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Chorng-Shyan Chern
Cheng-Kang Lee
Cheng Kuan
Kuan-Chaung Liu

Adsorption of BSA on the amphiphilic PEG graft copolymer-coated particles

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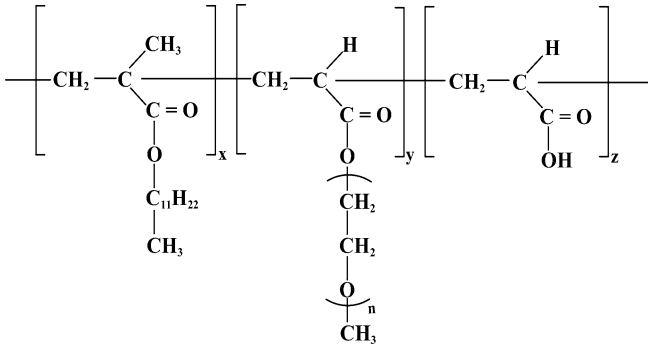
C.-S. Chern (✉) · C.-K. Lee
C. Kuan · K.-C. Liu
Department of Chemical Engineering,
National Taiwan University of Science and
Technology, 43 Keelung Road, Sec. 4,
Taipei, 106, Taiwan, ROC
E-mail: chern@ch.ntust.edu.tw
Fax: +886-2-27376644

Abstract The amphiphilic copolymers comprising several monomethoxy poly(ethylene glycol) (mPEG) and lauryl side chains were prepared and coated on the polystyrene (PS) particles to study the interactions between these particles and bovine serum albumin (BSA). The surface mPEG density and mPEG chain length were the primary parameters of interest. A significant fraction of the graft copolymer was washed away from the particle surface during five cycles of centrifugation-dispersion treatment, especially for the one with the smallest number of lauryl side chains. At pH 5, the BSA adsorption data did not follow the Langmuir isotherm model for the graft copolymer-modified particles. This was attributed to the presence of a surface mPEG layer that severely retarded the approach of BSA to the particle. The amount of the adsorbed BSA decreased with increasing the surface mPEG density. A mechanistic model was proposed to qualitatively describe the adsorption of BSA on the mPEG-containing particles and the native particles as well.

Keywords Protein adsorption · Amphiphilic PEG graft copolymer-coated PS particles · Hydrophobic interaction · Steric hindrance interaction

Introduction

Because of its unique volume exclusion property in aqueous solution [1, 2], poly(ethylene glycol) (PEG) can be used as a modifier for biomedical surfaces to reduce the protein adsorption and improve their biocompatibility [3, 4] and as a stabilizer for emulsion and dispersion [5]. In our previous work [6, 7], the amphiphilic graft copolymers containing both the monomethoxy PEG (mPEG) and lauryl (or stearyl) side chains were prepared and characterized. We propose using the negatively charged polystyrene (PS) particles coated with a layer of the mPEG graft copolymer (Scheme 1) to investigate the interactions between bovine serum albumin (BSA, pI is about 5) and the particles. The charge neutralization of the particles by the adsorption of protein, which carries the opposite net charge to that of the particles, would lead to strong flocculation [8, 9]. On the other hand, the electrostatic repulsive force between similar charged particles and protein retards the protein adsorption. Thus, the pH is set at 5 in order to minimize the electrostatic interaction between BSA with a nearly neutral net charge and particles with a negative net charge since the antifouling effect of the surface mPEG is the focus of this work. Another important issue is whether the steric stabilization effect [10, 11] provided by mPEG is strong enough to maintain adequate colloidal stability during the BSA adsorption. It is also interesting to study the effectiveness of the copolymer with several hydrophobic side chains in adsorbing on the PS particle surface.



Scheme 1 Molecular structure of the amphiphilic graft copolymers containing both the hydrophilic mPEG and hydrophobic lauryl side chains used to modify the PS particle surface

Experimental

Materials

mPEG (MW = 750 or 2,000), acryloyl chloride (AC), acrylic acid (AA), lauryl methacrylate (LMA), 2,2′-azobisisobutyronitrile (AIBN), triethylamine (TEA) and pyrene were obtained from Aldrich and used to prepare and characterize the amphiphilic PEG graft copolymers. Styrene (ST, Taiwan Styrene Co.), HS-10 (a reactive surfactant, $CH_2=CH–CH_2–C_6H_4–O–(CH_2CH_2O)_n–SO_3Na$, Daiichi), sodium bicarbonate (Riedel-de Haen), sodium persulfate (Riedel-de Haen), sodium chloride (Riedel-de Haen), hydrochloric acid (Nacalai Tesque), sodium hydroxide (Riedel-de Haen), and deionized water (Barnsted, specific conductance < 0.057 μS cm^{-1}) were used to prepare and characterize the latex particles. Other chemicals used include BSA (Sigma) and potassium hydrogenphthalate (KHP, Aldrich). ST and AC were distilled under reduced pressure, mPEG was dried in vacuo for 24 h, TEA was distilled from Na, and LMA and AIBN were purified by recrystallization from dry methanol and ethanol, respectively, before use. All other chemicals were used as received.

