width. This equation yields a variance, $\mu_2/\bar{\Gamma}^2$, called the polydispersity index (PDI). From the $\bar{\Gamma}$, the z-averaged diffusion coefficient, D, furthermore the corresponding hydrodynamic radius, R_h, can then be calculated using the following equations:

$$\bar{\Gamma} = Dq^2 \quad (2)$$

$$q = (4\pi n/\lambda)\sin(\theta/2) \quad (3)$$

$$R_h = \frac{k_B T}{(6\pi\eta D)} \quad (4)$$

Here q is the magnitude of the scattering vector, θ is the detection angle, k_B is the Boltzmann constant, T is the absolute temperature, and η is the solvent viscosity. The size distribution was estimated from the correlation function profile using the histogram method [11].

In the static light scattering (SLS) measurement, the light scattered by a dilute polymer solution at $\theta = 0$ that obtained by extrapolation is expressed as the following equations:

$$\frac{KC}{\Delta R_0} \approx \frac{1}{M_{w,app}} + 2A_2C \quad (5)$$

$$K = \frac{(4\pi^2 n^2 (dn/dc)^2)}{(N_A \lambda^4)} \quad (6)$$

where C is the polymer concentration, ΔR_0 is the difference of the Rayleigh ratio between the solution and the solvent, $M_{w,app}$ is the apparent weight average molar mass, A_2 is the second virial coefficient, dn/dc is the refractive index increment, and N_A is the Avogadro's number.

2.6. Spectroscopic measurements

Circular dichroism (CD) spectra were measured at two wavelength regions of 200–240 nm and 260–310 nm with a J-720W spectropolarimeter (Jasco, Tokyo, Japan) using a 0.1 cm quartz cell at 25 °C. All data were expressed as molar ellipticity [θ].

Fluorescence emission spectra were obtained upon excitation at 293 nm and measured with a PF-6500 spectrofluorometer (Jasco) using a 0.1 cm quartz cell at 25 °C.

The turbidity variations in lysozyme solution (1.0 mg/mL) with 40 or 50 v/v% of dioxane were monitored at 25 °C for 30 min at a wavelength of 500 nm using a V-550 spectrophotometer (Jasco). Ultraviolet (UV) absorption spectra were also obtained using the same instrument. Unless otherwise noted, all measurements in this study were performed at 25 °C.

2.7. Apparent enzymatic activity

Free lysozyme, un-crosslinked and crosslinked micelles with identical lysozyme concentration (=4.0 mg/mL; 0.14 μmol/mL), were separately reacted with NP-$(GlcNAc)_4$ stock solution at 25 °C. By monitoring the released amount of *p*-nitrophenol at 400 nm using the spectrophotometer V-550, apparent reactive velocity, v_{app}, of this reaction was determined. The NP-$(GlcNAc)_4$ concentration was adjusted to 37.5 μM in the mixtures. Extinction coefficient for the *p*-nitrophenol at 400 nm was calculated to be $1.14\times10^4\ M^{-1}$/cm from the calibration curve of *p*-nitrophenol phosphate solution at pH=7.4.

3. Results and discussion

3.1. Suitable GR values for crosslinked PIC micelles

The DLS and SLS measurements were performed for the PIC micelles that reacted with glutaraldehyde at GR ratios (=[aldehyde groups in glutaraldehyde]/[Lys residues in lysozyme]) of 0, 2, 4, 10, and 50, respectively, in order to monitor the variations in micellar size and light scattering intensity resulting from the core-crosslinking. As seen in Fig. 1, similar GR value dependences were observed for cumulant diameter (d_c) and light scattering intensity at a zero angle (ΔI_0), which is approximately proportional to the micellar $M_{w,app}$: an initial steep increase in these two parameters leveled off to the constant value beyond GR of approximate 4. Notably, as depicted in Fig. 2, the size distribution was still unimodale for these micelles even after the crosslinking event, indicating that crosslinking induces essentially no variations in micellar polydispersity. Since, there were subtle changes in micellar d_c, ΔI_0, and PDI at GR > 50 (data not shown), only the micelles with GR < 50 will be used in the following experiments.

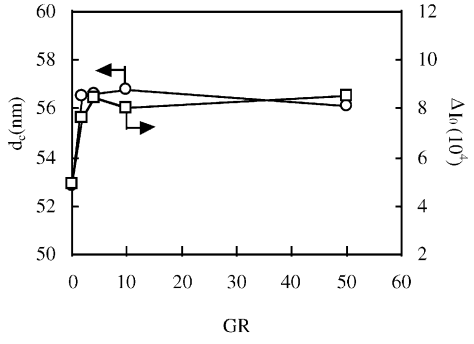


Fig. 1. Variations in d_c (○) and ΔI_0 (□) with increasing GR values obtained from DLS and SLS measurements for PEG–P(Asp)–NH_2/lysozyme micelles. Total concentration for each micelle system = 0.5 mg/mL; temperature = 25 ± 0.1 °C.

