Defining the Interactions between Proteins and Surfactants for Nanoparticle Surface Imprinting through Miniemulsion Polymerization

Chau Jin Tan,[†] Shalom Wangrangsimakul,[†] Renbi Bai,[‡] and Yen Wah Tong*[,†,§]

Department of Chemical and Biomolecular Engineering, Division of Environmental Science and Engineering, and Division of Bioengineering, National University of Singapore, 21 Lower Kent Ridge Road, Singapore 119077

Received August 3, 2007. Revised Manuscript Received October 29, 2007

Molecular imprinting has been considered one of the most promising techniques for the preparation of synthetic receptors. In spite of the ease of the conventional imprinting methodology and its associated success with the imprinting of small molecules, the approach has its limititation for the imprinting of protein macromolecules. This is primarily due to the limited diffusion associated with the bulkiness of the template macromolecules and the incompatibility between the fragile protein template and the imprinting conditions. To resolve these issues for the successful imprinting of proteins, miniemulsion polymerization has been employed for preparing protein surface-imprinted nanoparticles. Ribonuclease A (RNase A), bovine serum albumin (BSA), and lysozyme (Lys) were used as the template proteins while methylmethacrylate and ethylene glycol dimethacrylate were the functional and cross-linking monomers, respectively, to produce particles with sizes of about 40 nm. The RNase A surface-imprinted nanoparticles displayed favorable molecular selectivity and rebinding kinetics even in an aqueous medium. However, such a molecular recognition property was not observed for the BSA- and Lys-imprinted nanoparticles. By studying the template protein−surfactant interaction using circular dichroism, it was found that a certain degree of interaction between the template protein and the micelles is required to maintain the proteins at the particle surface. It was also found that such an interaction should not be too extensive to cause a significant conformational change, or denaturation, in the proteins to ensure the recognition of proteins in their native states. This study therefore defines the important parameters required for the successful application of a simple miniemulsion polymerization strategy for protein−surface imprinting.

Introduction

Molecular imprinting is a state-of-the-art technique for the preparation of tailor-made artificial receptors that can selectively bind predetermined target molecules. The technique is based on the effective template−monomer interaction, where complementary binding sites to the template molecules are formed in the polymeric matrix during polymerization in the presence of a cross-linker[1–4] as illustrated in Scheme 1.

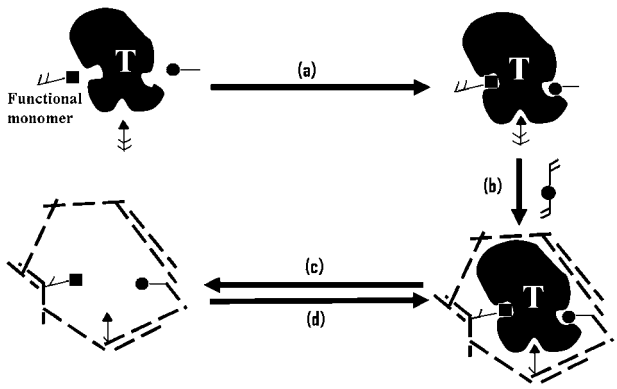
Molecularly imprinted polymers represent a new class of materials possessing a high affinity for the target molecule and form a promising substitute for biological antibodies in applications like catalysis,[5,6] immunoassay,[7] biosensing,[8,9] and drug delivery.[10–12] To date, successful application of the technique using the conventional approach has often been associated with small molecules[13–15] but is still limited for macromolecules like proteins and polysaccharides. This is primarily due to the difficulty of the template removal and the restricted accessibility of the binding sites embedded in

* Corresponding author. Tel: +65-6516 8467. E-mail: chetyw@nus.edu.sg.
[†] Department of Chemical and Biomolecular Engineering.
[‡] Division of Environmental Science and Engineering.
[§] Division of Bioengineering.

(1) O'Connor, N. A.; Paisner, D. A.; Huryn, D.; Shea, K. J. *J. Am. Chem. Soc.* **2007**, *129*, 1680–1689.
(2) Nicholls, I. A.; Andersson, H. S. In *Molecularly Imprinted Polymers: Man-made mimics of antibodies and their applications in analytical chemistry*; Sellergren, B., Ed.; Elsevier Science: Amsterdam, The Netherlands, 2001; Vol. 23, p 61.
(3) Kublickas, R.; Werner, C.; Jarienë, G.; Voit, B.; Laŝas, L. *Polym. Bull.* **2007**, *58*, 611–617.
(4) Simon, R.; Collins, M. E.; Spivak, D. A. *Anal. Chim. Acta* **2007**, *591*, 7–16.
(5) Volkmann, A.; Brüggemann, O. *React. Funct. Polym.* **2006**, *66*, 1725–1733.
(6) Pasetto, P.; Maddock, S. C.; Resmini, M. *Anal. Chim. Acta* **2005**, *542*, 66–75.
(7) Bossi, A.; Piletsky, S. A.; Piletska, E. V.; Righetti, P. G.; Turner, A. P. F. *Anal. Chem.* **2001**, *73*, 5281–5286.
(8) Lakshmi, D.; Prasad, B. B.; Sharma, P. S. *Talanta* **2006**, *70*, 272–280.
(9) Li, X.; Husson, S. M. *Langmuir* **2006**, *22*, 9658–9663.
(10) Cunliffe, D.; Kirby, A.; Alexander, C. *Adv. Drug Delivery Rev.* **2005**, *57*, 1836–1853.
(11) Sellergren, B.; Allender, C. J. *Adv. Drug Delivery Rev.* **2005**, *57*, 1733–1741.
(12) Duarte, A. R. C.; Casimiro, T.; Aguiar-Ricardo, A.; Simplício, A. L.; Duarte, C. M. M. *J. Supercrit. Fluids* **2006**, *39*, 102–106.
(13) Hall, A. J.; Quaglia, M.; Manesiotis, P.; Lorenzi, E. D.; Sellergren, B. *Anal. Chem.* **2006**, *78*, 8362–8367.
(14) Wang, H. Y.; Jiang, J. G.; Ma, L. Y.; Pang, Y. L. *React. Funct. Polym.* **2006**, *66*, 1081–1086.
(15) Zhang, D.; Li, S.; Huang, J.; Luo, G. *High Perform. Polym.* **2006**, *18*, 949–960.

10.1021/cm702174y CCC: $40.75
Published on Web 12/05/2007

Scheme 1. Schematic Illustration of Molecular Imprinting[a]



[a] (a) Prepolymerization complex formation between template T and functional monomers; (b) copolymerization with an excess of cross-linkers; (c) extraction of template from imprinted binding sites; (d) rebinding of template molecule to the imprinted sites.

the polymer matrices for large molecules.[16–18] To circumvent such limitations, the notion of surface imprinting has been put forward and applied for protein imprinting.[19–25] With the creation of imprinted binding sites on the polymer surface, the problem of diffusion can be alleviated. Another drawback of the traditional bulk imprinting methodology is the production of an inconsistent, irregular, and sharp-edged imprinted polymer. This limits its application in certain areas such as packings for chromatographic columns. A third significant disadvantage is the inability of the bulk polymerization to be employed on an industrial scale because of the poor thermal dispersion of the process. Together, these factors create the necessity for an alternative imprinting system for macromolecules.

