Magnetic Nano-Sponges for High-Capacity Protein Enrichment and Immobilization

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Protein adsorption and separation are crucial in many biotechnology applications, either for removal of unwanted high-abundant proteins or enrichment of desired low-abundant proteins.[1,2] For example, the concentration and detection of trace proteins, especially for the low-abundant cancer biomarkers, are of great significance for the early detection and diagnose of disease.[3-5] Additionally, the immobilization of functional proteins, such as enzyme, is also highly appealing.[6] Over the past decades, different driving forces[6-8] for protein adsorption have been discussed, such as antibody–antigen interaction, electrostatic, and hydrophobic interactions. Antigen–antibody interaction is the most important strategy for enriching specific proteins,[9] while sometimes nonspecific protein enrichment is as crucial as specific enrichment, especially for the unknown biomarker searching. All the proteins must be fished out from the intricate real samples to facilitate in-depth analysis of proteins in the entire blood or tissues. To this end, electrostatic interaction[10] is raised to enrich a class of proteins with opposite charges under the enrichment condition, which enlarges the enrichment varieties of proteins. Hydrophobic interaction[11-14] is another universal method for collecting hydrophobic proteins. In the reported enrichment protocols, the interaction was usually localized on the surface of the substrates (2D surface), especially for the functional microspheres,[13] so the enrichment capacity was low even reducing the substrate to nano size, which could not meet the demand of real applications.[15,16] Besides, the immobilization of the proteins on the hard flat surface usually influence the activity of the proteins.[17]

Utilizing the merits of RAFT (Reversible Addition-Fragmentation Chain Transfer) polymerization[18-20] on the surface of magnetic nanoparticles, we herein developed a new type of magnetic composite microspheres, which contained ultrasoft 3D functional shells for protein enrichment and immobilization. Usually, the chain transfer reaction in the free-radical polymerization system should be avoided because it will result in the annoying gel. In this paper, we deliberately utilized this gel effect integrated with the advantage of RAFT polymerization to obtain the ultrasoft 3D net-like shell as nano-sponges toward proteins on the magnetic core. The ultrasoft 3D net-like shell contained a large amount of negatively charged PAA chains, which promoted remarkable enrichment capability toward diverse proteins,[21,22] just like the traditional sponges toward water. Attractively, the magnetic nano-sponges possessed the following outstanding features: (1) An ultrahigh enrichment capacity toward various proteins (>1100 mg protein per g bead); (2) Excellent protein enrichment speed (less than 5 min); (3) Low protein detection limit (0.6 ng µL−1); (4) Efficient enrichment of diversely charged proteins from real biological samples such as human plasma and rabbit blood; (5) Enhanced catalytic activity of immobilized enzyme on the microspheres comparing with free enzyme (2.6 times of the free enzyme); (6) Facile operation process with quick magnet separation speed (1 min).

The synthesis process of the magnetic nano-sponge containing a Fe3O4 magnetic supraparticle (MSP) core, a cross-linked middle layer of PHEMA (poly(hydroxyethyl methacrylate)) and an outer net-like shell of PAA was schematically illustrated in Scheme 1. First, MSPs (about 150 nm) were prepared by a modified solvothermal reaction and decorated with MPS to form abundant double bonds.[22,24] Second, a layer of P(HEMA/MBA) was coated on the Fe3O4/MPS surface by reflux-precipitation polymerization of HEMA (hydroxyethyl methacrylate) and MBA (NN’-methylene-bis-acrylamide) to form abundant hydroxyl groups on the surface.[25,26] After that, carboxyl-containing RAFT reagent was added to react with the hydroxyl groups of PHEMA through esterification reaction, and ultrasoft PAA (poly(acrylic acid)) net-like shell was formed on the surface by RAFT polymerization and the chain transfer reaction.[27,28] The transmission electron microscopy (TEM) and scanning electron microscopy (SEM) images of Fe3O4/Fe3O4/PHEMA and Fe3O4/PHEMA-RAFT-PAA were shown in Figure 1. The thickness of intermediate layer PHEMA was ≈20 nm. After RAFT polymerization of PAA, the polymer thickness increased to ≈100 nm. The great increase of shell thickness showed huge amount of PAA chains were decorated to form nano-sponges.

