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BSA Adsorption and Immobilization onto Charged Monodisperse Polymer Nanoparticles

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Abstract

Controlling the behavior of adsorbed and immobilized proteins is essential for protein purification and a wide range of applications, including biosensors, biocatalysis and biomedical devices. In this study, monodisperse polymer particles were synthesized by soap free emulsion polymerization and the surface functional groups were directly introduced as a monomer or chemically modified with epoxy groups to enable protein immobilization through covalent bonding. Three kinds of surface charged particles having cationic, anionic, or both, groups were synthesized and characterized. Bovine serum albumin (BSA) was selected as a model protein to study the effect of pH of a buffer solution containing protein on the adsorption and immobilization amount. Protein adsorption was found to be strongly affected by pH and matching of the global protein and polymer particle charges, respectively. When pH was below the pI of either protein (pH 3.8), negatively charged polymer particles were found to adsorb a high amount of proteins. At maximum surface coverage of BSA on negatively charged polymer particles at pH 3.8 and after subsequent rinsing of the BSA-polymer particle complex with phosphate buffer (pH 7), ~50% of BSA was desorbed. Therefore, 50% of BSA was adsorbed by ionic interaction and the remaining fraction was immobilized covalently at pH 3.8. The remaining immobilized fraction was sufficient to completely shield the anionic charge of the polymer particles. Fluorescence spectroscopy suggests that at maximum immobilized amounts, the conformation of immobilized BSA appears to be the same as in aqueous solution.

Keywords: Monodisperse polymer particles; Protein conformation; Molecular spectroscopy; Protein immobilization; Bovine serum albumin

Introduction

A conformation change of protein is one of the most important studies for biochemistry and biotechnology. Protein conformation regulates its functions, such as interaction with other molecules, catalytic activity, binding against various specific targets, the formation of three dimensional constitution, motility characteristic of muscle or flagellum, and so on [1,2]. To utilize proteins for use of medicine, catalyst, bioactive reagents, the relationship between their conformation and their functions is important. However, it is very difficult to clarify a mechanism and pathway of the conformational change of various proteins including their denaturation. This was because the majority of micro-domain conformational change was depended on the amino acid sequence of protein [3] and these processes was controlled by many factors, such as steric hindrance, interaction with other amino acids residue or other organic compounds, hydrogen bonding, covalent disulfide bonding, folding behavior of helix and sheet micro domain, and so on. Nowadays, from the protein refolding and the studies using X-ray analysis, a collapse of regular and cyclic three dimensional conformations, such as α - helix and β - sheet, have been well studied using BSA [4], RNAse [5], and lysozyme [6] as a model protein. For the refolding study, protein conformation has various substantial states and the existence of intermediate has been proved [7]. From the study of properties of the intermediate states formed at equilibrium folding and unfolding of globular proteins, the structural description of these intermediates was studied and the existence of thermodynamically stable intermediates between the native and fully unfolded states was proved. This class of intermediate states has been called the "molten globule" intermediate state [7]. Conformational change between native form and molten globule is very slow and reversible. When protein attains molten globule form, it lacks quickly three dimensional conformation and becomes random coil conformation [8].

Enzymes are usually not stable at a high temperature and in the presence of organic solvents. Therefore, while enzymatic reactions in organic solvents or aqueous solutions containing organic solvents have several advantages [9], enzymatic reactions were limited within their stable reaction conditions. To overcome this disadvantage, enzyme immobilization is effective and many researches to develop thermal stable, pH stable, and organic solvent tolerant enzymes [10] have been taking place. Enzyme immobilization is regarded as the effective method to keep protein conformation native [11]. However, there is few studies concerning conformational change on the support and the effect of immobilization on the conformational change is still unclear. Furthermore, nowadays some disease, such as Alzheimer's and Parkinson's, were caused by the conformation change and their critical step was the formation of toxic multimeric, soluble β -sheet aggregates and subsequent precipitation of amyloid fibrils is the time-dependent α -helix → β-sheet assembly [12]. Some measurement systems for protein

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conformation change on a support have been developed. However, these methods had some restrictions for colloidal stability, low amount of immobilized proteins, dispersity of aqueous solution, and overlap of spectra with protein. Therefore, there is a need for a new system which can measure at various pH values, in the presence of organic solvent, and can immobilize large amounts of protein on its surface.

The objective of this work is to develop a support of protein immobilization with free restriction and the evaluation of conformation change of immobilized proteins on the support. For this purpose, three kinds of monodisperse polymer particles having cation, anion, or both ions were synthesized by soap free emulsion polymerization and chemical modification of surface function group as a first stage. Then, BSA was adsorbed and immobilized at various pH and protein concentrations. Finally, the amount of immobilized protein, zeta potential of immobilized protein on polymer particle at various pH, fluorescence spectra of immobilized protein were measured to analyze conformation change of immobilized protein.

Materials and Methods

2,2'-Azobis (amidino propane) dichloride salt was purchased from Wako Pure Chemical Co. (Osaka, Japan). Ammonia aqueous solution (25%) and hydrochloric acid were purchased from VWR PROLABO International (Paris, France). Ammonium persulfate, bovine serum albumin (BSA), 1,4-butanediol diglycidyl ether, chloroform, coomassie brilliant blue G-250, disodium hydrogen phosphate, divinyl benzene (80% mixture of isomers), glycidyl methacrylate (GMA), sodium tetraborate decahydrate, and styrene monomer were purchased from Sigma-Aldrich Co. (ST. Louis, MO, USA). Orthophosphoric acid (85%) and acetic acid were purchased from E. MERCK KG (Darmstadt, Germany). All of the reagents were used without purification.

