Talanta 93 (2012) 160–165

Contents lists available at SciVerse ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Amino acid side chain-like surface modification on magnetic nanoparticles for highly efficient separation of mixed proteins

Soo Youn Lee [1], Chi Young Ahn [1], Jiho Lee, Jeong Ho Chang *

Korea Institute of Ceramic Engineering and Technology, Seoul 153-801, Republic of Korea

ARTICLE INFO

Article history:
Received 24 November 2011
Received in revised form 1 February 2012
Accepted 3 February 2012
Available online 9 February 2012

Keywords:
Silica-coated magnetic nanoparticles (Si-MNPs)
Mixed protein separation
Bovine serum albumin
Lysozyme
Fluorescene

ABSTRACT

This work reports on the realization of specific functionalized silica-coated magnetic nanoparticles (Si-MNPs) for effective protein separation through surface modification with various amino acid side chain-like functional groups such as thiol (—SH), disulfide (—S—S—), carbon chain ($—C_n$), carboxyl (—COOH), amine ($—NH_2$), and aldehyde (—CHO). This study also suggests an improved and convenient method for the synthesis of functionalized Si-MNPs by hydrolysis condensation with silan-coupling agents. The protein adsorption effects in a coexistent mixed state are explored using various proteins, which have different isoelectric point (p*I*) values and molecular weights, in order to elucidate the binding performance of different proteins one solution. The adsorption efficiency of bovine serum albumin (BSA; 66 kDa; p*I* = 4.65) and lysozyme (LYZ; 14.3 kDa; p*I* = 11) is 70–100% with various amino acid side chain-like functional groups. However, the adsorption efficiency of a mixed protein solution of BSA and LYZ was different. Although the relatively bulky BSA molecule displayed 50% and 20% adsorption corresponding to pH 4.65, and pH 11, respectively, the smaller LYZ provided almost 100% adsorption at both pH 4.65 and pH 11.

1. Introduction

Over the past decade, magnetic nanoparticles (MNPs) have received increasing attention for application in bioseparation owing to their ease of operation, uniqueness, and nano-scale size [1]. The separation of proteins is important for numerous interdisciplinary scientific fields including biomaterials and biomedical science, biosensor development, and nanobiotechnology [2]. Recently, MNPs have been combined with polymers such as polystyrene and polysaccharide to create high-surface area and abundant protein adsorption sites [3]. However, the unique properties of MNPs arising from their nano-size and magnetization might be degraded during the sequential hybridization process [4].

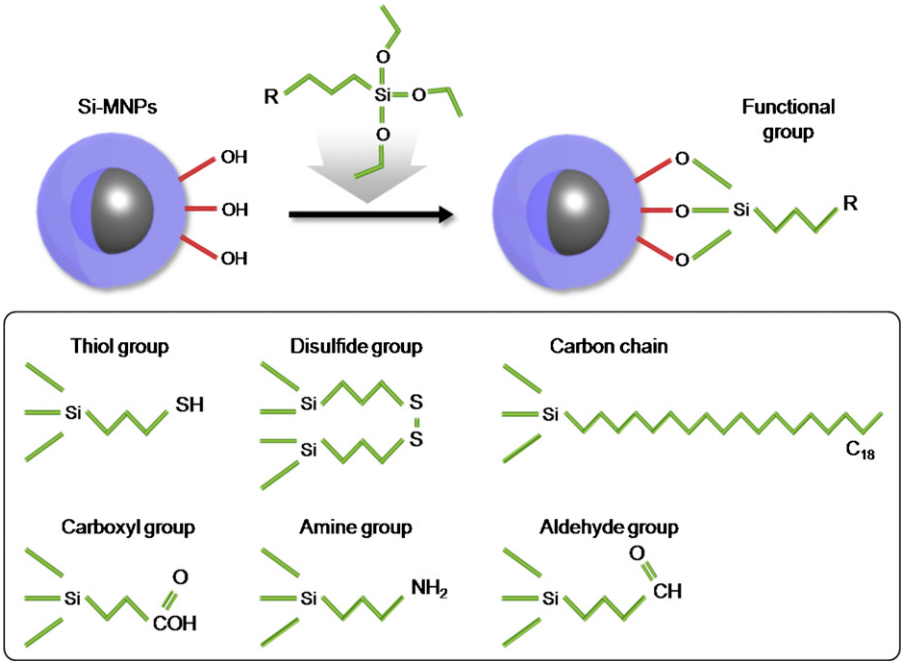
Meanwhile, silica-coated magnetic nanoparticles (Si-MNPs) facilitate control over the thickness of the coating on the particle surface, thereby allowing a means of adjusting the magnetic strength [3,5]. The hydroxyl group (Si—OH) of Si-MNPs has a surface that can provide versatile modification opportunities suitable for protein adsorption [5]. For example, complementary electrostatic and hydrophobic interactions between specially functionalized Si-MNPs and proteins can increase adsorption efficiency through their complex formation in solution [6]. Amino acids present a readily accessible source of electrostatic and hydrophobic elements found in proteins [7]. Therefore, the design of desirable functionalized Si-MNPs with well-defined surface properties remains an area of intense research interest.

