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Aggregation of biotinylated polymeric microspheres induced by interaction with avidin*

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Abstract: Monodisperse biotinylated poly(styrene-*co*-*N*-acryloxysuccinimide) microspheres were synthesized in aqueous solutions with a two-step method. Upon the addition of avidin solutions of different concentrations in phosphate buffer into the dispersed biotinylated microspheres, the microspheres aggregated rapidly due to the high binding affinity between biotin and avidin. The hydrodynamic diameter of the aggregates and the aggregation rate observed at given time intervals increased with increasing concentration of avidin. The composition of the microspheres and the incorporation of biotin were evidenced by Fourier transform infrared spectroscopy. The morphology, size distribution, and aggregation of the microspheres were studied by techniques such as scanning electron microscopy and dynamic light scattering.

Keywords: polymer microspheres; biotinylation; emulsion polymerization; bioconjugation; aggregation.

INTRODUCTION

The detection and analysis of biological molecules have attracted much attention over the past few years. The rapid and accurate determination of deleterious biomolecules is especially important due to the potential threat from toxins, bacteria, and viruses. Aggregations of nano- or microparticles via specific interactions between complementary biological components have been studied for the detection of biologically active molecules or building of complex nanostructured materials through a self-assembly process, including antigen and antibody [1], complementary DNA base pairing [2–8], enzyme and substrate [9], biotin and streptavidin or avidin [10–16].

Avidin, a 66-kDa glycoprotein, is a minor constituent of egg whites that could induce a nutritional deficiency in rats by forming a very stable noncovalent complex with the biotin ($K_a \sim 10^{15}$ M^{-1}) [17]. The biotin-(strept)avidin system has been utilized for many years in a variety of different applications. In particular, Hoffman, Stayton, and coworkers have studied extensively "smart" polymer bioconjugates based on the biotin-strepavidin system and their potential applications [18–23].

Controlling aggregation of biotinylated matrices arising from the presence of avidin or streptavidin would be a good method to detect certain target molecules through biological magnification. Inorganic nanoparticles were modified by biotin and then reacted with avidin or streptavidin to study

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the optical absorption spectra and the morphology of self-assembly [24–26]. For example, biotin-coated Au nanocrystals [27] underwent aggregation following the addition of streptavidin and formed a network structure. Recently, Costanzo et al. reported the aggregation of spherical inorganic nanoparticles surface-modified with biotin-functionalized poly(ethylene glycol) upon the addition of avidin into the initial dispersed nanoparticle aqueous solution [14]. They have also investigated the effect of nanoparticle size on the rate of aggregate formation using mixtures of various-sized SiO_2 nanoparticles [16]. Other biomacromolecules were used as biotinylated matrix to investigate the aggregations by biotin-(strept)avidin interactions including liposome [28], iron storage protein ferritin [11], DNA oligomers [29], and polysaccharides [30]. Furthermore, polymeric microspheres surface-coated with avidin or streptavidin could act as components to prepare self-assembling materials and biosensing system by specific interaction with biotin [31–34]. Biotinylated polymeric microspheres should be a good model system to explore the aggregation induced by avidin. The preparation of biotinylated particles is a basic and important component in such a system. Many biotinylation reagents have been employed to synthesize desired biotinylated particles, such as NHS-biotin analogs [11,31,35,36]. We report here the preparation of biotinylated polymeric microspheres and the formation of aggregates upon their interaction with avidin.

EXPERIMENTAL SECTION

Preparation of the microspheres

Poly(styrene-*co*-*N*-acryloxysuccinimide) [P(S-*co*-NAS)] microspheres have been synthesized by surfactant-free emulsion polymerization under a nitrogen atmosphere. 2.006 g of pre-distilled styrene (from Sigma) and 120 mL of water were placed into a four-necked round-bottom flask equipped with a mechanical anchor-type agitator, condenser, nitrogen inlet, and thermometer. 0.200 g of NAS (from Acros) dissolved in 2 mL *N*,*N*-dimethylformamide was added into the flask. Nitrogen was bubbled into the solution, and the mixture was stirred for 30 min at 300 rpm to remove oxygen from the system. Polymerization was initiated by adding 10 mL of an aqueous solution containing 0.192 g of potassium persulfate (KPS, from Tianjin Chemicals Co., China) at 70 °C and was allowed to proceed for 24 h under constant stirring. The resulting microspheres sample was purified by repetitive centrifugation (15 800 g), decantation, and redispersion for three times and dialyzed for a week, and then the sample was concentrated by centrifugation.

