Protein Multilayer Formation on Colloids through a Stepwise Self-Assembly Technique

Frank Caruso* and Helmuth Möhwald

Contribution from the Max Planck Institute of Colloids and Interfaces, D-14424 Potsdam, Germany

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Abstract: Multilayer films of the proteins fluorescein isothiocyanate-labeled bovine serum albumin (FITC-BSA) and immunoglobulin G (IgG) were assembled on 640 nm diameter polystyrene (PS) latex particles by their alternate deposition with either positively or negatively charged polyelectrolytes. The proteins and polyelectrolytes were deposited under conditions where they were oppositely charged to one another, thereby facilitating growth of the films through electrostatic interactions. The regular, controlled, stepwise assembly of the protein and polyelectrolyte multilayer films was followed by electrophoretic mobility (EPM), single particle light scattering (SPLS), and transmission electron microscopy (TEM) methods. EPM measurements reveal alternating negative and positive ζ-potentials with deposition of each protein and polyelectrolyte layer, respectively, for the FITC-BSA multilayer films. In contrast, no reversal of the surface charge occurred for the deposition of IgG and poly(sodium 4-styrenesulfonate) (PSS) in the construction of IgG multilayers, although an oscillatory behavior in ζ-potential was observed under the conditions studied. Both SPLS and TEM demonstrate that regular, stepwise protein multilayer growth occurs for both FITC-BSA and IgG. TEM also provides direct visual observation that the PS colloids are uniformly coated with protein. The stepwise, alternate assembly technique employed permits nanometer-level control over the thickness of the protein−polyelectrolyte shell surrounding the colloids. The formation of these novel, biologically functional, core-shell particles is expected to impact the areas of biotechnology and biochemical engineering.

Introduction

Multilayer films of proteins attached to solid surfaces are widely used in the areas of diagnostics, isolation, and localization in biotechnology.[1] Molecularly designed multilayer protein architectures with spatially organized proteins contain a high density of biomolecules, leading to enhanced sensitivities for the detection of biomolecular species, and in the case of multiprotein component films, they permit sequential biocatalytic reactions to occur.

One approach frequently employed to produce protein multilayer films is that which uses avidin and streptavidin as cross-linking agents; biotin/streptavidin pairs act as binding mediators between different molecular layers, allowing the fabrication of protein multilayer assemblies.[2−11] Using this strategy, protein multilayers composed of avidin and biotin-labeled alcohol oxidase (or biotin and biotinylated lactate oxidase) have been prepared on platinum electrodes by repeated deposition of the proteins.[7,8] More recently, multilayers of a biotin−protein conjugate and polymerized streptavidin have been produced by performing 20 alternating incubations of the conjugate and streptavidin.[9] In another approach, Shin et al.[11] constructed alternating multilayers of peroxidase-labeled IgG antibodies on a preadsorbed IgG antibody layer, and subsequently utilized these films for detection of the immunoreaction with the complementary antigen or other peroxidase-labeled antibodies. This multilayer antibody method was found to be more sensitive than the more conventional avidin−biotin complex approach.

An alternative method for the construction of protein multilayer films is that of the layer-by-layer (L-b-L) self-assembly technique.[12] The L-b-L method relies on the electrostatic attraction between alternately deposited charged species to produce multilayer films. Since its introduction in 1991 by Decher and co-workers,[12,13] the L-b-L method has been widely employed for the formation of multilayer films of a wide array of water-soluble proteins, alternately assembled with oppositely charged polyelectrolytes.[6,14−19] Protein/polymer hybrid multilayer films were constructed via the consecutive adsorption of

* To whom correspondence should be addressed. Fax: +49 331 567 9202. E-mail: f.caruso@mpikg-golm.mpg.de.

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biotinylated poly(L-lysine) and streptavidin.[6] Lvov et al.[15] assembled the proteins cytochrome *c*, myoglobin, lysozyme, histone f3, hemoglobin, glucoamylase, and glucose oxidase in alteration with polyelectrolytes to obtain protein-based multilayer systems on which enzymatic reactions could be performed. Regular protein growth, up to at least 10 protein layers, was observed. In subsequent studies it was shown that multilayer films of glucose oxidase and peroxidase,[16] and glucoamylase and glucose oxidase,[17] could be successfully used as specific enzyme reactors, leading to sequential enzyme reactions. In an immunosensing investigation, it was shown that anti-immunoglobulin G (anti-IgG) multilayers constructed via the L-b-L method displayed increased antigen binding capacities over monomolecular protein layers.[18] A Fourier transform infrared reflection−absorption spectroscopy (FTIR-RAS) study of the anti-IgG multilayers confirmed that the immobilized antibodies retained their biological function in the multilayer films.[19]

Although there have been numerous studies concerning protein multilayer films on solid, macroscopically flat charged surfaces, there are to date virtually no investigations on the controlled fabrication of *multilayers* of biological macromolecules *on colloidal particles*. This is despite the fact that the advantages and attractiveness of biofunctionalized colloidal particles have long been recognized.[20,21] Colloidal particles which have antibodies adsorbed on their surface (or immunomicrospheres) can react in a highly specific way with antigens, target cells, or viruses. Applications range from their use in immunoassays, in separation procedures, and as markers in electron or standard light microscopy.[20,21]

The preparation of protein multilayers on colloids permits the viable construction of functional films with a high density of biomolecules for their application in immunoassays, affinity separations, and catalysis. This is of particular importance where the signal, as a result of biological interaction, needs to be amplified for the successful detection of various species, or where a higher efficiency of product from enzyme−substrate reactions is required.[18,22−24] Therefore, fabrication of this new class of biofunctional supramolecular structures would provide novel systems for applications in the area of biotechnology.

