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Polyelectrolyte-Mediated Protein Adsorption: Fluorescent Protein Binding to Individual Polyelectrolyte Nanospheres

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We have used confocal fluorescence microscopy with single molecule sensitivity to characterize uptake and release of fluorescent protein (mEosFP) molecules by individual spherical polyelectrolyte brush (SPB) nanoparticles that were immobilized on a glass surface. The SPB particles consisted of a solid core particle of 100 nm diameter onto which long polyelectrolyte chains were affixed. They could be loaded with up to 30 000 mEosFP molecules in a solvent of low ionic strength. The concentration dependence of protein loading can be described with a simple bimolecular binding model, characterized by an equilibrium dissociation coefficient of 0.5 μM. Essentially complete release of the bound proteins was observed after increasing the ionic strength by adding 250 mM NaCl to the solvent. Fluorescence emission spectra and time-resolved fluorescence intensity decays were measured on individual, mEosFP-loaded SPB nanoparticles, and also on the dissolved mEosFP before and after adsorption. These results indicate that the mEosFP molecules remained structurally intact in this procedure. Hence, the present investigation demonstrates unambiguously that polyelectrolyte-mediated protein adsorption onto SPB particles presents a viable process for protein immobilization.

Introduction

Enzymes are proteins that function as versatile biocatalysts, capable of catalyzing many highly selective and stereospecific reactions. They can be employed to simplify complex syntheses and decrease the number of steps toward formation of a specific product. Advances in biotechnology have made enzymes much more available, including variants that are genetically modified for specific tasks, thus expanding their practical use in the conversion of chemicals and materials.[1] A major challenge for large-scale industrial applications of enzymes is the efficiency of recovery and repeated use. Another equally challenging requirement is that the immobilized enzymes retain their functionally active form.[2,3] Due to the relatively fragile and varied nature of proteins, their immobilization frequently leads to a marked loss of biological activity or complete denaturation.[4,5] Here we present a study on spherical polyelectrolyte brushes (SPBs), a novel class of carrier materials for the immobilization of proteins.[6−8] Their high storage density, good retention, and controlled uptake and release of the proteins make these colloidal nanoparticles promising devices for applications as drug delivery systems, biosensors, immunoassays, and biocatalysts.

SPBs consist of solid polymer cores, for example poly(styrene), onto which linear polyelectrolyte chains such as poly(styrene sulfonic acid) (PSS) or poly(acrylic acid) (PAA) are grafted (Figure 1).[9−11] Because of their nanoscopic size and brush structure, these particles exhibit a large interfacial area in solution so that a large amount of protein can be bound within the thin polyelectrolyte brush.[6] Surprisingly, even proteins carrying a net negative charge are strongly bound to negatively charged SPBs. Adsorption occurs only at low ionic strength of the solvent, whereas sufficiently high ionic strength prevents protein uptake.[6,7] This "polyelectrolyte-mediated protein adsorption" (PMPA)[4] was explained by the "counterion release force."[12] Patches of positive charge on the surface of the proteins become multivalent counterions of the polyelectrolyte chains attached to the surface of the SPB. To the best of our knowledge, adsorption of proteins to SPBs via the PMPA process provides the first example of a strong attraction of particles of the same

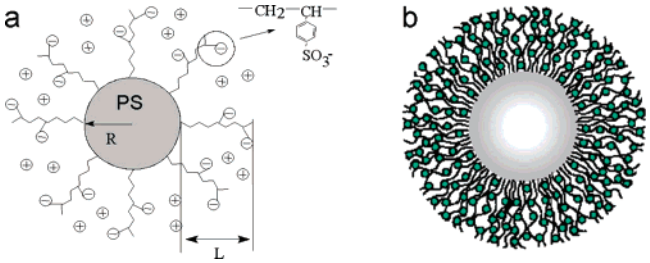

Figure 1. Studied interactions of SPB nanoparticles with the fluorescent protein mEosFP. (a) Schematic depiction of a spherical polyelectrolyte brush. The colloidal particles consist of a poly(styrene) (PS) core with radius *R* onto which long chains of poly(styrene sulfonate) (PSS) are grafted.[10] The thickness *L* of the brush depends on the ionic strength of the system. (b) Typical brush system with adsorbed protein molecules. The distribution of protein molecules within the polyelectrolyte shell was determined recently by small-angle X-ray scattering.[13]

