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Surface Protein Imprinted Core–Shell Particles for High Selective Lysozyme Recognition Prepared by Reversible Addition–Fragmentation Chain Transfer Strategy

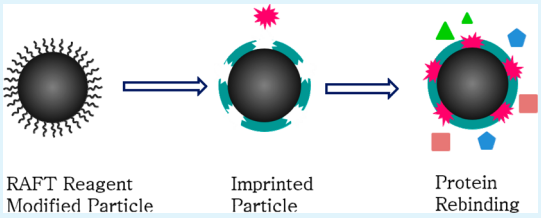
Qinran Li,[†,‡,§] Kaiguang Yang,[†,§] Yu Liang,[†] Bo Jiang,[†,‡] Jianxi Liu,[†,‡] Lihua Zhang,*[,†] Zhen Liang,[†] and Yukui Zhang[†]

[†]National Chromatographic R. &. A Center, Key Laboratory of Separation Science for Analytical Chemistry, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116023, China

[‡]University of Chinese Academy of Sciences, Beijing 100039, China

Supporting Information

ABSTRACT: A novel kind of lysozyme (Lys) surface imprinted core–shell particles was synthesized by reversible addition–fragmentation chain transfer (RAFT) strategy. With controllable polymer shell chain length, such particles showed obviously improved selectivity for protein recognition. After the RAFT initial agent and template protein was absorbed on silica particles, the prepolymerization solution, with methacrylic acid and 2-hydroxyethyl methacrylate as the monomers, and *N*,*N*′-methylenebis(acrylamide) as the cross-linker, was mixed with the silica particles, and the polymerization was performed at 40 °C in aqueous phase through the oxidation–reduction initiation. Ater polymerization, with the template protein removal and destroying dithioester groups with hexylamine, the surface Lyz imprinted particles were obtained with controllable polymer chain length. The binding capacity of the Lys imprinted particles could reach 5.6 mg protein/g material, with the imprinting factor (IF) as 3.7, whereas the IF of the control material prepared without RAFT strategy was only 1.6. The absorption equilibrium could be achieved within 60 min. Moreover, Lys could be selectively recognized by the imprinted particles from both a four-proteins mixture and egg white sample. All these results demonstrated that these particles prepared by RAFT strategy are promising to achieve the protein recognition with high selectivity.



KEYWORDS: *molecularly imprinting, RAFT, protein recognition, core–shell particles, lysozyme*

INTRODUCTION

Molecularly imprinting technology is an attractive mimetic approach to create specific binding sites geometrically and chemically complementary to the template molecules with nonbiological strategy.[1−5] However, the protein imprinting is still in the initial stage, mainly because of the weak binding for the macromolecule template anchorage and poor stability of the template protein during the polymerization.

Current protein imprinting can be divided into 2D and 3D according to how the template is presented.[6,7] Recently, the 2D imprinting strategy, with the binding sites on the material surface,[8,9] gains great popularity in macromolecule imprinting, because of the easy template removal and recognition accessibility. However, the recognition capacity for target proteins is still challenged by the hardly controllable polymer chain length of the imprinting layer,[10,11] which could hardly be controlled by the traditional free radical polymerization.[12,13]

In the past several years, various living/controlled radical strategies have been developed, among which reversible addition–fragmentation chain transfer (RAFT) allows the synthesis tailoring for macromolecular polymer with predetermined molecular weight (M_w), narrow M_w distribution, and terminal functionality.[12,14−17] For RAFT, the polymerization could be performed in various kinds of solvent. Although the aqueous-phase RAFT polymerization was developed recently, the polymerization in organic phase was still a mainstream. Additionally, due to the absent of the biological-unfriendly metal catalysts, this strategy has been used widely in the field of macromolecular-based material synthesis.[18−28] However, RAFT has been rarely applied in protein imprinting, especially in surface imprinting, which might be limited by the complexity of protein structure and the rigidity of polymerization conditions, such as room temperature or reaction time.

In our study, a novel kind of protein imprinting strategy based on RAFT strategy was proposed to prepare surface imprinted core–shell particles with the controllable polymer chain length of the shell, with lysozyme (Lys) as the template, 4-cyano-4-(phenylcarbonothioylthio) pentanoic acid (CPCP) as the RAFT initial agent, methacrylic acid (MAA) and 2-hydroxyethyl methacrylate (HEMA) as the monomers, and *N*,*N*′-methylenebis(acrylamide) (MBA) as the cross-linker. The obtained Lys imprinted particles demonstrated high selectivity

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to recognize the template protein from the complex samples, which demonstrated the great potential of RAFT strategy to improve the recognition capacity of protein imprinted materials.

