

Effects of PEG Molecular Weight on Its Interaction with Albumin^{*}

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Abstract The fluorescence property of bovine serum albumin was investigated in the presence of PEGs of different molecular weights. Based on our results, we propose that PEGs of different molecular weights bind to albumin in the pH 7.4 aqueous solution, but only when the polymer size is larger than the protein size, one polymer chain may bind to multiple proteins and cause the proteins to aggregate.

Keywords: Polyethylene glycol; Bovine serum albumin; Protein-polymer interactions; Fluorescence analysis.

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INTRODUCTION

The presence of polymers in the protein solution is known to affect the solubility, stability, and even the biological activities of the protein^[1, 2]. For example, poly(ethylene glycol) (PEG) is widely used to induce protein crystallization^[3, 4]. Polymers are also frequently grafted onto biomaterial surfaces to inhibit the protein absorption and consequently improve their *in vivo* performance^[5–8]. Moreover, synthetic polymers have been used to regulate the biological function of proteins and even act as the active therapeutic agents^[9, 10]. Therefore, the protein-polymer interactions in the aqueous environment is essential to understand the protein-polymer systems and to design functional polymers in various biomedical applications^[11–15].

However, even for the simplest system such as PEG and albumin, the polymer-protein interaction is not clearly understood. For example, Azegami *et al.* detected a blue shift in the fluorescence spectra of albumin in the aqueous PEG solution, and suggested that PEGs and albumin would form complex where several proteins bound to one PEG chain in a pearl-necklace-like manner^[16]. By using ANS (8-anilino-1-naphthalenesulfonate) as a fluorescence probe, Farruggia and co-workers found that albumin showed poor affinity to PEGs^[17]. On the other hand, PEG is one of most widely-used polymers in protein solutions to simulate the macromolecular crowding effect inside cells^[18–20], where it is assumed that no attraction or binding exists between PEG and proteins. The widely-used surface-grafting PEGs for protein adsorption inhibition also makes the same assumption for PEG-protein interactions.

We suspect that the confusion over the PEG-albumin interactions in the literature may be caused by the different molecular weight of PEGs used. Here, we used fluorescence spectroscopy and aimed to understand the effect of molecular weight on the PEG-albumin interactions. With two tryptophan residues, bovine serum

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albumin (BSA) has an absorption peak around 295 nm and shows a fluorescence emission maximum at about 340 nm in the aqueous solution^[21]. Moreover, the fluorescence emission is sensitive to the changes of the microenvironment around the BSA surface^[22]. We studied the changes of fluorescence property of BSA in the presence of PEGs of different molecular weights and concentrations, and tried to understand the role that molecular weight of PEG played in the PEG-albumin interactions.

EXPERIMENTAL

Materials

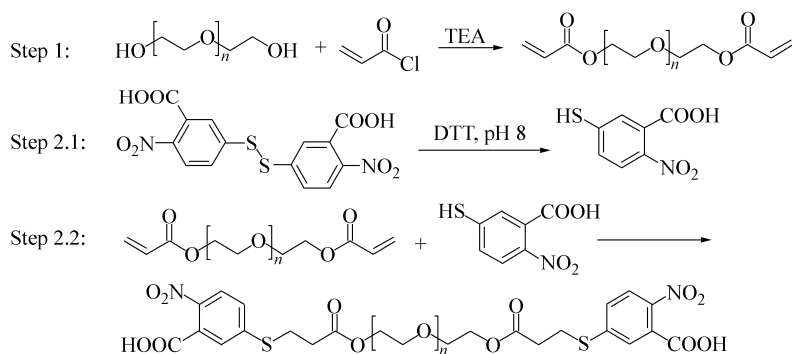
BSA (fraction V) and DTNB (5,5'-dithiobis-(2-nitrobenzoic acid)) were purchased from J&K Chemical Company. PEGs with molecular weight ranging from 400 to 20000 Da were purchased from Alfa Aesar. PEG with 35000 Da and ANS were purchased from Sigma Aldrich. DTT (DL-Dithiothreitol) was purchased from Amresco. Other reagents were purchased from Beijing Chemical Company.