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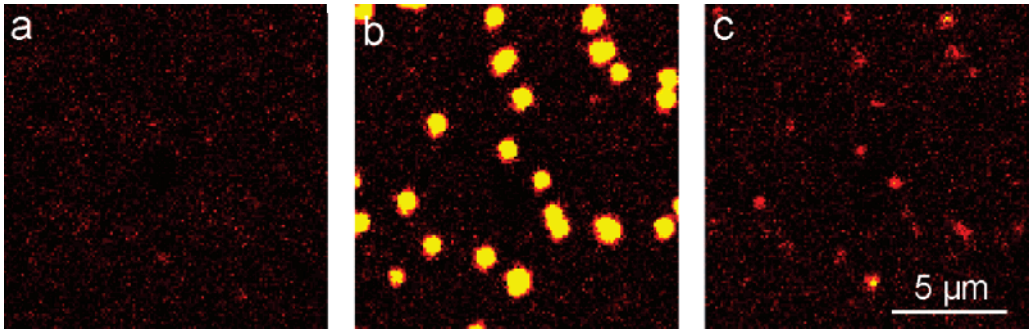

Figure 2. Confocal laser scanning microscopy images of a selected area, showing reversible binding of mEosFP protein molecules to individual SPBs. (a) Immobilized SPBs without protein, barely visible by their weak autofluorescence, (b) SPBs in the presence of 75 nM mEosFP in 7 mM MOPS buffer, and (c) after washing with 7 mM MOPS buffer containing 250 mM NaCl. The intensity scale is identical for all three images.

charge, mediated by a purely entropic force. Moreover, SPBs offer an entirely new approach to immobilizing proteins.

In this work, we focus on the controlled protein uptake and release by individual SPBs and specifically address the question as to whether protein immobilization in SPBs maintains the functionally competent protein structure. Our experimental approach is ultrasensitive confocal fluorescence microscopy, using the monomeric green fluorescent protein mEosFP as a model system.[14] This protein is a representative of the GFP family of proteins. They feature a unique structure, with an 11-stranded β-barrel forming the walls of a can. A helix runs in its center to which the chromophore, 4-(*p*-hydroxybenzylidene)-5-imidazolidinone, is tightly anchored.[15−17] The chromophore is strongly fluorescent in the protein but completely nonfluorescent in aqueous solution.[18] Evidently, the rigid microenvironment within the β-can, created by a tight hydrogen-bonded network around the chromophore, is essential for efficient fluorescence emission. Protein denaturation leads to disruption of the chromophore cage, with concomitant loss of the fluorescence.[19−21] By selecting mEosFP for our study, we exploit this exquisite sensitivity of the chromophore to the structural integrity of the protein. Moreover, in contrast to previous studies that used unspecific labeling of proteins with fluorescent dyes,[7] we ensure with mEosFP that each protein molecule carries a single fluorescent dye, which is a prerequisite for a precise determination of the loading capacity of the SPB particles.

Experimental Section

SPB Preparation. In this work, we have used SPBs consisting of a polystyrene/poly(styrene sulfonate) core/shell latex sphere (KpSS2) with a core diameter of 110 nm and a total particle diameter of 300 nm as determined by dynamic light scattering at pH 6.1.[22] Details of the preparation and characterization of the particles are given in refs 9−11.