Unique functional groups involved in the side chain of amino acids enable covalent conjugation with proteins [8]. Thus, there may be different forces driving the functional groups involved in adsorbing proteins via either hydrophobic or electrostatic interactions [9]. However, it is imperative to understand the inherent physical characteristics of proteins, such as the isoelectric point (p*I*) and molecular weight, even though the driving force of surface functional groups is suitable for protein adsorption. More specifically, the magnetic separation technique is underpinned by the implicit assumption that the behavior of an intact biological system consisting of a plurality of proteins reflects that of a coexistent mixed state [10].

To shed light on the adsorption efficiency of proteins, we report a facile synthesis approach, via hydrolysis condensation to prepare Si-MNPs that are surface modified with various amino acid side chain-like functional groups such as thiol (—SH), disulfide (—S—S—), carbon chain ($—C_n$), carboxyl (—COOH), amine ($—NH_2$), and aldehyde (—CHO) (Scheme 1) [11]. Furthermore, we discuss protein adsorption effects in mixed proteins with various p*I* values and molecular weights, derived from straightforward experiments of two binary proteins: bovine serum albumin (BSA) and lysozyme

* Corresponding author.
E-mail address: jhchang@kicet.re.kr (J.H. Chang).
[1] Authors with equal contributions.

0039-9140/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2012.02.003



Scheme 1. Versatile surface modification of Si-MNPs with amino acid side chain-like functional groups.

(LYZ) (Table S1 in the ESI†) [12]. The final aim of this work is to identify the optimal surface modification method of Si-MNPs for effective protein separation as well as to elucidate the binding performance of different binary proteins in one solution.

2. Experimental

2.1. Materials

Silane coupling reagents were purchased from Aldrich Chemical Co. and used as received. All other chemicals were of analytical reagent grade. Bovine serum albumin and lysozyme were obtained from Sigma. Alexa Fluor 488 and Texas Red fluorescence dyes were purchased from Molecular Probes Inc. The phosphate buffers (PBs) used here were a mixture of 2.17 mM KH_2PO_4 and 7.61 mM Na_2HPO_4. All aqueous solutions were prepared with double- distilled water, obtained from a Milli-Q water purifying system (18.3 MΩcm).

2.2. Instrumental analyses

The particle size and morphology of the Si-MNPs were determined by high resolution transmission electronic microscopy (HR-TEM) using a JEM-2000EX TEM (JEOL, Japan) and dynamic light scattering spectrophotometer (DLS) using a DLS-8000 (Otsuka Electronics, Japan). The magnetization of functionalized magnetic nanoparticles at room temperature up to 10 kOe was measured using a vibrating sample magnetometer (VSM 4179) (Oxford Instruments, UK). Identification and characterization of functional groups were carried out using JASCO FT-IR spectrometer in the range from 300 to 4000 cm^{-1} including an attenuated total reflectance Fourier transform IR (ATR-FTIR) technique and X-ray photoelectron spectroscopy (XPS) was also used to determine the surface functionalization with thiol and disulfide group (Sigma probe equipped with monochromatic Al source, 15 kV and 100 W) (Thermo Scientific, UK). Confocal microscopy was performed with a MultiProbe 2001 confocal scanning laser microscope, with an argon/krypton laser and ImageSpace Software from Molecular Dynamics, USA. The fluorescence intensity and absorbance of the samples were measured using microplate reader Infinite M200 (Tecan Ltd., Switzerland). The fluorescence was measured five times for each sample with a 20 μs integration time.