Recently, we developed a strategy whereby the L-b-L self-assembly technique can be successfully applied to utilize submicrometer- and micrometer-sized, charged colloidal particles as the adsorbing substrates.[25−30] Pure polyelectrolyte[25−27] and nanoparticle−polyelectrolyte[28] multilayer films have been fabricated on colloids using this approach. In an extension of this work, the use of decomposable colloidal cores as templates for the sequential deposition of alternately charged polymers, or nanoparticles and polymer, has allowed the fabrication of novel three-dimensional, hollow polymer capsules[29] and hollow inorganic or composite inorganic−organic hybrid capsules (adding structural rigidity to the capsule walls),[30] respectively.

In this paper we report on the construction of novel PS latex core-protein/polyelectrolyte multilayer shell particles by employing the L-b-L self-assembly strategy. Multilayers of fluorescein isothiocyanate-labeled bovine serum albumin (FITC-BSA), alternating with poly(diallyldimethylammonium chloride) (PDADMAC), and immunoglobulin G (IgG), alternating with poly(sodium 4-styrenesulfonate) (PSS), have been fabricated. BSA was chosen given its frequent use in adsorption studies[31] and IgG because it is widely used in solid-phase immunoassays.[32] We demonstrate that the L-b-L alternate adsorption method can be used to construct controlled and regular protein-shell structures on colloids. The advantages of the approach employed to fabricate the novel core-shell particles are as follows: (i) versatility, allowing nanometer-level control over the deposition of species; (ii) the ready fabrication of multilayers of various biomolecular species; (iii) the ability to tailor the size, topology, and composition of the biosystem to suit a given application; (iv) the ability to create multifunctional systems; and (v) the stability that can be imparted on the systems.

Experimental Section

Materials. Fluorescein isothiocyanate-labeled bovine serum albumin and sheep immunoglobulin G were obtained from Sigma. Poly(allylamine hydrochloride), M_w 8000−11000, poly(diallyldimethylammonium chloride), M_w <200000, and poly(sodium 4-styrenesulfonate), M_w 70000, were purchased from Aldrich. All proteins and polyelectrolytes were used as received, except for the 70000 M_w PSS, which was dialyzed against Milli-Q water (M_w cutoff 14000) and lyophilised before use. The negatively charged sulfate-stabilized polystyrene (PS) latex particles (diameter 640 nm) were prepared as described elsewhere.[33] Sodium chloride (AR grade) was obtained from Merck, 2-(*N*-morpholino)ethanesulfonic acid (MES) from Aldrich, and phosphate buffer (containing 0.025 M potassium dihydrogen phosphate and 0.060 M disodium hydrogen phosphate) (PBS) from Riedel-de Haën. The water used in all experiments was prepared in a three-stage Millipore Milli-Q Plus 185 purification system and had a resistivity higher than 18.2 MΩ cm.

Assembly of Protein Multilayers onto Colloids. Precursor polyelectrolyte multilayer films were first deposited to provide a uniformly charged surface and to facilitate subsequent protein adsorption. Typically, the precursor films were formed as follows: 0.5 mL of an aqueous 1 mg mL^{-1} PAH or PDADMAC solution containing 0.5 M NaCl was added to 0.2 mL of the negatively charged PS latex particles (approximately 10^{10} particles), the dispersion occasionally stirred, and 20 min allowed for polyelectrolyte adsorption. The dispersion was then centrifuged at ca. 10000 × *g* for 15 min, the supernatant removed, ∼2 mL of water added, and the particles redispersed by gentle shaking. The centrifugation/wash/redispersion cycle was repeated an additional 3 times to ensure removal of free polyelectrolyte from solution. Using the same procedure and conditions, additional polyelectrolyte layers were deposited by subsequent adsorption of PSS (1 mg mL^{-1}/0.5 M NaCl) and polycation onto the PAH- or PDADMAC-coated particles.

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(In all cases the polyelectrolyte concentration is much larger than that required for saturation coverage of the particles.)

Protein multilayers were fabricated by exposure of the polyelectrolyte-coated PS latex particles to protein solution under conditions where the protein and particles bear opposite charges, followed by alternate adsorption of polyelectrolyte and protein. FITC-BSA was deposited onto (PDADMAC/PSS/PDADMAC)-coated PS latex particles, and IgG onto $(PAH/PSS)_2$-coated particles. The particles have a positive surface charge when PAH or PDADMAC form the outermost layers, and a negative charge when PSS is the outermost layer (see Results section). FITC-BSA multilayers were formed by the alternate adsorption of FITC-BSA (0.5 mg/mL^{-1} in PBS buffer at pH 7.0, 30 min adsorption, or 1 mg/mL^{-1} in water at pH $\approx$ 5.6, 20 min adsorption) and PDADMAC (1 mg mL^{-1}/0.5 M NaCl, 20 min) onto the coated PS latex particles (5×10^9 particles). IgG multilayers were prepared by successive adsorption of IgG (1 mg/mL^{-1} in 0.05 M MES buffer at pH 6.0, pH adjusted using NaOH, 45 min adsorption) and PSS (1 mg mL^{-1}/0.5 M NaCl, 20 min) layers onto the coated PS latex particles (6×10^9 particles). (In all cases the concentration of the protein is approximately 10 times that required for saturation adsorption of the particle surface.) After each deposition of protein or polyelectrolyte layer, the samples were centrifuged at ca. $5000 \times g$ for 10 min, the supernatant removed, and at least three water washings performed.