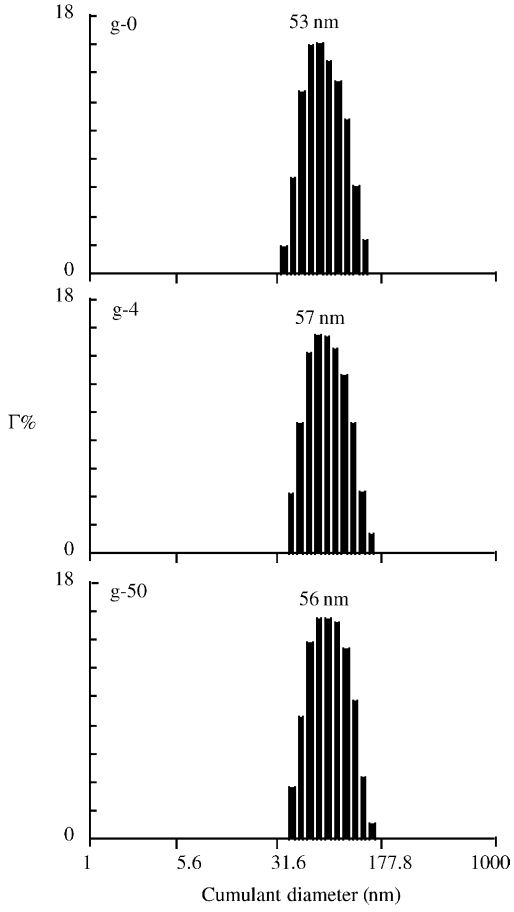


Fig. 2. Size distribution for g-0, g-4, and g-50 micelles, respectively. Total concentration for each micelle system = 0.5 mg/mL; temperature = 25 ± 0.1 °C.

3.2. Micellar stability versus ionic strength increase

Strong sensitivity to ionic strength is a well-known characteristic of polyion complexes due to the electrostatic shielding effect of salt that weakens the interactions between counter ions [12]. The crosslinking effect on the micellar stability against salt addition was estimated by comparing the changes in ΔI_0 and d_c with increasing NaCl concentrations for the micelles (1.0 mg/mL) with GR = 0, 2, 4, 10, 20, and 30, respectively.

The result in Fig. 3(a) (♦) shows monotonously decreased ΔI_0 values for un-crosslinked micelles (g-0), characteristic of micellar disintegration due to the decreased association force. d_c of g-0 micelles exclusively increased up to NaCl = 0.05 M (data not shown), and then became undetectable at NaCl > 0.05 M since increased NaCl concentration resulted in a drastically decreased photon count preventing the determination of the diffusion constant. Both of these results reflect the instability of g-0 micelles against salt addition. On the other hand, only a slight decrease in ΔI_0 and d_c values were observed for g-2 (▲) and g-4 (■) micelles, and at GR ≥ 10 (□, ○, △), both of these values reached a plateau until NaCl = 0.2 M (Fig. 3(a) and

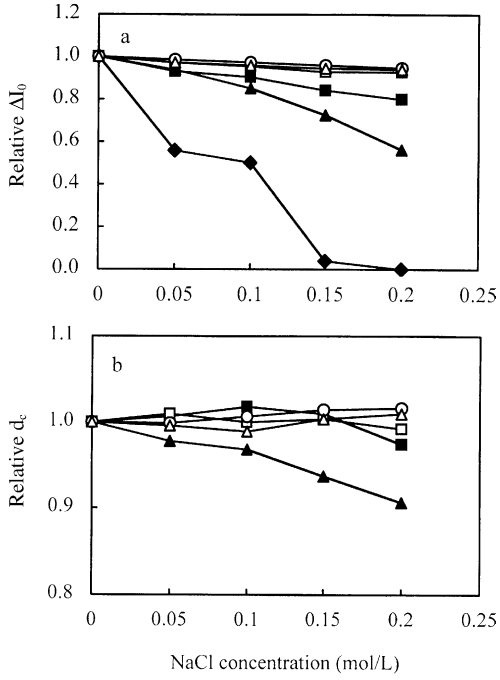


Fig. 3. Normalized variations in (a) ΔI_0 and (b) d_c values with increasing NaCl concentrations for g-0 (♦), g-2 (▲), g-4 (■), g-10 (□), g-20 (○), and g-30 (△) micelles, respectively. Total concentration for each micelle system = 0.5 mg/mL; temperature = 25 ± 0.1 °C.

(b)), indicating an improved micellar stability versus ionic strength due to the core crosslinking. Therefore, there exists a critical crosslinking degree over which PEG–P(Asp)–NH_2 block copolymers and lysozyme are fixed together physically and/or chemically due to the crosslinking event, which leads to an enhanced micellar stability against NaCl addition.