EXPERIMENTAL SECTION

Materials. Silica particles (5 μm, 70 Å) were obtained from Fuji Chemical (Kusugai, Japan). MAA, 3-aminopropyltriethoxysilane (APTES) and ammonium persulfate (APS) were obtained from Acros Organics (Fair Lawn, NJ, USA). Formic acid (FA), and *N*-(3-(dimethylamino)propyl)-*N*′-ehtylcarbodiimide hydrochloride (EDC) were purchased from Fluka Chemical (Buchs, Switzerland). Lys (M_w 14.2 kDa, pI 10.8), ribonuclease B (RNB, M_w 11.7 kDa, pI 8.8), albumin from procine (PSA, M_w 66 kDa, pI 5.1), myoglobin (Mb, M_w 17.5 kDa, pI 7.1), sodium bisulfite ($NaHSO_3$), MBA, *N*-hydroxysuccinimide (NHS), HEMA and CPCP were obtained from Sigma-Aldrich (St. Louis, MO, USA). Sinapinic acid (SA) was supplied by Bruker (Daltonios, Germany). HPLC-grade acetonitrile (ACN) was purchased from Merck (Darmstadt, Germany). Water was purified by a Milli-Q system (Millipore, Molsheim, France). All inorganic reagents were of analytical reagent grade, and all the other solvents were of HPLC grade, used without further purification.

Preparation of Lys Imprinted Particles. The procedure for preparing Lys surface imprinted core–shell particles by RAFT strategy was addressed in Figure 1. First, silica particles (100 mg) were homogenized in 2.0 mL methanol, and then 50.0 μL APTES was added to introduce amino groups on the surface of silica particles. This reaction was performed at 25 °C for 24 h with stirring. After centrifugation at 3000 rpm, the mixture was washed twice by methanol to remove residual APTES. Following desiccation at room temperature by vacuum, the amino-modified silica beads were obtained.

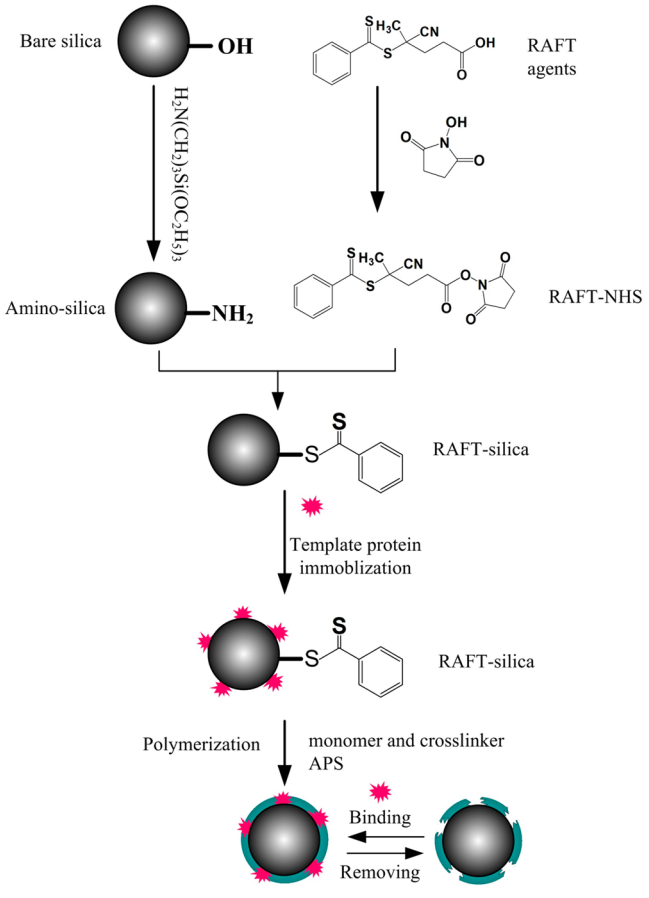

Figure 1. Preparation of surface Lys imprinted core–shell particles by improved RAFT strategy.