Electrophoretic Mobility (EPM) Measurements. Electrophoretic mobilities of the bare and coated PS latex particles were measured with a Malvern Zetasizer 4. The mobility u was converted into a ζ-potential by using the Smoluchowski relation $\zeta = u\eta/\epsilon$, where η and ϵ are the viscosity and permittivity of the solution, respectively. All ζ-potential measurements were performed on coated PS latex particles re-dispersed in air-equilibrated pure water (pH $\approx$ 5.6).

Single Particle Light Scattering (SPLS) Experiments. Details of the SPLS experimental system and measurement principle have been described elsewhere.[34,35] Briefly, the dispersion (of either uncoated or coated PS latex particles) is passed through a capillary with a 0.1 mm diameter orifice at the end. Hydrodynamic focusing is applied so that the dispersion stream is directed through a laser beam which is focused to allow only a single particle or aggregate in focus at a particular time. This requires particle concentrations of less than 3×10^8 particles mL^{-1}. The light scattered by the particles moving through the laser focus is recorded in the angular region of 5−10° in the forward direction. The intensity distributions, obtained with a resolution of 0.5%, are collected by a multichannel analyzer and then stored on a PC. The Rayleigh−Debye−Gans theory is used to obtain a particle size distribution of the dispersion.[36]

Transmission Electron Microscopy (TEM). TEM measurements were performed on a Philips CM12 microscope operating at 120 kV. Samples for TEM were prepared by deposition of aqueous solutions of the coated PS latex particles upon a carbon-coated copper grid. The mixtures were allowed to air-dry for 1 min and the extra solution was then blotted off.

Steady-State Fluorescence Measurements. Fluorescence spectra were recorded with a Spex Fluorolog 1680 spectrometer with excitation and emission bandwidths set at 1.0 nm. Typically, ca. 50−100 μL of the protein multilayer-coated PS latex suspension (ca. 10^9 particles mL^{-1}) was pipetted into 3 mL of water in a fluorimeter cell and the dispersion agitated for 0.5 min. The fluorescence spectrum of this dispersion was then recorded.

Results

The assembly of protein multilayers was first followed by EPM measurements. Prior to the formation of protein multilayers, a precursor three- or four-layer polyelectrolyte multilayer film was deposited onto the PS latex particles. The precursor film not only provides a uniformly charged surface which facilitates subsequent protein adsorption,[18] but also allows the surface charge to be altered (depending on whether the polycation or polyanion forms the outermost layer) so that the protein can be deposited under conditions where it is oppositely charged to the adsorbing surface. A three-layer (PDADMAC/PSS/PDADMAC) film was assembled onto the negatively charged PS latices prior to deposition of FITC-BSA, while a four-layer $(PAH/PSS)_2$ film was deposited onto the particles before IgG adsorption. The negatively charged (uncoated) PS latex particles have a ζ-potential of about −65 mV in water. The ζ-potential of the PDADMAC/PSS/PDADMAC-coated particles is ca. +50 mV, in accordance with the outermost layer being a polycation. For the $(PAH/PSS)_2$-coated PS latex particles, the ζ-potential is ca. −40 mV, consistent with the outermost layer being a polyanion. Figure 1 shows the ζ-potential (and electrophoretic mobility) as a function of layer number for the polyelectrolyte-modified PS latex particles coated with FITC-BSA/PDADMAC or IgG/PSS multilayers. The alternate assembly of FITC-BSA and PDADMAC causes a reversal in sign of the ζ-potential with each deposition up to 10 layers. When FITC-BSA forms the outermost layer, the ζ-potential of the coated particles is slightly negative (−10 to −20 mV). This indicates adsorption of FITC-BSA since the EPM measurements were conducted at a pH of approximately 5.6, which is above the isoelectric point of BSA (4.7),[37] and hence BSA bears an overall net negative charge at this pH. Subsequent adsorption of PDADMAC and FITC-BSA produces positive and negative ζ-potentials, respectively, suggesting stepwise growth of the multilayer films. For the IgG/PSS multilayer system, ζ-potential values close to 0 or slightly negative are observed when IgG is the outermost layer. The ζ-potential values decreased to ca. −40 mV with adsorption of PSS onto the IgG layers. Whereas charge reversal clearly occurs in the FITC-BSA/PDADMAC multilayer system with each FITC-BSA and PDADMAC deposition, the surface charge does not reverse in the buildup of the IgG/PSS multilayers under the conditions employed. Although no quantitative conclusions

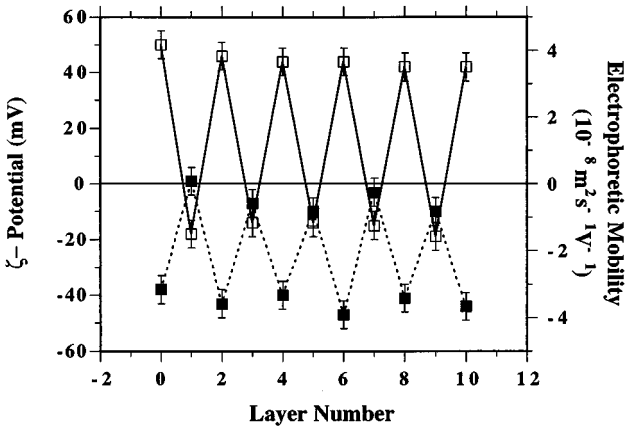

Figure 1. ζ-Potential and electrophoretic mobility as a function of polyelectrolyte layer number for (open squares) FITC-BSA/PDADMAC and (filled squares) IgG/PSS multilayers on polyelectrolyte-modified PS latex particles. FITC-BSA multilayers were formed on PDADMAC/PSS/PDADMAC-coated PS latex particles and IgG multilayers on $(PAH/PSS)_2$-coated particles. The odd layer numbers correspond to protein adsorption and the even layer numbers to polyelectrolyte deposition. The coated PS latex particles were re-dispersed in air-equilibrated pure water (pH $\approx$ 5.6) prior to measurement.