Functional monomers like methacrylic acid (MAA) and acrylamide have been commonly used for protein imprinting through the noncovalent (or self-assembly) approach. Favorable hydrogen bond formation between the functional monomers and the template protein molecules have often been used to achieve a satisfactory imprinting efficiency. Nevertheless, the recognition interactions will only be observed in an organic environment as water will interfere with the hydrogen bonding between the complementary functional moieties on the binding sites and the template protein.[26] It is, however, preferable to achieve protein recognition in an aqueous surrounding similar to biological systems where most applications are conducted.[27,28] In this work, we aimed to make use of the synergistic effect of the hydrophobic interaction and shape complementarity as the driving force to enable molecular selectivity of the resulting imprinted material in an aqueous environment.

Miniemulsion polymerization is a polymerization technique that can give monodispersed, regularly shaped polymeric nanoparticles of sizes between 50 and 500 nm. The difference from conventional emulsion polymerization lies in the addition of a high-shear homogenization step and the necessity for a cosurfactant. With excellent heat dispersion, this approach will be suitable for large-scale commercial production. This polymerization system serves as a potential imprinting system to solve the limitations posed by the traditional imprinting methodology. In fact, Vaihinger et al. had employed miniemulsion polymerization to prepare imprinted nanoparticles for amino acids,[29] but to date, this approach has not been widely used.

We hereby present an extension of our previous work[30] where redox-initiated miniemulsion polymerization was employed to prepare surface imprinted nanoparticles as a general method applicable for the whole class of protein macromolecules. Methylmethacrylate (MMA) and ethylene glycol dimethacrylate (EGDMA) were used as the functional monomer and the cross-linker, respectively. MMA is a common monomer used in miniemulsion polymerization, but because of its low water solubility, it is often not considered as a good candidate for hydrogen bond formation in conventional molecular imprinting. Hydrophobic binding cavities were found to form on the surface of the nanoparticles that enable favorable hydrophobic interactions between the shape-complementary binding sites and the target protein in an aqueous environment. Another reason why MMA was chosen is its similarity to MAA where its application in protein imprinting had been proved to be effective. Nevertheless, other hydrophobic monomers like pyridine can also be considered. A mixture of sodium dodecyl sulfate (SDS) and poly(vinyl alcohol) (PVA) had been used as the surfactant system. This was based on our previous study[30] on the template protein−surfactant interaction where the addition of the PVA helped the template Ribonuclease A (RNase A) to retain its structural configuration in spite of interacting with, and adsorbing to, the micelles. We have found that the RNase A surface-imprinted nanoparticles exhibited the desired molecular selectivity in the single and competitive protein adsorption systems in addition to the favorable rebinding kinetics. In this work, we examine proteins with different characteristics of size and hydrophobicity, such as bovine serum albumin (BSA) and lysozyme (Lys), to be used as the template molecules. The interactions between the surfactant and the template proteins were

(16) Turner, N. W.; Jeans, C. W.; Brain, K. R.; Allender, C. J.; Hlady, V.; Britt, D. W. *Biotechnol. Prog.* **2006**, *22*, 1474–1489.
(17) Wei, S.; Jakusch, M.; Mizaikoff, B. *Anal. Chim. Acta* **2006**, *578*, 50–58.
(18) Bossi, A.; Bonini, F.; Turner, A. P. F.; Piletsky, S. A. *Biosens. Bioelectron.* **2007**, *22*, 1131–1137.
(19) Shi, H.; Tsai, W. B.; Garrison, M. D.; Ferrari, S.; Ratner, B. D. *Nature* **1999**, *398*, 593–597.
(20) Nishino, H.; Huang, C. S.; Shea, K. J. *Angew. Chem., Int. Ed.* **2006**, *45*, 2392–2396.
(21) Hayden, O.; Haderspöck, C.; Krassnig, S.; Chen, X.; Dickert, F. L. *Analyst* **2006**, *131*, 1044–1050.
(22) Lin, H. Y.; Hsu, C. Y.; Thomas, J. L.; Wang, S. E.; Chen, H. C.; Chou, T. C. *Biosens. Bioelectron.* **2006**, *22*, 534–543.
(23) Rick, J.; Chou, T. C. *Biosens. Bioelectron.* **2006**, *22*, 544–549.
(24) Bonini, F.; Piletsky, S.; Turner, A. P. F.; Speghini, A.; Bossi, A. *Biosens. Bioelectron.* **2007**, *22*, 2322–2328.
(25) Yan, C. L.; Lu, Y.; Gao, S. Y. *J. Polym. Sci., Part A: Polym. Chem.* **2007**, *45*, 1911–1919.
(26) Silvestri, D.; Barbani, N.; Cristallini, C.; Giusti, P.; Ciardelli, G. *J. Membr. Sci.* **2006**, *282*, 284–295.
(27) Haupt, K.; Mosbach, K. *Tibtech* **1998**, *16*, 468–475.
(28) Janiak, D. S.; Kofinas, P. *Anal. Bioanal. Chem.* **2007**, *389*, 399–404.
(29) Vaihinger, D.; Landfester, K.; Kräuter, I.; Brunner, H.; Tovar, G. E. M. *Macromol. Chem. Phys.* **2002**, *203*, 1965–1973.
(30) Tan, C. J.; Tong, Y. W. *Langmuir* **2007**, *23*, 2722–2730.

characterized using circular dichroism (CD) and other methods to determine the factors essential to the success of protein surface imprinting through miniemulsion polymerization.

Experimental Section

Materials. RNase A from bovine pancreas and BSA and Lys from chicken egg white used in this study were purchased from Sigma (U.S.A.). MMA (99%), EGDMA (98%), SDS (minimum 98.5% GC), sodium bicarbonate (99.7–100.3%), sodium bisulfite (minimum 99%), and ammonium persulfate (APS, 98%) were from Aldrich (U.S.A.). PVA (80 mol % hydrolyzed, MW 6000) was obtained from Polysciences. All chemicals were used directly without further purification.

Preparation of the Surface-Imprinted Nanoparticles. The miniemulsion polymerization used for the protein imprinting process was used in our previous studies[30] and modified from the work by Miller et al.[31]

To form the first aqueous phase, 0.375 g of PVA, 57.7 mg of SDS, and 46.9 mg of sodium bicarbonate were first dissolved in 20 mL of deionized (DI) water. A second aqueous phase of 0.2 g of PVA and SDS were dissolved in 400 mL of DI water. A 0.8 mL volume of MMA and 4.2 mL of EGDMA were then mixed to form the oil phase. The monomer mixture oil phase was slowly added into the first aqueous phase, followed by homogenization at 24 000 rpm with a homogenizer (T25B, Ika Labortechnik, Germany) to form a miniemulsion. Subsequently, 1.87 μmol of the template protein were added to the miniemulsion and mixed with a magnetic stirrer for 30 min to allow effective monomer−template interaction. The miniemulsion with the template protein was then added into the second aqueous phase under stirring and transferred into a 1 L three-neck round-bottomed flask. The flask was mechanically stirred (RW20, Ika Labortechnik, Germany) at 300 rpm and slowly heated. After the prepolymerization mixture had reached 40 °C, the reaction vessel was prepurged with nitrogen gas for 15 min to displace the oxygen, and finally, sodium bisulfite (0.230 g), followed by APS (0.252 g), was added into the mixture for polymerization for 24 h.