Synthesis and characterization of the amphiphilic graft copolymers

The macromonomer, mPEG-acrylate, was prepared by acylation of mPEG (MW = 750 or 2,000, 8.5 mmol) with AC (23 mmol) in the TEA (73 mmol)-containing toluene under N_2 at 25 °C. The graft copolymers were prepared by the free radical copolymerization of mPEG-acrylate (MW(mPEG) = 2,000 used for preparing the copolymers P2k-1 and P2k-2 and MW(mPEG) = 750 for P750), LMA and AA in toluene using AIBN as the initiator at 70 °C. The molar ratio of AA : mPEG-acrylate : LMA was set at 70:15:15 except one recipe used to prepare the copolymer P2k-2 (60:20:20). The resultant copolymers were precipitated from n-hexane and the precipitates were collected and dried in vacuo. These copolymers were suspended in water and subjected to centrifugation (Beckman, J2-21) at 5,000 rpm for 10 min and the supernatants are collected. The supernatant aqueous solutions of these copolymers were then filtered to remove large undissolved particles, followed by ultrafiltration (Microcarbosep, MWCO 50000) until no mPEG peaks were shown by GPC and lyophilysed. The compositions were obtained from ^{1}H NMR spectra in $CDCl_3$ containing 1% (v/v) of tetramethylsilane (TMS) as the internal standard (Varian Gemin 2000, 500 MHz) [6, 7]. The number-average molecular weight (M_n) and the polydispersity index of molecular weight distribution (PDI(MWD) = M_w/M_n) were determined by GPC (Agilent 1100 series with a refractive index detector G1362A and PLgel columns 79911GP-503 and 79911GP-504). The calibration curve was established using PS as the standards (Polysciences). The purity of copolymers was checked by GPC and complete removal of unreacted species was confirmed. The critical micelle concentration (CMC) of copolymers in deionized water was determined by fluorescence measurement (Shimadzu, RF-5301PC). Excitation of pyrene in aqueous solutions containing various polymer concentrations was achieved at 336 nm and the emission spectra of pyrene were recorded. In all cases, the pyrene concentration was kept at 6×10^{-7} M, the solubility of pyrene in water. The ratio of the intensity of the third and first vibronic peaks (I_3/I_1) as a function of the polymer concentration was calculated and the point at which micelles started to form was then determined [12, 13]. Some physical properties of the copolymers are summarized in Table 1.

Preparation and characterization of the latex particles

Emulsion polymerization was used to prepare the PS particles. First, the emulsion consisting of 627.4 g H_2O, 0.48 g $NaHCO_3$, 0.06 g HS-10, 160.0 g ST was purged with N_2 for 30 min in a 1-l reactor equipped with a four-bladed fan turbine agitator, a thermometer and a reflux condenser. The first-stage polymerization was initiated by adding 0.96 g $Na_2S_2O_8$ dissolved in 20 g H_2O into the reaction mixture. The agitation speed, temperature and reaction time were set at 300 rpm, 80 °C and 8 h, respectively. After cooling and filtration (200-mesh), the resultant latex was used as the seed for the second-stage polymerization. The recipe used to produce the latex product was 355.3 g H_2O, 301.0 g seed latex, 0.27 g $NaHCO_3$, 87.8 g ST and 0.53 g $Na_2S_2O_8$.

Before characterization, the latex was subjected to centrifugation at 8,000 rpm for 60 min (Beckman,

Table 1 Characterization of amphiphilic graft copolymers and the graft copolymer-modified latex particles

Copolymer ID	P2k-1	P2k-2	P750
Molar ratio of AA:mPEG:LMA	89:4.7:6.3	66.5:15.4:18.1	75.8:11.9:12.3
$M_n \times 10^{-3}$ (g mol^{-1})	8.3	11.7	8.0
PDI(MWD)	1.46	1.37	1.26
N_{AA}, N_{mPEG}, N_{LMA}[a]	42.0, 2.2, 2.99	18.9, 4.4, 5.1	33.5, 5.3, 5.4
N_{mPEG}/N_{LMA}	0.74	0.85	0.97
CMC$\times 10^4$ (g ml^{-1})[b]	2.0	2.1	0.92
CMC$\times 10^4$ (g ml^{-1})[c]	2.1	2.6	1.0
CMC′$\times 10^4$ (g ml^{-1})[c]	11.0	12.0	11.0
$\Gamma_{p,0} \times 10^7$ (mol m^{-2})	2.5	1.9	2.9
$\Gamma_p \times 10^8$ (mol m^{-2})	6.7	9.3	0.11
$\Gamma_{mPEG} \times 10^7$ (mol m^{-2})[d]	1.5	4.1	5.9
F	0.73	0.51	0.62
D (nm)	3.4	2.0	1.7