Polymer particle synthesis

Polymer particles for protein adsorption support were synthesized by soap-free emulsion polymerization [13]. First, 1.8 g of GMA, 0.04 g of divinyl benzene, 1.2 g of styrene monomer and 95 ml of water were added to a 300 ml five-neck reaction flask. The reaction vessel was immersed in an oil bath at 70°C, and the reaction mixture was stirred at 100 rpm for 15 min. When the temperature of the reaction mixture reached 70°C, 0.06 g of initiator was dissolved in 5 ml of water and the resulting initiator aqueous solution was added to the reaction mixture. After 2 hours, 0.3 g of GMA was added to the reaction mixture. The reaction mixture was heated for 16 hours. Monomer conversion was measured by a gravimetric method, and the amount of epoxy groups on the polymer particles was measured by the hydrochloric acid-dioxane method [14]. Following polymerization, the reaction mixture was centrifuged at 13,000 rpm for 10 min, and the precipitated polymer particles were suspended in distilled water. Centrifugation, discarding the supernatant and suspending polymer particles with distilled water (hereafter referred to as particle wash) was repeated three times. In the case of synthesis of polymer particles having both anionic and cationic surface groups, 1 g of negatively charged polymer particles was dispersed in 3 M of ammonia solution using ammonium persulfate as an initiator and incubated at 70°C for 24 hours. Produced polymer particles were washed and dispersed in 100 ml of 100 mM 1,4-butanediol diglycidyl ether aqueous solution. The particle suspension was incubated for 24 hours at 30°C. After the reaction, the polymer particles were suspended in water and stocked with a refrigerator (4°C). The synthesized particles are hereafter referred to as cationic polymer particles (cationic PP), anionic polymer particles (anionic PP), and zwitterionic polymer particles (zwitterionic PP) depending on their surface charge.

Protein adsorption and immobilization on polymer particles

In this study, epoxy groups introduced on the polymer particle surface were reacted with amino groups on the protein [15], such as lysine, histidine and arginine residues, and protein was covalently adsorbed on the polymer particle. To investigate the effect of adsorption on the protein conformation, we chose BSA as a model protein. The pH of the protein solutions was regulated using either 10 mM of sodium acetate (pH 3.8), sodium phosphate (pH 7.0), or sodium borate (pH 9.0) as buffer solutions. In the adsorption step, 10 μM of protein stock solution in water, polymer particle suspended in buffer solution, 100 mM buffer stock solution, and Milli-Q water until the final buffer concentration was 10 mM were added to a 2 ml poly(propylene) sample tube. The final mixture contained 1 μM of BSA and 7.5 mg of polymer particle, respectively. The total volume of the mixture was adjusted to 2 ml. The adsorbed amounts at 4°C were measured after of 24 hour incubation. The mixture of protein and polymer particle was incubated at 4°C for times ranging from 5 min to 24 hours. Following adsorption, the sample tube was centrifuged at 14,500 rpm for 10 min and the remaining protein concentration in the supernatant was measured. Centrifuged polymer particles were washed and suspended in fresh buffer solution. The amount of adsorbed protein on the polymer particles was calculated by the difference between the initial protein concentration of a reference solution and the protein concentration remaining in the supernatant. Protein concentration was measured using the Bradford method [16].

Polymer particle characterization and analysis of adsorbed and immobilized protein

A 1000-fold diluted solution of the polymer particle stock solution (75 mg·ml^{-1}) with Milli-Q water was loaded into a 1 cm × 1 cm poly (styrene) cell, and polymer particle size and its distribution were measured using a Zetasizer 3000HSA (Malvern Instrument Ltd., Malvern, UK). The zeta potentials of the polymer particles, protein solution, and protein adsorbed on polymer particles were measured using a Zetasizer Nano ZS (Malvern). Here, 10 ml of the sample solution was prepared and loaded into the pH titration unit (MPT-2, Malvern). pH of the sample solution was varied from 3 to 10 using 0.25 M of sodium hydroxide or 0.25 M of hydrochloric acid. The pH adjusted sample solution was circulated in the Zetasizer and zeta potentials at various pH were automatically measured. FE-SEM microphotographs were taken using a JSM-6700FW (JEOL, Tokyo, Japan). The particle pore size and its distribution were measured by N_2 gas adsorption apparatus (Micromeritics, Norcross, GA, USA). Here, 0.5 g of sample was added to the glass sample tube and degaussed using a VacPrep 061 sample degaussing system. Particle pore size and its distribution were analyzed by BET isotherm theory [17], using the BJH method under the assumption of cylindrical pores [18]. Fluorescence emission spectra were measured using a Fluorolog-3 (Horiba Jobin Yvon, Paris, France). Protein solution or a mixture of protein and particle was added to the quartz cell and the detector was placed in the 90° direction against an excitation beam. The excitation and emission slit widths were set at 5 nm and the integration time was 5 sec. For calibration, fluorescence emission spectra of the particle suspension in various buffer solutions were also measured. In the case of adsorbed and immobilized protein, fluorescence emission spectra included particle contribution for fluorescence emission spectra. To obtain fluorescence emission spectra of immobilized protein, measured fluorescence emission spectra were numerically divided from the fluorescence emission spectra of particles.