For biotinylation, a solution of the P(S-*co*-NAS) microspheres at a concentration of 0.05 mg/mL was prepared by dispersing the microspheres in a phosphate buffer solution (PBS) (pH = 7.4). 40 mL of the above dispersion was added into 100 μL PBS solution containing 0.2 mg biotin hydrazide (from Fluka), and the reaction proceeded with magnetic stirring at room temperature overnight. The resulting solution was purified by dialysis against PBS for a week to remove the unreacted biotin hydrazide. PBS used was filtered through a 0.45-μm Millipore filter to remove any dust. For the Fourier transform infrared (FT-IR) measurements, the sample was dialyzed against water for two weeks and then freeze-dried. To avoid the nonspecific binding of the microspheres with protein, an excess of 2-aminoethanol was added into the dispersion of biotinylated microspheres to deplete the residual unreacted succinimide ester group and then the sample was dialyzed in PBS for several days to remove any unreacted 2-aminoethanol. The unbiotinylated microspheres were prepared by a reaction of P(S-*co*-NAS) microspheres with an excess of 2-aminoethanol in the absence of biotin hydrazide for the blank experiment.

Characterization

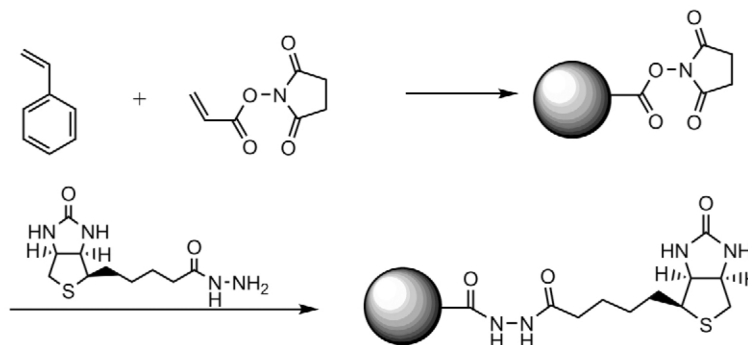
Scanning electron microscopy (SEM) was performed on a Hitachi S3500N microscope at 20 kV. Piror to the experiment, dispersion of the P(S-*co*-NAS) microspheres in water was lyophilized. FT-IR was carried out on a Bruker Tensor 27 FT-IR instrument with samples prepared with KBr pellet. Dynamic

light-scattering (DLS) measurements were performed on a laser light-scattering spectrometer (BI-200SM, Brookhaven Instruments) equipped with a BI-9000AT digital correlator at 532 nm.

For DLS experiments, the biotinylated microspheres dispersed in PBS were centrifugated to remove dust for 20 min at 1040 g. Then 1.0 mL of the sample was transferred to a clean scintillation vial with a clean pipette. 40 μL of avidin (from Sigma) solution of various concentrations in PBS (filtered through a 0.45-μm Millipore filter previously) was added to the above sample and the size of the aggregates was monitored over time by DLS. In all experiments, the final volume of the samples was 1.040 mL. The same processes were conducted with the dispersion of unbiotinylated microspheres.

RESULTS AND DISCUSSION

We have investigated a system containing surface-biotinylated P(S-*co*-NAS) microspheres and avidin in PBS. Biotin hydrazide was used as the biotinylation reagent to avoid the use of any organic solvents. The preparation of biotinylated polymeric microspheres involved a two-step process in an aqueous environment. The P(S-*co*-NAS) microspheres were synthesized by surfactant-free emulsion polymerization and then reacted with biotin hydrazide to form the biotinylated microspheres, as illustrated in Scheme 1.