[a] Number of monomeric units of AA and mPEG and lauryl side chains per macromolecule obtained from the data of molar ratio of AA:mPEG:LMA and M_n

[b] Determined in deionized water by the fluorescence measurement

[c] Determined at pH 5 (2 mM KHP) by the pendant drop digitization technique

[d] Surface concentration of mPEG after the treatment of five cycles of centrifugation-dispersion

J2-21). The precipitated particles were dispersed in water by ultrasonication (Delta DG-1). This procedure was repeated five times to remove the residual monomer, initiator and other impurities in the aqueous phase. The total solids content was determined gravimetrically. The z-average hydrodynamic particle diameter (d_p) determined by dynamic light scattering (DLS, Otsuka, Photal LPA-3000/3100) showed that the mechanical stability of the latex subjected to five cycles of centrifugation-dispersion was satisfactory. The reported d_p data represent the average of at least three measurements and their errors have been estimated to be 8% or less. The volume-average particle diameter ($d_v = 206$ nm) and the polydispersity index of particle-size distribution (PDI(PSD) $= d_w/d_n = 1.02$) were determined by TEM (JEOL, TEM-1200 EXII). The d_w and d_n represent the weight-average and number-average particle diameters, respectively.

The zeta potential (ζ) was determined by Zetamaster (Malvern). The pH and ionic strength of the sample were adjusted by 0.1 N NaOH and 0.1 N HCl and NaCl, respectively. The ionic strength and total solids content were kept constant at 0.01 M NaCl and 0.01%, respectively. Five measurements were made for each sample and the average of these measurements was reported as ζ. It is inappropriate to adopt the fluorescence technique to determine the point at which micelles start to form in the presence of particles (CMC′) because of the particle surfaces available for the pyrene adsorption. Thus, the surface tension (γ) versus polymer concentration data in the presence of particles (0.19 wt%) at pH 5 (2 mM KHP) were obtained from the pendant drop tensiometry [14, 15]. For reference, the CMC values of copolymers at pH 5 were also measured by this method (Fig. 1).

Adsorption of BSA on the graft copolymer-coated particles

First, the latex was treated with the graft copolymer at CMC′ (pH 5, 2 mM KHP) under stirring at 25 °C over 4 h. It was subjected to centrifugation at 8,000 rpm for 60 min (Beckman, J2-21) and the precipitate was dispersed in the pH 5 buffer solution by ultrasonication (Delta DG-1). This procedure was repeated five times to remove the residual copolymer in the aqueous phase. The difference between the CMC′ and CMC data in Table 1 represents the maximum amount of the copolymer that can be adsorbed on the particles. The actual quantity of the adsorbed copolymer was determined

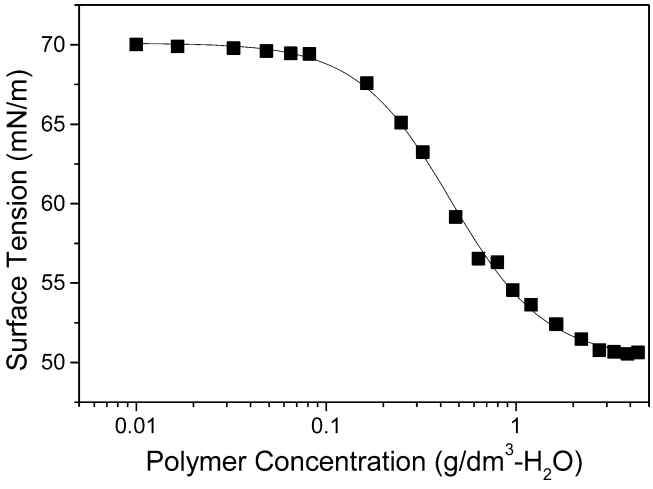


Fig. 1 A representative surface tension versus the graft copolymer (P750) concentration curve obtained from the pendant drop digitization technique