3.3. Importance of the ω-end amino groups in P(Asp) segments for micellar stabilization

In the case of PEG–P(Asp)–NH_2/lysozyme system, it is also possible that the ω-end amino groups in the P(Asp) segments may react with the glutaraldehyde. Thus, the contribution of these ω-end amino groups to the micellar stability was evaluated by comparing the micellar NaCl-stability for g-10 and g-10($COCH_3$) micelles, which were, respectively, formed from the PEG–P(Asp)–NH_2 and PEG–P(Asp)–NH–$COCH_3$ block copolymers. Worth noting, the g-10 and g-10($COCH_3$) micelles showed similar d_c and ΔI_0 values both before and after crosslinking at NaCl = 0 M (data not shown), emphasizing that the ω-end acetylation in the PEG–P(Asp)–NH–$COCH_3$ block copolymer had a negligible effect on the size and polydispersity of the micelles compared to g-10 micelles.

The NaCl-stability for g-10($COCH_3$) micelles was investigated using the same approach as in part 2, the result of which is shown in Fig. 4. At NaCl = 0.2 M, the ΔI_0 and d_c values both increased with time, and eventually precipitate

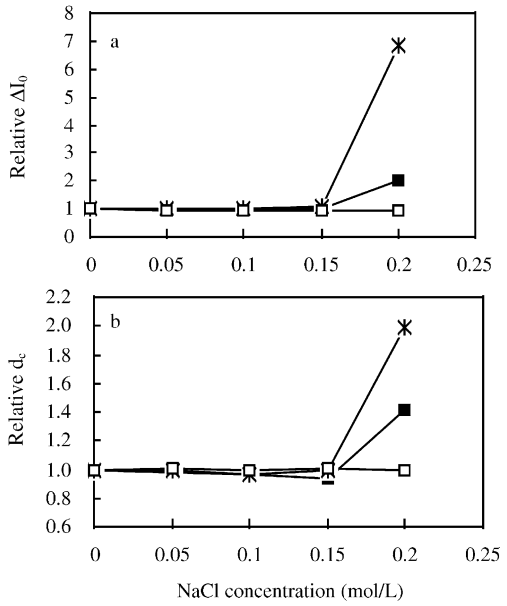


Fig. 4. Relative relationships of ΔI_0 and d_c with increasing NaCl concentrations for g-10 (□: 48 h) and g-10(COCH3) micelles (■: 24 h; ★: 48 h), respectively. Total concentration for each micelle system = 0.5 mg/mL; temperature = 25 ± 0.1 °C.

appeared in the g-10($COCH_3$) system (★ and ■; Fig. 4). These characteristics were obviously different from those of g-10 micelles, which showed constant ΔI_0 and d_c values at NaCl = 0.2 M even after 2 days (□; Fig. 4), indicating a decreased NaCl-stability for g-10($COCH_3$) micelles. In addition, in the UV absorption spectrum (Fig. 5) of the supernatant for g-10($COCH_3$) systems after formation of precipitate at NaCl = 0.3 M, the specific absorption around 280 nm for lysozyme could not be observed, consistent with the absence of lysozyme in the supernatant. Furthermore, ^1H NMR measurement revealed the existence of PEG–P(Asp)–NH–$COCH_3$ block copolymers in the supernatant (data not shown). Based on these UV and ^1H NMR results, it is reasonable to conclude that the PEG–P(Asp)–NH–$COCH_3$ block copolymers escaped from the g-10($COCH_3$) micelles

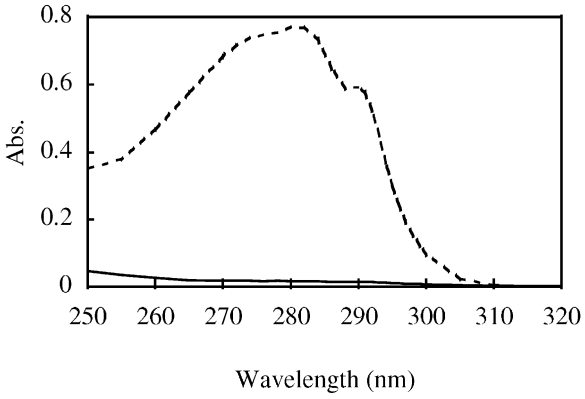


Fig. 5. UV absorption spectra for free lysozyme (0.3 mg/mL; broken line) and the supernatant from g-10(COCH3) system (solid line) at 0.3 M NaCl. Temperature = 25 °C.

at high salt concentration. Such event results from the diminished micellar association force and the absence of covalent bonding between the polymer and the lysozyme via glutaraldehyde bridges. Solubility of the system decreased as PEG–P(Asp)–NH–$COCH_3$ escaped into solution, and eventually, the crosslinked lysozyme precipitated. Hence, the ω-end amino groups in PEG–P(Asp)–NH_2 block copolymers, which may react with the glutaraldehyde, are indeed necessary to promote a better stability of crosslinked micelles against NaCl increase.