Results and Discussion

Synthesis and characterization of monodisperse polymer particles

Three types of charged monodisperse polymer particles having epoxy groups were synthesized by soap free emulsion polymerization of glycidyl methacrylate, styrene and divinyl benzene, with divinyl benzene acting as a cross-linking agent. Figure 1 illustrates the synthesis procedures employed here. It should be noted that the anionic polymer particles can also be synthesized using ammonium persulfate as initiator, and the cationic polymer particles can also be synthesized using 2,2'-azobis (amidino propane) dihydrochloride salt. The epoxy groups on the anionic polymer particles were further decomposed using aqueous ammonia, and cationic amino groups were introduced. In order to introduce epoxy groups onto the particles, one side of the epoxy group of 1,4-butanediol diglycidyl ether was reacted with a particle surface amino groups.

The particle size distributions were measured by dynamic light scattering. Average particle sizes of anionic PP, cationic PP, and zwitterionic PP were about 200 ± 20 nm with very narrow (~ monodisperse) size distributions. The particle surface appeared smooth and no surface pores could be observed from the SEM microphotographs.

From zeta potential measurements in Milli-Q water, the average zeta potential of anionic PP, cationic PP, and zwitterionic PP were –46, 27, and 10 mV, respectively. Table 1 shows mean zeta potentials of particles in 10 mM of various buffer solutions. Although there are some buffer effects on the zeta potential of the polymer particles, zeta potential value of anionic PP, cationic PP, and zwitterionic PP show that the anionic PP remained negatively charged throughout the entire pH region studied here. The cationic PP remained positively charged in the pH range between pH 3.8 and pH 8.0, with the value of the zeta potential decreasing with increasing pH values. When pH is 9.0, amidino group of cationic PP is neutralized by borate ions and its surface has a negative charge. For the zwitterionic PP, the cationic groups were dominant under acidic conditions, whereas the anionic surface groups dominated the charge behavior under basic pH conditions. The zwitterionic PP display an isoelectric point between pH 5.0 and pH 6.0 Table 1. Thus, we can introduce the desired charge on the particle surface, with all of the surface charges being homogeneous except for the zwitterionic PP.

From the surface area distribution of anionic PP measured by BET N_2 adsorption Table 2, there is some hint of mesopores between 8 nm and 48 nm in anionic PP. The total surface area of the pores ranging 2 nm to 43 nm was 15.7 m^2 (g-particles)$^{-1}$. The outer surface area of anionic PP was calculated to be 27.6 m^2 (g-particles)$^{-1}$ (one gram of particle was 2.17×10^{14} particle calculated by its density (1.1×10^3 kg m^{-3}) and the surface area per particle was calculated by multiplying this value with ($\pi \times (200\ nm \times 10^{-9})^2$). Both results are roughly similar value to the calculated outer surface area.

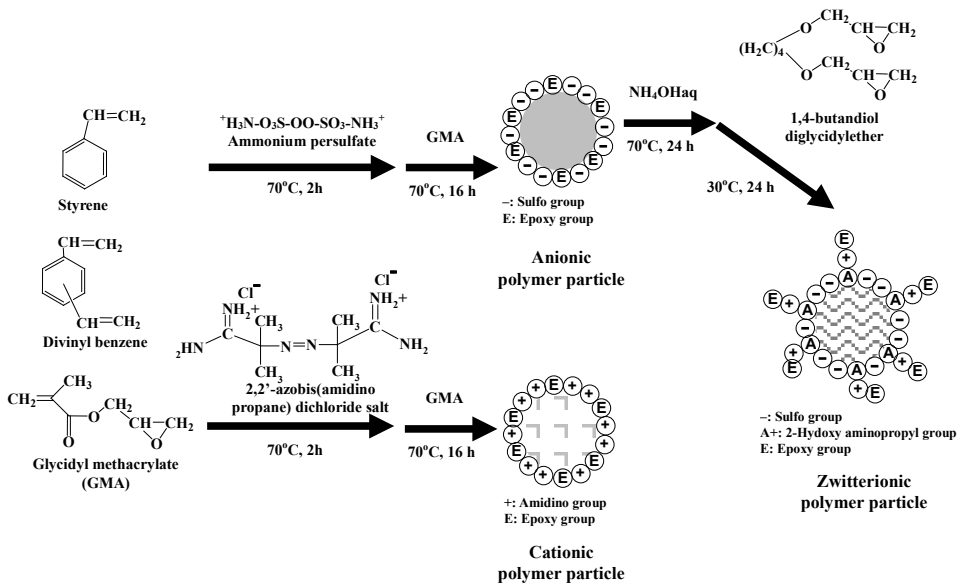

Figure 1: The synthesis procedures of polymer particle.

Particle	Zeta potential in 10 mM of buffer solution (mV)					
	Sodium acetate	Sodium phosphate	Sodium phosphate	Sodium phosphate	Sodium phosphate	Sodium borate
	pH (-)					
	3.8	5	6	7	8	9
Anionic PP	–33.4	–18.2	–19.4	–19.3	–19.4	–34.9
Cationic PP	31.5	6.3	6.4	5.7	4	–24.4
Zwitterionic PP	47.5	5.5	–2.7	–11.6	–24.8	–37.7

Table 1: Zeta Potential of polymer particles in various buffer solutions.

