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Quantitative Assessment of Protein Adsorption on Microparticles with Particle Mass Spectrometry

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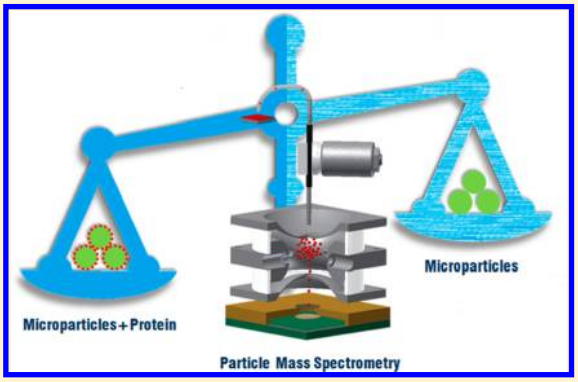
Supporting Information

ABSTRACT: In this paper, particle mass spectrometry (PMS), which consists of an aerodynamic desorption/ionization (AD) source, a quadrupole ion trap (QIT) mass analyzer, and a charge detector, was exploited to characterize the protein adsorption on microparticles based on the mass variations of microparticles before and after protein adsorption. This method is simple and has low sample cost. Importantly, its mass resolution is good enough to distinguish the microparticles with and without protein. For the adsorption of bovine serum albumin (BSA) on 3 μm porous poly styrene-divinylbenzene (poly S-DVB), the minimum mass increase that can be resolved by PMS corresponds to 128 fg (1.8 ng/cm^2) or 1.17 × 10^6 BSA molecules on each poly S-DVB particle. With PMS, the adsorption process of BSA on poly S-DVB spheres was successfully characterized, and the obtained maximum adsorption capacity q_m and dissociation constant K_d were consistent with that determined by the conventional depletion method. In addition, the influence of surface modification of silica particles on the enzyme immobilization was evaluated. Compared with C_4 (propyldimethylsilane), C_8 (octyldimethylsilane), and Ph (phenyldimethylchlorosilane), the CN (cyanoethyldimethylchlorosilane) functionalized silica particles were screened to be most beneficial for the immobilization of both lysozyme and trypsin.



Protein adsorption on microparticles is an important interface phenomenon, which involves the fields of biotechnology, biophysics, pharmaceutical sciences, etc.[1−3] By investigating the interaction between protein and microparticles, many biomedical applications such as protein purification/separation,[4−7] solid-phase immunoassay,[4,8,9] drug delivery,[4,10−12] etc. have been proposed. In order to control, predict, and manipulate the protein adsorption on microparticles, it is of significant importance to understand the adsorption process by quantitatively assessing the adsorbed amount of proteins. However, unlike that in flat and fixed surfaces, the protein adsorption on microparticles would be more efficient due to their irregular surface and relatively large surface area.[3] This feature increases the difficulty of quantifying the adsorbed amount on microparticles. Many techniques available for the flat and fixed surface, e.g., surface plasma resonance (SPR),[13] quartz crystal microbalance (QCM),[14] and total internal reflection fluorescence spectroscopy (TIRF)[15] become invalid to quantify the protein adsorption on microparticles.

In the last few decades, the protein adsorption on microparticles has been extensively studied, such as *in situ* monitoring of the adsorbed protein with fluorescent or radioactive labeling,[16,17] kinetic study with a flow cell,[6] the adsorbed layer study with dynamic light scattering (DLS),[18,19] etc. However, all these studies suffer from either poor reproducibility or poor accuracy. Most often, the rather primitive depletion method[20−25] and conventional gravimetric analysis[26] provide relatively reproducible and accurate results, but both of them require a large number of particles. Recently, the suspended microchannel resonator (SMR),[27−29] which can measure the mass of a single particle, was utilized to characterize the particle coating. It can greatly reduce the sample size and has a good sensitivity. However, it is an indirect assay. The absolute mass of particle needs to be converted from the measured buoyant mass, and the particle volume and solution density should be monitored throughout. These

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requirements increase the complexity of the experiment and limit the accuracy of this method.