3.4. Extent of crosslinking reaction

Since, TNBS only reacts with free amino groups [10c], the lysine residues involved in the crosslinking reaction on the surface of lysozyme can be estimated by subtracting the TNBS reactable ones from the total lysine residues. As described above, the ω-end amino groups in P(Asp) segments may also react with TNBS. Nevertheless, they are in a magnitude around 12-fold less than the lysine residues, and it may be acceptable to assume that essentially the lysine residues in lysozyme are estimated by this analysis method. A suitable reaction time was decided by evaluating the time dependence of N_{lysine} (= number of free amino groups per lysozyme molecule) for the reaction of free lysozyme with TNBS. N_{lysine} did not significantly vary over 72 h. Reasonably, 100 h was chosen as a suitable reaction time for free lysozyme and g-0 micelles, because of no crosslinked lysine residues in them, and 72 h for crosslinked micelles.

The calculated results are summarized in Table 1. N_{lysine} was found to be about 6 for both the free lysozyme and entrapped-lyzozyme in g-0 micelles. In other words, all the lysine residues on the surface of free [13] as well as micelle-entrapped lysozymes can react with the TNBS, clearly showing the suitability of this analysis method to the PIC micelles. In the case of crosslinked micelles, $N_{lysine} \approx 2.6$, 1.8, 1.6, and 1.2 were separately calculated for g-4, g-10, g-20, and g-30 micelles, respectively, indicating that a higher crosslinking degree is achieved at a higher GR ratio. Combined with the NaCl-stability results, $N_{lysine} \approx 2$ seems to be the critical value, below which crosslinked micelles may become stable even at NaCl = 0.15 M.

3.5. Micellar spectroscopic characteristics

Spectroscopic properties for g-20 micelles were studied in order to examine the possible conformational changes of entrapped lysozyme due to the crosslinking reaction. As seen in Fig. 6(a), the CD spectrum of g-20 micelles in the short wavelength region (200–240 nm), indicative of lysozyme secondary structure, was superimposed on those of g-0 micelles and free lysozyme. In the long wavelength region (260–310 nm) characteristic of the environments of aromatic side chains in lysozyme (Fig. 6(b)), both of the CD spectra for g-20 and g-0 micelles were similar in shape to

Table 1
Number of un-crosslinked lysine residues per lysozyme molecule (N_{lysine}) determined by TNBS analysis

	Lysozyme	g-0 Micelle	g-4 Micelle	g-10 Micelle	g-20 Micelle	g-30 Micelle
N_{lysine}	5.9	6.1	2.6	1.8	1.6	1.2

the one of free lysozyme, but [θ] decreased in the order of free lysozyme > g-0 > g-20 micelles. These CD measurements suggest that in g-20 micelles, the secondary structure of lysozyme remains unchanged but the tryptophane (Trp) residues experience a different local environment.

The UV absorption spectrum of g-20 micelles showed a blue shift in the absorption maximum wavelength (λ_{max}) from 280 nm to around 274 nm (Fig. 7(a)). There was also a marked increase in the absorbance over the whole spectrum for both of the g-20 and g-0 micelles compared with free lysozyme, pointing out some changes in the local environment of Trp residues that possibly result from the micellization and the core-crosslinking events.

Furthermore, the fluorescence emission spectrum of g-20 micelles upon excitation at 293 nm was appreciably blue-shifted in the maximum emission wavelength (338.8 nm) compared to the fluorescence emission of free lysozyme (343.0 nm) and g-0 micelles (340.8 nm) (Fig. 7(b)), which suggests that the crosslinking may generate a less polar environment around the Trp residues [14]. Simultaneously, a 75% decrease in the fluorescence emission intensity was observed for the g-20 micelles compared with the free lysozyme, but only a slight decrease in the g-0 micelles, which indicates that the fluorescence emission intensity of g-20 micelles was quenched. Notably, all these observed spectroscopic characteristics for g-20 micelles agreed well with those previously reported for glutaraldehyde-crosslinked proteins, e.g. glucoamylase [15]. However, the molecular mechanisms responsible for such properties are still unclear because of the complicated reaction mechanisms for glutaraldehyde [16a] and thus unpredictable reaction products [16b,c]. One of the possible products in proteins crosslinked by glutaraldehyde can contain the quaternary pyridinium structure [17] shown in Scheme 1. Pyridinium analogues may have λ_{max} = 266 nm (1,3,4,5-substitution) or 276 nm (1,2,3,5-substitution) [18] close to that observed for the g-20 micelles (274 nm). Additionally, it is known that this pyridinium moiety effectively quenches the fluorescence emission of aromatic groups probably by an electron transfer mechanism [19]. It should be noted that recent studies have shown that the pyridinium moiety could effectively quench the fluorescence emission of Trp residues in proteins and the fluorescence emission maximum was then also blue-shifted [20]. Hence, this type of pyridinium