Scheme 1 Preparation of biotinylated microspheres.

The SEM image of P(S-*co*-NAS) microspheres shown in Fig. 1 indicates that the microspheres are monodisperse and the average diameter of the microspheres is ca. 370 nm. Figure 2 shows the hydrodynamic diameter and the monodisperse distribution f(D_h) of the microspheres. The size observed from SEM was smaller than that measured by DLS since the former was observed in the dry solid state while the latter was measured in the swollen state in solution.

The FT-IR spectra of the PS, P(S-*co*-NAS), and biotinylated microspheres are shown in Fig. 3. In comparison to the spectrum of the PS microspheres, the presence of NAS active ester groups on the P(S-*co*-NAS) microspheres is confirmed by the absorption bands at 1744 and 1680 cm^{-1}, corresponding to carbonyl stretching vibration of ester and amide in succinimide groups, respectively. The biotinylated microspheres were synthesized by reacting the succinimide ester groups on the surface of the microspheres with the amino groups of biotin hydrazide. NAS was often used as a monomer to synthesize various linear polymers and microspheres for immobilizing biomolecules such as enzyme [37–39], peptide [40], and antibody [41], due to the high reactivity of succinimide ester group to primary amine group under mild conditions. The incorporation of biotin groups was identified from the FT-IR spectra shown in Fig. 3C. The peak at 1744 cm^{-1} assigned to carbonyl stretching vibration of succinimide ester groups has much reduced intensity in comparison to the same peak in Fig. 3B. The peak at 1630 cm^{-1} in Fig. 3C can be attributed to carbonyl stretching vibration of the amide groups formed during biotinylation, confirming the successful covalent attachment of biotin onto the microspheres. It is believed

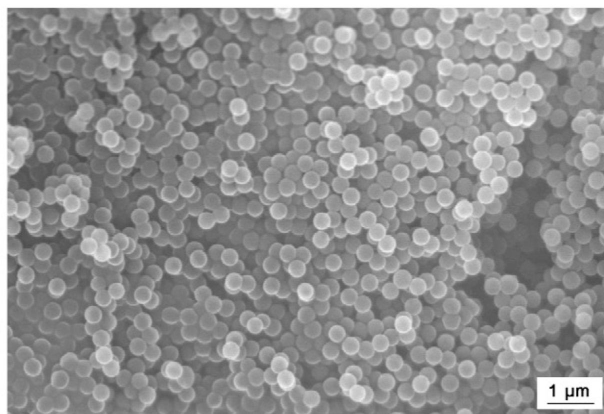


Fig. 1 SEM image of the P(S-*co*-NAS) microspheres prepared by surfactant-free emulsion polymerization.

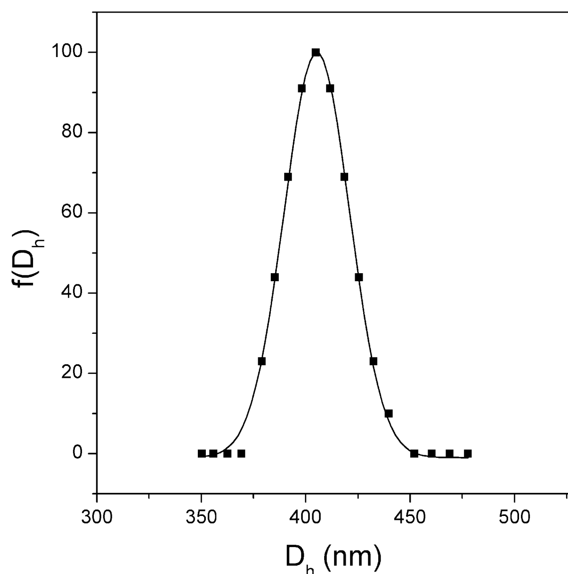


Fig. 2 The hydrodynamic diameter distribution f(D_h) of P(S-*co*-NAS) microspheres measured by DLS at a scattering angle of 90° at 25 °C.

that the immobilized biotin molecules are localized mostly on the surface of the spheres since the spheres were not swollen in the aqueous reaction medium during biotinylation. For subsequent interactions with avidin, only the biotin on the surface should be effective because of the large size of the protein molecules.