Subsequently, 46.17 mg of EDC, 22.60 mg of NHS, and 45.50 mg of CPCP were dissolved in 30 mL of ethanol, and the mixture was incubated with shaking for 3 h. Then amino-modified silica particles (400 mg) were added into the solution, and the reaction was performed for 18 h. After centrifugation at 4000 rpm and washed by ACN, ethanol, and water in sequence, the prepared CPCP-modified silica particles were desiccated at room temperature by vacuum. After washed twice by 10 mM phosphate buffer (PBS, pH 7.4), 1 mL of 1.0 mg/mL Lys solution was added, and the mixture was kept at 4 °C for 1 h. After incubation, the mixture was centrifuged at 3000 rpm, and washed twice by PBS to remove residual protein in the solution.

The prepolymerization mixture, composed of MAA (103 μL) and HEMA (200 μL) as the monomer (mol ratio ca. 5:4), and MBA (9.0 mg) as the cross-linker, was dissolved in 1.5 mL PBS (pH 7.4). After stirring, the mixture was purged with N_2 for 15 min. Then 20 mg of CPCP-modified silica particles with Lys absorption were homogenized in the prepolymerization mixture, followed by the addition of 5.0 μL 10% $NaHSO_3$ (w/v) aqueous solution and 5.0 μL 10% APS (w/v). The reaction was performed at 40 °C for 6, 12, 24, 36, and 48 h, respectively. After polymerization, the mixture was centrifuged at 3000 rpm to remove the unreacted reagents, followed by washing twice with water. Thereafter, the elution solution, containing 20% formic acid (v/v), 40% ACN (v/v) and 40% water, was used to remove the template from the surface of the imprinted particles. After template removal, 1.0 mL of 10% hexylamine in ethanol was added, and the mixture was kept for 12 h to remove the immobilized RAFT agent by destroy the dithioester group. After centrifuged at 3000 rpm, washed twice by ethanol and desiccated at room temperature by vacuum, Lys imprinted core–shell particles prepared by RAFT strategy were obtained. The nonimprinted particles (NIPs) were prepared by the same procedure but without Lys addition.

Additionally, as a control, the lysozyme imprinted particles were prepared by in situ free radical-initiated polymerization without CPCP modified. Other conditions of the control imprinted particles (control-MIP) preparation and polymerization, such as the initial temperature, ratio of the monomers and cross-linkers, were all same as the preparation by RAFT strategy.

Characterization. A JSM-6360LV scanning electron microscope (JEOL, Hitachi, Japan) was applied for scanning electron microscopy (SEM) imaging. Lys imprinted particles (2 mg) were washed with ethanol, and then dried at 70 °C for 2 h, followed by sputter coating with gold. Transmission electron microscopy (TEM) imaging was collected on a Tecnai G^2 Spirit microscope operated at 120 kV (FEI, Eindhoven, Netherlands).Zeta potential measurement was carried out on Malvern Nano Z Zetasizer (Worcestershire, UK). In our experiment, the particles were homogenized in water with the concentration of 1.0 mg/mL. The zeta potential of the particles was determined by three parallel sequential measurements.

HPLC Analysis. HPLC analysis was performed by using a Shimadzu HPLC system containing two LC-20AD Solvent Delivery Units, an SUS-20A gradient controller and an SPD-20A Detector (Shimadzu, Kyoto, Japan). The analytical column was obtained from Hrpersil (C8, 5 μm, 150 mm × 4.6 mm I.D., Dalian, China). A Chromatocorder 12 from SIC (Tokyo, Japan) was employed for data analysis. For chromatography analysis with gradient elution, mobile phase A was ACN/water (5:95, v/v, containing 0.1% trifluoroacetic acid (TFA), and mobile phase B was ACN/water (90:10, v/v, containing 0.1% TFA). The gradient was set as follows: 0–44.57 min, 20–98% B (v/v). The flow rate was 1.0 mL/min. The injection volume was 20 μL for all samples, and the wavelength of UV detector was set at 214 nm.

Adsorption Kinetics and Selectivity Study. The kinetic adsorption experiments were carried out by respectively incubating 10.0 mg of MIP or NIP particles with 0.50 mL of 0.40 mg mL^{-1} of Lys in PBS buffer (pH 7.4) at 4 °C. The concentrations of Lys in the supernatant were measured twice by HPLC at different time intervals.