2.3. Synthesis of surface modified Si-MNPs

2.3.1. Synthesis of MNPs (Fe_3O_4)

MNPs were prepared by coprecipitation with Fe^{2+} and Fe^{3+}. To prepare the Fe_3O_4 MNPs, a fresh mixture of 2 M $FeCl_2{\cdot}4H_2O$ and 1 M $FeCl_3{\cdot}6H_2O$ were added to 0.7 M ammonia solution with vigorous stirring. The obtained Fe_3O_4 MNPs were separated by magnet and the supernatant was decanted. The collected Fe_3O_4 MNPs were washed four times with ethanol and then air-dried.

2.3.2. Silica-coated magnetic nanoparticles (Si-MNPs)

Before the silica-coating process, the 100 mg Fe_3O_4 MNPs were dispersed in 100 mL cyclohexane under sonication for 3 h in the presence of 3.3 mL oleic acid. The 44 g Igepal CO-520 dissolved solution in 900 mM cyclohexane was added to Fe_3O_4 MNPs solution and then further stirred for 15 min. Then, 8 mL of aqueous ammonia solution was added drop wisely into the freshly prepared solution with stirring for 15 min. After stirring, a solution of 98% tetraethyl orthosilicate (TEOS) was added dropwise. The mixed solution was further stirred for 20 h. Methanol was added to the solution to form dark precipitates, which were collected through centrifugation after removal of supernatants. The dark precipitate was washed repeatedly with ethanol and then vacuum dried.

2.3.3. Surface modification of Si-MNPs

The Si-MNPs were then modified successively with six silane coupling agents to introduce functional groups. Briefly, 1 g of Si-MNPs was dispersed in 50 mL of anhydrous toluene containing 0.1 mg of (3-mercaptopropyl) triethoxysilane (—SH), bis [3-(triethoxysilyl) propyl]-disulfide (—S—S—), n-octadecyltriethoxysilane ($—C_{18}$), carboxyethylsilanetriol sodium salt (—COOH), 3-aminopropyl-triethoxysilane ($—NH_2$), and triethoxysilybutyraldehyde (—CHO), respectively, and the mixture was allowed react at 110 °C for 8h under dry nitrogen (Fig. 1). The resultants were separated by simple magnetic attraction and