Particle mass spectrometry (PMS)[30−37] is a mass spectrometric technique to measure the mass of a single particle with little sample consumption. To get the mean mass and mass distribution of particles, only several hundreds of particles are needed. Different from SMR, the absolute mass of individual particle can be directly determined by measuring both the mass to charge ratio (m/Z) and charge number (Z) of particle simultaneously. The obtained mass resolution and mass accuracy of PMS are ca. 100 and 1%, respectively.[38] To date, PMS has been successfully applied to distinguish the normal and abnormal cells,[30,33,34,36] study the cell endocytosis of nano/microparticles,[32] determine the specific surface area and size distribution of particle materials,[35] etc. In this work, the feasibility of the PMS (method) for characterizing the protein adsorption was investigated. First, the *in situ* sample preparation method compatible with PMS was modified to analyze the particles suspended in buffer solution. Then, the adsorption process of bovine serum albumin (BSA) on poly styrene-divinylbenzene (poly S-DVB) spheres was characterized with respect to adsorption kinetics and isotherm and the pH effect. Finally, the influence of surface modification of silica particles on immobilizing enzyme was analyzed, and CN functionalized silica particles were screened to immobilize both lysozyme and trypsin efficiently.

EXPERIMENTAL SECTION

Materials. Bovine serum albumin (BSA) was purchased from Amresco. Three μm poly styrene-divinylbenzene (poly S-DVB) spheres with pore size of 30 nm were purchased from Suzhou Nanomicro Technology Company Limited (China). The specific surface area of poly S-DVB particles was determined to be 554 m^2/g by the nitrogen adsorption test. BSA was adsorbed on 3 μm poly S-DVB spheres. Lysozyme, from chicken egg, and Trypsin, from bovine pancreas, were purchased from Sigma. Five μm porous silica particles with pore size of 10 nm and C_4 (propyldimethylsilane), C_8 (octyldimethylsilane), Ph (phenyldimethylchlorosilane), and CN (cyanoethyldimethylchlorosilane) bonded 5 μm porous silica particles with pore size of 10 nm were provided by Agela Technologies Inc. (China). Proper silica particles were screened for the immobilization of lysizyme and trypsin. Three μm polystyrene size standards, which were used for the calibration of PMS, were purchased from U.S. National Institute of Standards of Technology (NIST). This sample of NIST standards was a suspension of polystyrene spheres at a weight concentration of 0.25% in water. The certified average diameter of the spheres was 2.982 μm with a standard deviation (SD) of 0.016 μm.[33]

Protein Adsorption and Enzyme Immobilization. BSA was dissolved in 10 mM phosphate buffer (PB) solution at pH 4.7. The porous poly S-DVB particles were wetted with ethanol prior to use. After ethanol removal by centrifugation, poly S-DVB particles were washed with distilled water and then mixed with the protein solution. The adsorption was carried out by shaking the mixture at room temperature (24 °C) for ~2 h. The concentrations of BSA and particles were 1 and 2 mg/mL, respectively. To study the pH effect, the pH of buffer solution was adjusted by using 0.1 M HCl (pH < 4.7) or 0.1 M NaOH (pH > 4.7). The Langmuir isotherm was analyzed at various initial protein concentrations (0.25, 0.5, 0.75, 1, 2 mg/mL). After adsorption, the particles were washed 3 times with buffer solution to remove loosely attached protein molecules. Then, the particles were collected by centrifugation (2000 rpm) for the mass measurement. The Langmuir isotherm was also determined by UV−vis spectrophotometry, in which the BSA concentrations in supernatant before and after adsorption were measured at 280 nm.

Lysozyme and Trypsin were both dissolved in 10 mM PB solution at pH 7. CN-silica and Ph-silica particles were also wetted with ethanol prior to use. Due to the strong hydrophobicity of alkyl chain, after C_4-silica and C_8-silica particles were wetted by ethanol, they were dispersed in PB solution with 20% ethanol and then mixed with enzyme solution. The final concentrations of enzyme and silica particles were kept at 1 and 2 mg/mL, respectively. The enzyme and silica particles were incubated for 1 h, and then, the silica particles were thoroughly washed by PB solution for 3 times. For each PMS measurement, only a 100 μL aliquot of the particle suspension was needed (i.e., 0.1 mg of protein and 0.2 mg of microparticles).