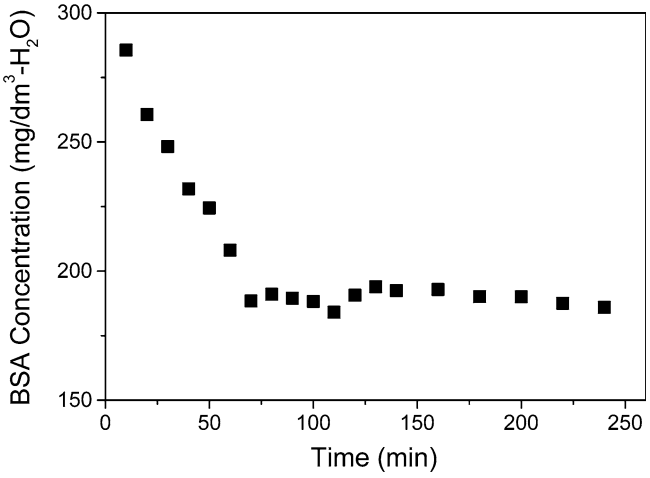


Fig. 2 Concentration of BSA in the supernatant phase as a function of time for the adsorption of BSA on native PS particles

by ^{1}H NMR spectra, based on a calibration curve of the integrated areas of the characteristic signal (3.65 ppm) for mPEG at different concentrations ([mPEG] (mg ml^{-1})) (area = 6.8623×[mPEG], coefficient of determination (r^2) = 0.9921). The fraction of copolymer that desorbed out of the particle surfaces during five cycles of centrifugation-dispersion treatment was then estimated.

The equilibrium adsorption of BSA on the copolymer-coated particles was then carried out by thoroughly mixing 0.14 g latex with 1 g aqueous solution with various concentrations of BSA (pH 5, 2 mM KHP) at 25 °C for 4 h. The weight percentage of particles was kept constant at 0.19%. This was followed by centrifugation of the sample at 13,000 rpm for 20 min (Hsiangtai, CNM) and filtration of the supernatant (0.22 μm PVDF membrane). The filtrate with a volume of 0.1 ml was then thoroughly mixed with 1 ml of Protein Assay (Bio-RAD) and allowed to stand for 5 min before determining the BSA concentration in the supernatant ([BSA], mg l^{-1})) by a spectrophotometer (Shimadzu UV-160A) at 595 nm along with a calibration curve (absorbance = 6.3×10^{-3} [BSA]−1.010^{-5} [BSA]^2, r^2 = 0.9923). The dynamic BSA adsorption data in Fig. 2 confirmed that 4 h were sufficient to assure the equilibrium condition.

Results and discussion

Both the numbers of mPEG and lauryl side chains for P2k-2 are about twice as large as those for P2k-1 (Table 1). The ratio of the number of mPEG side chains to that of lauryl side chains (N_{mPEG}/N_{LMA}) that is closely related to the hydrophile-lipophile balance should control the formation of micelles. As expected, slightly larger CMC values determined by two different methods were observed for P2k-2 in comparison with P2k-1