Fluorescence Measurement

All solutions were prepared in 50 mmol/L, pH 7.4 PBS solution, incubated for 4 h at room temperature before the fluorescence measurement. Fluorescence spectra were collected on a Hitachi F-7000 fluorescence spectrometer with excitation at 295 nm for BSA and 375 nm for ANS. The scan speed was set at 1200 nm/min, the slit for both excitation and emission was 5.0 nm and the PMT voltage was 400 V. The temperature was kept at 25 °C by using a circulating water bath.

Synthesis of PEG with Nitrobenzoic Acid End Groups (PEG-NB, Scheme 1)

Two grams of PEG (MW: 10 kDa, PEG10k) was dissolved in 40 mL of dichloromethane and stirred with nitrogen protection in an ice bath. Then, 0.28 mL triethylamine (TEA) and 0.16 mL acryloyl chloride were added slowly. The solution reacted at room temperature for 24 h, concentrated with rotary evaporation, precipitated with cold ethyl ether, and dried under vacuum to obtain acrylate-ended PEG (PEG10k-acrylate) for the next step.



Scheme 1 Synthesis of PEG-NB

Then, 77.1 mg of DTT and 396.4 mg of DTNB were dissolved in 20 mL pH 8 PBS solution and reacted at room temperature with the protection of nitrogen. After 1 h, 500 mg of the PEG10k-acrylate product was added and the reaction continued for 3 h. Then the solution was extracted with dichloromethane for three times. The dichloromethane solution was concentrated with rotary evaporation, precipitated with cold ethyl ether, and dried under vacuum to obtain PEG10k-NB. The same polymer but with the initial PEG MW of 20k Da (PEG20k-NB) was also synthesized with the same methods.

RESULTS AND DISCUSSION

We first studied the effect of PEG molecular weight, while kept the concentration of PEG and BSA constant at 10 mg/mL and 5 μmol/L, respectively. The PEG concentration was chosen to ensure the polymer solutions were

in the dilute solution range, even for the highest molecular weight used^[23]. The concentration of BSA was set to be 5 $\mu\text{mol/L}$ to avoid the self-association between BSAs, as it showed significant aggregation at concentrations above 10 $\mu\text{mol/L}$ ^[24]. In our study, the excitation wavelength was 295 nm to avoid the interference from non-tryptophan residues, and we monitored the fluorescence intensity at 340 nm. As shown in Fig. 1, for PEG molecular weight between 0.4 and 10 kDa, the presence of PEGs in the solution had little effect on the fluorescence intensity of BSA. However, the BSA fluorescence intensity increased significantly when PEGs of MW 20 and 35 kDa were added in the solution, clearly indicating a MW effect of PEG on BSA.

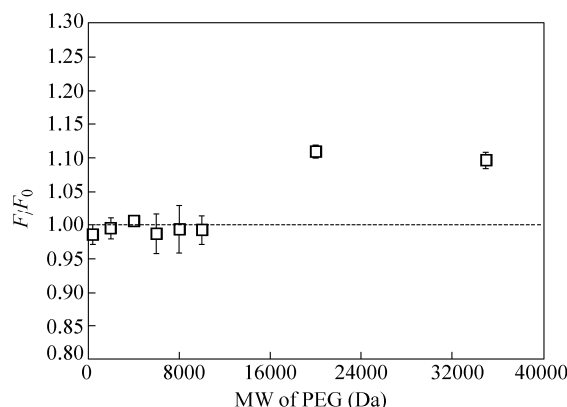


Fig. 1 The change of fluorescence intensity of 5 $\mu\text{mol/L}$ BSA solutions in the presence of 10 mg/mL PEGs of different molecular weight (MW), excitation: 295 nm; emission: 340 nm. The fluorescence intensity of each PEG-added sample (F) is compared with the intensity of the BSA solution (F_0). The error bars represent the standard deviation with 3 samples in each group.