Mass Measurement of Particles. The experimental setup of PMS is shown in Figure 1, which consists of an aerodynamic

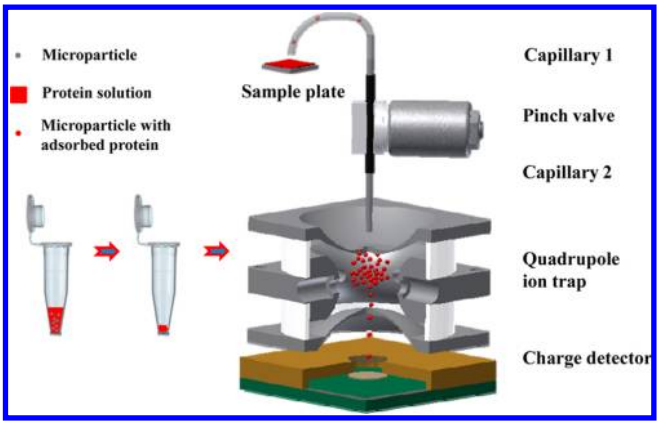

Figure 1. Schematic of the sample preparation process and the experimental setup which contains an AD ion source, a QIT mass analyzer, and a charge detector.

desorption (AD) ion source,[37] a quadrupole ion trap (QIT) mass analyzer,[39] and a charge detector.[30,31] To prepare the sample for PMS, the particles were first collected by centrifugation and then smeared on the Si wafer. In the AD source, stainless steel capillary 1 (i.d. of 0.02 in., o.d. of 1/16 in., and length of 5 cm) and stainless steel capillary 2 (i.d. of 0.04 in., o.d. of 1/16 in., and length of 22.5 cm) were connected via a silicone tube (i.d. of 1/16 in., o.d. of 1/8 in., and length of 3 cm), and the open/closed status of silicone tube was controlled by a pinch valve. The distance between the channel tip of AD and the QIT was about 5 mm. The QIT consisted of a hyperbolic ring electrode and two hyperbolic end-cap electrodes. The radius of the ring electrode was 10 mm. The pinch valve was open only 7 ms each time for the particle desorption and introduction. During the open time, the ions generated by the AD source were carried into QIT by gas flow through the hole drilled on the end-cap electrode and then trapped by a quadrupole electric field formed by applying a radio frequency (rf) voltage of 600 V (zero to peak) on the ring electrode with the end-cap electrodes electrically grounded. Helium buffer gas was applied to keep the pressure in the vacuum chamber at 20 mTorr under the evacuation of a mechanical pump (TRP-1).

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The mass analysis of particles was carried out by scanning the frequency of rf voltage linearly in 5 s from 450 to 100 Hz. During this process, the particle ions were ejected from the QIT according to their mass to charge ratio (m/Z) and subsequently detected by the charge detector, where the image charge induced by particles can be converted to the charge number Z carried by each particle. Figure 2a shows the typical spectrum (determined Z as a function of m/Z) of 3 μm poly S-DVB particles. Every peak in the spectrum represented a single particle with specific m/Z, and the peak height was the charge number Z. After measuring several hundreds of particles (usually >200), the mean mass and their mass distribution (Gaussian) were obtained. Three replicate measurements were made for each sample unless otherwise stated.