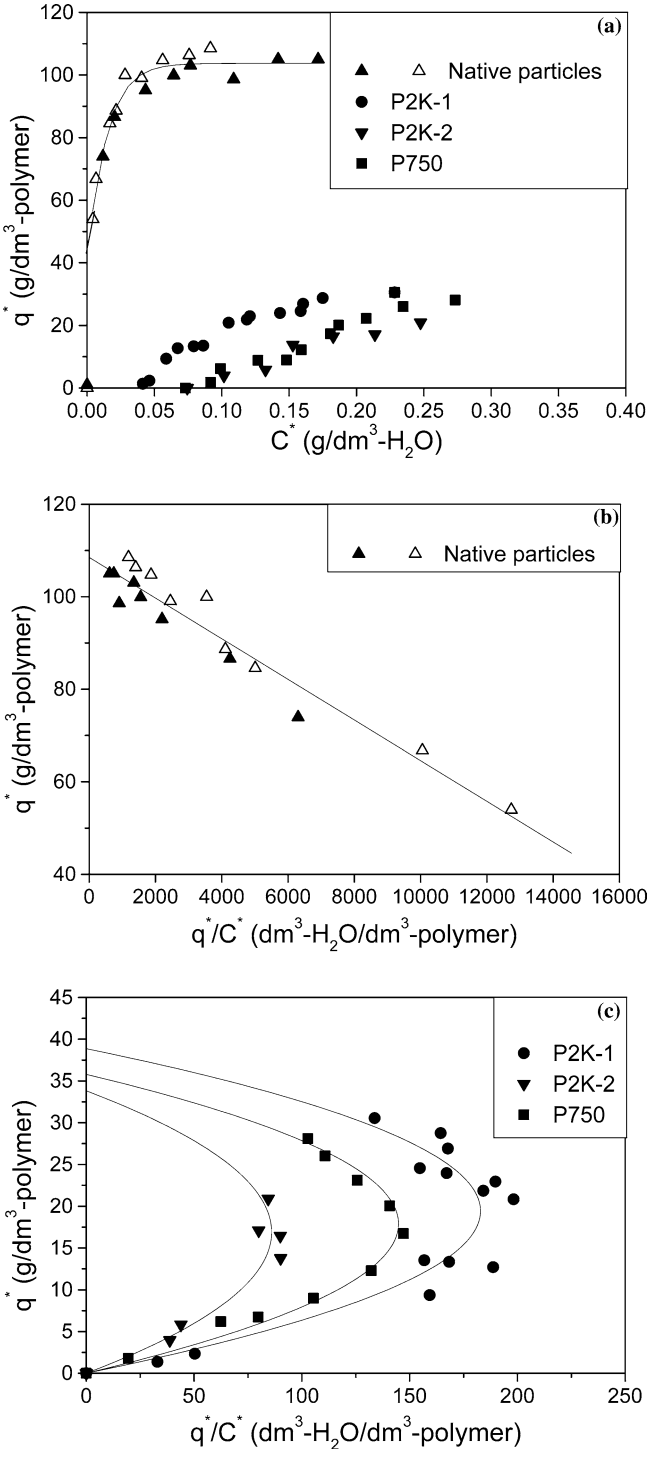


Fig. 3 **a** Langmuir isotherm curves and **b, c** Scatchard plots for BSA adsorbed on the particles. (*filled triangle*, *open triangle*) native particles; (*filled circle*) P2k-1-coated particles; (*filled inverted triangle*) P2k-2-coated particles; (*filled square*) P750-coated particles

because the N_{mPEG}/N_{LMA} of P2k-2 is larger than that of P2k-1 (Table 1). On the contrary, P2k-2 with a smaller N_{mPEG}/N_{LMA} has a higher CMC compared to P750.

This is because P2k-2 and P750 have quite similar numbers of mPEG and lauryl side chains. However, the mPEG chain length of P2k-2 is much longer than that of P750 and this will then make P2k-2 more hydrophilic.

The calculated surface concentration of the copolymer on the particles before five cycles of centrifugation-dispersion ($\Gamma_{p,0} = (CMC' - CMC) \times 10^3 / (M_n \pi d_v^2 N_p)$) and after the cleaning process (Γ_p) are listed in Table 1. For all the copolymers, the fraction of the copolymer that desorbed out of the particle surfaces during five cycles of centrifugation-dispersion ($F = (\Gamma_{p,0} - \Gamma_p)/\Gamma_{p,0}$) is quite large (Table 1). No apparent correlations between F and CMC ($r^2 = 0.0396$) and between F and N_{mPEG}/N_{LMA} ($r^2 = 0.2286$) can be observed, as shown by the very small r^2 obtained from the least-squares best-fitted linear relationships. Although the data are quite scattered ($r^2 = 0.6452$ for the F versus N_{LMA} data), the desorption of copolymer is most likely governed by N_{LMA}. The fact that P2k-1 has the smallest N_{LMA} (2.99) makes it easier for the copolymer to be removed from the particle surface ($F = 0.73$). To alleviate the problem associated with the weakly adsorbed copolymers, a polymeric structure containing more and/or longer hydrophobic side chains is highly recommended.

The Langmuir isotherm model [16–18] has been widely used to describe the equilibrium adsorption of protein on the particle surface:

$$q^* = q_{max} c^* / (K_d + c^*) \quad (1)$$

$$\text{or } q^* = q_{max} - (K_d q^* / c^*) \quad (2)$$

where q^* is the amount of adsorbed BSA per gram of particles, q_{max} the maximum amount of BSA that can be adsorbed on the particle surface, c^* the BSA concentration in aqueous solution and K_d is the dissociation constant for the BSA-binding-site pair. First, the equilibrium adsorption of BSA on the native particles was carried out twice and the reproducibility of the data was satisfactory (Fig. 3a). Eq. 2 predicts the BSA adsorption adequately ($r^2 = 0.9372$, Fig. 3b). On the other hand, the data do not follow the Langmuir isotherm model for the copolymer-modified particles (Fig. 3c). This is most likely due to the presence of a surface mPEG layer that severely retards the approach of BSA to the particle. The following empirical equation proposed by Suen [19] was used to fit the q^* versus q^*/c^* data:

$$q^*/c^* = q_{max}/K\prime_d q^* - 1/K\prime_d q^{*2} \quad (3)$$

where K_d' is the dissociation constant. The values of q_{max}, K_d and K_d' obtained from the least-squares best-fit are summarized in Table 2. The fact that q_{max} in decreasing order is Native $\gg$ P2k-1 > P750 > P2k-2 and K_d' in increasing order is P2k-1 < P750 < P2k-2 indicates that the presence of the mPEG-containing graft copolymers effectively reduces the BSA adsorption.