The polymer product was then washed five times each with DI water and a solution of SDS and acetic acid (10% w/v:10% v/v) to remove the template protein, four times with excess ethanol to remove the surfactant or any unreacted monomer and initiator, and six times with DI water. For each washing step, the solution was centrifuged (Universal 32R, Hettich Zentrifugen, Germany) at 9000 rpm for 1 h to isolate the polymeric product from the washing medium. UV spectrometry had been employed to verify the absence of the template protein in the washing solvent. The washed imprinted polymer was diluted with DI water to 200 mL and kept as a suspension under room temperature for further characterization. Three types of surface-imprinted nanoparticles were prepared, namely, the RNase A-imprinted (RMIP), the BSA-imprinted (BMIP), and the Lys-imprinted (LMIP) nanoparticles.

Preparation of Nonimprinted (NIP) Nanoparticles. NIP nanoparticles were prepared in a similar manner as above, except without the addition of the template protein. Upon completion of the polymerization reaction, the NIP polymer was washed in a similar way as that for the imprinted particles. The final polymer was kept as a suspension in water to be used as the control in the subsequent characterization phase.

Elemental Analysis. The RMIP nanoparticles had been subjected to elemental analysis (CHNS/O Analyzer Series II 2400, Perkin Elmer, U.S.A.) to verify the successful removal of the template protein molecules.

Size Measurement Using a Field-Emission Scanning Electron Microscope (FESEM). The sizes of the imprinted and NIP polymeric nanoparticles were measured through direct observations under a FESEM (JSM-6700F, JEOL, U.S.A.). Fifty particles from at least two different locations were counted for the size determination.

Surface Area Determination. Surface area measurements were carried out through a nitrogen sorption method with a NOVA 3000 series from Quantachrome Instruments, U.S.A., using a 7 point Brunauer−Emmett−Teller (BET) calculation.

Determination of Swelling Ratio (SR). The dispersed polymeric particles were recovered by centrifugation at 9000 rpm for 40 min. The supernatant was then removed, and the swollen weight (W_w) of the particles was measured. Subsequently, the particles were freeze-dried and weighed again to obtain the dry weight (W_d). The swelling ratio of the polymer was then calculated as follows:[32]

$$\mathrm{SR} = \frac{W_w - W_d}{W_d}$$

The measurements were performed in triplicates.

Batch Rebinding Test. Six samples of 5 mL of imprinted polymeric nanoparticles were measured and prepared in 15 mL centrifuge tubes. Then, 5 mL of protein solutions of different concentrations were added to make up to the various initial protein concentrations (0.8–1.6 mg/mL), and the samples were fitted onto a rotamix (RKVS, ATR Inc., Japan) and mixed (end-to-end) at room temperature for 24 h. The same procedure was carried out for the NIP nanoparticles. After 24 h, the samples were centrifuged (Universal 32R, Hettich Zentrifugen, Germany) at 9000 rpm for 60 min. The supernatants were then drawn and filtered with sterile 0.2 μm filter units before being sent for high performance liquid chromatography (HPLC) analysis. An HPLC system (Agilent 1100 series, U.S.A.) with a gel-permeation chromatography (GPC) column (Agilent Technologies Plgel Mixed-C, 300 × 7.5 mm, 5 μm, U.S.A.) was employed to determine the final protein concentration in the supernatant. The mobile phase used was a phosphate buffer saline at pH 7.4. The solvent flow rate was 1 mL/min, and the injection volume was 50 μL. A UV detector at a detection wavelength of 220 nm was employed. Protein standards for calibration had also been prepared. The amount of protein adsorbed onto the polymer was determined by the method of difference according to the following formula:

$$\text{Amount of protein adsorbed, } Q \text{ (mg protein/g polymer)} = \frac{(C_I - C_F)V}{m}$$

where C_I is the initial protein concentration, C_F is the final protein concentration, V is the total volume of the rebinding aliquot, and m is the mass of the polymer in each aliquot.

To determine the value of m, six 5 mL reference aliquots of well-stirred polymer suspension were measured and freeze-dried. They were subsequently weighed to obtain an average value of the mass of polymer in each sample. The test was carried out in triplicate.

Competitive Batch Rebinding Test. The selectivity of the prepared imprinted and NIP nanoparticles was examined in a competitive adsorption environment. In the binary protein competitive rebinding test, 5 mL of RMIP and NIP polymeric latexes were placed into 15 mL centrifuge tubes. A protein mixture of RNase A and BSA was prepared with DI water and added into the centrifuge

(31) Miller, C. M.; Sudol, E. D.; Silebi, C. A.; El-Aasser, M. S. *Macromolecules* **1995**, *28*, 2754–2764.

(32) Lu, S.; Cheng, G.; Pang, X. J. *Appl. Polym. Sci.* **2006**, *100*, 684–694.

tubes to make an initial protein concentration of 1.2 mg/mL each for RNase A and BSA. The rebinding test was then conducted as described earlier, and the adsorption results were obtained. The following equations[33] were used to evaluate the selectivity of the RMIP nanoparticles:

$$\text{Static distribution coefficient, } K_D = \frac{C_p}{C_s}$$

where C_p is the amount of ligand adsorbed and C_s is the free ligand concentration,

$$\text{Separation factor, } \alpha = \frac{K_{D1}}{K_{D2}}$$

where K_{D1} and K_{D2} are the static distribution coefficients of the template and the control molecules, and

$$\text{Relative separation factor, } \beta = \frac{\alpha_1}{\alpha_2}$$

where $\alpha_1$and α_2 are the separation factors of the RMIP and NIP nanoparticles.

Following that, a ternary protein system made up of RNase A, BSA, and Lys (with initial concentration of 1.8 mg/mL) was used as the competitive environment to further probe the selectivity of the various imprinted nanoparticles that had been prepared. All the studies were performed in triplicate.

Kinetics Study. Five milliliters of an RMIP polymer suspension was added into a centrifuge tube containing 5 mL of an RNase A solution. The initial RNase A concentration was 1.8 mg/mL. The centrifuge tube was then fitted onto the rotamix for adsorption to be carried out at ambient temperature. At different time intervals, 1 mL of the protein solution was drawn and sent for HPLC analysis to determine the RNase A concentration. The study was also carried out for the NIP nanoparticles as a control.