Preparation of Immobilized SPB Samples. To prevent unspecific protein adsorption,[3,23] glass cover slips were coated with poly(ethylene glycol) (PEG) according to the following procedure. In a first step, the surfaces were amino-functionalized with a commercial aminosilane, Vectabond (Vector Laboratories, Burlingame, CA). Subsequently, they were incubated with a solution of 100 mg/mL mPEG-SPA, MW 5000 (Nektar Therapeutics, Huntsville, AL) in 50 mM Na_2CO_3 buffer (pH 8.2) for 90 min in the dark, during which the succinimidyl functions at the ends of the PEG chains reacted with the amino groups on the silylated glass. To observe the SPBs under the fluorescence microscope while exchanging solvent, two 200-μm Mylar sheets were sandwiched between two cover slips so as to keep a 2 mm wide channel between the cover slips. A solution of SPB nanoparticles (6 mg/L) in 7 mM MOPS buffer at pH 5.5 was filled into the channel for 10 min. After washing multiple times with MOPS buffer at pH 7.3, a typical surface density of $\sim 10^5$ immobilized SPB particles/mm^2 was obtained. For binding of mEosFP, the sample cell was incubated for 10 min with mEosFP solutions in 7 mM MOPS buffer at pH 7.3. For protein release, the sample cell was washed with MOPS buffer containing an additional 250 mM NaCl.

Confocal Laser Scanning Fluorescence Microscopy. The 476-nm emission line from an argon ion laser (Innova Sabre ML14, Coherent, Santa Clara, CA) was used as the excitation source. The light was focused into a single-mode quartz fiber (QSMJ 488, OZ Optics, Carp, Canada) and fed into a home-built confocal microscope, based on a Zeiss Axiovert 35 (Carl Zeiss, Göttingen, Germany) frame, with two single-photon counting fluorescence detection channels and a piezoelectric sample scanning stage.[24,25] For mEosFP fluorescence detection, we used a dichroic mirror Q495LP (AHF, Tübingen, Germany) in the excitation pathway and an emission band pass HQ 535/70 (AHF). Protein loading experiments with the SPB particles were performed at an excitation power of 0.1 μW, whereas 5 μW was used for measuring the fluorescence from individual FPs.

Fluorescence Spectroscopy. Fluorescence lifetime measurements were performed in the confocal geometry by using time-correlated single photon counting (TimeHarp 200 with microtime software, PicoQuant, Berlin, Germany). For the protein within the brushes, we performed a selective analysis of the fluorescence bursts caused by the diffusing SPBs by intensity-gated lifetime analysis. The excitation light pulses (405 nm) were generated by a frequency-doubled (TP1B, Uniwave Technologies, Chatsworth, CA) argon ion laser pumped titanium: sapphire laser (Mira 900, Coherent, Santa Clara, CA). The fluorescence photons were detected with a thermoelectrically cooled PMT module (H7421, Hamamatsu Photonics, Ichinocho, Japan). For recording fluorescence emission spectra, a fiber-optic spectrometer (S2000, Ocean Optics, Dunedin, FL) was inserted into the microscope immediately after the detection pinhole.

Results and Discussion

By using confocal laser scanning microscopy with single molecule sensitivity,[24,25] we have observed mEosFP binding to individual SPB brushes at low ionic strength and an essentially complete release at high ionic strength. As an example, Figure 2 shows a typical sequence of three scan images of a particular sample area. In Figure 2a, the immobilized SPBs can hardly be recognized because of their weak autofluorescence due to impurities. After loading the particles with mEosFP dissolved in a buffer with low ionic strength (7 mM MOPS), extremely bright spots are visible, the sizes of which are governed by the