Next, we chose PEG10k and PEG20k (MW 10 and 20 kDa) as two typical molecular weights to study the effect of polymer concentrations. As shown in Fig. 2(a), when mixed with PEG10k, the fluorescence intensity of BSA only slightly decreased even at PEG concentration of 100 mg/mL, and no shift of the emission maximum was observed. In dramatic contrast, in the presence of PEG20k, the fluorescence intensity of BSA increased linearly with the increasing concentration of PEG (Fig. 2b), and a blue shift from 340 nm to 328 nm was observed for the emission peak location. PEG35k caused similar results to those of PEG20k (Fig. S1 in SI).

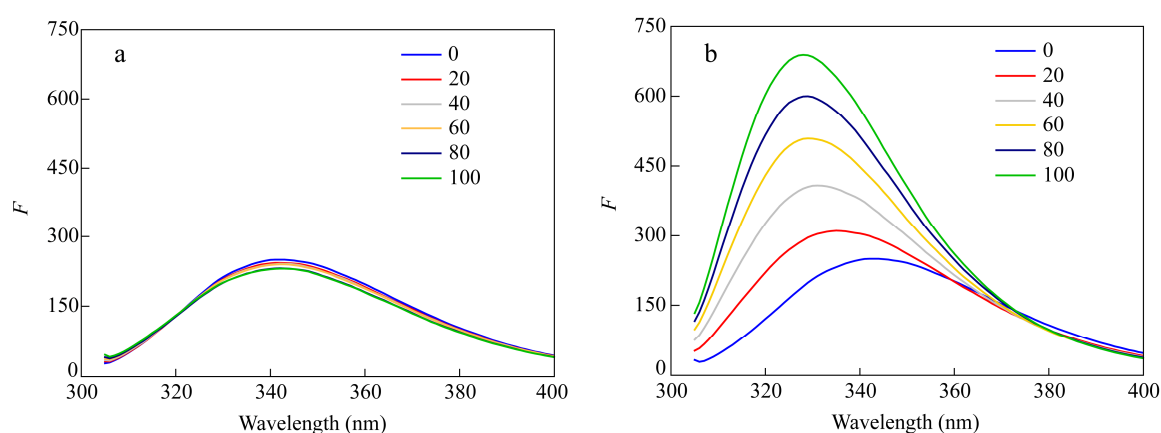


Fig. 2 Fluorescence spectra of 5 $\mu\text{mol/L}$ BSA solutions in the presence of (a) PEG10k, (b) PEG20k at different concentrations (unit: mg/mL), excitation: 295 nm

Therefore, it is clear that the increase of PEG molecular weight from 10 kDa to 20 kDa led to apparent change in its effect on BSA. According to the literature on tryptophan fluorescence^[25], both the increase in the

fluorescence intensity and the blue shift in the emission peak location can be explained as the tryptophan residues moving to a more hydrophobic micro-environment. For example, similar blue shift in the BSA fluorescence was observed in literature, which was induced by pH and was believed to be caused by the aggregation of protein and hence less exposure to the aqueous solution^[26, 27]. In our PEG-BSA system, two possible scenarios may make the BSA micro-environment more hydrophobic and/or less exposed to water: one is the PEG binding onto BSA and cover part of the protein surface, and the other is BSA aggregation. In the first scenario, the observed PEG molecular weight effect (Figs. 1 and 2) would mean, when molecular weight is larger than 10kDa, PEGs bind to BSA surface, while PEGs with lower molecular weight do not. If the second scenario is correct, the results would mean that only PEG with larger molecular weight will promote the aggregation of BSA at the concentrations studied.

