Measurement of the Average Mass of Proteins Adsorbed to a Nanoparticle by Using a Suspended Microchannel Resonator

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ABSTRACT: We assessed the potential of a suspended microchannel resonator (SMR) to measure the adsorption of proteins to nanoparticles. Standard polystyrene beads suspended in buffer were weighed by a SMR system. Particle suspensions were mixed with solutions of bovine serum albumin (BSA) or monoclonal human antibody (IgG), incubated at room temperature for 3 h and weighed again with SMR. The difference in buoyant mass of the bare and protein-coated polystyrene beads was calculated into real mass of adsorbed proteins. The average surface area occupied per protein molecule was calculated, assuming a monolayer of adsorbed protein. In parallel, dynamic light scattering (DLS), nanoparticle tracking analysis (NTA), and zeta potential measurements were performed. SMR revealed a statistically significant increase in the mass of beads because of adsorption of proteins (for BSA and IgG), whereas DLS and NTA did not show a difference between the size of bare and protein-coated beads. The change in the zeta potential of the beads was also measurable. The surface area occupied per protein molecule was in line with their known size. Presented results show that SMR can be used to measure the mass of adsorbed protein to nanoparticles with a high precision in the presence of free protein. © 2014 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci

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INTRODUCTION

The use of nanoparticles for biomedical applications has been a major area of study in the last decade and continues to be a rapidly growing field of research. Nanoparticles are rigorously researched in the field of drug delivery and imaging not only because of their size, shape, or high surface to volume ratio, but also because they are looked at as platforms that offer versatile possibilities for modification with functional moieties ranging from small chemical groups to large macromolecules.[1] Drugs, proteins, fluorescent dyes, and targeting ligands are examples of functional moieties that are attached to nanoparticles for specific functions. In addition, the surface of nanoparticles is often coated with molecules such as polyethylene glycol (PEG) to stabilize the particle suspension or reduce its unspecific interaction with proteins and other components of the biological environment.[1,2]

The abovementioned moieties are generally immobilized on the surface of nanoparticles either through a covalent linkage or by a simple adsorption process. Regardless of the method of immobilization, it is a challenge to quantify the amount of immobilized moiety on the surface of a nanoparticle.[3,4] Most of the used quantification methods are based on measurement of the depletion of the component of interest in the solution that is used to coat the nanoparticles.[5] These so-called indirect methods have several drawbacks, including: (1) they do not provide a proof that the moiety is actually coated on the nanoparticles, as it could well be adsorbed to other interfaces encountered in the adsorption or sample preparation process; (2) they are susceptible to the presence of unbound impurities or contaminants; (3) they are inaccurate when only a small fraction of the moiety is coated; and (4) the overall quantification requires knowing the exact number of nanoparticles coated in the process. There are also methods that aim for direct measurement of the amount of immobilized components. The majority of such methods are, however, only suitable for qualitative or semiquantitative assessments and do not provide precise indicates of the amount of immobilized components. For instance, fluorescent labeling of proteins has been frequently used to evaluate the adsorption of proteins to the surface of gold nanoparticles.[5] Other than being nonquantitative, such methods employ modified versions of the immobilized component (e.g., fluorescently labeled protein) that may influence the immobilization behavior and lead to inaccurate indications.[3] Generally used sizing methods such as dynamic light scattering (DLS) and nanoparticle tracking analysis (NTA) have also been used to evaluate the immobilized components on the surface of nanoparticles.[3,6,7] These methods provide information regarding the hydrodynamic thickness of the adsorbed layer and not directly about the adsorbed amount. Several other techniques such as X-ray photoelectron spectroscopy or zeta potential measurements can offer proof on the presence of a coating on the nanoparticles without providing quantitative data. Scanning and transmission electron microscopy and atomic force microscopy may also be useful for evaluation of the coatings; however, these methods have an extremely low throughput and often require interfering treatments (such as drying, freezing, or deposition of a conductive layer) before an image can be made.[3,4,8]

Recently developed suspended microchannel resonators (SMR) enable the measurement of the mass of single nanoparticles with precision in the range of femtogram to attogram.[9–11] In these systems, a suspension of particles is flushed through a microchannel inside the resonator. The resonance frequency of the suspended microchannel is highly sensitive to the

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presence of particles whose density differs from that of the flushed fluid. Passage of a particle with a density higher than that of the fluid will cause a decrease in the resonance frequency of the microchannel. This decrease in frequency is proportional to the buoyant mass of the particle in the surrounding fluid. SMR systems have been previously used for studying the mass of various micro- and nanoparticles including protein particles and also for quantitative differentiation of protein particles and silicone oil droplets.[11,12]

The aim of this study was to evaluate the potential of the SMR, also referred to as resonant mass measurement,[12] for measuring the mass of proteins adsorbed to nanoparticles. To this end, proteins in different formulations were allowed to adsorb to model polystyrene beads and the resulting coated beads were weighted by using SMR. For comparison, DLS, NTA, zeta potential, and BCA protein assay measurements were also performed. Our data show that SMR provides a precise estimate of the average mass of adsorbed protein per nanoparticle without the need for separation from unadsorbed protein.