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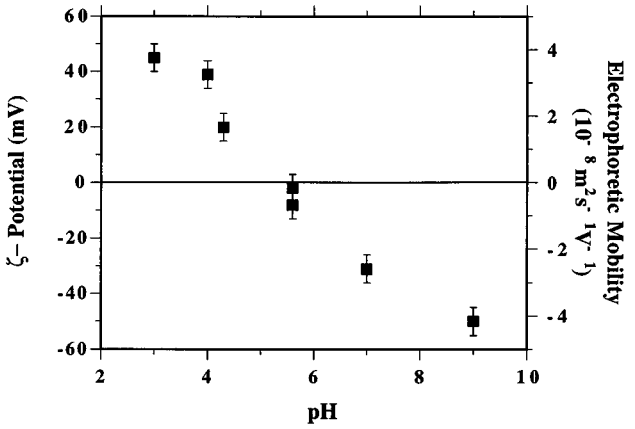

Figure 2. ζ-Potential and electrophoretic mobility as a function of pH for IgG adsorbed on $(PAH/PSS)_2$-coated PS latex particles. IgG was deposited from MES buffer at pH 6.0. The coated PS latex particles were re-dispersed in air-equilibrated pure water (pH ≈ 5.6) prior to measurement.

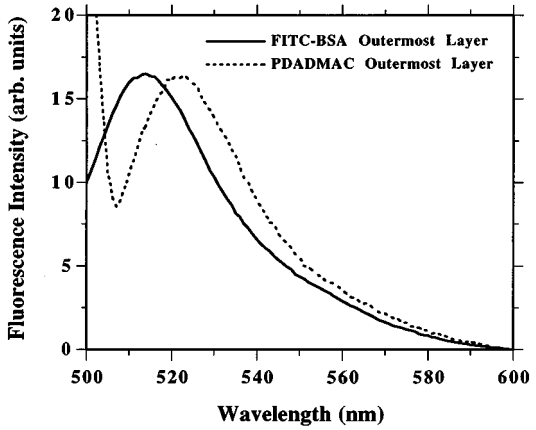

Figure 3. Typical fluorescence spectra of FITC-BSA in multilayer films of FITC-BSA/PDADMAC assembled onto PDADMAC/PSS/PDADMAC-coated PS latex particles. The solid line corresponds to the spectrum of the multilayer film when FITC-BSA forms the outer layer, and the dashed line to that of the same film with PDADMAC deposited on top. Excitation wavelength = 492 nm.

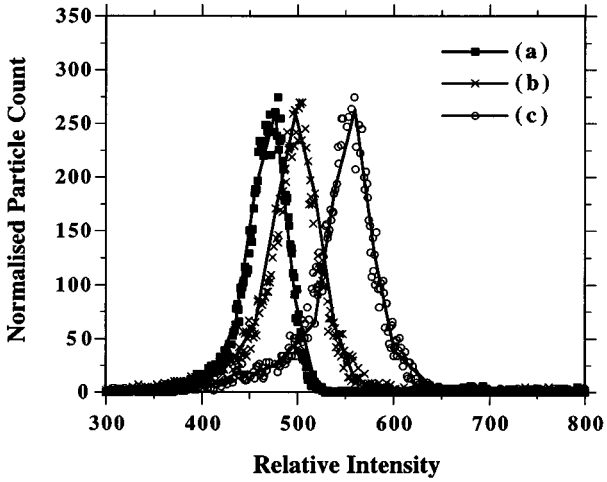

Figure 4. Normalized single particle light scattering intensity distributions of PDADMAC/PSS/PDADMAC-coated PS latex particles (a) and the same particles with one (b) and three (c) multilayers of FITC-BSA/PDADMAC. The final multilayer film structures on the colloids are the following: [PDADMAC/PSS/PDADMAC/(FITC-BSA/PDADMAC)$_N$], where $N = 0$ (a), 1 (b), and 3 (c). FITC-BSA was adsorbed from pure water.

are made from the ζ-potential values obtained,[38,39] the alternating changes observed in the magnitude of the ζ-potential for the IgG/PSS system also suggest that protein multilayer growth occurs on the particles.

EPM measurements, where the ζ-potential was recorded as a function of pH, were conducted on $(PAH/PSS)_2$-modified PS latex particles coated with *one* IgG layer to confirm the presence of IgG on the particles (Figure 2). At moderately high protein surface coverage on colloids, the isoelectric point (iep) of the coated colloid is expected to be shifted to pH values close to that of the protein. The iep of *adsorbed* IgG is approximately 5.5, a value that is slightly lower than the iep of IgG in solution (iep ≈ 6−7).[40−42] The ζ-potential−pH curve obtained shows that at pH values less than 5.5, adsorbed IgG is positively charged and at pH values greater than 5.5 it is negatively charged.