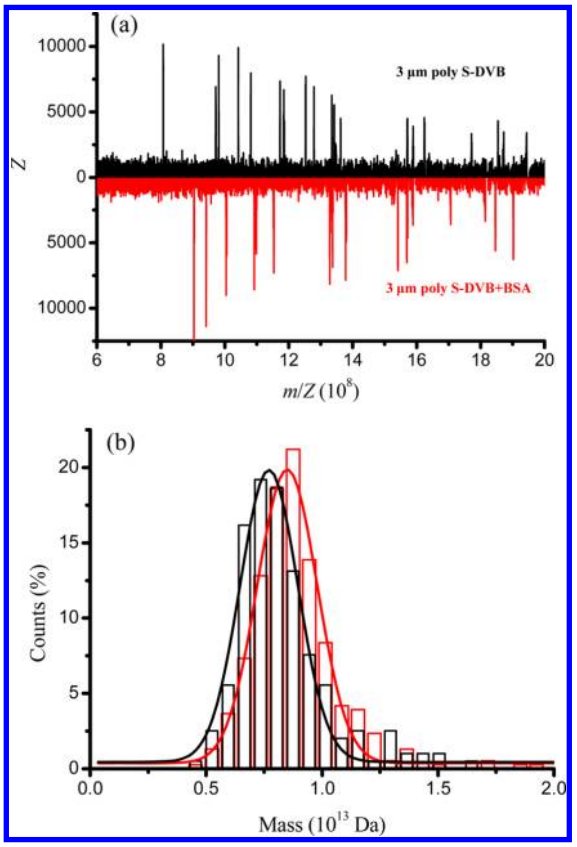

Figure 2. Determined charge number Z versus m/Z (a) and the corresponding mass profile (b) of 3 μm poly S-DVB particles before (black line) and after (red line) BSA adsorption. The BSA was adsorbed at pH 4.7 for 2 h, and the initial concentrations of BSA and poly S-DVB particles were 1 and 2 mg/mL, respectively.

RESULTS AND DISCUSSION

Calibration of PMS and Modification of Sample Preparation for PMS. The accuracy of PMS was calibrated by 3 μm polystyrene size standards having a known mass (molar mass of 8.81×10^{12} Da) and small mass variation (mass coefficient of variance (CV) is 1.6%).[38] The mass histogram of 3 μm polystyrene spheres measured by PMS was shown in Figure S1, Supporting Information. Its mass distribution was characterized by CV = 16.5%. Since this observed mass distribution was a result of the convolution of source and instrumental function, the instrumental function of PMS was determined to have CV_i = 16.4% according to the following relationship:[33]

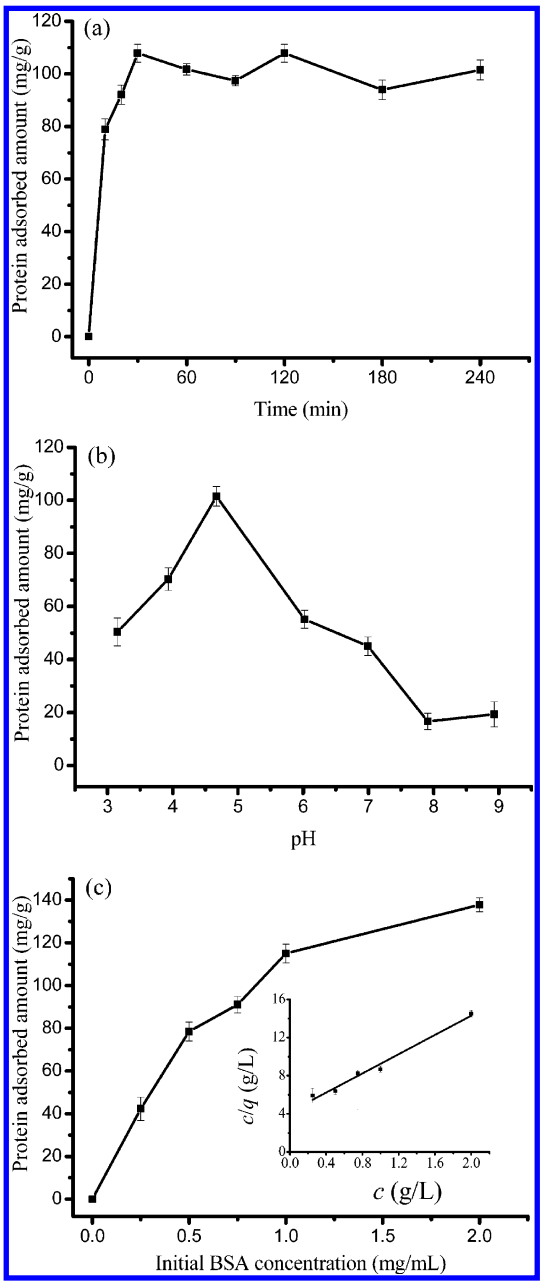

Figure 3. Variation of BSA adsorbed amount on 3 μm poly S-DVB particles with the incubation time (a), buffer pH (b), and initial BSA concentration (c). The concentration of poly S-DVB particles was 2 mg/mL for (a–c). In (a), BSA was adsorbed at 1 mg/mL initial concentration at pH 4.7; in (b), the initial BSA concentration was 1 mg/mL and the incubation time was 2 h; in (c), BSA was adsorbed at various initial protein concentrations at pH 4.7 for 2 h, and the inset is the linear Langmuir plots between c/q and c.