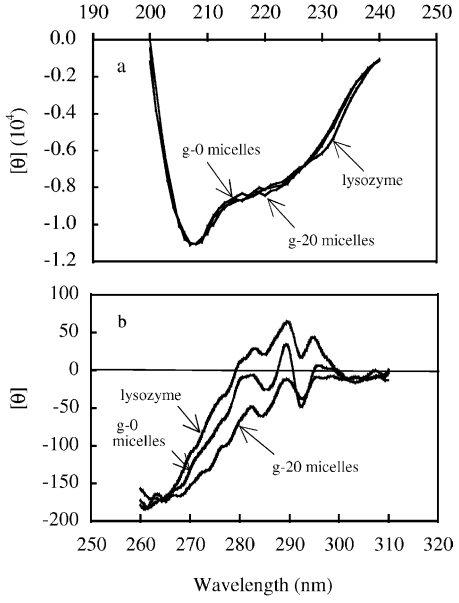


Fig. 6. CD spectra for free lysozyme, g-0, and g-20 micelles, respectively, (a) lysozyme = 0.2 mg/mL (lysozyme = 0.4 mg/mL for g-0 micelle system, total concentration of which was higher than cac); (b) lysozyme = 2.0 mg/mL; temperature = 25 °C.

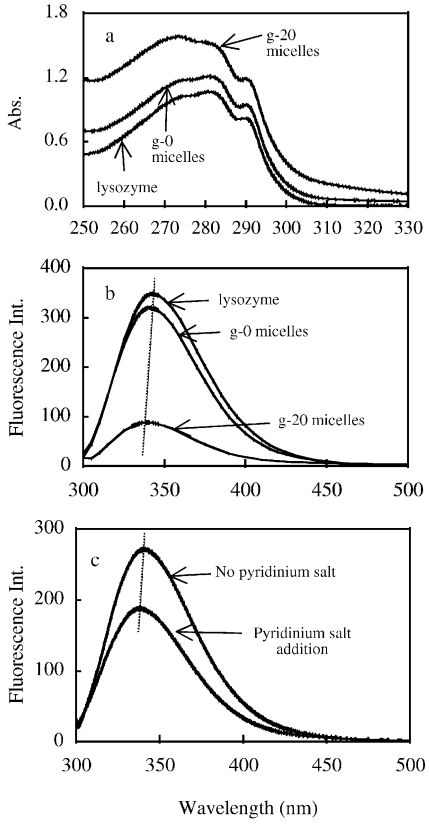
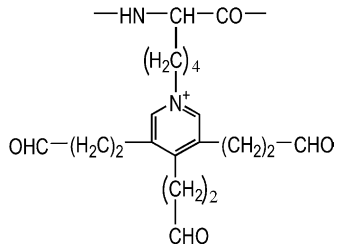


Fig. 7. Both of the UV absorption spectra; (a, lysozyme = 2.0 mg/mL) and fluorescence emission spectra; (b, lysozyme = 0.5 mg/mL) for free lysozyme, g-0, and g-20 micelles, respectively. And c is the fluorescence emission spectra (lysozyme = 0.5 mg/mL) for g-0 micelle before and after pyridinium salt addition. Temperature = 25 °C.



Scheme 1.

structures may also form in the crosslinked PIC micelles. Concrete evidence was obtained upon addition of a 500-fold excess of pyridinium chloride per lysozyme molecule to the g-0 micelles solution (lysozyme concentration = 0.5 mg/mL). Note that no significant micellar dissociation occurred at this salt concentration. The fluorescence spectrum measured after 24 h incubation at 25 °C is shown in Fig. 7(c). Obviously, a blue shift of the fluorescence emission wavelength maximum as well as a quenched fluorescence emission were observed for the g-0 micelles after pyridinium salt addition, which consistently support the pyridinium structure formation in the crosslinked micelles.

3.6. Critical association concentration (cac) for crosslinked PIC micelles

It has been reported that the critical association concentration (cac) of micelles might be calculated from the Debye plot of SLS (Eq. (5), a $KC/\Delta R_0$ curve against C) [8a,b]. However, for the core-crosslinked micelles, the dn/dc value, which is necessary to determine the K parameter, is not easily obtained by the method described previously [8a], because the amount of residual glutaraldehyde inside micellar core after dialysis is unknown. Hence, $C/\Delta I_0$ values, which are proportional to the $KC/\Delta R_0$ ones, were alternatively used and the respective $C/\Delta I_0$ curves plotted against C for g-0, g-10, and g-20 micelles obtained as demonstrated in Fig. 8, where all the $C/\Delta I_0$ values at 1 mg/mL were set to unity as controls. For g-0 micelles, the $C/\Delta I_0$ values increased with dilution due to the progressive decrease of the ΔI_0 values, indicative of micellar dissociation.