BET N_2 absorption isotherm		
Average pore size (nm)	Incremental pore area (m^2/g-particles)	Cumulative pore area (m^2/g-particles)
42.7	3.28	15.7
29	3.75	12.4
20.6	2.58	8.63
16.1	1.25	6.05
13.3	0.828	4.8
11.2	0.574	3.97
9.6	0.443	3.4
8.4	0.298	2.96
7.5	0.242	2.66
6.7	0.171	2.42
6.1	0.136	2.25
5.5	0.096	2.11
5.1	0.085	2.01
4.7	0.088	1.93
4.3	0.089	1.84
4	0.075	1.75
3.7	0.069	1.68
3.5	0.074	1.61
3.2	0.084	1.53
3	0.111	1.45
2.8	0.105	1.34
2.6	0.114	1.23
2.5	0.14	1.12
2.3	0.169	0.98
2.25	0.054	0.811
2.2	0.065	0.757
2.15	0.059	0.692
2.1	0.049	0.633
2.05	0.064	0.584
2	0.06	0.52
1.96	0.054	0.46
1.93	0.064	0.404
1.9	0.071	0.34
1.85	0.083	0.269
1.8	0.085	0.186
1.7	0.101	0.101
> 1.7	0	0

Table 2: Pore area distribution measured by Bet N_2 adsorption.

BSA adsorption and immobilization on monodisperse polymer particles

In addition to charged groups, the monodisperse polymer particles synthesized here were also functioned with epoxy groups which can react with amino groups such as lysine on the protein surface (Figure 1). We expected that the surface charge density matching between protein and polymer strongly affects the reaction probability between epoxy groups on the particles and protein amino groups. BSA is one of the most well-characterized proteins with respect to both structure and properties, and is known to reversibly change its conformation with pH [4]. The pH-dependent BSA conformations have been classified as native form (around pH 7), fast migrating form produced abruptly (pH 3.5 - 4.3), expanded form (pH < 3.5), and basic form (pH > 8) [4,19]. Moreover, albumin is the most abundant plasma protein. Therefore, it was a convenient model for the fundamental studies of protein adsorption and conformational change on the polymer particles. For BSA, the number of acidic amino acid residues (asparagine and glutamine) was 99 and the number of basic amino acid residues (lysine and arginine) was 82 [20]. An abundance of acidic amino acid residues results in an acidic pI, whereas basic amino acid residues form covalent bonds with epoxy group on the polymer particle. pIs of BSA are 4.8-5.0. From global charge considerations, we expected anionic PP to be the most efficient below pI, while cationic PP is most efficient above pI, with zwitterionic PP being capable of adsorption throughout the entire pH range studied here. To prevent pH changes attributed to the counter ions of the ionic polymer particles, three kinds of buffer systems were adopted here; at pH 3.8 (sodium acetate buffer), 7.0 (sodium phosphate buffer), and 9.0 (sodium borate buffer). The maximum adsorbed amounts of BSA on the three kinds of polymer particle and three pH conditions studied here are shown in Table 3. To rule out non-specific protein adsorption, the protein-polymer particle constructs were precipitated by centrifugation and resuspended in a 10 mM buffer solution. This particle wash procedure was repeated three times. From the protein amount of removed supernatant and dilution ratio calculated from the added amount of buffer solution, the amount of liberated protein was negligible.

The amount of adsorbed protein is controlled by surface charge density matching between protein and polymer particles. When BSA was adsorbed on the anionic PP in acetate buffer (pH 3.8), the polymer particles precipitated slowly and reversibly in the course of 5 minutes. This indicates that the protein-particle constructs formed by the anionic PP softly aggregated, with the positive charge of adsorbed BSA acting as a binder between negatively charged particles.

In the case of cationic PP, amino groups derived from initiator fragments can react with epoxy groups on the polymer particle surface, lowering the number of surface epoxy groups [21]. The total epoxy amount of cationic PP was found to be 95% of that of anionic PP. The decomposition of epoxy groups around the surface slightly contributed to the lower adsorbed amount of BSA. The adsorption mixture of cationic PP and BSA in 10 mM phosphate buffer (pH 7.0) also precipitated, whereas at pH 9, only slight precipitation could be observed. However, at pH 9, the adsorbed amount was small compared to that at pH 7 (Table 3), resulting in a low protein-induced precipitation rate. This can be ascribed to negatively charged BSA adsorbed on the surface of cationic PP acting as a binder between cationic PP.

In the case of zwitterionic PP, epoxy groups of anionic PP were first reacted with ammonia, and the introduced 2-hydroxy amino group was further reacted with one end group of 1,4-butanediol diglycidyl ether Figure 1. Therefore, unreacted epoxy groups from 1,4-butanediol diglycidyl ether remained on the particle. Due to significant steric hindrance in the polymer network, the reaction between amino-

Particle	Maximum adsorbed amount (μg-protein/mg-particle) pH (-)		
	3.8	7	9
Anionic PP	28.86[A]	0.79[R]	0.33[R]
Cationic PP	1.00[R]	2.02[A]	0.96[R]
Zwitterionic PP	2.27[R]	1.26[R]	2.92[R]

[A]: attractive forces act between polymer particle and protein

[R]: repulsive forces act between polymer particle and protein

A or R is judged by the pI of protein and Zeta potential of polymer particles in various buffer solution as shown in Table 1.