Evidence for the growth of FITC-BSA/PDADMAC multilayers was provided by fluorescence measurements. The fluorescence spectra for FITC-BSA/PDADMAC multilayers, for the cases where FITC-BSA or PDADMAC form the outermost layer, are shown in Figure 3. The emission maximum occurs at 515 nm when the outer layer is FITC-BSA. This spectrum resembles that of FITC-BSA in pure water, both in shape and in position of the emission maximum. When PDADMAC is the outer layer, this maximum red-shifts by about 6−7 nm to 521−522 nm. Reproducible, oscillating maxima were observed for each deposition of FITC-BSA and PDADMAC. A similar behavior was earlier reported for FITC-PAH/PSS multilayers on colloidal particles:[25] in that study the emission spectrum of FITC-PAH blue-shifted upon the subsequent deposition of PSS. The explanation for such shifts in both studies may be that the polarity (i.e., environment) of the FITC label is altered as a result of polyelectrolyte deposition on top of the adsorbed layers containing FITC.[25]

To obtain quantitative evidence of stepwise protein multilayer growth, the technique of SPLS was employed. SPLS is a sensitive optical technique that enables determination of the thickness of layers assembled onto colloids, as well as the state and degree of the coated colloids with respect to aggregation. By passing a dispersion of the coated particles through a capillary and hydrodynamically focusing the dispersion, the light scattered from one particle at a given moment in time is recorded. Repeating this process yields a histogram of particle number versus scattering intensity: Figure 4 shows the normalized SPLS intensity distributions for (PDADMAC/PSS/PDADMAC)-modified PS latex particles (a), and the same particles coated with one (b) and three (c) FITC-BSA/PDADMAC multilayers. There is a systematic shift in the SPLS intensity distribution (in the *x*-axis direction) with increasing multilayer layer number, confirming the growth of FITC-BSA/PDADMAC multilayers on PS latex particles. Similar SPLS intensity distributions were obtained for the IgG/PSS multilayers on PS particles. The peaks seen in the SPLS curves shown in Figure 4 correspond to singlets, i.e., unaggregated protein multilayer-coated particles. Small intensity peaks were also observed at higher intensities in the SPLS intensity distributions for the

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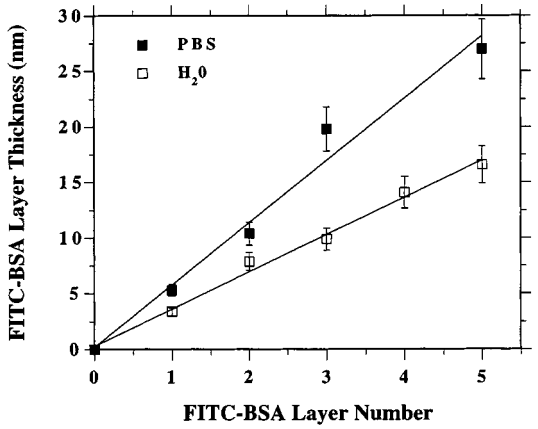

Figure 5. FITC-BSA layer thickness (determined from SPLS) as a function of protein layer number for FITC-BSA multilayers assembled on PDADMAC/PSS/PDADMAC-coated PS latex particles. The FITC-BSA multilayers were deposited alternately with PDADMAC. Data are shown for multilayer systems where FITC-BSA was adsorbed from pure water or PBS buffer (pH 7.0). The thickness of the intermediate PDADMAC layers is approximately 0.5 nm.

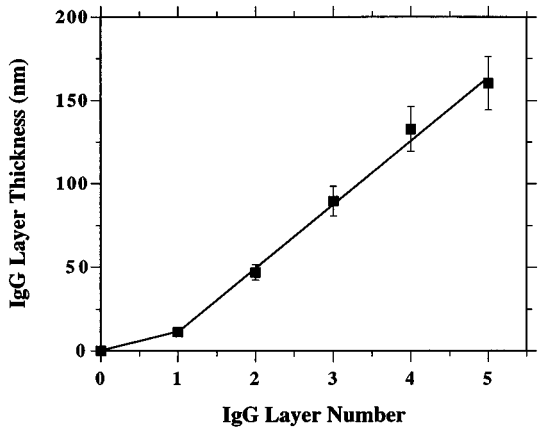

Figure 6. IgG layer thickness (determined from SPLS) as a function of protein layer number for IgG multilayers assembled on $(PAH/PSS)_2$-coated PS latex particles. The IgG multilayers were deposited alternately with PSS.

protein multilayer systems studied (data not shown), indicating that some particle aggregation occurs. Analysis of the data revealed that the coated particles exist predominantly as singlets, with less than 20% of the particles as doublets (an aggregate of two particles). The fraction of doublets was considerably reduced (<5%) when the polyelectrolyte was the outermost layer. This indicates that adsorption of the polyelectrolyte separates some of the weakly and reversibly flocculated protein multilayer-coated particles. In addition, the stability of the coated particles increased when the polyelectrolyte forms the outer layer (see later). This is in part due to the electrostatic stabilization of the particles conferred by the polyelectrolyte. A separate study that will deal with the immunoreactivity of such systems will further address the issue of aggregation in protein multilayer-coated colloids.