To demonstrate the recognition specificity of prepared MIPs to Lys, 15 mg of MIP and NIP particles were respectively incubated in a-four-protein mixture (PSA, RNB, Lys, and Mb, 0.40 mg/mL for each protein) dissolved in 10 mM PBS buffer (pH 7.4) at 4 °C for 24 h.

After incubation, the supernatant was separated, and the imprinted particles were washed three times by 1.0 mL of 40% ACN (v/v), followed by 5 min elution with 0.40 mL buffer composed of 20% formic acid (v/v), 40% ACN (v/v) and 40% water (v/v). The elution was collected and analyzed by HPLC under conditions mentioned above. The concentration of each protein in solution was averaged by two parallel HPLC experiments.

Lys Recognition from Real Samples. Chicken egg white, separated from a fresh egg and diluted 1000 times with PBS buffer (10 mmol/L, pH 7.4), was taken as the sample. Twenty miligram imprinted particles were added into 2.5 mL sample at 25 °C. The conditions of incubation, washing, elution, and analysis by HPLC followed those mentioned above. The eluted solution was 0.3 mL. Furthermore, the eluted component of the peak at the retention time about 16.5 min was collected and concentrated by vacuum and analyzed by MS. A total of 0.5 μL of this eluant was dropped onto a MALDI plate, to which 0.5 μL of SA support solution (20.0 mg/mL, 0.1% TFA in 60% CH_3CN aqueous solution) was added. All mass spectra were taken from an Ultraflex III MALDI-TOF/TOF MS instrument (Bruker, Daltonios, Germany). The laser intensity was kept constant for all samples.

RESULTS AND DISCUSSION

Design of Lys Imprinted Core–Shell Particles by RAFT strategy. With the consideration on the fragile inherent characteristics of proteins, we developed a new method to prepare Lys imprinted core–shell particles by RAFT in aqueous phase at 40 °C with an oxidable-reductive initiator, which was different from the RAFT strategy applied for the small molecule imprinting in organic phase with a thermo-initiator.[29] Due to the good thermal stability[30] and the immobilization on silica particle by electrostatic attraction at pH 7.4, Lys could keep its conformation at under such conditions.

CPCP, a hydrophilic diothioester was chosen as the RAFT agent because of its solubility in polar solvent and absence of long alkyl chain, which could not only have minor influence on protein configuration, but also be easily removed after polymerization under basic condition. As shown in Figure 1, the RAFT agent was first covalently immobilized on the surface of silica particles through the reaction between carboxyl and amino groups with the addition of EDC and NHS.[31,32] Meanwhile, EDC could keep the reaction solution weakly acidic, favored to keep the stability of the RAFT reagent.[33]

The immobilization of CPCP was monitored by the change of zeta potential and the color of functionalized silica particles. For bared silica beads, the zeta potential was about −27.8 mV, whereas that of amino-modified silica was about 7.57 mV. With RAFT group modified, the zeta potential turned to 32.6 mV. The amount of modified diothioester groups on the particles was controlled and optimized by the amount of CPCP added in the reaction solution with equal mole of EDC and NHS. Super abundant CPCP adding may cause the excessive density of diothioester modified, which had adverse effect on the formation of effective imprinted shell to recognize the proteins.

To decrease the nonspecial adsorption and increase the recognition specificity toward the target protein, the formed imprinting polymer should be hydrophilic. Therefore, HEMA was chosen as the main monomer for polymerization to provide hydrogen bonding interaction with the template protein. A small amount of MAA was added to provide moderate electrostatic interaction distributed over the surface of polymer shell to form selective recognition sites.[34,35] Additionally, MBA was used as the cross-linker to ensure the formation of the hydrophilic imprinted polymers with ignorable effect on the native structure of Lys.[36] Herein, the best mole ratio of MAA and HEMA was about 4:5 and that of total monomers and cross-linker was about 5:1, which was optimized by the ability and selectivity of prepared particles.

After polymerization and the removal of unreacted monomers and cross-linker, the protein elution solution containing 20% FA, 40% ACN and 40% water was applied to remove Lys by disrupting the intramolecular hydrogen bonds within the protein molecule.[37] No chromatographic peak of Lys observed in the eluted buffer was applied to judge the complete removal of Lys from MIPs. Furthermore, 10% (v/v) of hexylamine in ethanol was used to deoxidize the RAFT reagent groups to thiolene,[38,39] which would decrease the nonspecial protein binding by avoiding the strong hydrophobic interaction between the protein and the phenyl group of the initiator. The decreased zeta potential of prepared particles from 32.6 mV to −1.83 mV indicated that the R group of RAFT agent was removed clearly.