The dispersions of biotinylated P(S-*co*-NAS) microspheres were prepared, and various concentrations of avidin were introduced. The stable dispersion of the biotinylated microspheres in PBS became unstable and started to aggregate when avidin in PBS was added. Because avidin has four biotin binding sites, it can act as a biological cross-linking agent and initiate the formation of microsphere aggregates. The average hydrodynamic diameter of the aggregates increased with time, as monitored by DLS. In all the experiments, the concentration of microspheres and total volume of the solution were kept constant to allow for direct comparison. In the experiments, avidin concentrations varied from 3.8×10^{-8} to 1.5×10^{-5} M with a total of 6 concentrations. We have also verified the reversibility of the

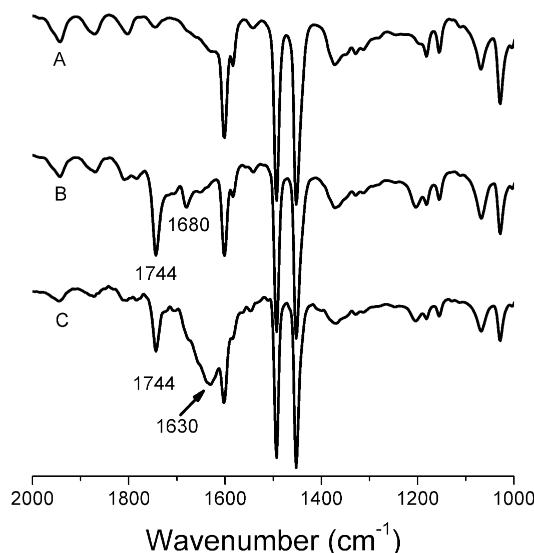


Fig. 3 The FT-IR spectra of the PS microspheres (A), P(S-*co*-NAS) microspheres (B), and biotinylated P(S-*co*-NAS) microspheres (C). The peaks of carbonyl stretching vibration of ester and amide in the NAS ester groups appear at 1744 and 1680 cm^{-1}, respectively (B). The carbonyl stretching vibration of the amide group formed during the biotinylation process appears at 1630 cm^{-1} (C).

aggregation by the addition of free biotin in PBS, no disruption of the aggregation was observed even when an excess of biotin was added. Considering the limited solubility of biotin in aqueous media, we have also used biotin hydrazide, a derivative of biotin with much better solubility than biotin in water, into the mixture, the aggregation of the microspheres was not disrupted. All this indicates the good stability of the aggregates formed between avidin and the biotinylated microspheres.

To determine the specificity of the system to avidin, blank and control experiments were performed. The blank experiment involved the use of unbiotinylated microspheres of the same concentration and 40 μL avidin solution with a concentration of 7.6×10^{-6} M. The hydrodynamic diameter of the microspheres did not change during the time of the experiment. This indicates that there was no interaction between particles and avidin because there were no binding sites of avidin on the microspheres. Furthermore, this experiment also proved that biotin had been incorporated on the microspheres. Although the amount of biotin on the microspheres cannot be determined directly, the immobilization is unequivocally confirmed by the marked difference between the biotinylated and unbiotinylated beads.

The specificity of this approach was also demonstrated by the addition of a nonspecific protein, bovine serum albumin (BSA), with a molecular weight (66 kDa) similar to that of avidin, to the dispersion of biotinylated microspheres. The control experiment was performed with the biotinylated microspheres of the same concentration and volume and 40 μL BSA solution with a concentration of 1.5×10^{-5} M. The size of the dispersed biotinylated microspheres also remained unchanged, and no aggregation formed suggesting the target specificity of this system.