By using the Rayleigh−Debye−Gans theory,[36] and refractive indices (n) of 1.43 and 1.47 for the protein[18] and polyelectrolyte[43,44] layers, respectively, the average thickness of the protein/polyelectrolyte multilayers on the PS latex particles (d) can be determined. For the FITC-BSA multilayers, the layer thickness increases linearly with the number of protein layers deposited (Figure 5). (The thickness of each polyelectrolyte interlayer is approximately 0.5 nm.) The calculated average layer thickness increment for the FITC-BSA layers is 3.3 nm when FITC-BSA is adsorbed from pure water, and 5.8 nm when adsorbed from PBS. These data clearly show that BSA multilayers can be grown by the stepwise adsorption of BSA and PDADMAC onto PS latex particles. The difference in thickness is attributed to the different conditions from which the protein was deposited. The larger thickness observed for FITC-BSA deposited from the PBS buffer (pH 7) may be ascribed to the higher percentage of charged groups on the protein at pH 7, and hence the greater degree of electrostatic interaction with the PDADMAC surface onto which it adsorbs.

Figure 6 shows the IgG layer thickness of IgG/PSS multilayers assembled on $(PAH/PSS)_2$-coated PS latex particles as a function of IgG layer number. Regular, stepwise multilayer growth is observed. The total multilayer film thickness (i.e., when a polyelectrolyte layer is deposited on top of adsorbed IgG so that the outermost layer is PSS) is the same, within experimental error, as when the outermost layer is IgG. This indicates that the polyelectrolyte layer thickness is negligible in comparison with the thickness of the protein layers. The IgG multilayer film growth is linear after the first deposition step, as shown in Figure 6. The thickness of the first IgG layer deposited is approximately 11 nm. The IgG average thickness increment after the first deposition cycle (of IgG and PSS) is 37 nm. This thickness is considerably larger than any of the dimensions of IgG (10 × 14 × 5 nm),[45−47] suggesting aggregation of the protein on the surface. It should be pointed out that the first protein layer deposition is on a four-layer precursor polyelectrolyte film, while subsequent protein layers are deposited on a single PSS outermost layer, which has in turn been deposited onto a protein layer. The PSS layer deposited onto IgG may exhibit a significant degree of surface roughness, thereby producing a greater area for subsequent adsorption of IgG, and also promoting aggregation.[18,19]

Direct visualization of the protein multilayer growth process is provided by TEM. Figure 7 shows the TEM micrographs of PDADMAC/PSS/PDADMAC-modified PS latex particles (a and c) and the same particles coated with $(FITC\text{-}BSA/PDADMAC)_2$/FITC-BSA multilayers (b and d). The polyelectrolyte-coated PS latex particles closely resemble uncoated PS latices in appearance: they exhibit a smooth surface. The thickness of the polyelectrolyte coating (PDADMAC/PSS/PDADMAC) is approximately 3−4 nm (from SPLS experiments). The presence of FITC-BSA multilayers on the PS latices produces both an increase in surface roughness and an increase in the diameter of the polyelectrolyte-coated PS latices (b and d). The increase in surface roughness is most notable at higher magnification (compare images c and d). The diameter increase of the particles with $(FITC\text{-}BSA/PDADMAC)_2$/FITC-BSA layers is approximately 20 nm, corresponding to a layer thickness increase of about 10 nm. This value is in close agreement with the SPLS thickness for the same multilayer (11 nm). At the outer surface of the protein multilayer-coated particles, where the particles are in contact, some particle "coalescence" is observed (b). This is attributed to aggregation of the proteins forming the outermost layer on each particle upon drying (sample drying is required

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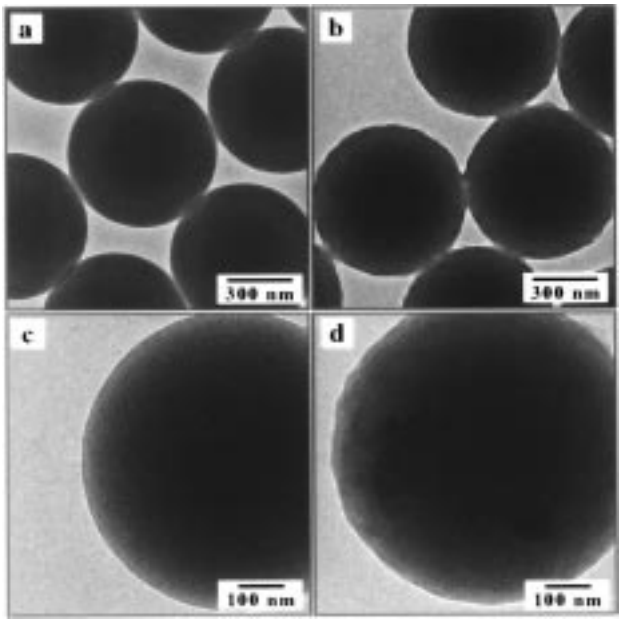

Figure 7. TEM micrographs of PDADMAC/PSS/PDADMAC-coated PS latex particles (a and c) and the same particles additionally coated with [(FITC-BSA/PDADMAC)$_2$/FITC-BSA] (b and d). The presence of FITC-BSA protein on the surface is evident from the increase in roughness of the surface texture seen for the protein multilayer-coated particles, as well as the increase in diameter of the coated particles (images b and d). Note the "coalescence" at the surface where the particles are in contact; this is a result of sample drying, which is required before TEM measurement. FITC-BSA was adsorbed from pure water.