Morphology characterization. SEM imaging was performed to observe the size, shape and surface structure of Lys imprinted particles prepared by the improved RAFT strategy. As shown in Figure 2a, the MIP particles were regular spherical

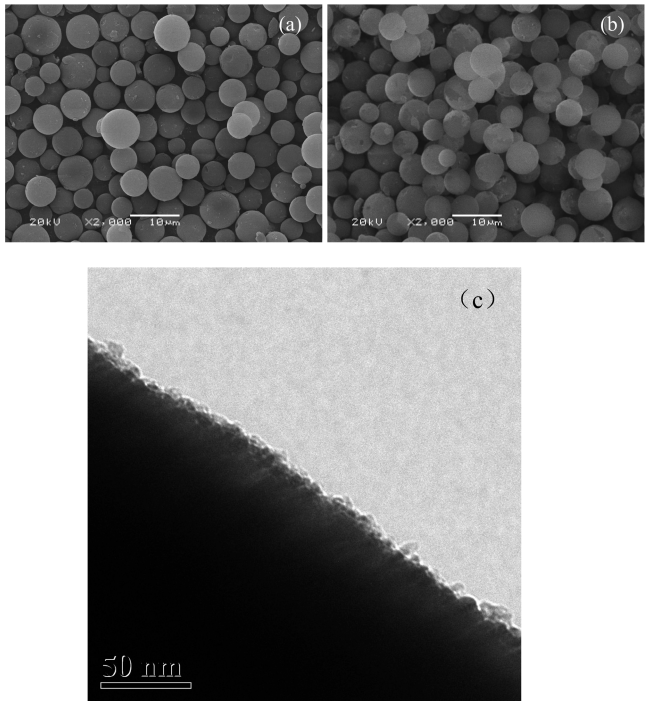

Figure 2. SEM images of (a) RAFT-MIP and (b) control-MIP, and TEM images of RAFT-MIP (c): (a, b) magnification ×2000; (c) magnification ×360 000.

and no aggregation was observed among the core–shell particles. The surface of the imprinting particles was smooth. As a control, as shown in Figure 2b, the imprinted particles prepared by the classic free radical initiation without RAFT strategy were of rugged surface and slightly aggregated. The morphology discrepancy between RAFT-MIP and control-MIP indicated that the applied RAFT strategy was beneficial to ensure the control shape of the imprinted shell, which could further influence the recognized ability and selectivity toward the template protein.

TEM image of the imprinting particles by RAFT strategy is shown in Figure 2(c). A thin shell around the silica core,

regarded as the surface imprinted polymer, was observed. For the comparison, as shown in Figure S1 in the Supporting Information, similar TEM image of the RAFT-NIP was observed. The thickness of the imprinted polymer layer was about 8 nm, which was nearly close to the summation of the protein approximate diameter (~3.2 nm) and the truss arm (~5 nm). This controllable shell thickness regulated by RAFT strategy would favor the recognition sites to bind the template protein with high selectivity.

Optimization of Polymerization Time. To investigate the regulatory role of RAFT strategy in the formation of imprinted shell, the growing characteristic of the polymer shell was explored first by the relationship of zeta potential and reaction time. As shown in Figure 3, the linear relationship was

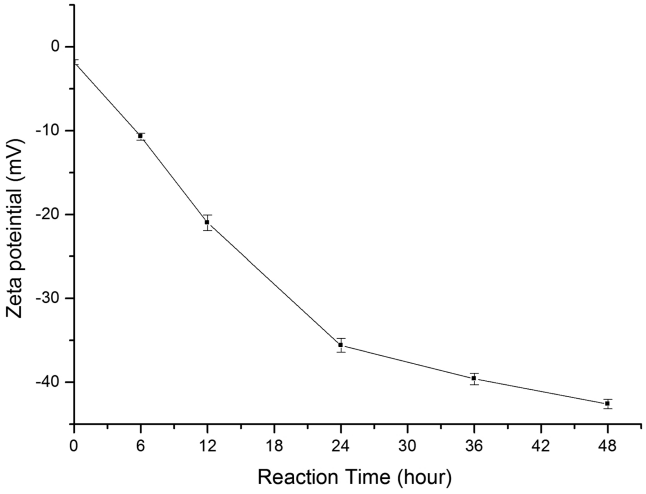

Figure 3. Relationship of polymerization time and zeta potential of RAFT-MIP.