The efficient modification of RAFT reagent[29] to Fe3O4/PHEMA (Fe3O4/PHEMA-RAFT) was proved by FT-IR (fourier transform infrared spectra) and TGA (thermogravimetric analysis). The surface-initiation of RAFT polymerization...
was performed and monitored by DLS (dynamic light scattering) and TGA. With RAFT reaction time prolonging to 1, 6, 12, 24, and 36 h, the hydrodynamic diameter ($D_h$) of Fe$_3$O$_4$/PHEMA-RAFT-PAA increased gradually (Figure S2a, Supporting Information). Meanwhile TGA determined the weight loss enhanced to 57.5%, 65.5%, 69.8%, 76.2%, and 83.3% (Figure S2b, Supporting Information) respectively, which showed living characteristic of the polymerization. The final thickness of PAA was about 100 nm, and the degree of polymerization (DP) was larger than 1000, while traditional RAFT polymerization could not reach that high DP in 36 h, which suggested the formed PAA was slightly crosslinked instead of linear ones. In fact, we did not get linear PAA chains through hydrolysis of the soft polymer shell due to the chain transfer reaction and self-crosslinking, the polymer shell remained intact after hydrolysis in 1 M NaOH solution for 12 h (Figure S3, Supporting Information), showing that they formed a slightly crosslinked network instead of linear chains.

FT-IR spectra with a strong peak of $\text{COOH}$ at 1715 cm$^{-1}$ (Figure S4b, Supporting Information) also demonstrated the decoration of PAA shell. In addition, the whole graft-from process was characterized by monitoring the zeta potential of microspheres in water as well. Fe$_3$O$_4$/PHEMA possessed a slightly negatively charged surface ($-6.8$ mV) and after RAFT polymerization the magnetic nano-sponges turned to $-40.1$ mV. The magnetic properties of the nano-sponges were obtained using a vibrating sample magnetometer. Saturation magnetization ($M_s$) value of Fe$_3$O$_4$ was 69.7 emu g$^{-1}$. After coating P(HEMA/MBA), the $M_s$ value decreased to about 52.8 emu g$^{-1}$. Upon the RAFT polymerization of PAA shell, the $M_s$ value strikingly reduced to 12.8 emu g$^{-1}$. The magnetic responsiveness of the final nano-sponges is strong enough to facilitate the separation of particles from solution (within 1 min) using a magnet (Figure S4d, Supporting Information).

The separation of various proteins is one of the most important issues in biotechnology. Generally, in order to enrich all the proteins without selectivity, people usually use the hydrophobic enrichment, while the recovery efficiency of the adsorbed proteins is not good and the enrichment capacity is limited. Herein, we introduce a new concept of 3D ultrasoft shell as nano-sponges to enrich all the proteins based on the counter-ion evaporation effect. The specific mechanism is presented in Scheme S2 in the Supporting Information. When the solution is with low ionic strength, the polyelectrolyte PAA chains possess numerous positive counter-ions confined within the polymer network and create a high osmotic pressure. Because the surface of the proteins exhibits both positively and negatively charged patches, the positively charged patches act as multivalent counter-ions of the negatively charged PAA chains when the proteins enter into the ultralow crosslinked (ultrasoft) polymer network, thus releasing large amount of positive counter-ions, which is driven by entropy increase. In this way, the proteins could be desorbed through increasing the ionic strength or using acid solution with high recovery. The whole enrichment process was similar to using sponges to adsorb water, in a quite rapid and reversible way. The protein enrichment capability of nano-sponges was testified through the following adsorption experiments (the sample of magnetic nano-sponge used in the following part is Fe$_3$O$_4$/PHEMA-RAFT-PAA-24 h). The standard proteins BSA (bovine serum albumin) (66 kDa, pI = 4.7), HRP (horseradish peroxidase) (44 kDa, pI = 7), MYO (myoglobin) (17 kDa, pI = 7.2), and LYS (lysozyme) (14 kDa, pI = 10.8) were used to investigate the separation efficiency of the nano-sponges toward various proteins. After enrichment, all the proteins were isolated and released from the nano-sponges (Figure S6a, Supporting Information), showing a higher recovery rate than the hydrophobic materials. In order to test the enrichment sensitivity, we diluted the protein concentration to 10 ng µL$^{-1}$ each (1000 µL deionized water containing 10 µg BSA, 10 µg HRP, 10 µg MYO, and 10 µg LYS), and 0.1 mg magnetic nano-sponges were incubated with 1000 µL stock solution. After incubation and magnetic separation, the proteins were enriched and showed a much higher...
concentration than the stock solution (Figure S6b, Supporting Information). We further lowered the protein concentration to 6, 3, 2, 1.2, and 0.6 ng µL$^{-1}$, the proteins still could be enriched and eluted efficiently (Figure S7, Supporting Information), which facilitates the detection of low-abundant biomarkers.