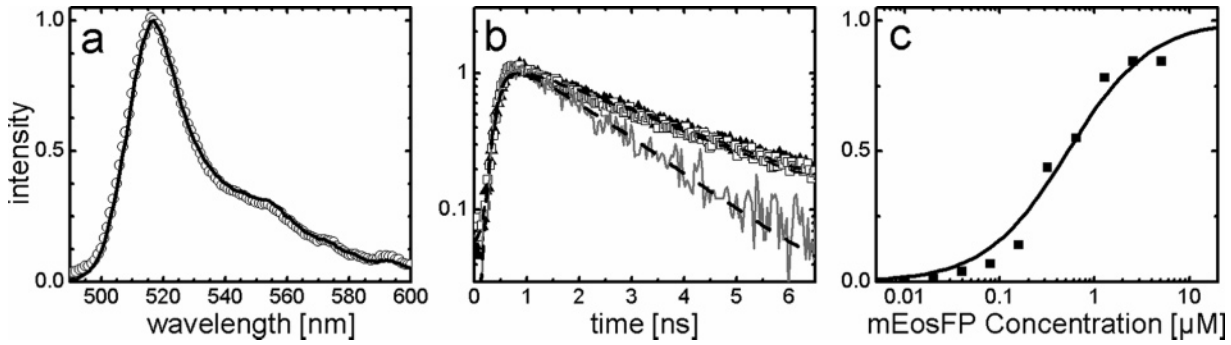

Figure 3. Photophysical properties of mEosFP interacting with SPB nanoparticles. (a) Normalized fluorescence spectra of mEosFP in MOPS buffer (line) and immobilized in SPBs (circles). (b) Fluorescence lifetime measurements of mEosFP in MOPS buffer (solid triangles), bound in SPBs (grey curve) and after release from the SPBs in MOPS buffer with 250 mM NaCl (squares). The dashed lines represent fits with single-exponential model functions. (c) Average fluorescence per SPB particle as a function of mEosFP concentration, scaled between 0 and 1. The experimental data (squares) were taken from the same image area exposed to different protein solutions. The solid line represents a fit with a bimolecular binding curve.

convolution of the particle size with the optical resolution of the microscope (Figure 2b). After washing with buffer of high ionic strength (7 mM MOPS and 250 mM NaCl), the fluorescence emission from the spots decreases to less than 1% (Figure 2 c), indicating efficient release of the bound mEosFP molecules under these conditions.

To assess the structural integrity of the protein upon binding to the SPBs, we investigated the photophysical properties of mEosFP before, during, and after its interaction with the SPB nanoparticles (Figure 3). The emission spectrum of the fluorescent protein was unchanged upon binding to the SPB (Figure 3a), suggesting that the protein remains properly folded within the SPB brush. In addition, we performed fluorescence lifetime measurements as they are more sensitive to environmental influences on the fluorophore (Figure 3b). For mEosFP in MOPS buffer, an exponential decay of the fluorescence was observed with a lifetime of (2.7 ± 0.1) ns. After release from the brushes, the fluorescence lifetime was identical within the experimental error, (2.6 ± 0.1) ns, indicating that the protein molecules retained their native conformations. In densely loaded SPB particles, we observed an accelerated fluorescence decay, yielding a lifetime of (1.3 ± 0.2) ns. As a consequence of the shorter lifetime, the fluorescence emission intensity from mEosFP molecules inside the SPBs is quenched by ~50%.

We have also measured the fluorescence emission of individual SPBs as a function of the mEosFP concentration by scanning the identical sample area while exposing the SPBs to different protein concentrations in the surrounding solvent. Figure 3c shows the resulting binding curve. It shows a steep increase for protein concentrations in the $0.1-1$ μM range and saturation above. A simple bimolecular binding model describes the data within the experimental error, indicating only weak energetic coupling between individual protein binding events. The fit yields an equilibrium dissociation coefficient of (0.5 ± 0.2) μM and a saturation intensity (maximum count rate) of (1600 ± 300) kHz. A single mEosFP molecule, immobilized on a PEG surface by a biotin-streptavidin linkage, emits at a specific rate of 1 kHz/μW, which corresponds to a rate of 100 Hz under the experimental conditions used here.[14] Taking into account the quenching of the fluorescence to ~50% upon binding inside the brushes, we determine a loading capacity of $\sim 3 \times 10^4$ mEosFP molecules per SPB particle.

In conclusion, our studies on individual SPBs suggest that the PMPA process provides a new and powerful approach to protein immobilization. SPBs fulfill the essential requirements for industrial applications as protein (enzyme) carriers, including loading at high protein density and controlled release while preserving the structural integrity of the proteins during and after immobilization.

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