observed at the range from 0 to 24 h, with the square of linearly dependent coefficient R^2 as 0.9944, which indicated that the polymer chains grew linearly by the improved RAFT strategy, different from the traditional radical polymerization, with free radical termination occurred easily and irreversibly without regulation. However, the zeta potential of prepared MIP changed slowly with the further increase of the reaction time, which might be caused by the fact that the unavoidable decomposition of dithioester or other side reactions in the long time reaction caused the decreasing of regulated ability by RAFT agent.[40] As a result, the RAFT-regulated reaction time in our study should be within 24 h.

Furthermore, the relationship of the reaction time to the binding capacity and imprinting factor (IF) of the imprinted particles prepared by RAFT strategy was also investigated. As shown in Figure 4, the binding capacity increased linearly with the reaction time from 12 to 48 h, which indicated that the imprinting sites began to form after 12 h polymerization. When the reaction time was 24 h, the highest IF (3.7) was obtained. Although the binding capacity increased with the prolonged polymerization time, IF decreased after 24 h, which was caused by the increased nonspecific binding with the excessive proliferation of polymer chains. Therefore, with the comprehensive consideration on the controlled polymerization, binding capacity and IF, the optimized reaction time in this study was 24 h.

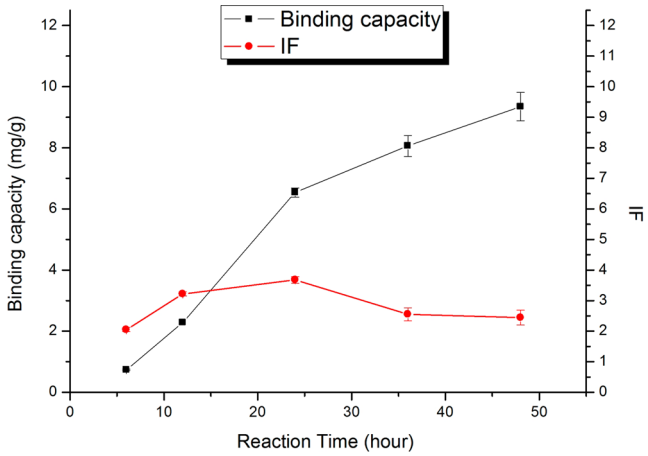

Figure 4. Relationship of reaction time of the RAFT imprinting particles preparing with the binding capacity and imprinting factor for Lys recognition.

Binding Capacity and Kinetic Study. The binding capacity and kinetic study of the Lys imprinted core–shell materials prepared by the improved RAFT strategy under the optimized conditions were investigated. As shown in Figure 5,

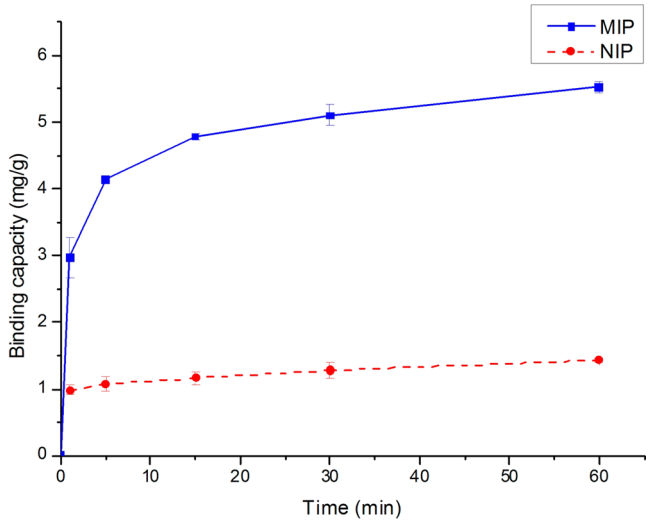

Figure 5. Kinetics of RAFT-MIP (blue points) and NIP (red points) toward the template protein, Lys.

for MIPs, the binding amount toward the template protein, Lys, could reach 75% of the maximum within 5 min, and the saturated adsorption, 5.6 mg/g, could be achieved within 60 min. The short equilibrium time and fast rebinding speed were benefited from the surface imprinting, which would favor the accessibility of the recognition sites. Furthermore, the adsorbed capacity of NIP was 1.5 mg/g, much less than that of MIP. The imprinting factor of Lys was calculated as 3.7, whereas the IF of control material prepared without RAFT strategy was only 1.6. Although the binding capacity is lower than that in some previous publications,[2,34,41] the recognition coefficient could reach to a quite high level in the field of protein imprinting. This was attributed to the low nonspecific binding of the Poly(MAA-HEMA-MBA) matrix and the specific binding of the recognition sites prepared by RAFT strategy.