Desorption Study. The RMIP nanoparticles were first subjected to a 24 h batch rebinding test as described above in an RNase A solution at an initial concentration of 1.8 mg/mL. The amount of the RNase A adsorbed onto the nanoparticles was determined in a similar fashion. Upon the completion of the batch adsorption, the RMIP nanoparticles with the adsorbed protein were isolated by centrifugation at 9000 rpm for 1 h. Following that, the nanoparticles were dispersed and rotary (end-to-end) mixed for 24 h in two different types of solvents (2 mL), namely, water and acetonitrile/water (1:1). Finally, the amount of RNase A desorbed was determined using HPLC.

CD Study. The protein solution of interest was prepared using DI water at a concentration of 5 mg/mL. To investigate the interaction between the surfactant and the proteins, in some protein samples SDS (10 mM) or SDS (mM)/PVA (1.5% w/v) had been added. The samples were loaded into a 5 mm round cuvette and analyzed using a spectropolarimeter (JASCO, model J-810, U.K.), with a continuous mode, at a scan speed of 50 nm/min. The range of wavelength scanned was 180–300 nm.

Statistical Analysis. Standard deviation calculations and Student's *t* tests were carried out using Microsoft Excel (Seattle, WA) for statistical comparisons between pairs of samples. One-way analysis of variance (ANOVA) with Tukey HSD post hoc analysis have also been applied for multiple samples analysis.

Results and Discussion

Morphological Features. FESEM had been used to capture the microscopic images of the nanoparticles prepared.

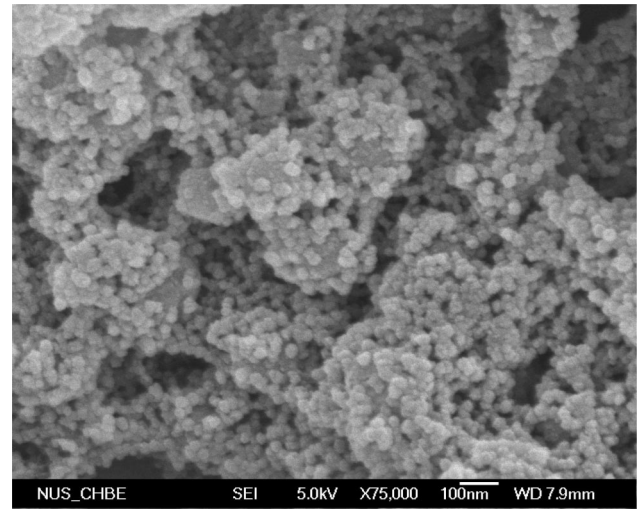


Figure 1. FESEM images of RMIP nanoparticles.

As shown in Figure 1, highly monodispersed, regularly shaped nanoparticles were synthesized. It can be seen here that the RMIP particles are of sizes around 40 nm, and there were no significant morphological differences between the RMIP particles and the other nanoparticles (NIP, BMIP, and LMIP images not shown). In molecular imprinting, highly cross-linked imprinted polymers are usually prepared for effective maintenance of the imprinted sites formed during the imprinting polymerization. The SR values are direct indications of the extent of the cross-linking of the polymeric nanoparticles.[32] In this work, the SR values obtained for various nanoparticles prepared (around 3–5) are close to those obtained by Lu et al.,[32] illustrating that sufficient amount of cross-linking had been achieved. Additionally, the nitrogen sorption BET results showed that there were no significant differences in the specific surface areas of the NIP, RMIP, and LMIP nanoparticles, which averaged about 26 m^2/g. Nevertheless, it was interesting to note that the specific surface area of the BMIP particles (about 14 m^2/g) was significantly lower than the others. The exact reason for this discrepancy is unclear at this moment, but it is hypothesized to be due to the difference in the type of interactions between BSA and the micellar system in the imprinting polymerization system (see protein−surface interaction below). It is interesting to note that the specific surface areas observed for these nanoparticles were not as high as those obtained by Vaihinger et al.[29] (about 58.0 m^2/g) despite the size of the particles (about 200 nm) being much bigger. This could be attributed to the difference in the porosity of the nanoparticles prepared in the respective works, as no observable pores can be seen from the FESEM images. Additionally, agglomeration is generally observed for nanoparticles in the dried state; thus, when the nanoparticles were lyophilized for BET measurement, it was highly possible that they agglomerated extensively resulting in the smaller measured specific surface area. Nevertheless, although having a larger surface could be advantageous for loading and rebinding kinetics, this could in fact compromise the selectivity of the imprinted nanoparticles because of the increase in the nonspecific adsoption on the non-imprinted areas.

(33) Lu, S.; Cheng, G.; Pang, X. J. *Appl. Polym. Sci.* **2006**, *99*, 2401–2407.

Table 1. Results of the Elemental Analysis

polymer	N composition (wt %)
NIP (before removal)	0[a]
NIP (after removal)	0[a]
RMIP (before removal)	0.659
RMIP (after removal)	0[a]

[a] Below the detection limit of the elemental analyzer.

Elemental Analysis. Template removal has been one of the most important challenges in protein imprinting because of the bulkiness of the template protein molecules. The solvent for template removal employed in this study had previously been used[34–36] and had been found to be the optimum for this purpose.[28] To verify the successful removal of the template protein molecules in the fabrication of the imprinted nanoparticles, the RMIP nanoparticles had been used as the model sample and subjected to elemental analysis. The results are shown in Table 1. From the elemental analysis, it was found that after the washing procedure the template RNase A molecules had been effectively removed and could not be detected by the analyzer.

Batch Rebinding Tests. The nanoparticles were subjected to single protein batch rebinding tests to elucidate their adsorption behavior. The results of the rebinding test carried out using RNase A, as shown in Figure 2a, had been performed with a series of different initial concentrations (0.8–1.6 mg/mL). It can be seen that the RNase A uptake by various polymeric nanoparticles increased with the increase in the initial RNase A concentration. Most notably, the RMIP nanoparticles displayed a significantly preferential uptake of the protein compared to others at various initial protein concentrations. This illustrated the selectivity of the RMIP nanoparticles toward the template RNase A used in its fabrication and the successful imprinting of the protein as previously observed.[30] The RNase A loadings of the RMIP particles ranged from around 10 to 60 μmol/g. On the other hand, while the batch rebinding was conducted with BSA as the adsorbate protein (Figure 2b), it is surprising to observe that the BMIP nanoparticles failed to exhibit any molecular recognition property toward the protein, indicating that the imprinting efficiency for the BMIP nanoparticles was very poor. Generally, the BSA loadings of the nanoparticles were lower than those for RNase A, and this could be attributed to the difference in size of the two proteins. In addition, the BSA uptake also showed a similar trend as the RNase A adsorption with the increase in the initial BSA concentration, except for RMIP nanoparticles at certain initial concentrations. The third batch rebinding test was performed in a Lys solution (Figure 2c), where only the LMIP and NIP nanoparticles were used. It can be observed that, on average, the LMIP nanoparticles did give us a certain degree of molecular selectivity as compared to their NIP counterparts. However, such differential Lys uptake was not statistically significant, and it would be difficult to conclude that a good imprinting efficiency had been achieved.