Table 2 Maximum amounts of adsorbed BSA and dissociation constants for the graft copolymer-modified latex particles

Latex	Native	P2k-1	P2k-2	P750
q_{max} (g dm^{-3})	108.5	38.9	33.8	35.8
K_d (g dm^{-3})	0.0044	–	–	–
K_d' (g dm^{-3})^2	–	2.07	3.33	2.21
r^2	0.9372	0.9588	0.9865	0.9825

Furthermore, the reduction of the BSA adsorption is closely related to the average distance between two adjacent mPEG chains on the particle surface (D), which is a measure of the surface mPEG density. At constant mPEG chain length, the higher the surface mPEG

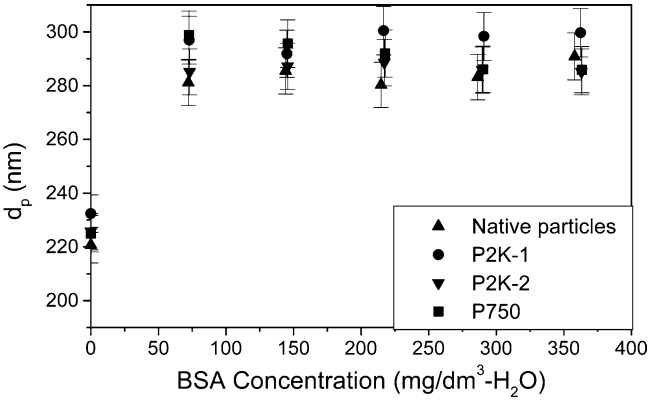


Fig. 4 Hydrodynamic particle diameter as a function of the BSA concentration in aqueous solution. (*filled triangle*) native particles; (*filled circle*) P2k-1-coated particles; (*filled inverted triangle*) P2k-2-coated particles; (*filled square*) P750-coated particles

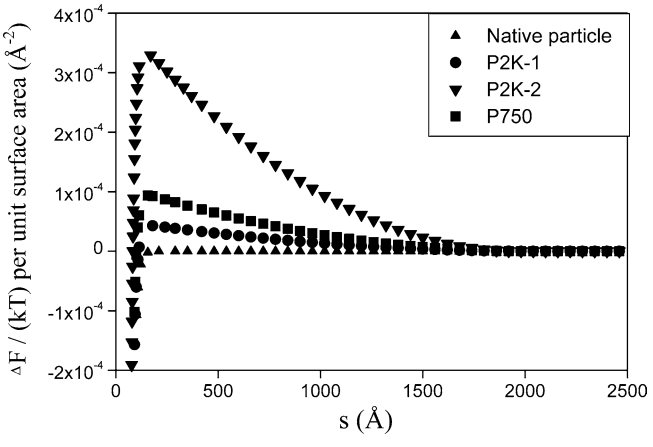


Fig. 5 Total free energy per unit surface area as a function of the separation distance between two hydrophobic surfaces. (*filled triangle*) native particles; (*filled circle*) P2k-1-coated particles; (*filled inverted triangle*) P2k-2-coated particles; (*filled square*) P750-coated particles