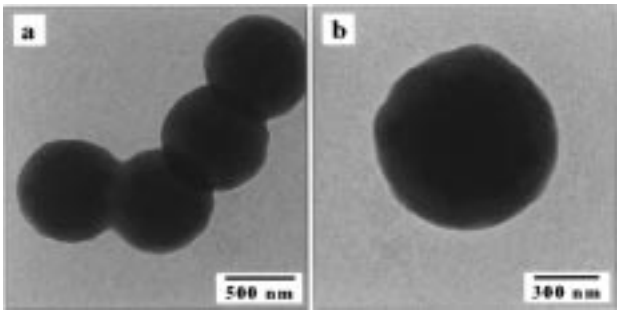

Figure 8. TEM micrographs of IgG multilayers assembled onto (PAH/PSS)$_2$-coated PS latex particles. The final multilayer film structure on the particles is [(PAH/PSS)$_2$/(IgG/PSS)$_2$/IgG]. Both images show the uniformity of the protein multilayer coating. The coalescence of two IgG multilayer coated particles (doublet) can be seen in image a (lower two particles) while overlap of the upper two particles results from drying the samples on a TEM grid. Image b is a higher magnification showing the roughness of the surface due to the presence of IgG multilayers.

for TEM analysis); the aggregation seen is not due to preformed aggregates in solution. The TEM images for FITC-BSA multilayer-coated PS latex particles confirm that a uniform coating of the particles is obtained with deposition of FITC-BSA multilayers (Figure 7, b and d).

TEM micrographs for the IgG/PSS multilayer coated PS latex particles (Figure 8) clearly demonstrate a regular coating of the particle surface by the protein multilayers. Particle coalescence is observed for the two lower particles in image a. Such aggregates (doublets) are readily detected by SPLS (see earlier). The diameter of the IgG multilayer particles is significantly larger than the PS latices. The deposition of one, three, and five IgG layers resulted in an increase in diameter of approximately 16, 164, and 296 nm, respectively. These values correspond to layer thickness increases of 8, 82,and 148 nm, and are in excellent agreement with those calculated from SPLS measurements (11, 90, and 160 nm for 1, 3, and 5 IgG layers, respectively).

Discussion

EPM, SPLS, and TEM data demonstrate the stepwise, regular formation of protein multilayers on PS latex particles. The protein layer thickness increases linearly with protein layer number for FITC-BSA and IgG (after one IgG/PSS deposition cycle). This finding is in agreement with the data reported for protein multilayers constructed on *planar surfaces*, where it was also found that a linear relationship exists between protein layer thickness and protein layer number.[15,18]

The protein layer thickness data can be converted into adsorbed protein amounts on the particles (Γ, mg m^{-2}) by using de Feijter's relation:[48]

$$\Gamma = \frac{d(n_f - n_b)}{dn/dC} \quad (1)$$

where d is the thickness of the adsorbed layer(s), n_f and n_b the refractive indices of the adsorbed layer(s) and protein-buffer solution (1.334), respectively, and dn/dC the refractive index increment with concentration of the proteins. Values of dn/dC for the proteins[49] and polyelectrolytes[25,44] are 0.188 and 0.196 cm^3 g^{-1}, respectively. The amount of protein adsorbed for each FITC-BSA layer is calculated as 1.7 $\pm$ 0.6 and 3.0 $\pm$ 1.3 mg m^{-2} for BSA adsorption from water and PBS, respectively. Assuming a closely packed monolayer of unperturbed molecules in a side-on orientation, a surface loading of 2.5 mg m^{-2} is calculated from the dimensions of BSA (4 $\times$ 14 nm).[50] The surface coverage for BSA adsorbed from water is similar to that often found for BSA adsorption onto sulfate PS latices (approximately 2 mg m^{-2}),[50−52] which corresponds to monolayer coverage. Higher values (up to 3.5 mg m^{-2}) have also been obtained, with explanations for high surface coverages ranging from the asymmetry of the charge distribution in BSA molecules to the flexibility of BSA.[50,53]

In the construction of the IgG multilayers, the surface coverage for the first layer of IgG deposited is about 5 mg m^{-2} (layer thickness $\approx$ 10 nm). This value is larger than that theoretically calculated for a close-packed IgG monolayer in an end-on orientation with repelling F_{ab} fragments (3.7 mg m^{-2}).[41] The average IgG coverage for layers 2−5 of the IgG multilayer films is 18 $\pm$ 4 mg m^{-2}. Clearly, this indicates the formation of protein aggregates on the surface of the particles. The above findings are in agreement with a previous investigation on the formation of identical IgG multilayers on *planar* gold substrates: a surface coverage of ca. 4 mg m^{-2} was obtained for the adsorption of the first anti-IgG layer onto a (PAH/PSS)$_2$ precursor surface, and 14 $\pm$ 4 mg m^{-2} for subsequently adsorbed IgG layers (layers 2−5) separated by PSS.[18] (Similar deposition conditions as those used in the current investigation were employed.) In that study, aggregation of IgG

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for layers 2−5 was confirmed by scanning electron microscopy and atomic force microscopy measurements.[19] In the present investigation, IgG aggregation is evidenced by the considerable increase in diameter of the PS latex particles with IgG multilayers (Figure 8). It was shown that in similar multilayer films, protein aggregation can be avoided by separation of the IgG layers by five, rather than one, polyelectrolyte layers.[18,19]