MATERIALS AND METHODS

Materials

Polystyrene standard beads with diameters of 600 and 1000 nm were purchased from Fisher Scientific (Landsmeer, The Netherlands). Monoclonal human antibody of the IgG1 subclass (IgG) was kindly provided by Boehringer Ingelheim (Biberach, Germany). Bovine serum albumin (BSA) and other chemicals were purchased from Sigma–Aldrich (Steinheim, Germany). Ultrapure water (18.2 MΩ cm water) was dispensed by using a PURELAB Ultra water purification system (ELGA LabWater, Ede, The Netherlands).

Adsorption Experiments

The polystyrene bead suspensions (diameter of 600 and 1000 nm) were diluted 2000× or 1000×, respectively, in 10 mM phosphate buffer (PB), pH 7.0 to reach a concentration that was optimal for measurements, that is, about 10^7 beads/mL. BSA solutions (1, 0.1, and 0.01 mg/mL) in PB were prepared. IgG solution (0.1 mg/mL) in PB was also prepared. Two milliliter of the bead suspension was mixed with 2 mL of the protein solution and the mixture was incubated for 3 h (long enough to reach saturation of the surface, as indicated by preliminary experiments) at room temperature after which the measurements were performed.

SMR Measurements

Suspended microchannel resonator measurements were performed using an Archimedes system (Malvern Instruments, Malvern, UK). A microsensor chip with internal microchannel dimensions of $8 \times 8\ \mu m^2$ was used for all experiments. Prior to experiments, the sensor was calibrated with 1.034 μm polystyrene size standards as instructed by the manufacturer. The sensor was rinsed with 2% sodium dodecyl sulfate solution and washed thoroughly with several runs of ultrapure water before each measurement. The sample was loaded to the sensor for 30 s. The limit of detection was fixed to 0.018 Hz, which was well above the noise level and found to be optimal for detection of the nanoparticles used herein. The measurement was continued for 10 min at room temperature, which allowed detection of at least 2000 particles. The buoyant mass of particles before (bare polystyrene beads) and after (BSA-coated and IgG-coated polystyrene beads) adsorption of protein was measured. The distribution of particles with a buoyant mass within the range of 0–40 femtogram was plotted by choosing a bin size of 0.25 femtogram. The average buoyant mass of a certain population of particles was calculated by averaging the mass of particles under the peak associated to that population in the distribution graph. The PB and BSA solution were also tested as control samples. In order to investigate whether the presence of unbound protein would influence the measurement of the mass of particles, two control measurements were conducted: (1) the beads were spun down by using a centrifuge (Microfuge 18; Beckman Coulter Inc., Brea, California) at 10,000*g* for 10 min, resuspended in PB and weighed again and (2) the mixture was diluted 10-fold with PB and measured again. For each condition, three independent measurements with separately prepared mixtures of polystyrene beads and proteins were performed.

The average buoyant mass of the adsorbed protein layer to a single nanoparticle (i.e., the average change in the buoyant mass of beads) was converted to the real mass of the protein layer according to Eq. 1, assuming the density of the protein to be 1.35.[13] The densities of different fluids were measured by SMR and were statistically equal to the density of ultrapure water. Therefore, the value of 1 g/mL was used as the density of fluid in all calculations.

$$M = \frac{M_B}{(1 - \rho_{fluid}/\rho_{particle})} \quad (1)$$

From the calculated average mass of proteins adsorbed to a nanoparticle and the known molar mass of the protein, the average number of protein molecules adsorbed to a single nanoparticle was calculated. Subsequently, the average surface area per each protein molecule was calculated, by assuming a monolayer of protein on the nanoparticle surface.

Dynamic Light Scattering

Dynamic light scattering measurements were performed with a Malvern Zetasizer Nano ZS (Malvern, Herrenberg, Germany) equipped with a 633-nm He–Ne laser and operating at an angle of 173°. The software used to collect and analyze the data was the Zetasizer Software version 7.03 from Malvern. Five-hundred microliter of each sample was measured in single-use polystyrene half-micro cuvettes (Fisher Emergo, Landsmeer, The Netherlands) having a path-length of 10 mm. The measurements were made at a position of 4.65 mm from the cuvette wall with an automatic attenuator and at a controlled temperature of 25°C. For each sample, 10 runs of 15 s were performed. The Z-average (Z-ave) diameter, polydispersity index, and peak center of the intensity distribution were obtained from the autocorrelation function by using the "general purpose mode" analysis model. These parameters were calculated from three independent measurements with separately prepared samples.

Zeta Potential Measurements

The particles' zeta potential was determined by laser Doppler electrophoresis with the same instrument as used for DLS measurements. The average and standard deviation of the zeta potential were calculated from three independent measurements with separately prepared samples.

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