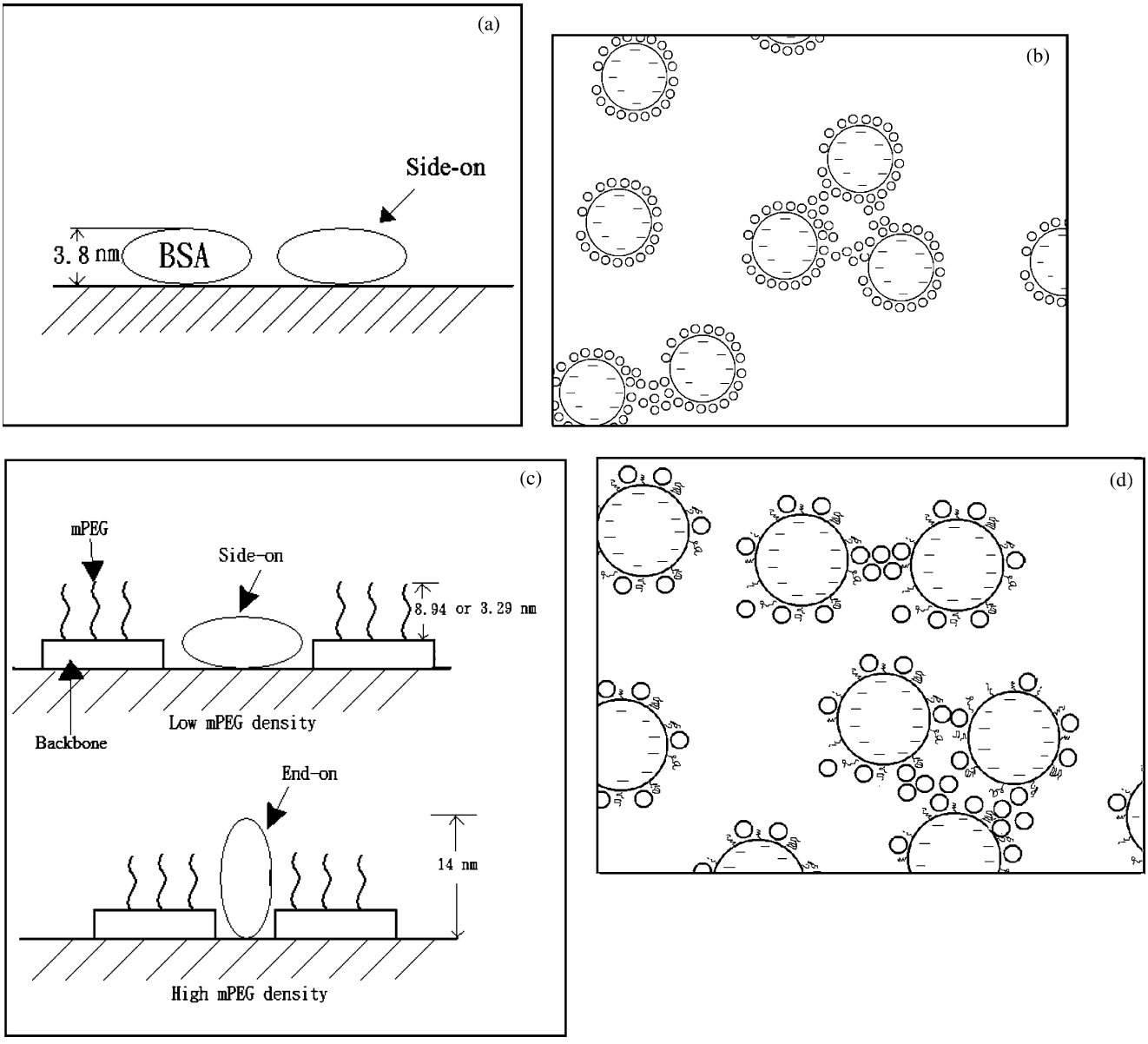


Fig. 6 Schematic representation of (**a**) the adsorbed BSA on the native particle surface, **b** the bridging aggregation of the native particles during the BSA adsorption, **c** the adsorbed BSA on the graft copolymer-modified particle surface and **d** the bridging aggregation of the graft copolymer-modified particles during the BSA adsorption. According to the meander model, the chain length is 8.9 and 3.3 nm for the mPEG 2000 and mPEG 750, respectively

density is, the smaller the q_{max} is (see the data for P2k-1 and P2k-2 in Tables 1 and 2). However, P750 with a smaller D results in a larger q_{max} than P2k-2. This is because P750 has a shorter mPEG chain length and, therefore, it exhibits inferior steric repulsive force toward the approaching BSA to the P2k-2 counterpart.

The driving forces for the BSA adsorption include van der Waals and hydrophobic interactions [20–25]. Assuming that van der Waals force is negligible (hydrophobic force ≫ van der Waals force at a separation distance below 20 nm [25]), Eqs. 4, 5, 6 were used to study the interactions between BSA and the particle surface [10, 11, 26]:

$$\Delta F_h/(kT) = -13.59e^{-s/14}\left(nm^{-2}\right) \tag{4}$$

$$\Delta F_s/(kT) = 4\pi kTC_{mPEG}^2(0.5-\chi_1)/\left(3V_1d_{mPEG}^2\right) \times [\delta-(s-2\delta)/2]^2[3r+2\delta+(s-2\delta)/2] \tag{5}$$

$$\Delta F/(kT) = \Delta F_h/(kT) + \Delta F_s/(kT) \tag{6}$$

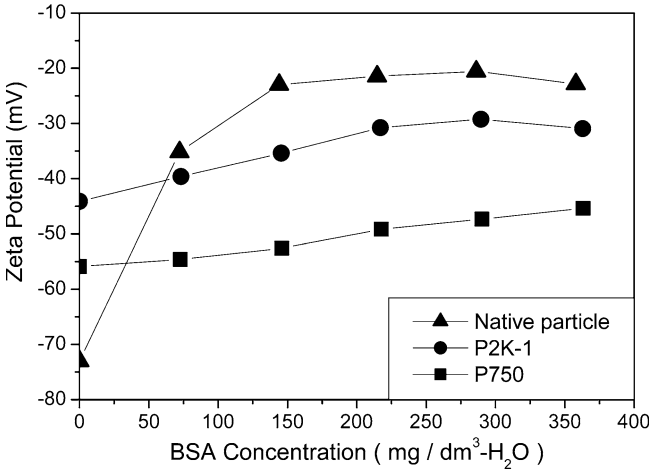


Fig. 7 Zeta potential of the colloidal system as a function of the BSA concentration. (*filled triangle*) native particles; (*filled circle*) P2k-1-coated particles; (*filled square*) P750-coated particles