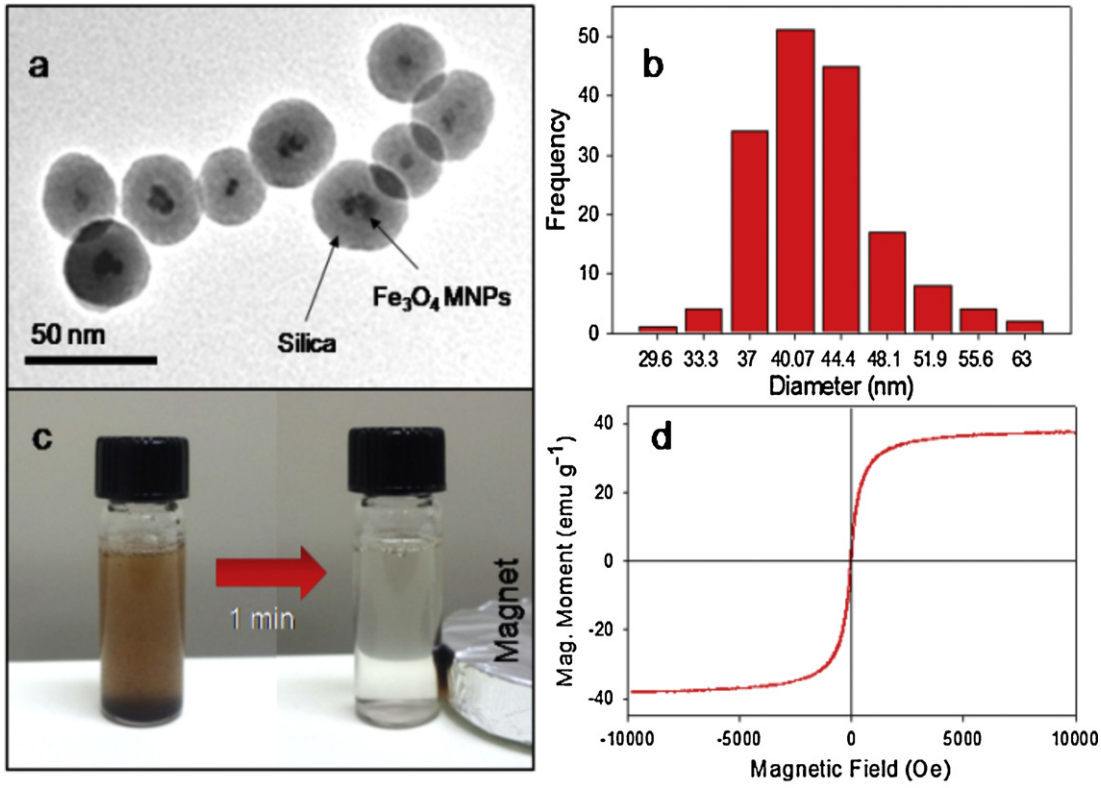


Fig. 1. (a) TEM image, (b) particle size distribution, (c) pictures showing the magnetic attraction and (d) hysteresis loop showing the superparamagnetic properties of Si-MNPs.

washed repeatedly with toluene, and finally vacuum dried for further use.

2.4. Protein adsorption and desorption experiments

A 0.1 M PBs buffer solution of pH 4.65 was prepared by dissolving sodium phosphate monobasic monohydrate 6.876 g and sodium phosphate dibasic 0.0465 g in 500 mL deionized water. A buffer solution of pH 11.0 was prepared by adding 41 mL of NaOH solution (1 g NaOH in 250 mL deionized water) to 500 mL of 0.05 M Na_2HPO_4 solution (sodium phosphate dibasic 3.549 g in 250 mL deionized water).

Protein adsorption experiments were carried out in 1 mL of protein solution dissolved in 100 mM phosphate buffer (pH 4.65 and pH 11) were added to disperse the functionalized Si-MNPs (10 mg), according to the conventional method described elsewhere. The initial concentrations of BSA and LYZ solutions were both 5 μM. The reaction mixtures were incubated at room temperature for 10 min with continual agitation to the suspending particles. After remove particles by magnetic attraction, the protein concentrations in the supernatants were determined by UV–vis-spectrophotometer measurement at 278 nm. For the sequential separation experiments, the particles were suspended using 0.1 M glycine solution, which can desorb the proteins attached to the surface. After sharply stirring, the particles in the suspensions were separated with magnetic attraction and the supernatants containing the desorbed proteins were collected for SDS-PAGE analysis. The efficiency of protein adsorbed by the particles was calculated from the following formula:

$$E_{ad} = \frac{C_i - C_f}{C_i} \times 100$$

where E_{ad} (%) is the efficiency of protein adsorbed by unit mass of dry particles, C_i (μM) and C_f (μM) are the protein concentrations of the initial and final solutions, respectively. All the tests were conducted in triplicate.

2.4.1. Fluorescence labeling of proteins in vitro

For the fluorescence labeling of BSA and LYZ, Alexa Fluor 488 and Texas Red protein labeling kit (Molecular Probes, UK) were used according to the manufacturer's instructions. 50 μL of 1 M sodium bicarbonate solution were added into 0.5 mL of 100 μM BSA and LYZ solutions each other. The protein solutions were mixed with 0.5 mL of reactive Alexa Fluor 488 and Texas Red dyes, respectively, for 1 hr at room temperature. After the reaction, remaining traces of free dyes were removed by column chromatography.

2.4.2. Analysis of fluorescence labeled-proteins

In mixed protein solution, the adsorption of fluorescence labeled BSA and LYZ to functionalized Si-MNPs were observed by confocal microscopy. Fluorescence spectra were measured using a microplate reader. The lasers provided excitation of Alexa Fluor 488 for BSA and Texas Red for LYZ at 494 nm and 595 nm, respectively, and emitted fluorescent lights were detected at 519 nm and 615 nm, respectively.

3. Results and discussion

3.1. Synthesis and characterization of amino acid side-chain like modified Si-MNPs

The synthesis of Si-MNPs involved three steps: the formation of a Fe_3O_4 magnetic core and the coating of the silica shell. The Fe_3O_4 magnetite core was synthesized via chemical precipitation of Fe^{2+} and Fe^{3+} salts (with a molar ration of 1:2) in a basic solution. The Si-MNPs were directly produced by the sol–gel reaction of the TEOS precursor. Fig. 1 shows the generated Si-MNPs,