Selectivity of Lys Imprinted Particles. To investigate the selectivity of Lys imprinted core–shell particles prepared by

improved the RAFT strategy, we actualized the competitive recognition under the optimum conditions (10 mM PBS buffer, pH7.4) in a mixture of four proteins with equal concentration, composed of Lys, RNB, PSA, and Mb, with various physical and chemical parameters. As shown in Figure 6, in the specific

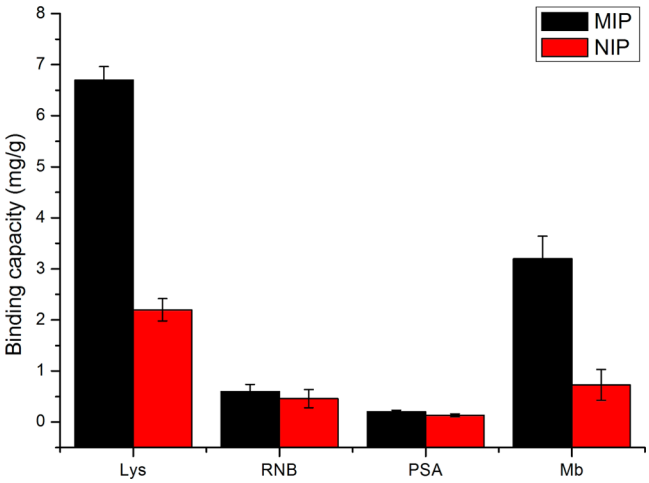

Figure 6. Eluted concentrations of proteins from MIP and NIP particles prepared by RAFT strategy. Incubation lasted 24 h in PBS buffer with pH 7.4.

elution fractions, the eluted amount of Lys from MIP and NIP were respectively 6.7 and 2.2 mg/g, with IF about 3.0, close to that obtained in single protein rebinding. Besides, the eluted amount of PSA and RNB from MIP and NIP was neglectable, while that of Mb was also much less than Lys. These results indicated that the imprinted particles had good selectivity toward the template protein, and low nonspecial absorption toward the interferential proteins in competitive recognition. To investigate further, we noted that the condition of pH 7.4, similar to the physiological condition, was suitable for the template protein recognition. Under such condition, the charge of polymer surface was negative, mainly because of the presence of carboxyl groups inside the copolymer network skeleton. Lys (pI 10.8) and RNB (pI 8.8), which were electron positive, could be attached by the nonspecial adsorption through charge interaction. As contrast, the acidic competitive protein, PSA (pI 5.1) or Mb (pI 7.0), had much lower absorption because their negative charge under recognition condition caused charge exclusion between protein and polymer shell. Additionally, the hydrophilicity of this material, arising from the hydrophilic monomers and cross-linker, was proved by the much lower nonspecial absorption toward the hydrophobic protein than Lys, such as Mb, under our optimized condition for protein competitive recognition, which indicated that hydrophilic interaction also played an indispensable role in protein binding and recognition.

For comparison, core–shell MIP particles were also prepared by traditional radical polymerization strategy. As shown in Figure 7, Lys eluted from the control MIP was much less than that of RAFT MIP, which indicated that the shell around control MIP may have weaker imprinting effectiveness without the RAFT agent regulated. Nearly no selectivity toward the template Lys appeared in the recognition of the control-MIP prepared by the same conditions as the RAFT MIP only without diothioester adding. Additionally, RNB eluted from the control MIP was also less than that from RAFT-MIP, but for