Ideally, the two scenarios can be differentiated by looking for the aggregation particle formation in the solutions. However, because of the low protein concentrations of this system, neither the direct particle size analysis nor the viscosity measurement gave reliable results (data not shown). Alternatively, we used a dye-displacement study as an indirect method to detect if PEGs of different molecular weights bind to BSA surface. ANS was widely used to study the ligand-albumin binding^[28]. It has an absorption peak at around 375 nm and a fluorescence emission peak at about 470 nm. When bound to BSA in the aqueous solution, ANS shows much stronger fluorescence emission than the free ones. If PEG is added to the solution and if it does bind to BSA, ANS may be displaced from the BSA surface, as indicated by a significant decrease of the fluorescence intensity at 470 nm. As shown in Fig. 3(a), at the concentration of 10 mg/mL, neither PEG10k nor PEG20k made significant changes in the albumin-bound ANS fluorescence. When the concentration of PEGs increased to 100 mg/mL, the fluorescence of ANS decreased significantly and, once again, there was little difference between the PEG10k and PEG20k. These results suggest that both PEG10k and PEG20k have similar capability to displace albumin-bound ANS dyes and hence might bind to BSA surface with similar affinity.

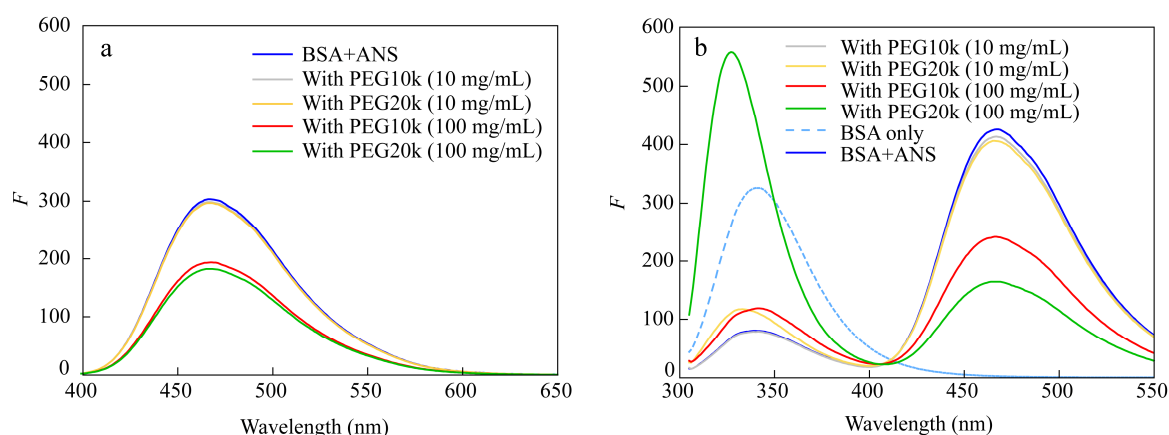


Fig. 3 Fluorescence spectra of BSA (5 $\mu\text{mol/L}$)/ANS (5 $\mu\text{mol/L}$) mixture solutions in the presence of PEGs: (a) excitation: 375 nm and (b) excitation: 295 nm

However, when the excitation wavelength was set at 295 nm instead of 375 nm, then the tryptophan residues of BSA will first be excited, and since the absorbance spectrum of ANS overlaps with the emission spectrum of tryptophan, the bound ANS will be subsequently excited through energy transfer^[28]. Therefore, the fluorescence emission of BSA and albumin-bound ANS can be monitored simultaneously. As shown in Fig. 3(b), without PEGs, the characteristic emission maximums of BSA and ANS (at about 340 nm and 470 nm, respectively) were observed, and the emission of BSA at about 340 nm was much lower than the one without ANS due to the fluorescence resonance energy transfer (FRET) effect. In the presence of PEGs, the fluorescence of the BSA-bound ANS (around 470 nm) showed the same results as those in Fig. 3(a), while the fluorescence changes around 340 nm were consistent with the results in Fig. 2, again suggesting that PEGs with both

molecular weights might bind onto BSA, but the one with MW 10k had little effect on the BSA fluorescence, while the one with MW 20k changed both its intensity and the emission peak location.