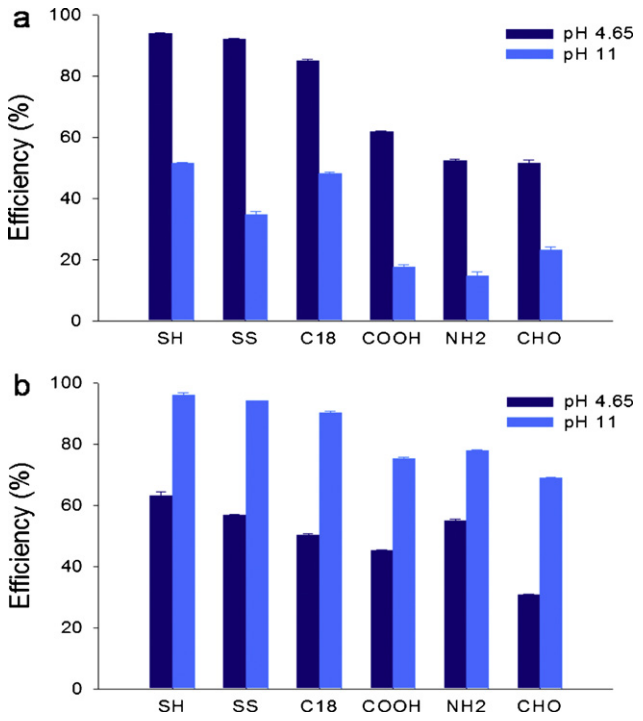


Fig. 2. Adsorption efficiency of (a) BSA and (b) LYZ on the six functionalized Si-MNPs at pH 4.65 and pH 11.

which have a well defined core/shell morphology and an average diameter of 40 nm. The magnetic property of Si-MNPs was estimated to be about 40 emu g^{-1} of the saturated magnetization at 300 K. The Si-MNPs were dispersed in water by shaking or sonication, and then easily separated with a magnet within 1 min. The coating of the silica shell onto the Fe_3O_4 magnetite core provides a modifiable surface for the introduction of various functional groups for protein adsorption. In this study, various functionalized silanes were used to modify the surface of the Si-MNPs via a hydrolysis condensation reaction. In addition, the surface-modified Si-MNPs were characterized by Fourier transform-infrared spectroscopy (FT-IR) and X-ray photoelectron spectroscopy analyses (Figs. S1 and S2 and Table S2 in the ESI†). For example, the X-ray photoelectron spectroscopy spectrum of Si-MNPs@SH showed that Si electronic configuration peaks were detected at 149 and 100 eV for 2s and 2p, respectively. The most prominent feature of the Si-MNPs@SH was a S electronic configuration peak detected at 165 eV for 2p. Consequently, the XPS data verified that thiol groups were successfully coupled with the hydroxyl groups of the silica surface.

3.2. Magnetic separation for single protein with amino acid side-chain like modified Si-MNPs

The conformation of proteins as a function of pH adsorbed at a surface is more compact when the pH is close to the p*I* of the native proteins [13]. This is due to the proteins having a hydrophobic property with no net electric charges at the pH value [9]. Hence, the six as-prepared functionalized Si-MNPs can be used to investigate the effects of different pH range on the adsorption of BSA (p*I* = 4.65) and LYZ (p*I* = 11) as a single-component solution [12]. As shown in Fig. 2, the adsorption efficiencies of two proteins at pH values corresponding to their own p*I* were more enhanced. For instance, when separating with the Si-MNPs@SH, the adsorption efficiency of BSA at pH 4.65 was 1.8 times higher than at pH 11 (Fig. 2a) but the adsorption efficiency of LYZ at pH 11 was 1.5 times higher than at pH 4.65 (Fig. 2b).

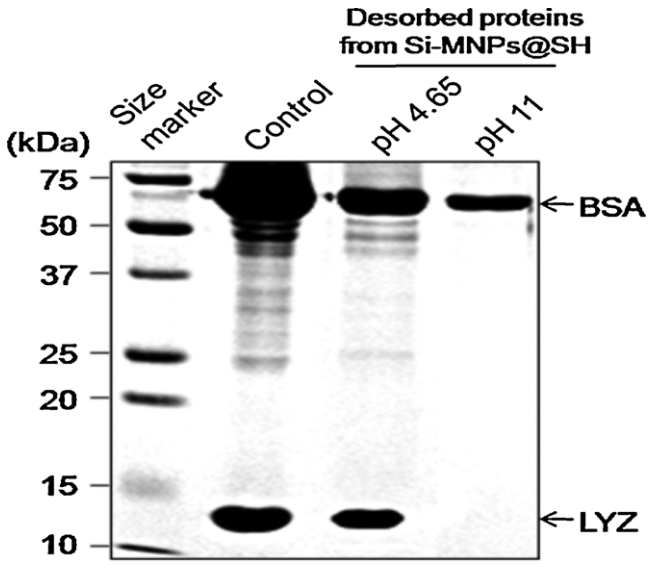


Fig. 3. Protein gel electrophoresis of separated BSA (66.7 kDa) and LYZ (14.6 kDa) by the Si-MNPs@SH.