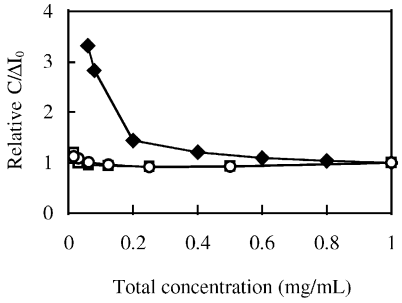


Fig. 8. Relative relationships of $C/\Delta I_0$ values with total concentration for each micelle system obtained from SLS measurements for g-0 (◆), g-10 (□), and g-20 (○) micelles, respectively. Temperature = 25 ± 0.1 °C.

Apparently, a different tendency was observed for the crosslinked micelles (g-10 and g-20), in which no significant increase in $C/\Delta I_0$ values appeared during dilution, suggesting that the cac value for crosslinked micelles is too low to be determined by the SLS measurements. Consequently, dilution stability of the crosslinked micelles was dramatically improved only by the crosslinking of micellar core.

3.7. Micellar pH-sensitivity

Micellar pH-sensitivity was also examined by the DLS and SLS measurements for g-0 (lysozyme concentration = 1.0 mg/mL) and g-20 (lysozyme concentration = 0.5 mg/mL) micelles at various pH values, which were separately prepared by 0.1 M HCl or NaOH addition to avoid significant deviations in the micellar concentration and the ionic strength of medium. Fig. 9 shows the pH dependence of d_c (Fig. 9(a)) and ΔI_0 (Fig. 9(b)) values for these two micelles. For an easy comparison, all the d_c and ΔI_0 values at pH = 7.4 were made to be unity as controls. As seen in Fig. 9(a), the d_c for g-0 micelles (◆) slightly decreased with decreasing pH values down to pH = 4.0, and then sharply increased to become five times higher than that at pH = 7.4. Conversely, with increasing pH values from 7.4, a notable increase in d_c was observed at pH > 10.5, close to the isoelectric point of lysozyme (= 11) [8a]. As seen in Fig. 9(b), similar but more remarkable variations in the ΔI_0 for g-0 micelles (◆) were observed, along with a steep decrease in ΔI_0 at pH > 10.5 and pH < 3.5. Both of the d_c and ΔI_0 results confirm the instability of g-0 micelles in acidic and basic environments, possibly as a result of the attenuated electrostatic interactions between the P(Asp) segments in block copolymer and lysozyme, due to the progressive protonation of COO^- groups in the P(Asp)

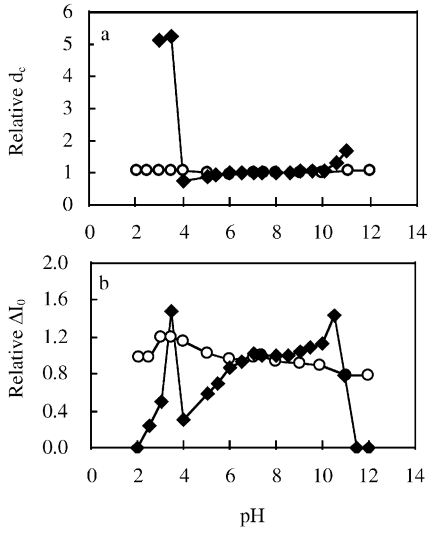


Fig. 9. Normalized variations in d_c (a) and ΔI_0 (b) with pH values, respectively, for g-0 (◆; 1.0 mg/mL) and g-10 micelles (○;0.5 mg/mL). Temperature = 25 ± 0.1 °C.

segments at pH < 7.4 [21], and the deprotonation of NH_4^+ groups of the lysine residues in lysozyme at pH > 7.4 [22]. At pH > 10.5 and pH < 3.5, the g-0 micelles dissociated because of the diminished association force.

On the other hand, for g-20 micelles, the fluctuations in d_c and ΔI_0 values (○) were obviously subtle compared with the g-0 micelles, suggesting that the crosslinking in the core substantially contributed to preserve the micellar structure even in conditions generated weak electrostatic interactions. Also, the resistance of g-20 micelles against pH decrease suggests that no azomethine bonds were formed in the crosslinking reaction of glutaraldehyde as such connectivity readily dissociates in acidic environment [23].