Table 1. Measured Langmuir Coefficients[a]

method	K_d (g/L)	q_m (mg/g)	R^2
PMS	0.83	198.4	0.98
conventional depletion method	0.95	162.5	0.99

[a] K_d: dissociation constant (g/L); q_m: maximum protein adsorption (mg/g); R^2: correlation coefficient.

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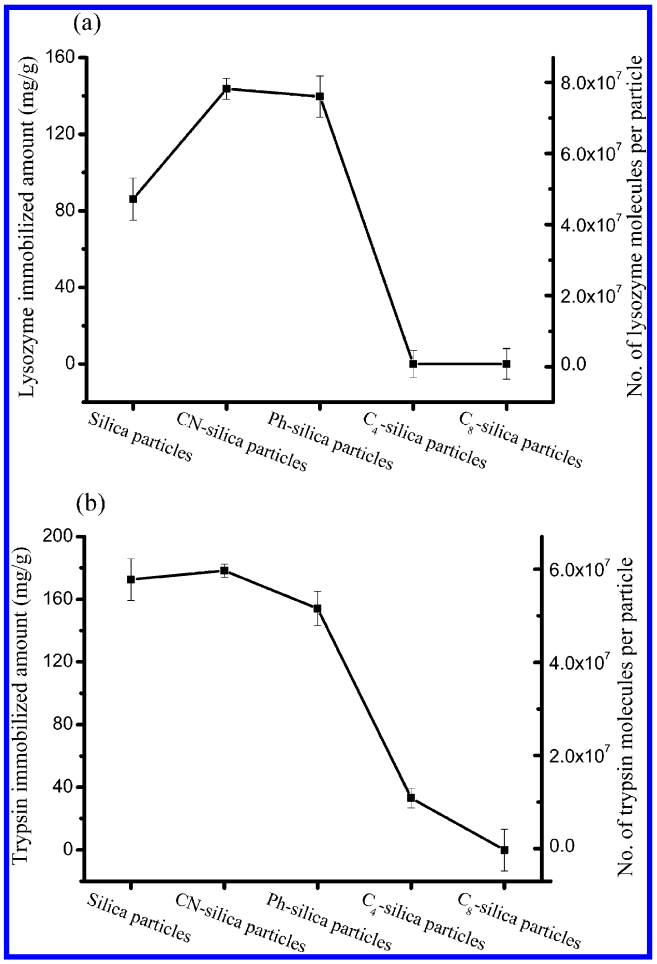

Figure 4. Immobilization of lysozyme (a) and trypsin (b) on porous silica particles. The CN-silica, Ph-silica, C_4-silica, and C_8-silica particles were functionalized by cyanoethyldimethylchlorosilane, phenyldimethylchlorosilane, propyldimethylsilane, and octyldimethylsilane, respectively.

$$SD^2 = SD_s^2 + SD_i^2 \quad (1)$$

where SD_s and SD_i were the standard deviation of the source and the instrumental function, respectively.

In the protein adsorption, the porous particles were suspended in buffer solution, which was crucial to maintain the adsorption equilibrium. When the particle suspension was directly deposited on Si wafer (conventional method), salt would crystallize in the pore of the particles during the air-drying process. This would cause significant interference for the mass measurement by PMS. By suspending the 3 μm poly S-DVB spheres in PB solution only, the variation of determined mean mass of particles with the salt concentration was studied. As shown in Figure S2, Supporting Information, when the sample was prepared by the conventional method (black line), the determined mean mass of particles increased gradually with the increase of salt concentration, and the mass increase level was 3.9%, 5.4%, and 10% for 10, 20, and 30 mM PB solution, respectively. Therefore, a modified sample preparation method was proposed for analyzing the protein adsorption by PMS. In the modified method, the particles collected by centrifugation were directly smeared on the Si wafer. Since most of the buffer solution was removed, the mass increase caused by the residual salt was only 0.6%, 0.5%, and 1% for 10, 20, and 30 mM PB solution, respectively (red line in Figure S2, Supporting Information). Note that the concentration of PB solution used on the following experiments was only 10 mM, and the small mass interference in the modified sample preparation method can be neglected.

