Protein Separation

DOI: 10.1002/anie.201405849

Magnetic Separation of Proteins by a Self-Assembled Supramolecular Ternary Complex**

*Avik Samanta and Bart Jan Ravoo**

Abstract: *The easy and effective separation of proteins from a mixture is crucial in proteomics. A supramolecular method is described to selectively capture and precipitate one protein from a protein mixture upon application of a magnetic field. A multivalent complex self-assembles in a dilute aqueous solution of three components: magnetic nanoparticles capped with cyclodextrin, non-covalent cross-linkers with an adamantane and a carbohydrate moiety, and lectins. The self-assembled ternary complex is precipitated in a magnetic field and readily redispersed with the aid of a non-ionic surfactant and competitive binding agents. This strategy to purify proteins by supramolecular magnetic precipitation is highly selective and efficient.*

Magnetic iron oxide (Fe_3O_4, γ-Fe_2O_3) nanoparticles (MNPs) have received significant attention owing to the promise they bring into the biomedical and therapeutic fields.[1] In particular because of their superparamagnetism, relative chemical stability, and inherent biocompatibility, MNPs have become the leading contender in wide variety of biomedical applications, such as contrast enhancement agents for magnetic resonance imaging[2] probes for bacteria capture,[3] drug delivery,[4] biofunctional molecular imaging,[5] and cell sorters.[6] Another field of interest is the separation and purification of cells and biomolecules in bioprocesses.[7] MNPs have several superior characteristics for biotechnological separations in comparison with conventional micrometer-sized beads or resins, such as the fast and effective binding of biomolecules, good dispersion, and rapid as well as reversible flocculation owing to their small size and high surface area. In the last decades, a limited number of studies have explored the potential of magnetic nanomaterials in the field of protein purification. Recently, Xu and co-workers demonstrated that Ni-nitrilotriacetic acid (Ni-NTA) conjugated MNPs are excellent agents to sequester histidine-tagged (His-tagged) proteins.[8] Mirkin and co-workers established an efficient and selective procedure to separate His-tagged proteins using Ni-containing nanorods with a diameter of about 300 nm.[9] Furthermore, Philips Magnotech technology exploits the dynamic properties of MNPs in magnetic fields to establish immunoassays in blood, serum, and saliva.[10]

A supramolecular approach to immobilize proteins on surfaces and nanoparticles by using the host–guest inclusion of β-cyclodextrin (CD) and adamantane derivatives was described by several groups.[11] In 2012, we reported a light-responsive supramolecular complex to capture lectins on the basis of CD vesicles and azobenzene guests.[12] Important advantages of a supramolecular strategy to capture proteins are:

1) orthogonal interactions which allow the same system to be used for very different biological applications without having to change the CD platform;
2) a lack of denaturing interactions between proteins and solid supports and creation of a protective hydrophilic microenvironment around the protein by the CD moieties;
3) multivalent interaction of proteins through non-covalent supramolecular association.

Herein, we demonstrate a simple dynamic supramolecular system to selectively capture and separate a desired protein from a mixture of proteins in a magnetic field. Two orthogonal non-covalent motifs operate simultaneously in this system: β-CD-adamantane host–guest inclusion and protein–ligand interaction. As these non-covalent interactions are inherently weak (binding constant for a single interaction $K_a \approx 10^3$–$10^4\ L\,mol^{-1}$), multivalent binding is required to form a stable complex in aqueous medium ($K_a \approx 10^6\ L\,mol^{-1}$ for a divalent interaction, $K_a \approx 10^9\ L\,mol^{-1}$ for a trivalent interaction, $K_a \approx 10^{12}\ L\,mol^{-1}$ for a tetravalent interaction, and so on).[13] Herein we report a novel type of hydrophilic MNP capped with β-CD and their use as support for selective supramolecular capture of lectins. We demonstrate an unprecedented and highly effective approach to magnetic purification of proteins in aqueous medium.

The β-CD capped magnetic nanoparticles (MNP-CDA) were synthesized in two steps that comprise the preparation of bare MNPs by alkaline co-precipitation of Fe^{II} and Fe^{III} salts followed by ligand stabilization with per-6-deoxy(carboxylpropyl)thio-β-cyclodextrin (CDA). The binding affinity of typical functional groups to a magnetite surface follows the order: phosphonate > carboxylate > hydroxy > sulfonate.[14] We designed a ligand containing seven carboxylic acid functions that can bind to the MNP surface in multivalent fashion without affecting the superparamagnetic properties (Figure 1 A). The two-step synthesis of the CDA ligand is described in the Supporting Information. The size and shape of MNP-CDA was investigated by transmission electron

[*] M. Sc. A. Samanta, Prof. Dr. B. J. Ravoo
Organic Chemistry Institute and Graduate School Chemistry
Westfälische Wilhelms-Universität Münster
Corrensstrasse 40, 48149 Münster (Germany)
E-mail: b.j.ravoo@uni-muenster.de

[**] TEM was performed by Dr. Martin Peterlechner at the Institute of Materials Physics of the Westfälische Wilhelms-Universität Münster. TGA measurements were carried out by Jessica Hüsker in the laboratory of Prof. Dr. M. Winter (MEET, WWU Münster). A.S. acknowledges a fellowship of the Graduate School of Chemistry.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201405849.