3.8. Micellar stability in the presence of organic solvents

Generally, proteins are instable in organic solvents because of destruction of their peculiar conformation [24]. To thoroughly estimate the properties of the crosslinked micelles, their tolerance against organic solvents addition was also evaluated. Hamaguchi et al. have shown the effects of dioxane addition on the thermal denaturing temperature of lysozyme (T_m) [25]. The T_m linearly decreased with an increase in dioxane volume, and down to 40 °C with 40 v/v% of dioxane, from which the T_m might be predicted down to around 30 °C with 60 v/v% of dioxane. To determine the dioxane volume that may denature lysozyme at 25 °C, turbidity measurements were undertaken for lysozyme solutions with different volumes of dioxane. A constant and an exclusively increased turbidity, respectively, corresponded to 40 and 50 v/v% of the dioxane within 30 min, showing that the lysozyme immediately denatured and precipitated in 50 v/v% of dioxane at 25 °C. According to this result, both of 50 and 60 v/v% of dioxane were separately added into g-0 and g-20 micelle solutions, and incubated in water bath at 25 °C over 24 h before DLS analysis, the results of which are summarized in Fig. 10 (g-0 micelle) and Fig. 11 (g-20 micelle), respectively. Notably, d_c increased for g-0 micelles after dioxane addition from 53 nm (dioxane content: 0 v/v%) to 451 nm (dioxane content: 50 v/v%) and 608 nm (dioxane content: 60 v/v%) (Fig. 10). Concomitantly, the size distribution became bimodal. In Fig. 11, although the d_c for g-20 micelles with 50 v/v% (51 nm) and 60 v/v% (50 nm) of dioxane slightly decreased from that in buffer only (56 nm), a unimodal size distribution was still present. Additionally, after removal of the added dioxane by dialysis against PBS solution, the d_c for g-20 micelles was recovered to the original value of 57 nm (50 v/v% dioxane → 0 v/v% dioxane) and 59 nm (60 v/v% dioxane → 0 v/v% dioxane). It is likely that the subtly decreased d_c for g-20 micelles upon dioxane addition may reflect a slight shrinking of PEG shell due to the decreased compatibility with dioxane [26], and/or the viscosity effect on the R_h (Eq. (4)) since dioxane has a higher viscosity index than water [27]. Consequently, crosslinking micellar core may improve the micellar stability against dioxane addition, therefore, inhibiting lysozyme denature that might occur for un-crosslinked micelles with 50 or 60 v/v% of dioxane.

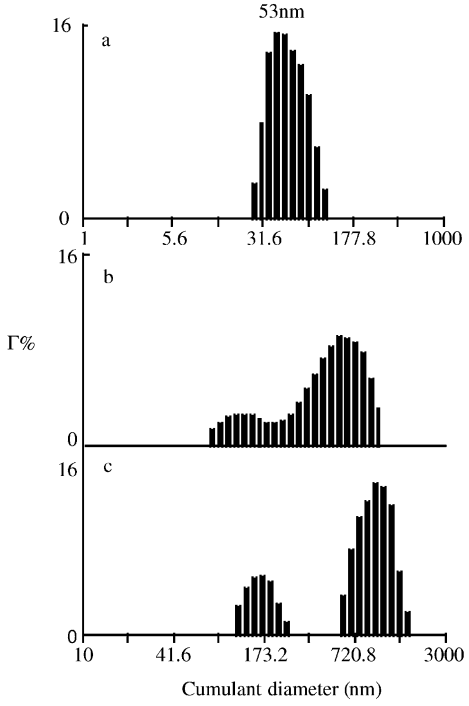


Fig. 10. Size distribution obtained from DLS measurements for g-0 micelles at 0 v/v% (a, total concentration = 1.0 mg/mL, PDI = 0.083), 50 v/v% (b, total concentration = 0.5 mg/mL, PDI = 0.244), and 60 v/v% (c, total concentration = 0.4 mg/mL, PDI = 0.278) of dioxane, respectively. Temperature = 25 ± 0.1 °C.

3.9. Apparent enzymatic activity

To specify the lysozyme activity within crosslinked PIC micelles, a synthesized substrate NP-$(GlcNAc)_4$ was employed and considering its small size, it can favorably diffuse into the micellar core, where it can be hydrolyzed [8c]. Here, only the apparent reactive velocity, v_{app}, calculated from the released amount of *p*-nitrophenol was evaluated, and more detailed information will be supplied elsewhere.

Fig. 12 shows the relative v_{app} values, respectively, for g-0, g-4, g-20 micelles and the v_{app} for free lysozyme set as unity. Obviously, the v_{app} values for both of the crosslinked and un-crosslinked micelles were around 2.5 times higher in magnitude than that for free lysozyme, suggesting that micellization facilitates the apparent lysozyme activity, and that core-crosslinking does not inhibit the enzymatic activity of the entrapped lysozyme at this NP-$(GlcNAc)_4$ concentration. The reason for this has been explained in our recent reports [8d,e]: PEG shell around the micelles favors substrate accumulation in micellar core, thus the substrate concentration in the core is higher than that around the micelles.