Quantitative Analysis of the BSA Adsorption on Poly S-DVB Particles. Figure 2a shows the determined charge number Z as a function of m/Z for 3 μm poly S-DVB particles before (black line) and after protein adsorption (red line). The adsorption was conducted at pH 4.7 for 2 h, and the initial concentrations of BSA and poly S-DVB particles were 1 and 2 mg/mL, respectively. The m/Z range in Figure 2a was from 6×10^8 to 2×10^9 Th. The charge number carried by each particle, typically larger than 5000 with a good signal-to-noise ratio (S/N > 8, noise was around 600 e), was measured by the charge detector. It can be seen that the observed mass peaks of 3 μm poly S-DVB particles with adsorbed BSA (red line) were shifted to higher m/Z values compared with that of 3 μm poly S-DVB particles without BSA (black line). By multiplying the acquired m/Z values by the corresponding charge number Z on each particle, the absolute masses of analyzed particles were obtained. Figure 2b shows the corresponding mass histogram of 3 μm poly S-DVB particles before (black line) and after protein adsorption (red line). The mean masses of poly S-DVB particles before and after the protein adsorption were determined to be 7.72×10^{12} Da (centroid error, 0.05×10^{12} Da) and 8.55×10^{12} Da (centroid error, 0.04×10^{12} Da), respectively. The 11% mass shift indicated about 1.26×10^7 BSA molecules were adsorbed on each poly S-DVB particle. Additionally, the observed mass distribution of the 3 μm poly S-DVB spheres shown in Figure 2b was determined to have CV = 16.7%. Assuming the PMS had the same instrumental function throughout the mass analysis, the intrinsic mass distribution of poly S-DVB particles was determined to have CV_s = 3% according to eq 1, which was consistent with that given by the manufactory (2.99%). It can be seen that the instrumental function of PMS resulted in a much broader mass distribution compared with the intrinsic mass distribution of particles. Therefore, the poor mass resolution of PMS for the mass analysis of single particle can be represented by the instrumental function, which was mainly attributed to the measurement error of charge number Z by using charge detector.[31,33] However, in this work, the mean masses of particles before and after protein adsorption were compared. For the mean, the corresponding error caused by the instrument function would be much smaller after several hundreds of particles were detected. Assuming the intrinsic mean mass of particles was uniform, the mass resolution of PMS can be regarded as the CV of the determined mean mass of particles according to eq 1. After five replicate measurements of the mean mass of poly S-DVB (more than 200 particles were measured for each time), the mass precision of PMS was determined to be 1.01% (i.e., the mass resolution of PMS was about 100).[32] Therefore, the minimum mass variation that can be resolved by PMS corresponded to 128 fg (1.8 ng/cm^2) or 1.17×10^6 BSA molecules on each poly S-DVB particle, and this mass resolution of PMS (1.01%) was sufficient to distinguish the mass shift caused by BSA adsorption on 3 μm poly S-DVB spheres.

Figure 3a shows the adsorption kinetics of BSA on poly S-DVB spheres. The initial BSA concentration was 1 mg/mL, and the pH of buffer solution was 4.7. Two adsorption domains can be identified: (i) the first domain (0−30 min) showed a fast adsorption from the very beginning and (ii) the second domain

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(after 30 min) exhibited a well-defined adsorption plateau (about 100 mg/g), which represented the adsorption equilibrium state. The kinetic study indicated that the adsorption equilibrium of BSA on poly S-DVB spheres can be established within half an hour. Nevertheless, to ensure the complete adsorption equilibrium was achieved, the mixture of BSA and poly S-DVB particles was incubated for 2 h in the following experiments.