Competitive Batch Rebinding Test. To further probe the molecular recognition property of the fabricated nanoparticles, the batch adsorption run was next performed in a competitive environment. As RMIP nanoparticles had shown the most significant molecular selectivity in the previous single protein batch rebinding test, the RMIP and control NIP nanoparticles were exposed to a protein mixture of RNase A and BSA with an initial concentration of 1.2 mg/mL, respectively, in the first binary protein competitive adsorption test. The results are shown in Figure 3. As observed, the NIP nanoparticles adsorbed more RNase A (1.205 μmol RNase A/g NIP) than BSA (0.394 μmol BSA/g NIP) in the protein mixture. However, under identical conditions, when the test was conducted for the RMIP nanoparticles, a marked increase of about 80% in the binding of the template protein to the polymer was observed while the BSA loading (0.412 μmol BSA/g RMIP) remained constant as that for the case of NIP nanoparticles. It was deduced that the protein uptake by NIP nanoparticles was mostly nonspecific, and the marked increase observed in the adsorption of RNase A to RMIP nanoparticles was a strong proof that the recognition property had been imparted onto the imprinted nanoparticles.[30] The values of K_D, α, and β were calculated and are presented in Table 2. In this case, the RMIP nanoparticles achieved a separation factor that was almost twice as high as that by the NIP nanoparticles.

Extensive nonspecific adsorption is commonly observed for small particles with very large surface areas, and this could possibly mask the recognition property of the imprinted nanoparticles.[37] However, in this study, high template loading and good selectivity were detected through the routine rebinding tests in spite of the small sizes and the moderate BET surface areas of the RMIP nanoparticles. This indicated that effective imprinting had been achieved and that the nanoparticle surface was mostly occupied by imprinted sites for the template RNase A.

In most cases, surface adsorption data is fitted to the Langmuir isotherm (Scatchard plot) to determine the saturation binding capacity (Q_s) as well as the association constant (K_a). These two values will characterize the binding properties. In this work, the rebinding data did not fit any commonly known adsorption isotherms such as the Langmuir and Freundlich isotherms. In Rushton et al.'s work,[38] the limitations of these isotherms had been discussed, and the reasons for the unsuccessful fitting of the rebinding data may include the incorrect assumptions made for the isotherms[39] and the inappropriate concentration range under study.

To further investigate the molecular selectivity of the fabricated nanoparticles, the latter were subjected to a ternary protein solution consisting of RNase A, BSA, and Lys at a respective initial concentration of 1.8 mg/mL for competitive

(34) Pang, X.; Cheng, G.; Li, R.; Lu, S.; Zhang, Y. *Anal. Chim. Acta* **2005**, *550*, 13–17.
(35) Hawkins, D. M.; Stevenson, D.; Reddy, S. M. *Anal. Chim. Acta* **2005**, *542*, 61–65.
(36) Hawkins, D. M.; Trache, A.; Ellis, E. A.; Stevenson, D.; Holzenburg, A.; Meininger, G. A.; Reddy, S. M. *Biomacromolecules* **2006**, *7*, 2560–2564.
(37) Whitcombe, M. J.; Vulfson, E. N. *Adv. Mater.* **2001**, *13*, 467–478.
(38) Rushton, G. T.; Karns, C. L.; Shimizu, K. D. *Anal. Chim. Acta* **2005**, *528*, 107–113.
(39) Norde, W.; Haynes, C. A. In *Proteins at interfaces 2: fundamentals and applications*; Horbett, T. A., Brash, J. L., Eds.; ACS Symposium Series 602; American Chemical Society: Washington, DC, 1995; pp 26–40.

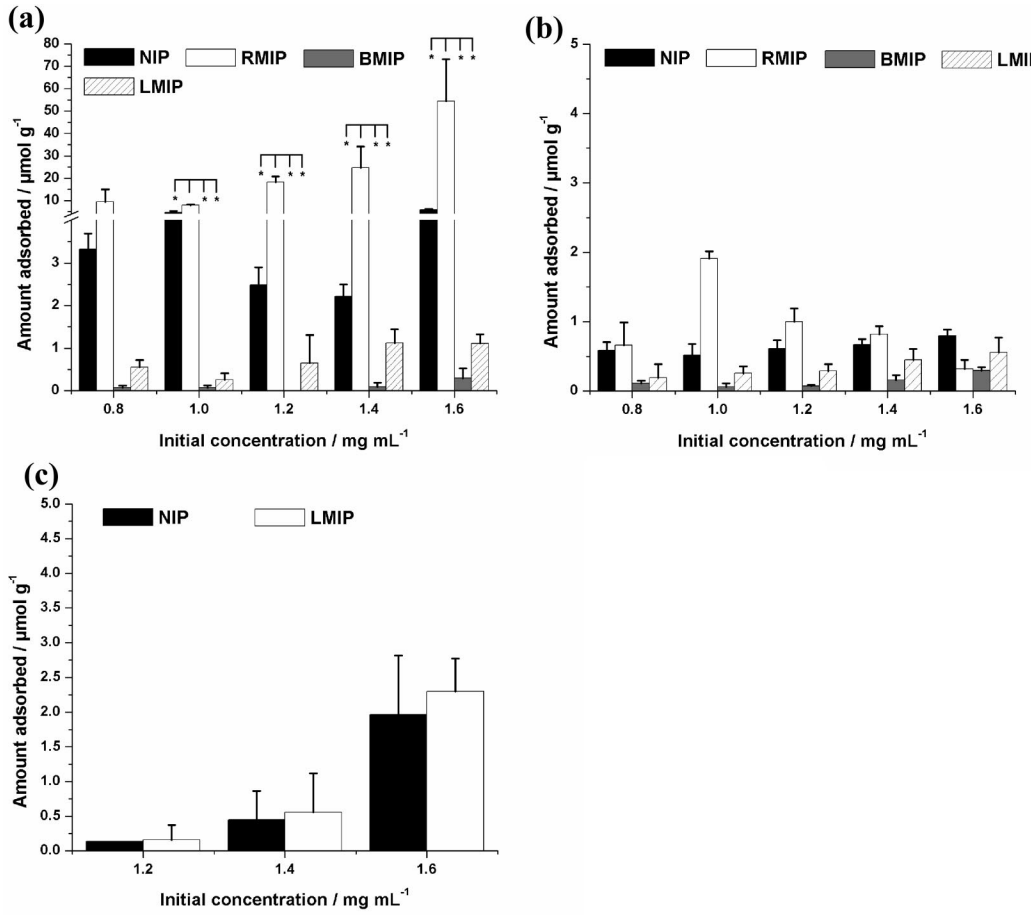


Figure 2. Results of batch rebinding tests in (a) RNase A, (b) BSA, and (c) Lys protein solutions. Statistical significance (*) was determined using one-way ANOVA with Tukey HSD post hoc analysis with $p < 0.01$.