To further prove that both PEG10k and PEG20k bind to BSA surface, PEG chains with nitrobenzoate (PEG10k-NB and PEG20k-NB, Fig. 4) were synthesized. The UV absorption spectrum of the nitrobenzoate residue overlaps with the fluorescence emission spectrum of BSA, and hence can efficiently quench the fluorescence of the latter if the two molecules are close to each other within the distance of 4 nm^[29–31]. Therefore, PEG-NB might be a straightforward model PEG molecule to show whether or not the PEG chains bind to the BSA surface. The ¹H-NMR result of PEG10k-NB is shown in Fig. 4, indicating successful conversion of the end groups to nitrobenzoate (conversion percentage > 90%). The result for PEG20k-NB was similar (data not shown).

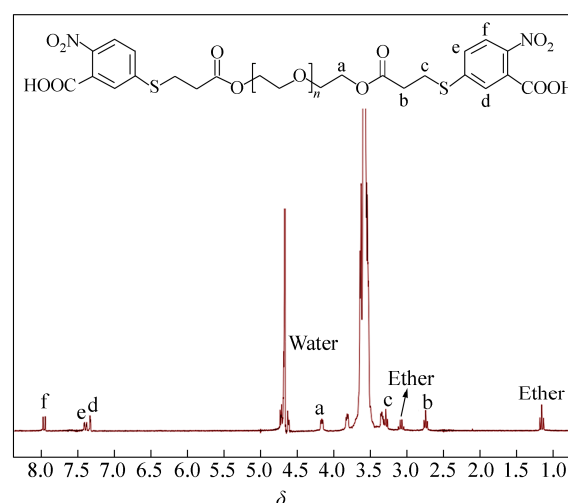


Fig. 4 ¹H-NMR (400 MHz, D₂O) of PEG10k-NB

The end group conversion is calculated to be more than 90% by comparing the integrals of aromatic group and PEG backbone.

As shown in Fig. 5, the increase of the PEG-NB concentration dramatically decreased the fluorescence emission of BSA, indicating that PEGs were close enough for the NB end groups to quench the fluorescence of BSA. For example, in the solution with 5 μmol/L BSA and 0.05 mmol/L PEG10k-NB (about 0.1 mmol/L NB groups), the average BSA-PEG surface-to-surface distance would be larger than 10 nm without binding between the two species. However, the significant quenching of the BSA fluorescence suggested that the two polymers are much closer, *i.e.*, PEG-BSA binding occurred. More importantly, PEG10k-NB and PEG20k-NB showed similar efficiency after the polymer concentrations were normalized with the end group concentrations, suggesting their similar binding affinity to BSA.

Therefore, all our data suggest that PEGs of both 10 kDa and 20 kDa molecular weight bind to BSA surface in the aqueous solutions. From a mechanistic perspective, the hydrophobic patch and the positive charge regions on the BSA surface can serve as the binding sites for the PEG segments^[32]. However, the binding of PEGs with MW 10 kDa and 20 kDa does cause difference in the BSA micro-environment, which needs to be explained. In the aqueous solution, the particle size of BSA is about 7 nm in diameter^[21], and the coil sizes of PEG10k and PEG20k are 6 nm and 9 nm, respectively^[33]. Thus, a critical molecular weight exists between PEG10k and PEG20k, at which the PEG chains will have the same size as BSA. A possible reason for the MW effect on the BSA fluorescence is as follows (Fig. 6): for PEG20k, which is larger than BSA, one PEG chain may bind with multiple proteins, act as a bridge to link BSAs and promote the aggregation of BSAs. As a result, the surface of BSA will be less exposed to the aqueous environment, which causes the blue shift and increase of the emission. On the other hand, one PEG10k chain can only bind to one BSA, and the binding sites may not be close enough to the tryptophan residue to cause its fluorescence change.