Figure 4 shows that the average hydrodynamic diameter of the aggregates measured by DLS increased rapidly once avidin was introduced, suggesting the biotinylated microspheres aggregated because of the interaction between biotin on the surface of the microspheres and avidin. Avidin acted as biochemical cross-linkers for the microspheres, which resulted in the formation of aggregates. Moreover, the rate of the formation of aggregates depended on the concentrations of avidin, and a high concentration of avidin led to a faster aggregation.

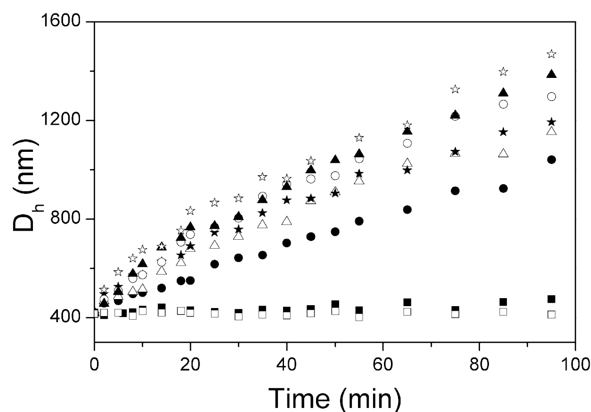
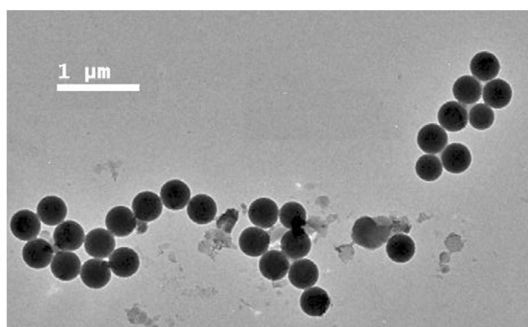
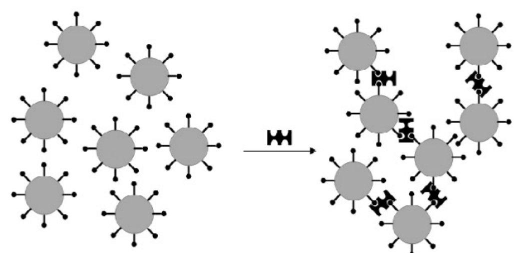


Fig. 4 The plot of average hydrodynamic diameter (D_h) of the aggregates vs. time measured by DLS at a scattering angle of 90° at various concentrations of avidin: 3.8×10^{-8} M (closed circle); 3.8×10^{-7} M (open triangle); 1.9×10^{-6} M (closed pentagram); 3.8×10^{-6} M (open circle); 7.6×10^{-6} M (closed triangle); 1.5×10^{-5} M (open pentagram). The blank experiment (closed square) was performed with unbiotinylated microspheres and 7.6×10^{-6} M of avidin, and the control experiment (open square) was performed with biotinylated microspheres and 1.5×10^{-5} M of BSA.

Figure 5A shows the transmission electron microscopy (TEM) image of the aggregation of the biotinylated microspheres induced by the addition of 40 μL of aqueous solution of avidin at a concen-



(A)



(B)

Fig. 5 (A) The TEM image of the aggregation of biotinylated microspheres upon the addition of avidin; (B) The illustration of aggregation process of biotinylated microspheres induced by the addition of avidin.

tration of 7.6×10^{-6} M in the sample. The possible mechanism of the aggregation is illustrated in Fig. 5B, showing the cross-linking effect of the avidin in such interactions.

CONCLUSION

The polymeric microspheres with succinimide ester groups can be modified successfully to obtain surface-biotinylated microspheres. The addition of avidin solutions into the dispersion of the biotinylated microspheres in PBS caused the rapid aggregation of the particles. The average hydrodynamic diameter of the aggregates increased with time, and the aggregation became more rapid with increasing concentration of avidin. Control experiments with unbiotinylated microspheres or with a protein other than avidin confirmed the specificity of the binding. The prototypical biotin–avidin interaction should provide a basis of a simple diagnostic technique for biochemical analytes with potential applications in the field of targeted detection and biosensing.

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