where $\Delta F_h/(kT)$, $\Delta F_s/(kT)$ and $\Delta F/(kT)$ are the hydrophobic attraction free energy, steric repulsion free energy and total free energy per unit surface area, respectively. k is the Boltzmann constant, T the absolute temperature and s is the separation distance between two hydrophobic surfaces. C_{mPEG} is the adsorbed mPEG concentration (28.8, 82.2 and 43.6 kg m^{-3} for P2k-1, P2k-2 and P750, respectively), estimated by the amount of the adsorbed mPEG (^{1}H NMR) in combination with the difference in d_p between the particles with and without the surface mPEG layer (i.e., the surface mPEG layer thickness ($\delta = 1.02\times10^{-8}$, 9.85×10^{-9} and 9.92×10^{-9} m for P2k-1, P2k-2 and P750, respectively), see the d_p data at [BSA] = 0 in Fig. 4)). χ_1 is the mPEG–water interaction parameter $(=\chi_s + V_1 [(\delta_{1d}-\delta_{2d})^2 + (\delta_{1p}-\delta_{2p})^2]/(RT) = 0.34$, where χ_s is the entropic term (0.34),δ_{1d} and δ_{2d} are the dispersive solubility parameters ($J^{1/2}$ $m^{-3/2}$) for mPEG (1.62×10^4) and water (1.55×10^4), respectively, δ_{1p} and δ_{2p} are the polar solubility parameters ($J^{1/2}$ $m^{-3/2}$) for mPEG (9.21×10^4) and water (1.60×10^4), respectively [27], R is the gas constant, and V_1 is the molar volume of water). d_{mPEG} is the density of the surface mPEG layer (1×10^3 kg m^{-3}) and r is the particle radius. The native particles exhibit the strongest protein adsorption (Fig. 3a), as shown by the negative values of $\Delta F/(kT)$ in Fig. 5. Furthermore, the free energy barrier against the BSA adsorption in decreasing order is P2k-2 > P750 > P2k-1. As expected, the larger the $\Delta F/(kT)$ is, the smaller the q_{max} is. The BSA adsorption is dependent on the surface mPEG density and mPEG chain length, which is consistent with the theoretical work of Jeon et al. [28].

Considering the values of D and the dimension of BSA (14×3.8×3.8 nm) [29], it seems unlikely for BSA to adsorb onto the copolymer-coated particles. However, the data in Figs. 3 and 4 provide supporting evidence that the volume exclusion effect provided by mPEG cannot completely retard the BSA adsorption. It should be noted that either the surface mPEG layer thickness ($\delta\sim10$ nm) or the BSA monolayer with a thickness smaller than 14 nm alone cannot sustain the increase of d_p during the BSA adsorption (ca. 70 nm). For the native particles, the q_{max} value for a layer of spherical BSA molecules (7.2 nm in diameter [30]) with a thickness of 35 nm and a minimum void volume fraction of 0.26 is estimated to be 900 g dm^{-3}, that is one order of magnitude greater than the q_{max} data (108.5 g dm^{-3}). On the other hand, a loosely packed BSA layer with a thickness of 35 nm and a void volume fraction of 0.9 is required to accommodate this amount of BSA. This is unreasonable because the Scatchard equation describes the BSA adsorption adequately (Fig. 3b). The most probable structure is the small particle aggregates with the adsorbed BSA in the side-on form only, as shown in Fig. 6a, b. The following mechanism is proposed for the formation of the network structure. There are positive and negative groups along with the hydrophobic patches distributed over the BSA surface. In an aqueous environment close to the pI of BSA, a net charge near zero minimizes the electrostatic repulsion force between two BSA molecules. This may result in the intimate contact of BSA molecules. Furthermore, the minimum electrostatic repulsion force makes the intermolecular hydrophobic attraction among the interactive BSA species more important. Under the circumstances, BSA may form dimmers or even oligomers and the adsorbed BSA dimmers or oligomers may bridge the negatively charged PS particles or the copolymer-modified particles into the limited aggregates. The limited bridging flocculation of the particles during the BSA adsorption is supported by the changes in d_p (Fig. 4) and zeta potential (ζ) with increasing [BSA] (Fig. 7). The ζ (i.e., electrophoretic mobility) of the particles with the adsorbed BSA is expected to decrease with the increasing particle size. For the copolymer-modified particles, the distance between two surface mPEG chains may be shorter than those reported in Table 1 since the mPEG side chains are tied to the backbone of the copolymer. Thus, the space between two copolymer molecules may be sufficient for BSA to adsorb on the particle surface. Depending on the surface mPEG density, the adsorbed BSA may be in the side-on or end-on form (Fig. 6c, d). The higher the surface mPEG density is, the larger the probability of the adsorbed BSA in the end-on form is. Finally, in comparison with the native particles, the smaller reduction in the zeta potential implies a lower level of BSA adsorbed on the copolymer-modified particles (Fig. 7).

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