Table 3: Maximum adsorbed amount of BSA on polymer particles at various pH.

and epoxy groups was restricted to the particle surface [13]. The amount of epoxy groups (0.4 mmol (g-particle)$^{-1}$) introduced to the particles as described here was severely limited by comparison with the introduction as a monomer (4 mmol (g-particle)$^{-1}$). However, the resulting adsorbed amount of BSA was high considering that the total epoxy amount of zwitterionic PP was about 10% of that of anionic PP (Table 3). Moreover, pH did not affect the amount of protein adsorbed on the zwitterionic PP due to the presence of both amino groups and sulfo groups. As shown in Table 3, protein adsorption is strongly dependent on the relationship between the surface charge of the polymer particle (A or R) and the global protein charge. Here, protein can access oppositely charged polymer particles, allowing amino groups on the protein to react with epoxy groups on the polymer particle surface. In other words, the reaction between epoxy groups on the polymer particles and amino groups on the protein is inhibited by the same charged ion barrier.

The adsorbed amount of BSA of anionic PP at pH 3.8 was the highest in all tested in this study and 10-20 times higher than finding at other conditions. This can be attributed to a severe increase of protein hydrophobicity close to the pI (BSA; 4.8–5.0), with concomitant formation of dimers or multiple protein aggregates [22]. BSA adsorption can be well explained by global charge density matching and their multimer formation. This can be explained either by multilayer formation of BSA on anionic PP, or by optimized packing of the protein layer under these conditions. Since a small portion of liberated protein could be detected from the precipitated and washed anionic PP in the presence of high concentration of salt and the majority of adsorbed protein was remaining on anionic PP, we conclude that within the ~1% detection sensitivity of this method, all of the remaining protein was covalently bound to the polymer particles. Fluorescence results, described below, are also consistent with this interpretation. As anionic PP at pH 3.8 was found to result in maximum protein adsorption, these conditions were adopted for the remainder of this study.

Protein adsorption kinetics

When 7.5 mg of polymer particles was added to 2 ml of 1 μM of BSA in 10 mM of buffer solution and equilibrated for 24 h at 4°C, free protein was detected in the supernatant after centrifugation (14500 rpm, 5 min). This was because all of the available epoxy groups on the surface were occupied by the adsorbed protein, blocking additional free protein from further access to epoxy groups by centrifugation. Since BSA has 82 of lysine and arginin residues, respectively, multipoint covalent bonding could be formed at low fractional surface coverage, likely resulting in conformational changes in the adsorbed protein. Typically, the rate of conformational change after adsorption and aggregates formation of adsorbed protein (on a particle or between particles) are relatively slow compared to the reaction between protein amino groups and epoxy groups on the polymer particle. Therefore, a change in long term period was measured.

When the incubation time of polymer particles with protein solution was shortened to 3 hours, the adsorbed amounts were almost the same values as shown in Table 3. This indicates that the reaction between epoxy groups on the polymer particle and protein amino groups was complete after 3 hours. To further investigate the kinetics of protein adsorption, we measured time-dependent adsorption of BSA at the conditions of maximum protein adsorption (anionic PP at pH 3.8) from 5 min to 60 min. Since it took 5 min to precipitate polymer particles using centrifugation, we could not take obtain data points at shorter times than 5 min. The adsorbed amounts of BSA from 5 min to 60 min were almost the same within the experimental error, indicating that the adsorption was completed within 5 min and the effect of conformational change in adsorption amount was negligible. Using unsteady state diffusion theory [23], we can estimate that the adsorption was finished within 0.35 - 3.49 seconds (it depended on the absorption amount of BSA on the particle). These results support that adsorption occurs almost instantly and maximum adsorption amount was attained within 5 minutes in our experimental condition in which excess amount of protein exists in the supernatant.

Protein desorption from polymer particles

BSA was adsorbed on three kinds of polymer particles, with the adsorbed amount being affected by pH as shown in Table 3. To clarify whether the proteins were immobilized or adsorbed, i.e.; covalently or electrostatically attached, polymer particle-protein constructs were rinsed with 1 M of NaCl aqueous solution after protein adsorption. When protein is adsorbed on ion-exchange resins, desorption is accomplished using aqueous solutions containing high concentration of salt [24]. Therefore, desorption of protein reveals whether ion interaction or epoxy group-amino group conjugation is predominant for protein adsorption on the polymer particles used here. Prior to the desorption experiment, protein desorption via use of centrifugation and buffer change was investigated, with neither process yielding any measurable desorption.

Under the conditions used in this study, the detection limit for the Bradford method was found to be a difference of 2 μg (ml)$^{-1}$. For a system consisting of 7.5 mg polymer particles with an adsorbed amount of 28.86 μg (mg-particle)$^{-1}$, rinsing with 100 μl of salt containing buffer resulted in 0.46% desorption of the adsorbed BSA. However, for systems with low immobilization amounts, such as 0.33 μg (mg-particle)$^{-1}$ (BSA, pH 9.0, Anionic PP), 10% desorption of adsorbed protein could be detected. Under conditions of maximum adsorbed amounts of BSA onto anionic PP (at pH 3.8; 28.86 μg (mg-particle)$^{-1}$), addition of 1M NaCl to the aqueous buffer resulted in ~15% desorption of BSA. Interestingly, no desorption of BSA (except at pH 3.8) from polymer particle could be detected upon addition of 1M NaCl to the buffer solutions, indicating that with the exception of BSA adsorbed onto anionic PP at pH 3.8, BSA was covalently immobilized on the polymer particles.