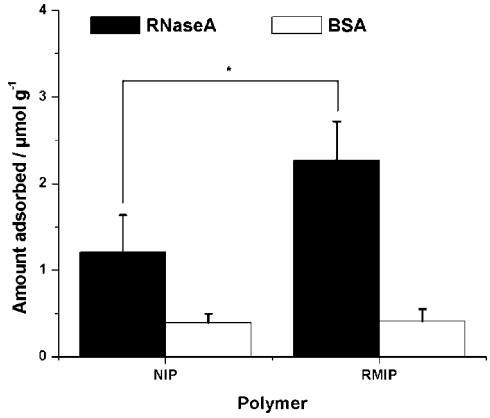


Figure 3. Results of the binary protein competitive batch rebinding test. Student's *t* test, *: $p < 0.12$.

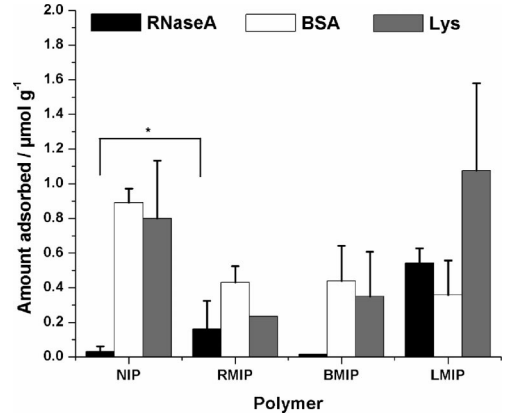


Figure 4. Results of the ternary protein competitive batch rebinding test. Student's *t* test, *: $p < 0.06$.

Table 2. Calculated Separation Factors of the NIP and RMIP Nanoparticles Based on the Competitive Rebinding Test

	RNase A		BSA					
polymer	C_p (μmol/g)	C_s (μmol/mL)	C_p (μmol/g)	C_s (μmol/mL)	K_{D1} (mL/g)	K_{D2} (mL/g)	α	β
RMIP	2.268	0.08569	0.4124	0.01783	26.5	23.1	1.147	1.82
NIP	1.205	0.08642	0.3935	0.01780	13.9	22.1	0.630	–

rebinding. Because of the presence of an additional competitive protein in this ternary protein system, the protein uptake for the nanoparticles had decreased to less than 1 μmol/g (Figure 4) as compared to that for the single or binary protein adsorption systems. In this competitive environment, the RMIP nanoparticles still recognized and adsorbed more RNase A than the NIP and BMIP nanoparticles. This illustrated the high imprinting efficiency achieved with the imprinting of the RNase A through miniemulsion polymerization. As for the BMIP nanoparticles, the competitive rebinding results coincided with those from our earlier single protein batch rebinding test where the expected affinity for the BSA protein was totally absent. In the case of the LMIP nanoparticles, on average, the Lys uptake was higher than all other nanoparticles although it was not statistically

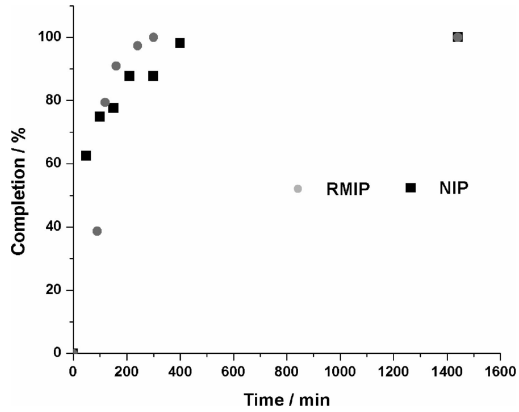


Figure 5. RNase A adsorption profiles of the NIP and RMIP nanoparticles.

significant. Thus, combined with our single protein batch rebinding results obtained for the LMIP above, we concluded that the imprinting efficiency was only modest. Nevertheless, it was interesting to note that the uptake of RNase A by the LMIP nanoparticles was higher than that for the RMIP particles. This unusual result is explained by a hypothesis as provided in conjunction with the CD analysis below.

Rebinding Kinetics. From the various rebinding tests conducted, it was found that the RMIP nanoparticles exhibited the most obvious molecular selectivity toward the template RNase A molecules used in their fabrication. One of the significant problems faced in protein imprinting is associated with the restricted diffusion due to the bulkiness of the template protein. Therefore, we studied the rebinding kinetics of the RMIP nanoparticles for RNase A as shown in Figure 5. The rebinding kinetics of the NIP nanoparticles has also been included as a control. Generally, there were no significant differences between the rebinding profiles of the NIP and those of the RMIP nanoparticles. The RNase A adsorption was observed to slowly increase for the first 100 min, after which it increased more rapidly in the next 50 min. After about 175 min, the rate of the rebinding started to decrease, and by 250 min, it had almost reached the equilibrium. The rapid initial protein adsorption was attributed to the presence of a large amount of empty, high-affinity binding sites on the surface of the nanoparticles. When most binding sites were filled up, the rate of adsorption dropped significantly and the equilibrium protein loading was eventually achieved. The rebinding profiles obtained are typical of those reported by other investigations.[40,41] The adsorption kinetics of our RMIP nanoparticles was comparable to the work performed by Pang et al.[40] in terms of the time taken to reach equilibrium. Such rapid rebinding kinetics are highly desired, and this will enhance their application in the fields of separation, biosensing, and analytical chemistry.

Desorption Study. The regeneration of the adsorbent nanoparticles was also investigated by desorbing the adsorbed protein. For the same reason as the above rebinding kinetics study, the RMIP nanoparticles had been chosen for this study.

Table 3. Results of the Desorption Study Using Different Solvents

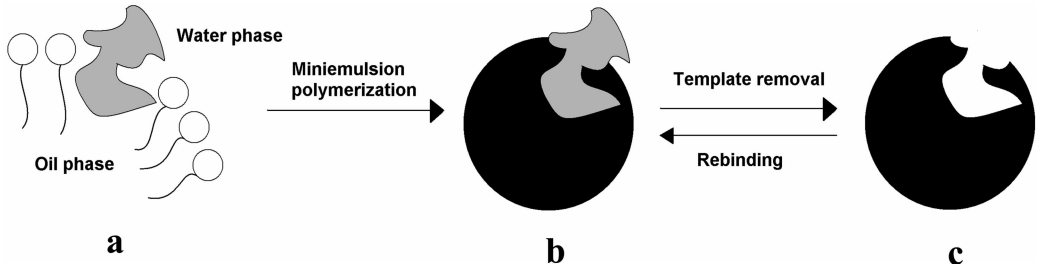
solvent	amount of RNase A desorbed (%)
water	38.0
water/ACN (1:1)	62.3

Two different types of desorbing solvents were used to remove the adsorbed RNase A from the RMIP nanoparticles, and the results are shown in Table 3. When water was used as the desorbing solvent, only about 38% of the adsorbed RNase A was removed, whereas the value increased to 62.3% when a 50% organic modifier (acetonitrile) was added to the solvent. The results shed some light on the type of interactions involved between RNase A and the RMIP nanoparticles. Apparently, hydrogen bonding did not play a critical role in the adsorption process as is illustrated by the low desorption efficiency of water. Comparatively, by increasing the nonpolarity of the solvent with acetonitrile (ACN), the desorption efficiency significantly increased, proving that the hydrophobic interaction was more significant in the RNase A uptake by the RMIP nanoparticles. To completely remove the adsorbed proteins, it is believed that a more hydrophobic solvent would be required, although this might denature the proteins being desorbed. Therefore, a more complete study on the proper desorption solvent would be necessary to enable these nanoparticles to be reusable.