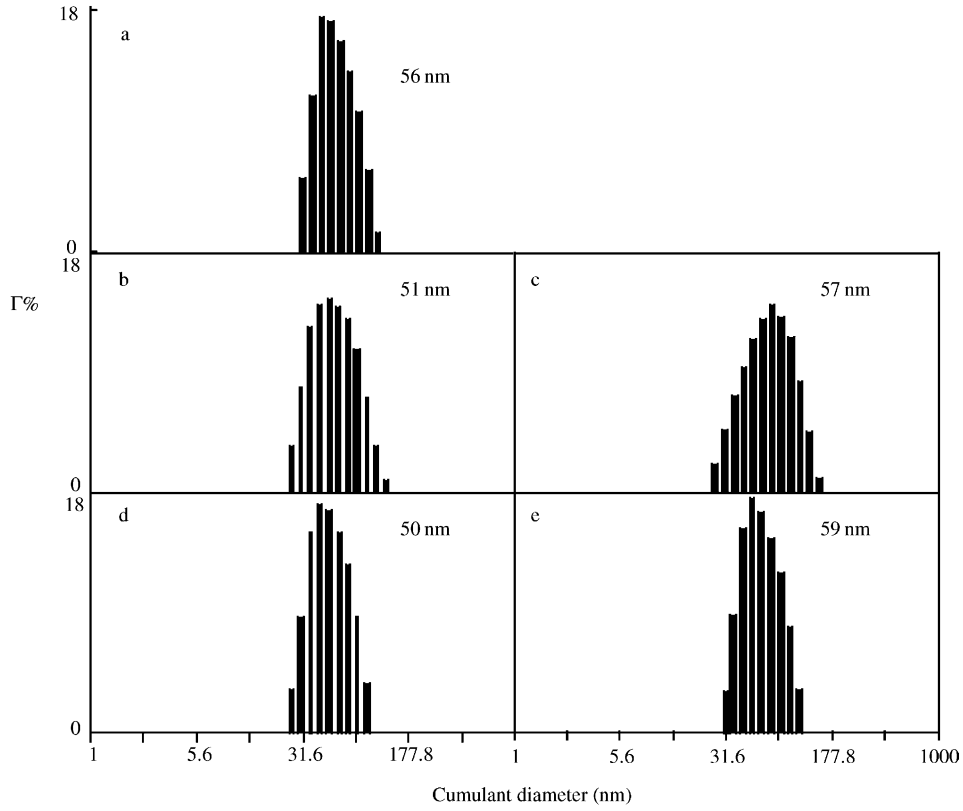


Fig. 11. Size distribution obtained from DLS measurements for g-20 micelles at 0 v/v% (a, total concentration = 0.5 mg/mL, PDI = 0.061), 50 v/v% (b: before dialysis, total concentration = 1.0 mg/mL, PDI = 0.046; c: after dialysis, total concentration was unknown because of significant variations in volume, PDI = 0.059), and 60 v/v% (d: before dialysis, total concentration = 0.8 mg/mL, PDI = 0.072; e: after dialysis, total concentration was unknown because of significant variations in volume, PDI = 0.068) of dioxane, respectively. Temperature = 25 ± 0.1 °C.

4. Conclusions

Simple glutaraldehyde addition to PEG–P(Asp)–NH_2/lysozyme micelles led to a successful crosslinking of the micellar core with insignificant changes in the micellar size and size distribution. Notably, both of the ω-end amino groups in PEG–P(Asp)–NH_2 block copolymers and lysine residues on the surface of lysozyme participated in the reaction with glutaraldehyde, resulting in a tightly fixed micellar core-shell structure responsible for the enhanced micellar stability not only towards dilution and ionic strength increase, but also dioxane addition and pH variation. A critical crosslinking degree for micelle stabilization requires an average reaction of 4 out of the 6-lysine residues with glutaraldehyde in each lysozyme molecule. Pyridinium analogues are expected to form in the crosslinking reaction between glutaraldehyde and the amino groups, as suggested by the blue shifted λ_{max} and quenched fluorescence emission for crosslinked micelles. More interestingly, the crosslinking of the micellar core has no significant interference with the apparent lysozyme activity in the micelles, keeping it even higher than that for free lysozyme.

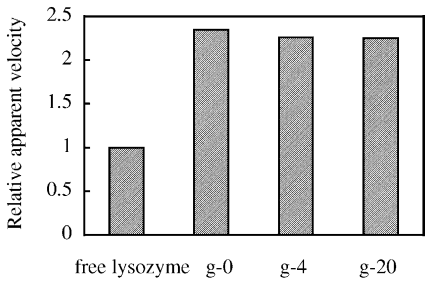


Fig. 12. Relative apparent reactive velocities, respectively, for free lysozyme, g-0, g-4, and g-20 micelles with identical lysozyme concentration (2.0 mg/mL) calculated from the released amount of *p*-nitrophenol at 400 nm. Temperature = 25 °C.

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