Since BSA desorption was detected for BSA adsorption onto anionic PP at pH 3.8, the effect of pH on the BSA desorption was further studied. After preparation of BSA adsorbed on anionic PP (28.86 μg (mg-particle)$^{-1}$), BSA-polymer particle constructs were rinsed with 10 mM of acetate buffer, precipitated by centrifuge and resuspended in 10 mM of phosphate buffer (pH 3.8, 4.2, 4.7, 5.0, 6.0, 7.0, 8.0, or 9.0) or 10 mM of borate buffer (pH 9.0). In the region pH 6 - 10, ~ 50% desorption of BSA was measured, whereas at lower pH values, the percentage of desorbed BSA was measured to be 0, 0, 22.4, and 43.6% at pH 3.8, 4.2, 4.7, and 5.0, respectively. This result indicates that the reversibility of BSA adsorption, i.e.; desorption is strongly linked to the solution pH relative to the pI of BSA (4.8), and that ~ 50% of BSA adsorbed onto anionic PP at pH 3.8 was desorbed by inversion of surface charge. This result also indicates that at pH 3.8, ~ 50% of BSA adsorbed onto anionic PP was adsorbed by ionic interaction and the remaining fraction was immobilized covalently. The desorption percentage resulting from addition of 1M NaCl was significantly lower that resulting from a pH change in a 10 mM buffer. When 10 mM of NaCl was added to the suspension of anionic PP at pH 3.8 on which 28.86 μg (mg-particle)$^{-1}$ of BSA was adsorbed, the desorbed amount is 50% of the adsorbed amount. Therefore, the high concentration of salt affects the inhibition of BSA desorption, and hydrophobic interactions

attributed to salting out may be enhanced by the addition of salt.

Next, the effect of BSA surface concentration on desorption was studied. BSA was adsorbed onto anionic PP at pH 3.8, and surface concentrations were varied from 1.88 μg (mg-particle)$^{-1}$ to 28.86 μg (mg-particle)$^{-1}$. After the rinsing and centrifugation steps, BSA-PP constructs were resuspended in a 10 mM phosphate buffer (pH 7.0). Percentages of desorption for surface-bound BSA at surface concentrations of 28.86, 19.48, 10.75, 5.05, and 1.88 μg·(mg-particle)$^{-1}$ were 50, 23.7, 0, 0, and 0%, respectively. As expected, desorption is strongly dependent on the surface concentration, and the remaining amount of BSA was calculated to be 14-15 μg (mg-particle)$^{-1}$. Since the excess adsorbed BSA on anionic PP at pH 3.8 reached the same molar amount with that of immobilized BSA, we make the assumption that BSA forms dimers at pH 3.8. Rezwan et al. studied BSA adsorption on colloidal Al_2O_3 particles, and an adsorption model of BSA in which BSA forms dimers around pH 5.0 (near pI of BSA) was presented [25]. The driving force of BSA dimer formation is hydrophobic interactions between the hydrophobic domain III of BSA molecules, which are enhanced close to the pI. At pH 3.8, the strongly positive charged domains I and II, where many of the ε-amino group of Lys are localized, approach the surface of the polymer particle by charge-charge interaction with sulfo groups on the anionic PP, placing the hydrophobic domain III in a reverse position against the particle surface in a side-on monolayer [25]. In turn, the exposed hydrophobic domain III of adsorbed BSA molecules is favorably positioned for interaction with hydrophobic domain III of free BSA, leading to surface-induced dimer formation. In our case, masking of surface charges attributed to sulfo groups on anionic PP by side-on monolayers was not complete, and 15% of adsorbed BSA residing as surface-bound dimers from charge-charge interaction with sulfo groups was desorbed by the addition of salt. As shown in Figure 2, the difference between free BSA and adsorbed and immobilized BSA in pH-zeta potential profile existed. Since 1M of NaCl addition to buffer solution enhanced hydrophobic interaction, the remaining 35% of adsorbed BSA remained as a dimer layer. Upon increasing the pH above the pI of BSA, the global BSA charge changes from positive to negative, resulting in complete desorption of the outer, weakly bound layer of the BSA dimers.

