Bovine Serum Albumin—Poly(methyl methacrylate) Nanoparticles: An Example of Frustrated Phase Separation

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ABSTRACT: A new protein—polymer conjugate made of denatured bovine serum albumin (BSA) covalently bonded to poly(methyl methacrylate) (PMMA) is synthesized by attaching PMMA to acryloylated BSA followed by nanoparticle precipitation. Depending on the BSA to PMMA ratio, these conjugates self-assemble into uniform spherical nanoparticles which show “island” growth on the surface of the nanoparticles. This growth is promoted or retarded by exposing the nanoparticles to different solvents, causing the two components to undergo incipient phase separation. Incipient phase separation of the BSA—PMMA conjugate two-component system was observed in single nanoparticles, resulting in “island” growth on the surface of the nanoparticles. Incipient phase separation of the BSA—PMMA conjugate two-component system was observed in single nanoparticles, resulting in “island” growth on the surface of the nanoparticles.

KEYWORDS: Protein—polymer conjugate, nanoparticles, phase separation

The large surface-to-volume ratio of nanoparticles provides one important reason why nanoparticles have many advantages over the same material in bulk. For example, surface properties can regulate stability, solubility, and functionality, which play an important role in many applications. In the present study, we have synthesized a new protein—polymer conjugate made of denatured bovine serum albumin (BSA), which is a nutrient to cells, and poly(methyl methacrylate) (PMMA), which is a synthetic thermoplastic polymer. Under certain composition conditions, the resulting BSA—PMMA nanoparticles may be highly spherical in shape and can display incipient phase separation of the two immiscible components, which cause “islands” to appear on the surfaces of the nanoparticles.

Protein—polymer conjugates, which consist of proteins and polymers covalently linked together, have been previously demonstrated to have significant applications in the area of therapeutic protein delivery and biocatalysis. Protein—polymer conjugates could be synthesized either by attaching a synthetic polymer to protein or by growing synthetic polymer chains from protein. In this study, PMMA chains were directly grown from acryloylated protein by radical polymerization to form the branched copolymer (Scheme 1).

Each BSA was confirmed to be modified with 51 acryloyl groups as determined by MALDI-TOF mass spectrometry. The content of BSA in the conjugate was varied to be 4, 69, or 82 wt % as determined by 1H NMR. The conjugate in acetonitrile (2.5 mg/mL) was injected into a phosphate buffered saline (pH 7.4) solution to precipitate nanoparticles. As the content of BSA increased from 4 wt % to 69, 82 wt %, the average size of the nanoparticles increased from 30 to 60 to 90 nm (Figure 1), as determined by scanning electron microscopy (SEM). Dynamic light scattering (DLS) revealed a different set of measurements; the average size decreased from 477 to 336 to 321 nm (also a peak with particle size around 100 nm was shown indicating well-separated nanoparticles presented in this sample). The DLS measurements suggest that in aqueous solution aggregates are formed with low content of BSA while with 82 wt % of BSA in the conjugate the nanoparticles are very well dispersed and readily more separate from each other.

More interesting is the morphology of the nanoparticles, which also changed with conjugate composition. At 4 wt % of BSA, irregularly shaped nanoparticles with some agglomeration are formed (Figure 1a). At 69 wt % of BSA, very uniform spherical nanoparticles with smooth surface are formed (Figure 1b). At 82 wt % of BSA, small “islands” were observed to form on the surface of the uniform nanospheres (Figure 1c). Because we...
concentrate on the last case, we introduce in what follows the symbol BSA\textsubscript{82}/C\textsubscript{0}PMMA\textsubscript{18} for nanoparticles formed with 82% BSA and 18% PMMA.

We have concentrated our attention to the surface morphology of the BSA\textsubscript{82}/C\textsubscript{0}PMMA nanospheres having high BSA content. We found from SEM that the amount of "islands" on the surface of the BSA\textsubscript{82}/C\textsubscript{0}PMMA nanoparticles gradually increased over a period of 2 weeks when the nanoparticles were incubated in aqueous PBS solution (pH 7.4) without any treatment (Figure 2). During this time, the hydrodynamic size and the zeta potential of the nanoparticles remained unchanged, indicating no obvious degradation of the nanoparticles, a conclusion that is also consistent with the SEM observations.

To gain more information about the surface morphology, we chemically attached FITC to the BSA component of the conjugate, followed by the same nanoprecipitation procedure to make the BSA\textsubscript{82}/C\textsubscript{0}PMMA\textsubscript{18} nanoparticles in aqueous PBS (pH 7.4). The fluorescent spectrum was recorded (Figure 3) during the incubation period of 2 weeks. The maximum emission wavelength shifts from 538 nm (day 1) to 526 nm (day 14). Along with the blue shift, the fluorescent intensity was also observed to increase. Self-quenching of FITC dye is one of the oldest observations in fluorescence spectroscopy\textsuperscript{10} and is caused by resonance energy transfer (RET) between FITC molecules. In the case of FITC the Forster distance for RET is about 42 Å.\textsuperscript{11} Thus, from the fluorescence spectroscopy result, we propose that at the beginning of the incubation, the BSA component with FITC attached to it was confined in a relatively small region where self-quenching of FITC molecules occurred, resulting in a decrease of fluorescent intensity. During the incubation period, the BSA component gradually transferred from the inside region to the outer region of the nanoparticles, which resulted in a more separated FITC molecules and thus a decrease of self-quenching.