Interestingly, the adsorption efficiencies of the two proteins with Si-MNPs@SH, Si-MNPs@SS, and Si-MNPs@C_{18} were more enhanced than with Si-MNPs@COOH, Si-MNPs@NH_2, and Si-MNPs@CHO at conditions of pH 4.65 for BSA and pH 11 for LYZ. According to the Pauling scale, a difference in electronegativity between atoms can change the value of the molecular electrostatic potential bond [14]. The thiol (—SH), disulfide (—S—S—), and carbon chain (—C_{18}) group may possess less electronegativity. Based on this theory, they would have higher hydrophobicity than the carboxyl (—COOH), amine (—NH_2), and aldehyde (—CHO) group. Thus, when conducting a nonspecific protein-binding process, protein separation using surface-functionalized Si-MNPs with hydrophobic (nonpolar) groups such as the thiol (—SH), disulfide (—S—S—), and carbon chain (—C_{18}) group is more effective than separation with hydrophilic (polar) groups [9]. In addition, the thiol group is known to play a significant role in cross-linking proteins, which increases the rigidity of proteins [15]. The strongest nucleophile present in natural proteins is the cysteine thiol groups [8]. From our results, we notably observed that the highest protein adsorption efficiency, through the separation process of both BSA at pH 4.65 (93.9 ± 0.4%) and LYZ at pH 11 (96.0 ± 0.8%) was provided by Si-MNPs@SH (Fig. 2). Generally, the driving forces of protein adsorption originated from chemical bonding, such as electrostatic and hydrophobic interactions between the protein and the nanomaterials surface [9], as well as structural rearrangement of the absorbed protein [2].

3.3. Microscopic characterization for mixed protein separation with amino acid side-chain like modified Si-MNPs

In order to investigate the adsorption behavior of mixed proteins, identical mole ratios of BSA and LYZ in a binary mixed solution were adsorbed with Si-MNPs@SH. After the protein adsorption process, the protein adsorption patterns were observed by a protein gel electrophoresis analysis. As shown in Fig. 3, the quantity of adsorbed proteins was estimated through desorption of the proteins from magnetic nanoparticles by 0.1 M glycine solution. In the case of BSA, increased protein binding was observed at pH 4.65 as compared to pH 11, as expected. However, unexpectedly, the protein band of LYZ could not be distinguished at pH 11. Indeed, our preliminary experiments showed that the desorption efficiency of LYZ drastically decreased to 9.3 ± 1.69% at pH 11, as compared