Influence of the Protein−Surfactant Interaction. The main objective of this work is to design a one-step, easy-to-apply system for the preparation of protein surface-imprinted nanoparticles; thus, the miniemulsion polymerization had been chosen for this purpose. The exact mechanism involved in the protein imprinting miniemulsion polymerization is not known. However, it is hypothesized here that the success of the protein imprinting depends on the interaction between the micellar system and the surface-active template protein molecules. In a study by Moore et al. on protein−surfactant interactions,[42] it was proposed that the water-soluble protein molecules tend to be bound to the surface of the micelles formed by the surfactants. It was thus similarly hypothesized that when amphiphilic template protein molecules such as RNase A were added into the prepolymerization miniemulsion, they were adsorbed to the micelles and partitioned across the phase boundary. The polymerization reaction was then initiated with the template molecules trapped on the micelle surfaces. Upon completion and subsequent template removal, binding sites of the complementary protein hydrophobic sections were formed on the nanoparticle surfaces. During rebinding in an aqueous environment, protein molecules with the complementary shape to the binding sites would bind preferentially to the imprinted nanoparticles through hydrophobic interactions, as illustrated in Scheme 2. Although hydrophobic interaction is less specific compared to covalent, ionic, or hydrogen interactions, its ease of application and the applicability in an aqueous medium, supplemented with shape complemen-

(40) Pang, X.; Cheng, G.; Lu, S.; Tang, E. *Anal. Bioanal. Chem.* **2006**, *384*, 225–230.
(41) Fu, G.; Zhao, J.; Yu, H.; Liu, L.; He, B. *React. Funct. Polym.* **2007**, *67*, 442–450.
(42) Moore, P. N.; Puvvada, S.; Blankschtein, D. *Langmuir* **2003**, *19*, 1009–1016.

Scheme 2. Illustration of Protein Surface Imprinting through Miniemulsion Polymerization[a]



[a] (a) Adsorption of template protein molecule to the micelle; (b) molecular imprinting on the surface of the nanoparticles; (c) removal of the template RNase A molecules frees the imprinted cavities.

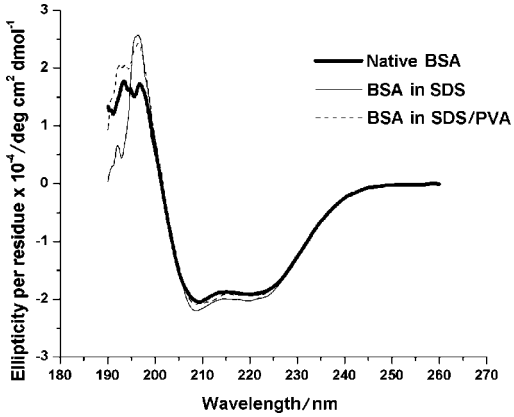


Figure 6. Solvent-corrected CD spectra of BSA in different types of surfactant systems, illustrating the lack of protein−surfactant interaction.

tarity, enables it to be an optimum type of interaction for molecular imprinting.[17,43,44]

Nevertheless, it is known that the adsorption of a protein molecule to surfactants can result in the unfavorable unfolding of the protein, causing it to lose its structural configuration.[45] From our earlier study[30] and some published works,[46,47] it was found that the structural integrity and rigidity of the template molecule plays a crucial role in the success of molecular imprinting. This is especially important for flexible template molecules like proteins. To verify our above hypothesis, we studied the structural conformation of the template protein molecules in surfactant solutions through CD measurements. This study would help to elucidate the interaction between the template proteins and the surfactants used in the imprinting polymerization. The CD spectra for BSA are shown in Figure 6. Native BSA has two characteristic negative signals at 209 and 222 nm that correspond to the absorption of the α-helices of BSA. It was found that even with the addition of SDS or SDS/PVA there were surprisingly no observable configurational changes from the CD spectra. Although the interactions between BSA and ionic surfactants like SDS are well-documented,[48–50] such interactions were not observed in our study. In a study[48] on BSA−SDS interaction, it was proposed that the denaturation of BSA by SDS proceeds through different stages. At low SDS concentrations, the SDS monomers bind to BSA molecules through electrostatic and hydrophobic interactions, loosening but not denaturing the protein. When the SDS concentration slowly increases, they continue to bind to BSA and start to promote denaturation. At this stage, rather than acting cooperatively as monomers, the SDS starts to form micelle-like clusters with the BSA molecules. Finally, when the SDS concentration increases beyond the critical micelle concentration (CMC), the BSA molecules are largely unfolded. Thus, the BSA−SDS interaction is a stepwise process.[49] In our current imprinting process, the template BSA molecules were directly exposed to an SDS/PVA solution at a concentration higher than the CMC. This could have prevented the complexation between the BSA and the SDS monomers and thereby deprived the BSA of the chance to interact with the surfactants in a stagewise fashion. Given the thermodynamically well-folded, compact, and globular structure of BSA, without the structure-loosening step by surfactant monomers in the preliminary stage of the interaction, it is probably not easy for BSA to interact directly with the surfactant micelles. This explains the absence of any conformational changes to BSA in the presence of the surfactants.

Another plausible explanation for the lack of protein−surfactant interaction is the electronic charge on the protein molecules in the imprinting polymerization mixture. The isoelectric point (pI) of BSA is 4.7, whereas the pH of our system is about 7.2. In this environment, the BSA molecules would have a net negative charge that would result in an electrostatic repulsion between the anionic SDS surfactant and the BSA molecules. On the basis of our hypothesis, a certain degree of interaction between the template protein and the surfactant would be required to ensure the success of the protein surface imprinting. The lack of interaction between BSA and the surfactant could be a main reason for the absence of the molecular selectivity observed for the

(43) Molinelli, A.; O' Mahony, J.; Nolan, K.; Smyth, M. R.; Jakusch, M.; Mizaikoff, B. *Anal. Chem.* **2005**, *77*, 5196–5204.
(44) Silvestri, D.; Barbani, N.; Cristallini, C.; Giusti, P.; Ciardelli, G. *J. Membr. Sci.* **2006**, *282*, 284–295.
(45) Dickinson, E. *Colloids Surf., B* **1999**, *15*, 161–176.
(46) Mahony, J. O.; Nolan, K.; Smyth, M. R.; Mizaikoff, B. *Anal. Chim. Acta* **2005**, *534*, 31–39.
(47) Karim, K.; Breton, F.; Rouillon, R.; Piletska, E. V.; Guerreiro, A.; Chianella, I.; Piletsky, S. A. *Adv. Drug Delivery Rev.* **2005**, *57*, 1795–1808.
(48) Ding, Y.; Shu, Y.; Ge, L.; Guo, R. *Colloids Surf., A* **2007**, *298*, 169–169.
(49) Takeda, K.; Shigeta, M.; Aoki, K. *J. Colloid Interface Sci.* **1987**, *117*, 120–126.
(50) Lu, R. C.; Cao, A. N.; Lai, L. H.; Zhu, B. Y.; Zhao, G. X.; Xiao, J. X. *Colloids Surf., B* **2005**, *41*, 139–143.