Figure 3b presents the effect of pH on the equilibrium BSA adsorption capability on poly S-DVB particles. The initial BSA and particle concentrations were 1 and 2 mg/mL, respectively. As expected, the maximum BSA adsorption was observed at pH 4.7, which was in the vicinity of the isoelectric point of BSA (5.0).[40] This result confirmed that hydrophobic interaction between BSA and the poly S-DVB spheres dominated. At the isoelectric point of BSA, the water solubility of BSA was at a minimum, and thus, the amount of protein adsorbed by the hydrophobic interaction would be at a maximum. At higher or lower pHs, BSA would be negatively or positively charged, which would weaken the hydrophobic interaction and reduce the adsorption capability.

Figure 3c shows the adsorption isotherm of BSA on poly S-DVB spheres under different initial BSA concentrations varying between 0.25 and 2.0 mg/mL at pH 4.7. Assuming 1:1 matching of BSA molecule and active site on poly S-DVB particles, the adsorption process can be described by the Langmuir isotherm equation:[2,22]

$$\frac{c}{q} = \frac{K_d}{q_m} + \frac{c}{q_m} \quad (2)$$

where c, q, q_m, and K_d were the BSA concentration in the solution (mg/mL), the mass ratio of adsorbed BSA to particle (mg/g), the maximum adsorption capacity (mg/g), and the dissociation constant (mg/mL), respectively. The data shown in Figure 3c were analyzed by the linear regression between c/q and c. The corresponding linear Langmuir plot was shown in the inset. The correlation coefficient R^2 of this regression was 0.98. By solving the linear regression equation, the values of K_d and q_m were calculated as 0.83 g/L and 198.4 mg/g, respectively. As a comparison, the depletion method, in which the BSA concentration in the supernatant was determined by UV−vis spectrophotometry at 280 nm, was also exploited to determine the K_d, q_m, and R^2. The measured values of these two methods were listed in Table 1. It can be found that the measured q_m value in the depletion method was smaller than that in the PMS method. This may be attributed to the solvent adsorption on particles and the concentration of residual BSA solution in the depletion method. While in PMS, the adsorbed solvent had been eliminated during the sample preparation. Therefore, the PMS method was more reliable for characterizing the protein adsorption on microparticles.

The Application of PMS on Rapid Screening of Proper Particles for Enzyme Immobilization. Enzyme immobilization is the best strategy known for improving the stability, recovery, and recyclability of enzyme, which plays an important role in biocatalysts for chemical production.[41−44] However, the enzyme immobilization capacity is influenced significantly by the morphology, composition, and surface property of supporting material. Proper supporting materials, which can immobilize the enzyme efficiently, are expected to be screened out. In our study, the performances of five porous silica particles with different surface chemistries including silica, CN-silica, Ph-silica, C_4-silica, and C_8-silica particles for immobilizing lysozyme and trypsin were compared by using PMS. Figure 4 shows the variation of enzyme immobilized amount on different silica particles. Figure 4a,b represents the results for trypsin and lysozyme, respectively. Considering that the isoelectric points of trypsin (10.5) and lysozyme (11.0) were much higher than the pH of PB solution used for enzyme immobilization (7.0), the main driving force in the enzyme immobilization process was electrostatic interaction. In Figure 4, it can be seen that the enzyme immobilized amounts of both lysozyme and trypsin decreased in the following order: CN-silica > Ph-silica > C_4-silica > C_8-silica. This was consistent with the polarity trend of the function groups: CN > Ph > C_4 > C_8 based on electrostatic interaction. Compared with silica particles, CN-silica particles exhibited a higher enzyme immobilization capability for both trypsin and lysozyme. The results indicated that CN-silica particles would immobilize both trypsin and lysozyme more efficiently.

CONCLUSION

In conclusion, PMS is a novel technique to characterize protein adsorption on microparticles. The protein adsorbed amount on microparticles can be measured with high accuracy and little sample consumption. With PMS, not only the adsorption process in terms of adsorption kinetics, the influence of solution property, and adsorption isotherm can be analyzed, but also proper particles with good adsorption capability can be screened. Therefore, PMS provides a powerful tool to study the protein adsorption on microparticles.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This information is available free of charge via the Internet at http://pubs.acs.org/.

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Notes

The authors declare no competing financial interest.

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