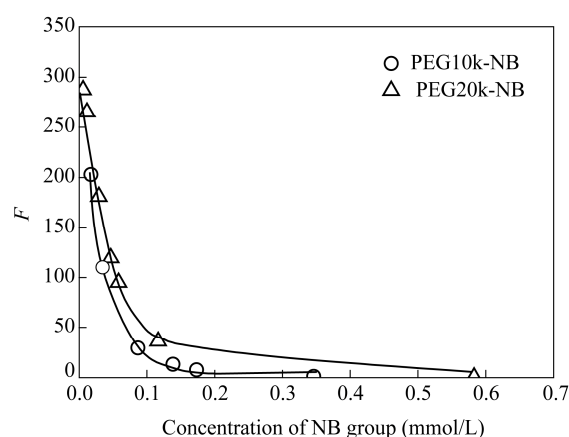


Fig. 5 Quenching effect of PEG-NB in 5 $\mu\text{mol/L}$ BSA solution, excitation: 295 nm; emission: 340 nm. The fluorescence intensity of BSA decreased exponentially with the increasing concentration of PEGs.

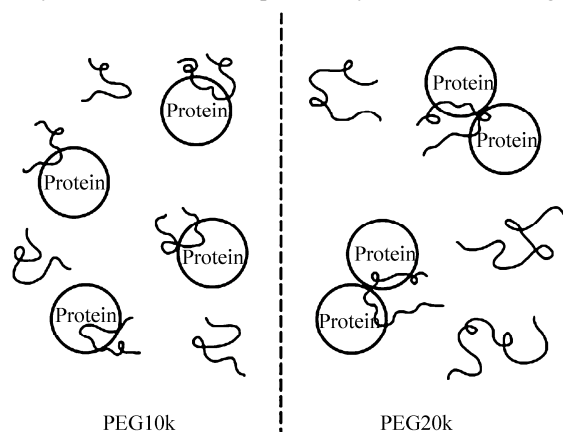


Fig. 6 The proposed mechanism of the PEG molecular weight effect

When the molecular weight of PEG is large enough, as shown in the right side, it is possible for one PEG to bind with multiple BSAs, and result in the aggregation of BSAs. In contrast, as shown in the left side, most PEG10k may only interact with one BSA, and thus will not significantly change the micro-environment around the BSA surface.

For comparison, we also studied the dextran-BSA system. Different from PEG, there is no sign of binding between BSA and dextran in the aqueous solutions^[18]. As a consequence, dextran did not show notable MW effect on the fluorescence spectra of BSA, *i.e.*, neither enhancement nor blue shift was observed for the dextran molecular weight studied (10 kDa and 20 kDa, data not shown). This indirectly supports our hypothesis that the MW effect of PEG on the BSA fluorescence spectra is the results of binding between the polymer and the protein.

The proposed picture may help us to understand the literature a little better. As mentioned in the introduction section, Azegami *et al.*^[16] proposed that several albumin molecules bound simultaneously with one PEG chain and formed a pearl-necklace complex in the aqueous solutions. In their study, the molecular weights of PEG chains ranged from 13 kDa to 428 kDa, *i.e.*, PEG chains were long enough to act as bridges between albumins. In contrast, Faruggia *et al.*^[17] used shorter PEG chains (1–8 kDa), and found those PEG chains were able to displace albumin-bound ANS, but the displacement extent was low even when 12 wt% of PEGs was added. Hence, their results suggested a real but poor affinity between short PEG chains and albumin in the aqueous solutions, which is consistent with our proposed picture. In the research area of macromolecular crowding, the most commonly-used PEGs are of molecular weight 6 kDa and 8 kDa, and for these short PEG chains, protein aggregation *via* PEG bridging would be unlikely.

CONCLUSIONS

In summary, we found that PEGs of different molecular weights bind to the surface of BSA, but showed different influence on the fluorescence property of BSA, suggesting the MW of PEG is an important factor in the PEG/BSA solution system. These results suggest that when the coil size of PEG is larger than the size of protein, the PEGs could bind to multiple proteins and cause their aggregation. It would be interesting to see if similar phenomena could be observed for other polymer-protein systems.

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