In addition, the separation of proteins from human plasma and rabbit blood was conducted. In Figure 2, all the proteins were enriched and released from the magnetic nano-sponges. As a contrast, the microspheres before RAFT polymerization ($\text{Fe}_3\text{O}_4$/PHEMA-RAFT) could hardly enrich any proteins. The rabbit blood (diluted by 40 fold) before and after enrichment with magnetic nano-sponges or $\text{Fe}_3\text{O}_4$/PHEMA-RAFT were also investigated, the excellent performance of the magnetic nano-sponges offered a new robust method to high-capacity protein enrichment from complex biological systems (Figure S8, Supporting Information). The binding capacity toward BSA, HRP, MYO, LYS, and Cyt c were 1498, 1178, 1555, 1233, and 1590 mg g$^{-1}$ (protein/beads), respectively (Figure S9, Supporting Information), which were much higher than that through electrostatic methods (478 mg proteins per g beads) method$^{[13]}$ or hydrophobic (410 mg proteins per g beads)$^{[14]}$. In comparison, $\text{Fe}_3\text{O}_4$/PHEMA-RAFT-PAM and $\text{Fe}_3\text{O}_4$/PHEMA-RAFT-PHEMA without any charged polymer chains were prepared through similar method and then utilized to enrich proteins, while hardly any proteins could be enriched (less than 20 mg g$^{-1}$).

Considering the effect of the mesh size of the polymer net-like shell, the magnetic microspheres ($\text{Fe}_3\text{O}_4$/PAA) with different PAA crosslinked densities (cross-linking degree varied from 5% to 20%, MBA as crosslinker, TEM shown in Figure S14, Supporting Information) were also prepared$^{[31]}$ and utilized to enrich BSA or MYO, their enrichment capacities were compared with the magnetic nano-sponges (Figure 3a,b). Considering both the enrichment capacity of the whole nano-sponges and the pure PAA shell, the magnetic nano-sponges exhibited much higher capacity than the cross-linked $\text{Fe}_3\text{O}_4$/PAA. That is because the traditional polymerization using crosslinker leads to high crosslinked density of the polymer shell instead of the ultrasoft one. In Figure 3a,b, it suggested that the higher crosslinking degree of PAA shell led to the lower adsorption capacity toward proteins. The self-crosslinked ultrasoft shell of the nano-sponges contained the crosslinking degree of lower than 0.5%$^{[32]}$ which was suitable for the proteins to pass through, so the whole shell was available for enrichment, resulting in much higher enrichment capacity (2250 mg protein/g PAA). While the traditional crosslinked polymer network were too dense, the high-crosslinked and poor-stretchable shell was not good for the proteins to enter into. Thus, the magnetic nano-sponge with ultrasoft shell is the perfect material for high-capacity removal of high-abundant proteins due to
the ultrahigh enrichment capacity and universal interaction toward diverse proteins.