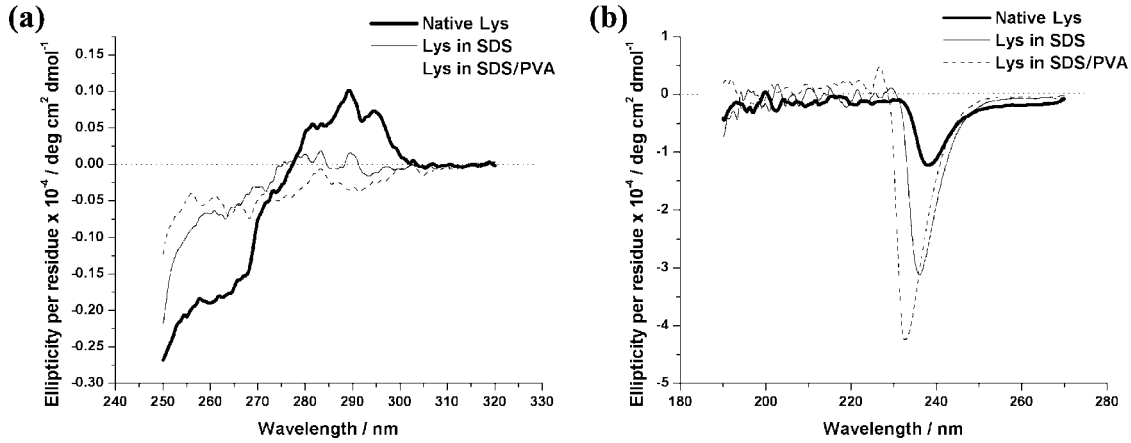


Figure 7. Solvent-corrected (a) near-UV and (b) far-UV CD spectra of Lys, illustrating the change in the protein structure in the presence of surfactants.

BMIP nanoparticles in our earlier investigation as the BSA would remain completely in the water phase. This explanation is also consistent with the results of the rebinding tests on the RMIP and LMIP nanoparticles. The pIs of the RNase A and Lys are 9.45 and 11.0, respectively, giving the two template molecules a net positive charge during the imprinting process. The resulting electrostatic attraction between the protein molecules and the SDS would promote protein−surfactant interaction, and thereby surface imprinting is more likely to be achieved.

On the other hand, Lys interacted significantly with the surfactant such that its conformation had been totally changed. This is as illustrated in the CD spectra obtained for Lys (Figure 7). In the near-UV region (250–300 nm, Figure 7a), the native Lys has a characteristic positive "head-and-shoulders" signal at 290 nm (head), 283 nm (shoulder), and 293.5 nm (shoulder),[51] which represents the tertiary structure of the protein. In the presence of surfactants, it was apparent that the positive CD signal vanishes, showing that Lys had been unfolded by the surfactants. Drastic conformational changes caused by the surfactants were further illustrated by the far-UV spectra obtained (Figure 7b). From this study, it was found that the template Lys interacted extensively with the surfactants used in the imprinting polymerization reaction and thereby lost its conformational features, resulting in the modest imprinting efficiency observed for the LMIP nanoparticles. In addition, because of such drastic change in the template Lys structure, the "incorrect template" that had been imprinted could be the reason for the unexpectedly high uptake of RNase A in the ternary protein competitive adsorption test performed earlier. This was highly possible as the RNase A and Lys have very similar molecular weights and sizes.

The CD spectra for RNase A in the surfactant solutions are shown in Figure 8. Compared to those for BSA and Lys, the presence of both the SDS and the PVA surfactants caused only a slight red-shift in the RNase A characteristic CD signal at around 218 nm. This indicates an interaction between the protein and the surfactant. Nevertheless, the general shape of the CD spectrum for RNase A in the SDS/PVA solutions remains similar to that for the native RNase A. Such optimum

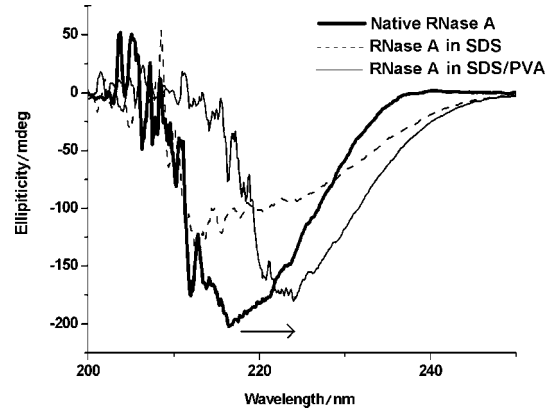


Figure 8. Solvent-corrected far-UV CD spectra of RNase A in surfactant solutions, illustrating an optimum level of protein−surfactant interaction for protein imprinting through miniemulsion polymerization.[30]

extent of protein−surfactant interaction was probably the main reason for the high imprinting efficiency achieved for the RMIP nanoparticles as we have shown in the previous work.[30]

Conclusions

In this work, the use of the redox-initiated miniemulsion polymerization for protein surface imprinting has successfully been illustrated with the significant molecular recognition property demonstrated by the RMIP nanoparticles. Nevertheless, when the technique was applied to imprint Lys and BSA, it was found that the molecular selectivity was only modest for the LMIP nanoparticles and totally absent for the BMIP nanoparticles. With the subsequent investigation on the protein−surfactant interactions, it was proposed that such interactions play an essential role in the success of protein imprinting using the miniemulsion polymerization system. A certain degree of interaction is required between the template protein molecules and the surfactant micelles to partition the template molecule at the micelle interface (as shown by the BMIP nanoparticles), but it is important that the interaction not be so extensive that the structural configurations of the protein molecules are changed drastically (as shown by the RMIP nanoparticles). A certain optimum interaction level should be achieved to balance these two factors. To enable the surface imprinting of proteins

(51) Goux, W. J.; Hooker, T. M., Jr. *Biopolymers* **1980**, *19*, 2191–2208.

other than RNase A, such as BSA and Lys, the use of this miniemulsion polymerization system based on the above considerations is currently being carried out to further test this hypothesis. We believe that because of its ease of application, its excellent heat dispersion, and its production of regularly sized imprinted nanoparticles with favorable features, the miniemulsion polymerization system is suitable to be employed at the industrial scale. Furthermore, the polymerization system provides a convenient means of incorporating desired properties, like drug-releasing and superparamagnetism,[52] into the imprinted nanoparticles. With such features in addition to their favorable selectivity and adsorption kinetics, the nanoparticles can act effectively as enzyme mimics and be possibly used in the fields of bioseparation, drug delivery, biosensing, and bioimaging.

Acknowledgment. This work was funded by the National University of Singapore under Grant R279000129112.

CM702174Y

(52) Tan, C. J.; Tong, Y. W. *Anal. Chem.* **2007**, *79*, 299–306.