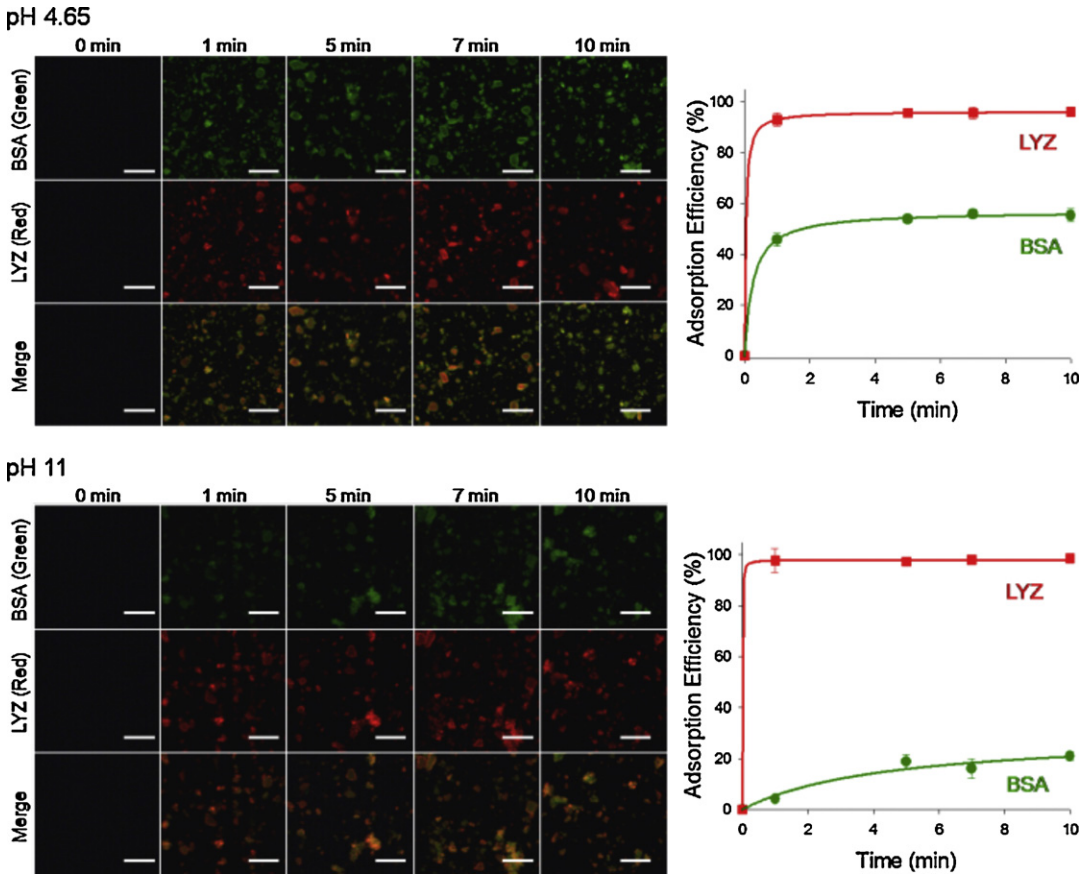


Fig. 4. Confocal images of BSA (green) and LYZ (red) adsorbed onto Si-MNPs@SH and fluorescence intensity measurements.

with 47.3 ± 2.06% at pH 4.65 (data not shown). This result may indicate that the desorbed smaller and globular protein (LYZ) on the magnetic nanoparticles may have re-adsorbed under desorbing conditions in the protein mixture owing to its high structural stability and the favorable charge interactions between the protein and surface. [16].

To further elucidate this phenomenon, BSA and LYZ were labeled with a green and red fluorescence dye, respectively, for observation via a confocal laser scanning microscope. Fig. 4 shows the microscope images and their quantified results for both BSA and LYZ molecules bound onto the Si-MNPs@SH surfaces, when coexisting in a binary mixed solution. Compared with the binding performance of the two proteins in single-component solutions (Fig. 3), the adsorption efficiency of BSA was only about 50% at pH 4.65, and barely 20% at pH 11. On the other hand, the adsorption efficiency of LYZ was almost 100% at both pH 4.65 and pH 11. There are some factors that may account for why the binding effects of BSA and LYZ show such a discrepancy between

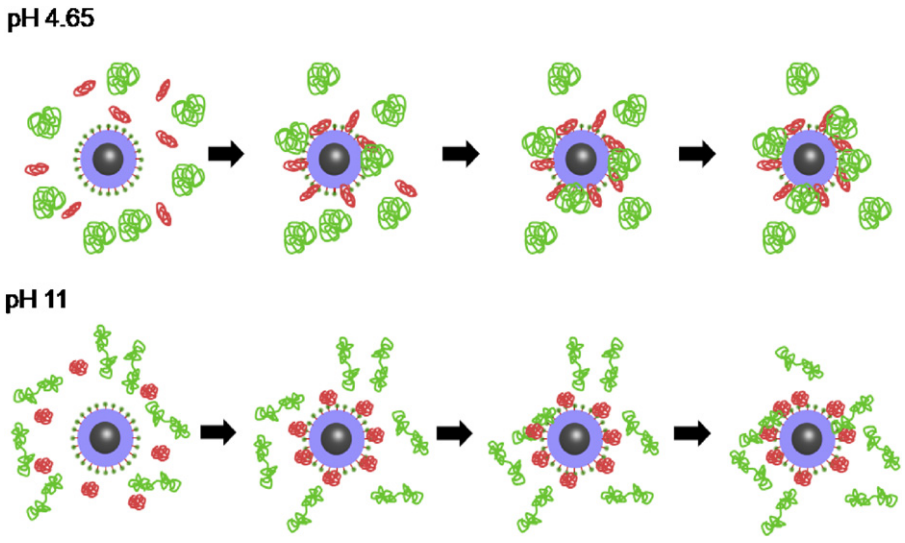


Fig. 5. Illustration for adsorption of mixed binary proteins, BSA (green) and LYZ (red), onto Si-MNPs at pH 4.65 and pH 11.