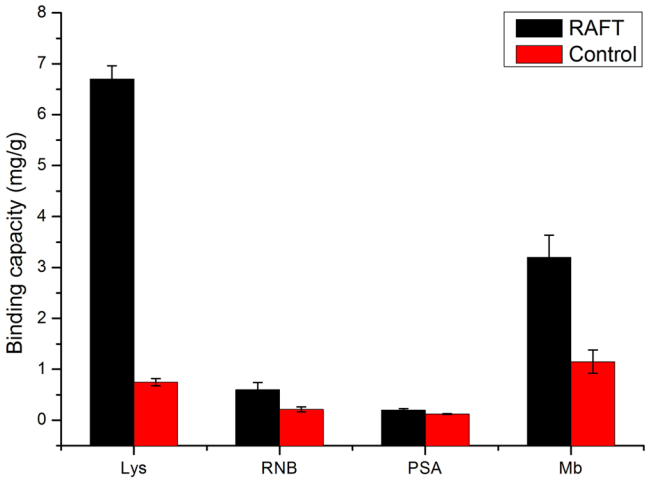

Figure 7. Eluted concentrations of proteins from MIP particles prepared by RAFT strategy and conventional polymerization as control. Incubation lasted 24 h in PBS buffer with pH 7.4.

the other two competitive proteins, PSA and Mb, eluted from RAFT-MIP and control-MIP were nearly equal. This fact indicated that the selectivity of RAFT-MIP toward the template protein was much better than that of control-MIP, which further deduced that the growth of polymer chains regulated by RAFT strategy was beneficial to improve the selectivity of protein imprinted particles. It was also noted that the eluted amount of Mb from the control-MIP higher than that from the RAFT-NIP (Figure 6). Deductively, heterogeneous shell could be formed without the RAFT agent regulation, which would lead to the silica core bareness. Then, strong nonspecial absorption toward the hydrophobic protein Mb could be observed.

Lys Recognition from the Chicken Egg White. To further investigate the applicability of Lys imprinted core–shell particles prepared by the improved RAFT strategy, we used chicken egg white as the sample, in which the mass fraction of Lys about 3%.[42,43] The chromatograms of egg white diluted by 1000 times and the eluant from the imprinted particles are shown in Figure 8. The obvious peak with the retention time

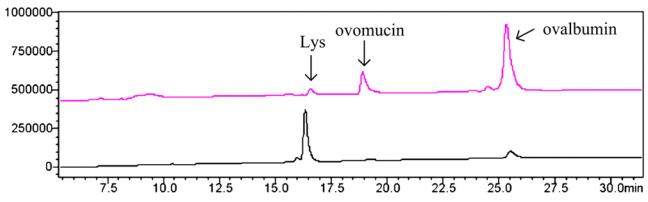

Figure 8. Chromatograms of the 1000-fold chicken egg white (the top line) and eluant from RAFT-MIP (the bottom line).

~16.50 min was identified as Lys, which was further confirmed by MALDI-TOF/TOF MS (see Figure S2 in the Supporting Information). The high recovery of Lys enriched from egg white was achieved, ca, 98.9%, collected from the imprinted particles. Other proteins in egg white, such as ovomucin (with the retention time ~19.2 min) and ovalbumin (with the retention time ~25.7 min), displayed little adsorption on the imprinted particles, but did not interfere with the binding of Lys. These results showed the potential of Lys imprinted core–shell particles for protein recognition from the complex biological samples.

CONCLUSIONS

A novel strategy combining molecularly imprinting and RAFT strategy was developed to prepare Lys imprinted core−shell particles with controllable imprinted shell in aqueous solution, with MAA and HEMA as the hyodrophilic functional monomers, and MBA as the cross-linker. The results demonstrated that the prepared protein imprinted particles showed good recognition selectivity, high binding capacity and rapid mass transfer toward the template protein, no matter in protein competitive recognition and real samples. With the advantages such as the consideration on the selectivity or environmentally friendly fabrication protocols, this proposed strategy could become a general and straightforward method for developing artificial antibodies to capture proteins, especially for proteins whose antibodies could not be produced hardly by current biological processes.

ASSOCIATED CONTENT

Supporting Information

(1) Information regarding the TEM images of RAFT-NIP (Figure S1). (2) Information regarding the MALDI-TOF/TOF MS spectrum of the fraction with the retention time 16.5 min in the experiment of Lys Recognition from egg white samples (Figure S2). (3) Information regarding the regulatory role of RAFT agent in polymerization was further investigated by the relationship between reaction time and template binding capacity in competitive recognition and shown in Figure S3. All these materials are available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*E-mail: lihuazhang@dicp.ac.cn. Phone & fax: +86 411 84379720.

Author Contributions

§Q.L. and K.Y. contributed equally.

Notes

The authors declare no competing financial interest.

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