Let us consider the Flory–Huggins theory of mixing\textsuperscript{12} as applied to this system. We denote by $\phi$ the volume fraction of BSA chains, $N$ the number of lattice sites occupied by each molecule ($N_{\text{BSA}}$ for the BSA chains; $N_{\text{PMMA}}$ for the PMMA chains), and $\chi$ the Flory interaction parameter. Then the free energy of mixing is given by

$$\Delta G_{\text{mix}} = kT \left[ \frac{\phi}{N_{\text{BSA}}} \ln \phi + \frac{1-\phi}{N_{\text{PMMA}}} \ln(1-\phi) + \chi \phi(1-\phi) \right]$$

(1)
The second derivative of the free energy gives the spinodal and consequently the critical interaction parameter for phase separation, which is expressed as

$$\chi_c = \frac{\nu_0}{2} \left( \frac{1}{\sqrt{v_{\text{BSA}}} + \frac{1}{\sqrt{v_{\text{PMMA}}}}} \right)^2$$  \hspace{1cm} (2)

where $\nu_0$ is the lattice site volume and $v_i$ is the molecular volume of polymer chain $i$. Knowing the dimension of a single BSA molecule\(^\text{13}\) ($v_{\text{BSA}} = 135 \text{ cm}^3 \text{ mol}^{-1}$) and a MMA monomer\(^\text{14}\) ($v_{\text{MMA}} = 78 \text{ cm}^3 \text{ mol}^{-1}$), and assuming the lattice site volume $v_0$ equals the volume of MMA monomer, for the BSA\(^\text{82}/ \text{PMMA}18\) conjugate (with a molar ratio of repeating units of PMMA/C0 equals the volume of MMA monomer, for the BSA\(^\text{82}/ \text{PMMA}18\) nanoparticles in acetonitrile for 14 days; (d) SEM of the same BSA\(^\text{82}/ \text{PMMA}18\) nanoparticles. Scale bars are 100 nm.

To further understand the self-organization process, we observed the BSA\(^\text{82}/ \text{PMMA}18\) nanoparticles after incubation of 14 days in aqueous PBS solution by transmission electron microscopy (TEM), as shown in Figure 4a. This image indicates that an incipient phase separation is occurring in the nanoparticles. A clear delineation of core−shell structure from the periphery of the nanoparticles is caused by the large difference in scattering between BSA and PMMA parts. While most of the PMMA chains are located at the cores of the nanoparticles, in the shells of the nanoparticles BSA chains and PMMA chains interweave together in a complex pattern. This behavior seems to be consistent with the complex surface morphology observed by SEM as shown in Figure 4b.

In sharp contrast, the behavior of the BSA\(^\text{82}/ \text{PMMA}18\) nanoparticles in acetonitrile (Figure 4c,d) is markedly different. Unlike the nanoparticles incubated in aqueous solution which are shown in panels a and b of Figures 4, we cannot discriminate between the BSA and PMMA parts in the nanoparticles in acetonitrile both immediately after dissolving the conjugate in acetonitrile and after incubating it in acetonitrile for 14 days. We conclude that the distribution of two components is relatively uniform and no obvious structure change occurred in acetonitrile.

Figure 5 illustrates a proposed mechanism for frustrated phase separation. A clear delineation of core−shell structure is caused by the large difference in scattering between BSA and PMMA parts. While most of the PMMA chains are located at the cores of the nanoparticles, in the shells of the nanoparticles BSA chains and PMMA chains interweave together in a complex pattern. This behavior seems to be consistent with the complex surface morphology observed by SEM as shown in Figure 4b.

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The mechanism accounts for the structural changes observed by TEM, as shown in Figure 2. It also explains the fluorescence experiments shown in Figure 3, which indicates that the BSA gradually migrates from the hydrophobic confined environment to the outer-layer hydrophilic environment.

In conclusion, we synthesized the denatured BSA−PMMA conjugate, from which uniform spherical nanoparticles were prepared by a simple self-assembling process. The nanoparticles exhibit unique phase separation phenomena in aqueous solution. What use this frustrated phase separation behavior might be put to remain an open question. It is easy to imagine using it to enhance nanoparticle labeling with yet another conjugate. To our knowledge, however, this type of behavior has not been previously reported and could be of interest to applications involving the fine-tuning of the surface properties of the nanoparticles.
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