The enrichment capacity over time was further demonstrated, and adsorption kinetics curves were obtained in Figure 3c,d. In about 5 min, the enrichment capacity reached the plateau value for both BSA and MYO (the dosages of both proteins were 1800 mg g\(^{-1}\)). The adsorption capacity varied with different protein dosages when keeping the incubation time constant in 5 min (Figure S10, Supporting Information). The recovery rate of proteins released from nano-sponges was tested by comparing the stock, supernatant, and eluate solution, and over 86% of the proteins could be eluted from the nano-sponges (Figure S11, Supporting Information). This reflects a much higher protein release efficiency than that through hydrophobic enrichment (about 50%). \[13\]

In order to test the influence of enrichment solution, we studied the parameters of pH and ionic strength for the enrichment capacity toward BSA and MYO (Figures S12 and S13, Supporting Information). At pH ranging from 4 to 11, the enrichment capacity maintained pretty high without obvious fluctuation. At pH = 3, the enrichment capacity decreased due to the increase of H\(^+\) ionic strength and the inhibition of counter-ion evaporation. This broad pH scope rendered the nano-sponges with high capacity toward proteins directly from real samples in physiological condition. Ionic strength is also considered as one of the most vital factors in the enrichment process, the enrichment capacity also could be influenced by the ionic strength (Figure S13, Supporting Information), which is because the large amount of ions in the solution strikingly lowered the evaporation of counter-ions, namely no entropy increase could be obtained and the enrichment driving forces are negligible.

The retention of the activity is another key point of the protein enrichment and immobilization. Here we inspected the catalytic activity of the immobilized Cyt c (cytochrom c). Cyt c is a hemoprotein and has several advantages for use as a peroxidase mimic.\[33,34\] Because its enzyme activity is not high, researchers tried converting cytochrome c into a peroxidase-like metalloenzyme by molecular design to improve their catalytic activity.\[35\] In our experiment, we used the magnetic nano-sponges to immobilize Cyt c. TMB (3,3',5,5'-tetramethylbenzidine) coloring solution was used as the substrate, which could be oxidized and changed to blue color by peroxidase. The enzyme activity of Cyt c was determined by testing the UV–vis of solution after catalyzing TMB at different reaction time either in the free state or immobilized on the nano-sponges. Cyt c-immobilized nano-sponges showed much higher catalytic activity (2.6 times) than free Cyt c (Figure 4, the higher intensity means higher catalytic activity). The catalytic activity varied with different ratio of Fe\(_2\)O\(_4\)/PHEMA-RAFT-PAA:Cyt c, and it reached the highest when the weight ratio was 40:1, showing that the synergistic effect was optimized. The similar enhanced catalytic performance could also be seen when changing Cyt c to HRP (Figure S16, Supporting Information), the possible reason is the adjacent spatial arrangement and high localized concentration of enzymes on the nano-sponges, which is more favorable than free enzyme toward TMB. Besides, the enzyme-immobilized nano-sponges were negatively charged and could bind positively charged TMB easily, facilitating the contact of enzyme and substrate, which promoted the catalytic reaction.\[36\]

In summary, the magnetic nano-sponges containing highly charged net-like shells through surface-initiated RAFT
polymerization and chain transfer reaction were prepared. The enrichment capacity is unprecedentedly high and almost can enrich all kinds of proteins, which is quite favorable for various proteins enrichment and desalting directly from biological samples. Besides, the immobilized enzyme on the nano-sponges showed much higher catalytic activity than free enzyme which is quite inspiring and economical in real application. Moreover, it also implies vital significance for potential creation of column and membrane using this concept, which opened a promising approach to industrial and biomedical application.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Figure 4. a) The UV adsorption intensity of catalytic product using free enzyme Cyt c or Cyt c immobilized magnetic nano-sponges as catalyst toward TMB solution at different reaction time (UV was measured at the wavelength of 451 nm) and b) relative catalytic activity comparing with free enzyme Cyt c (We set free enzyme Cyt c activity as 100%). (i) free Cyt c as catalyst, (ii–viii) Cyt c immobilized magnetic nano-sponges, the ratios of magnetic nano-sponges: Cyt c are 2:1, 2:1, 10:1, 20:1, 40:1, 80:1, 120:1, respectively. For all the samples, the Cyt c concentration is 16 µg mL⁻¹ and the TMB solution concentration is the same.