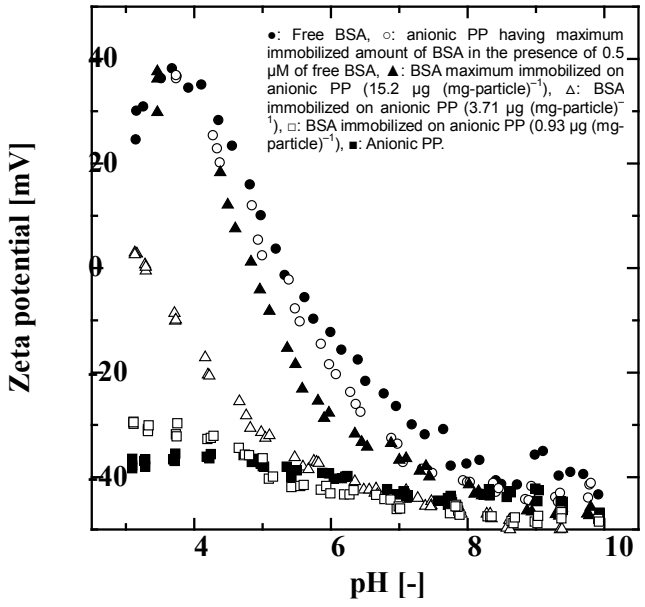

Figure 2: The effect of pH on the zeta potential of BSA immobilized anionic PP. BSA was immobilized with 37.5 mg of by anionic PP at pH 3.8 under various BSA concentrations. Anionic PP was washed with Mili-Q water the centrifuge. Wash was repeated 3 times and the anionic PP was suspended in 10 ml of Mili-Q water. pH was adjusted at 3.0 using 0.1 M of HCl aqueous solution.

Effect of surface protein coverage on zeta potential of anionic PP

To study whether the polymer particle surfaces were completely covered with protein and whether conformational changes occurred in the adsorbed protein, zeta potentials of anionic PP-protein constructs were measured in the pH interval between 3.0 and 10.0. The amount of adsorbed protein was varied from 5-100% of maximum surface coverage as described above and adsorbed protein was immobilized to anionic PP at these immobilization amounts. After adsorption, the anionic PP - protein constructs were washed with Milli - Q water to remove excess (free) protein and suspended in Milli - Q water. After adjusting pH to 3.0 with aliquots of 0.1 M HCl, the zeta potentials of free protein, anionic PP, and anionic PP-protein constructs were measured as a function of pH. Figure 2 shows the zeta potential titration curves of free and immobilized BSA. The zeta potential of anionic PP was strongly negative, ranging from - 48 to - 35 mV in this pH region. For BSA only, the zeta potential is strongly positive at low pH (pH 3 - 4), with the absolute value decreasing with increasing pH. At pH 5.6, the zeta potential of BSA reaches zero, while at higher pH the protein shows a negative charge. The difference between pI reported by previous works [26] and that determined in this study may be attributed to the ionic strength of water, which is known to affect the pI of proteins [27]. When anionic PP - BSA constructs with maximum surface coverage were dispersed in the presence of 0.5 μM free BSA, the resulting pH - zeta potential profile is very similar to that of anionic PP - BSA constructs at maximum surface coverage without any excess BSA, with a pI residing between anionic PP - BSA at maximum surface coverage and that of free BSA. For this sample, the measured signal is the sum of contributions from free and immobilized BSA, yielding an intermediate pH - titration profile. At low pH values, the zeta potential of anionic PP - BSA constructs is shifted from positive to negative values with decreasing immobilization amounts, indicating that BSA completely blocks the anion charge of the polymer particle at maximum immobilization amount in this study as described in protein desorption from polymer particles.

From the surface area of anionic PP (1.27×10^{-2} m^2 (mg-particles)$^{-1}$ (pore diameter is between 8.4 nm and 42.7 nm in Table 2) as calculated from the BET adsorption isotherm and the molecules number of 15.2 μg of BSA (composed of 1.35×10^{14} molecules), we calculated the area occupied by one adsorbed BSA molecule to be 9.41×10^{-17} m^2 (BSA molecule)$^{-1}$. Here, we assume that BSA has an approximate spherical shape in aqueous solution. The hydrodynamic diameters of BSA at pH 3.8 were 7.9 ± 0.9 nm measured by Zetasizer. We estimated the mean molecular projection area of one BSA molecule to be 4.90×10^{-17} m^2 (BSA molecule)$^{-1}$. These estimated the occupied areas were almost the same level as that of occupied area, indicating that BSA completely covered the surface of anionic PP at 15.2 μg (mg-particles)$^{-1}$ of BSA immobilization amount.

Steady-state fluorescence spectra of protein immobilized on anionic PP

Fluorescence spectroscopy is regarded as a good technique with which to evaluate BSA conformational change. BSA has two tryptophan (Trp) residues, one of which resides close to the surface and one which is buried in the hydrophobic interior. Changes in the