A number of groups have investigated the adsorption of proteins onto colloidal particles. Such studies have essentially been limited to investigations of single protein layer adsorption.[37,42,50−52,54−57] Schmitt et al.[54] reported that a monomolecular layer of BSA was adsorbed onto PS latex particles from solutions of pH ≈ 5, which is in agreement with the FITC-BSA multilayer data obtained in the present study for adsorption of BSA onto PS latices from water. Kamyshny and Magdassi[55] studied the adsorption of native and hydrophobically modified IgGs on hydrophobic polystyrene latex beads. They reported that under conditions where both the latex particles and IgG molecules are negatively charged, the IgG had a high affinity for the particle surface. This affinity increased with increasing hydrophobicity of the IgG molecules, leading to the conclusion that the main driving force for IgG adsorption on polystyrene beads is hydrophobic binding. Hydrophobic binding was also reported to be the dominant force for IgG binding to polyelectrolyte surfaces in a recent study where IgG/PSS multilayers were assembled onto planar surfaces.[18] This is consistent with the EPM data obtained in the present study (Figure 1), which shows that under the conditions which the IgG multilayer film is fabricated, the net surface charge for adsorbed IgG is slightly negative or close to zero (pH ≈ 5.6, Figure 2), but nonetheless PSS can be deposited and IgG/PSS multilayers successfully constructed.

Two most commonly studied aspects in adsorbed protein−colloid systems are colloidal stability and immunoreactivity of the proteins after adsorption onto the particle surface.[37,42,54−57] An understanding of these is of vital importance because of the requirements for protein-coated particles in areas such as clinical diagnostics. A good indicator of colloidal stability is the electrophoretic mobility of the protein-coated particles. As mentioned earlier, particles covered by a monolayer of BSA have ζ-potentials of −10 to −20 mV when the coated particles are re-dispersed in pure water at pH ≈ 5.6. These values are in fair agreement with those reported in an earlier study for BSA on sulfonated PS latex particles: ζ-potentials of about −20 mV were obtained at pH 5, and re-dispersing these particles at pH 7 resulted in a decrease of the ζ-potential to ca. −50 mV, indicating a greater mobility of the BSA-coated particles and hence greater colloidal stabilization.[37] It should be mentioned that colloidal stabilization of the BSA-coated PS latices may, in part, also be due to steric stabilization, since at ca. −10 mV the electrostatic contribution to the stabilization is small. PS particles covered by IgG have an iep at pH 5.5 (Figure 2). The IgG-coated particles display a relatively high mobility at basic pH and at pH < 4 (the absolute magnitude of the electrophoretic mobility is important), indicating colloidal stability of the coated particles at these pH values. The data in Figure 1 also suggest that the protein-coated particles can be better stabilized by having polyelectrolyte as the outermost layer: higher electrophoretic mobilities are observed for these particles when the polyelectrolyte forms the outermost layer. Furthermore, the protein-multilayer-coated particles with an outermost polyelectrolyte layer are stable at neutral pH values.

The colloidal stability of IgG-coated latex particles has also been shown to be increased by coadsorption with BSA.[56] In that work, anti-CRP (C-reactive protein) IgG was coadsorbed with BSA onto latex particles and the immunoreactivity of the coated microspheres with the complementary antigen, CRP, was also studied.[56] Under specific incubation conditions, the particles were found to have good immunoreactivity to the CRP antigen, as studied by following the changes in the turbidity after the addition of CRP. The adsorption of BSA also suppresses nonspecific interactions of the complementary antigen, as it covers nonoccupied parts of the latex by adsorption.

Immunosensing and FTIR studies of IgG multilayer films on *planar* gold substrates (identical to those constructed in this work) revealed that the protein function was preserved in the multilayer film.[18,19] Therefore, it is expected that the biological activity of the protein multilayers assembled on the colloids would also be retained. It was demonstrated that proteins can diffuse through such multilayers because of the porous nature of the network, allowing interaction with immobilized proteins (such as antibodies) in the films.[18,19] The creation of related multilayers for enzyme catalysis is also of special interest because of the ability to increase the catalysis efficiency by depositing enzyme multilayers on colloids; the substrates could readily diffuse through the film and react with the immobilized enzyme, thereby producing product.

Preliminary work has shown that the quantity of dodecyl glucoside, the product of enzymatic catalysis of dodecyl alcohol and glucose in the presence of β-glucosidase (GLS), increases with increasing number of GLS layers deposited onto PS latex particles by the L-b-L method. The GLS layer growth on colloids and its enzymatic activity will be reported on in a separate paper.

Conclusions

Protein multilayer films of FITC-BSA and IgG have been constructed by the stepwise adsorption of protein and polyelectrolyte onto PS latex particles. The multilayer growth process was found to proceed similarly to that which occurs on planar substrates, provided conditions are chosen to stabilize the colloidal dispersion, therefore avoiding aggregation of the coated colloids. Linear growth of the protein layer thickness with the number of protein layers deposited onto the particles was observed. SPLS revealed that more than 80% of the particles exist as singlets when the outermost layer is protein, and approximately 95% when the outer layer is polyelectrolyte (under the conditions used). Uniform protein coatings were observed on the particles by TEM. The finding that protein multilayers can be successfully formed with use of either PDADMAC or PSS as the interlayer polyelectrolyte between protein layers demonstrates the general nature of the approach employed.

It is anticipated that realization of the successful fabrication of biomultilayer assemblies on colloids, as shown in this work, will stimulate further research in the area of producing microspheres with biologically active species for applications in immunosensing, catalysis, and novel separation procedures.

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Furthermore, employment of the versatile assembly strategy outlined, coupled with the ability of polyelectrolytes to stabilize colloidal systems, provides outstanding opportunities to produce tailored and optimized systems for numerous applications in the biotechnology area.

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