single-component and mixed proteins. First, the 3-dimentional structure of LYZ is more stable than that of BSA under pH variation [17]. Second, from a mixed protein solution, protein adsorption occurs through a complex series of adsorption-displacement steps where low-molecular-weight proteins that arrive first at a surface are displaced by relatively high-molecular-weight proteins arriving later, until a steady state is reached [10]. Moreover, as shown in Fig. 5, LYZ in the protein mixture would tend to dominate the initial adsorption process at all pH conditions. This suggests that, for LYZ, which is a small and rigid structured protein, binding to the magnetic nanoparticles is mostly driven by the hydrophobic interaction, which has a greater influence than the net charge in the aggregation process in the protein mixture.

4. Conclusion

In conclusion, this work reported the fabrication of Si-MNPs and their use in a surface modification method for effective protein separation in mixed proteins. For this purpose, the binary proteins BSA and LYZ, which have different molecular weights and p*I* values, were used as model proteins for protein separation via Si-MNPs surface-modified with amino acid side chain-like functional groups. The hydrophobic group of the modified Si-MNPs was shown to be more suitable for protein separation than the hydrophilic group of functionalized Si-MNPs, perhaps due to its high adsorption affinity against the amino acid residues of proteins. In addition, compared with single-component proteins, the adsorption performance of BSA and LYZ was governed not only by pH, but also by the molecular weight of each protein in the mixed protein solution. The phenomenon was visualized by a confocal microscope analysis of protein-bound Si-MNPs. We anticipate that the present findings may have important implications in the rule of binding mixed proteins onto nano-sized particles, and should also contribute to the future application of magnetic nanoparticle-based technologies to separate proteins from intact biological samples.

Acknowledgments

This work was supported by a grant from the Fundmental R&D Program for Core Technology of Materials funded by the Ministry of Knowledge Economy, Republic of Korea.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2012.02.003.

References

[1] P.S. Doyle, J. Bibette, A. Bancaud, J.L. Viovy, Science 295 (2002) 2237.
[2] Z. Liu, M. Li, X. Yang, M. Yin, J. Ren, X. Qu, Biomaterials 32 (2011) 4683.
[3] K. Kang, J. Choi, J.H. Nam, S.C. Lee, J.K. Kim, S.W. Lee, J.H. Chang, J. Phys. Chem. B 113 (2009) 536.
[4] A.-H. Lu, E.L. Salabas, F. Schüth, Angew. Chem. Int. Ed. 46 (2007) 1222.
[5] S.Y. Lee, S. Lee, I.H. Kho, J.H. Lee, J.H. Kim, J.H. Chang, Chem. Commun. (2011), doi:10.1039/c1cc11664a.
[6] M. Valerio, A. Colosimo, F. Conti, A. Giuliani, A. Grottesi, C. Manetti, J.P. Zbilut, Proteins 58 (2005) 110.
[7] M. De, C.-C. You, S. Srivastava, V.M. Rotello, J. Am. Chem. Soc. 129 (2007) 10747.
[8] A.J. de Graaf, M. Kooijman, W.E. Hennink, E. Mastrobattista, Bioconjug. Chem. (2009) 1281.
[9] J.H. Chang, J. Lee, Y. Jeong, J.H. Lee, I.J. Kim, S.E. Park, Anal. Biochem. 405 (2010) 135.
[10] A. Krishnan, C.A. Siedlecki, E.A. Vogler, Langmuir 20 (2004) 5071.
[11] S.Y. Lee, J. Lee, J.H. Chang, J.H. Lee, BMB Rep. 44 (2011) 77.
[12] Z.G. Peng, K. Hidaja, M.S. Uddin, J. Colloid Interface Sci. 281 (2005) 11.
[13] D.-H. Tsai, F.W. DelRio, A.M. Keene, K.M. Tyner, R.I. MacCuspie, T.J. Cho, M.R. Zachariah, V.A. Hackely, Langmuir 27 (2011) 2464.
[14] C.H. Suresh, N. Koga, J. Am. Chem. Soc. 124 (2002) 1790.
[15] D.-H. Lee, S.-G. Kim, D.-H. Kweon, J.-H. Seo, BMC Biotechnol. 9 (2009) 1.
[16] J.W. Chidlow, J. Stephen, H. Smith, Biochem. J. 117 (1970) 49.
[17] Z.G. Estephan, P.S. Schlenoff, J.B. Schlenoff, Langmuir 27 (2011) 6794.