microenvironment surrounding the Trp residues of BSA result in a shift in the fluorescence emission as the residues become solvated. Figure 3 shows fluorescence emission spectra of immobilized BSA on anionic PP in 10 mM acetate buffer at pH 3.8. BSA concentrations were varied from 0.4 μM to 1.0 μM. Anionic PP-BSA constructs were washed and suspended in a 10 mM acetate buffer at pH 3.8 after immobilization. The fluorescence intensities were directly correlated to BSA concentration at all pH (data not shown) and the fluorescence intensities of adsorbed BSA were proportional to adsorbed amounts. However, at high adsorbed amounts, the correlation between BSA adsorption amount and emission intensity was lost. We attribute this to static quenching from the anionic PP [28]. The maximum emission peak positions of the maximum immobilized BSA on anionic PP, (a) - (d), and free BSA at pH 3.8 were 358, 355, 354, 353, and 353 nm, respectively. The maximum emission peak was increased with decreasing the immobilization amount. Red shift of maximum emission peak was attributed to the strong conjugation with anionic PP at a low immobilization amount. When BSA is present in excess relative to the amount required for complete surface coverage on the particles, the rate of BSA deposition was quite high as estimated in the section on protein immobilization kinetics, resulting in immobilization of BSA without conformational changes. However, at low fractional surface coverage, free epoxy groups remain after immobilization of BSA, which may result in conformational changes of the immobilized protein due to several covalent attachment points. Multipoint conjugation between epoxy groups on the surface and protein amino groups resulted in strong conjugation between BSA and the particle. The larger changes observed for lower fractional surface coverages are in agreement with the zeta potential titration curve trends observed for both proteins, as discussed above. This is also in agreement with general theory for adsorption of macromolecules. Briefly, each protein molecule must go through the following steps during immobilization: (1) transport towards the surface, (2) attachment, and (3) reorientation/spreading, with the latter step being responsible for conformational changes in the protein. Let τ_i be the time required to transport and immobilize proteins on the particle surface, and let τ_s be the time required for the protein to reorient or spread following initial attachment. If $\tau_i > \tau_s$, each protein molecule can reorient and/or spread before it is surrounded by other molecules. Conversely, if $\tau_s > \tau_i$, each protein molecule will be enclosed by neighbors before it has time to reorient or spread. For a specific protein-surface interaction, τ_s will likely be constant. However, as shown by Glomm et al. [26], τ_i is tunable via bulk protein concentration and resulting fractional surface coverage. At or close to complete surface coverage, the protein is tightly packed and more likely to retain its native structure, whereas low fractional surface coverage in this case enables multipoint conjugation with available epoxy groups. In turn, this increases the degree of conformational changes, as indicated by the relationship between fractional surface coverage, zeta potential profiles and tryptophan emission spectra.

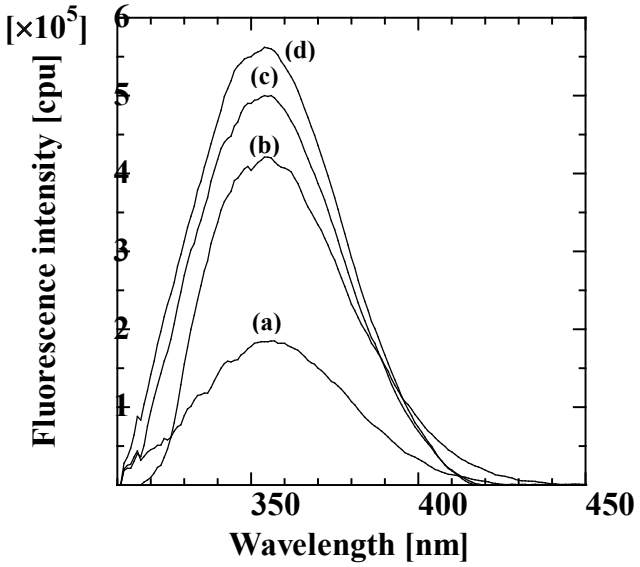

Figure 3: Fluorescence emission spectra of immobilized BSA in acetate buffer (pH 3.8). 3.75 mg of Anionic PP and BSA were added to acetate buffer solution (pH 3.8) and incubated for 24 h at 4oC. BSA concentrations were 0.4, 0.6, 0.8, and 1.0 μM, respectively. Immobilized BSA was washed and suspended with acetate buffer. The adsorption and immobilization amounts of BSA against anionic PP were (a) 4.73, (b) 8.96, (c) 13.8 and (d) 18.2 μg (mg-particle)–1. Fluorescence excitation was took place at 295 nm.

Conclusion

We have studied the synthesis and characterization of monodisperse charged nanosized polymer particles and concomitant protein immobilization on anionic, cationic and zwitterionic polymer particles, respectively. The synthesized polymer particles were not porous and their surface charge was controlled via the synthetic method. When the sign of the particle surface charge was the same as the global protein charge, the maximum adsorption amount was low while the maximum adsorption amount was high in the case of oppositely charged particles and protein. Protein adsorption was found to be complete within 5 min and all of the feed protein was adsorbed until maximum adsorption amount. When BSA was adsorbed onto anionic PP at pH 3.8 and surface concentrations were varied from 1.88 μg (mg-particle)$^{-1}$ to 28.86 μg (mg-particle)$^{-1}$, BSA was immobilized on the polymer particle surface until about 15 μg (mg-particle)$^{-1}$, after which an excess of BSA forms dimers on immobilized proteins. When excess BSA was adsorbed on polymer particle and protein-polymer particle complex was dipped in buffer solution in which global protein charge was reversed, protein desorption occurred.

From estimation of immobilized amount of protein and zeta potential measurements, the surface of the anionic PP were completely covered with BSA at the maximum immobilized amount in 10 mM of acetate buffer at pH 3.8, with BSA completely shielding the negative charge of the anionic PP. Since the pH dependent conformations of immobilized BSA were found to be indistinguishable from that of free BSA, immobilized BSA kept native like conformation and packed and concentrated immobilization on the particle surface made BSA conformation stiff and keeping away from quencher access. Fluorescence emission spectra of immobilized BSA on anionic PP in 10 mM acetate buffer at pH 3.8 revealed that the strong conjugation of BSA with anionic PP at a low immobilization amount and the state of BSA immobilized on anionic PP was similar to that of free BSA for maximum surface coverage. Therefore, immobilized BSA kept native like conformation. The role of global protein charge density matching in adsorption and immobilization is very important and we can reasonably explain the adsorption and the immobilization mechanism of BSA against anionic PP, cationic PP, and zwitterionic PP.

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