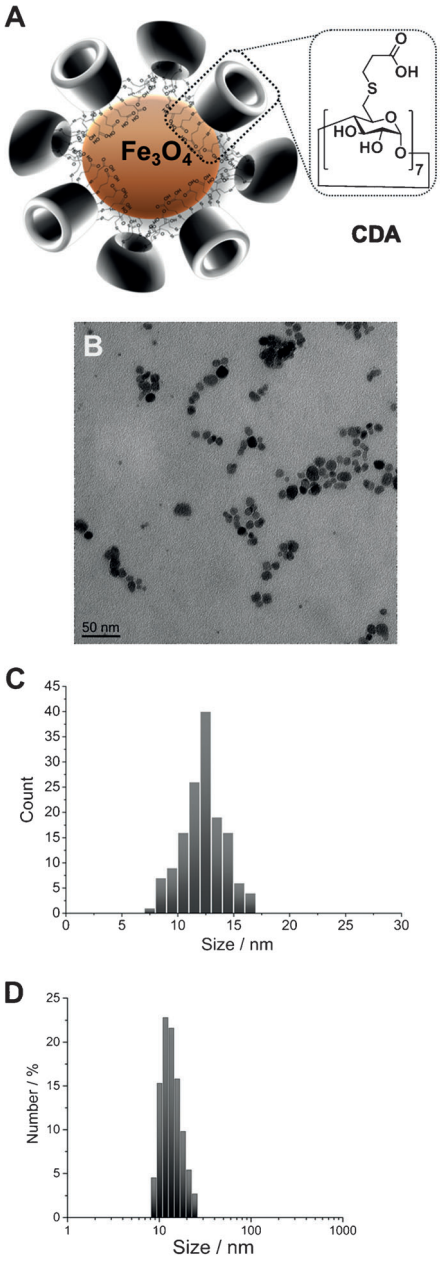

Figure 1. Representation and characterization of hydrophilic β-CD -coated magnetic nanoparticles (MNP-CDAs). A) Illustration of MNP-CDA and the molecular structure of ligand CDA (inset). B) TEM image of MNP-CDA. C) Particle size histogram derived from TEM images. D) Particle size distribution according to DLS.

microscopy (TEM). The TEM images and size distributions (Figure 1B,C) confirm that the MNPs are monodisperse and uniform in shape. The size distribution shows that the average diameter of MNP-CDA is 12 ± 5 nm which also agrees with the average diameter obtained from dynamic light scattering (DLS; Figure 1D). The ζ-potential of MNP-CDA is −43 mV, which is due to the hydroxy groups present at the secondary side and residual non-complexed carboxylate groups of immobilized CDA ligands on the MNP surface (Supporting Information, Figure S1). The FTIR spectrum of MNP-CDA exhibits bands consistent with the main vibration modes of CDA such as the C–H vibration at 2916 cm^{-1} (at 2924 cm^{-1} in case of free CDA), CH_2 scissoring band at 1400 cm^{-1} (at 1398 cm^{-1} for free CDA), characteristic strong C=O (carboxylate) stretching vibration at 1557 cm^{-1} (at 1564 cm^{-1} for free CDA), and a broad weak band around 3263 cm^{-1} (at 3274 cm^{-1} for free CDA) owing to vibration of the hydroxy groups (Supporting Information, Figure S2). There is no such band observed in case of bare MNPs, which indicates the immobilization of the CDA ligand at the surface of the MNP. Furthermore, thermogravimetric analysis (TGA) was performed on bare and functionalized MNPs (Supporting Information, Figure S3). In the case of bare MNPs, a weight loss of 1.0% was detected in a temperature range of 100 to 600 °C that is due to the residual water. The additional weight loss for MNP-CDA was found to be 17.1%, which is attributed to the loss of CDA monolayer from the MNPs. The majority of the weight loss in case of MNP-CDA and CDA occurred in the temperature range of 150 °C to 400 °C (Supporting Information, Figure S3). Based on TGA measurements it is possible to calculate the number of CDA per MNP: We calculated the molar mass of the MNPs by multiplying the average volume (derived from the diameter determined by TEM) with the bulk density of magnetite (2.64 $g\,cm^{-3}$). The complete calculation is provided in the Supporting Information. For MNP-CDA we find about 1.97×10^3 CDA per MNP. The experimental values can be compared with theoretical values achieved from simple geometrical consideration. In that case, the CDA ligands immobilized on the surface are simplified to flat disks with a diameter of 1.53 nm that cover the MNP surface in a hexagonal closed packing. According to theoretical estimation ca. 0.94×10^3 CDA are required to cover a single MNP. Our experimental result shows that the number of CDA per MNP is the same order of magnitude as the theoretical estimation but substantially higher. This difference can be explained by the fact that the MNPs are not perfectly spherical, which leads to a larger available surface area. Furthermore, CDA can bind in a "side-on" fashion rather than "head-on", which decreases the effective footprint of the ligands on the MNP surface.

The selective separation of protein from a protein mixture was investigated by using MNP-CDA and adamantane–carbohydrate conjugates, as shown in Figure 2. Two lectins were selected: peanut agglutinin (PNA) and concanavalin A (ConA). Both lectins form tetramers at neutral pH and have four carbohydrate binding sites.[15] ConA binds exclusively to α-D-glucose and α-D-mannose and their glycosides. PNA binds exclusively to β-D-galactose and its glycosides. We have previously coined the term "artificial glycocalix" for the self-assembled multivalent display of carbohydrates on CD vesicles.[16] The guest molecules G1 and G2 each contain two orthogonal molecular recognition sites. The adamantane group forms a 1:1 inclusion complex with β-CD ($K_a \approx 10^4\ L\,mol^{-1}$).[16] The adamantane–carbohydrate conjugates have a tetraethyleneglycol spacer between the adamantane and the carbohydrate. The surface of MNP-CDA can be decorated with specific carbohydrates simply by adding the adamantane–carbohydrate conjugates to the nanoparticles. We investigated the characteristics of MNP-CDA decorated with adamantane–carbohydrate conjugates in presence of

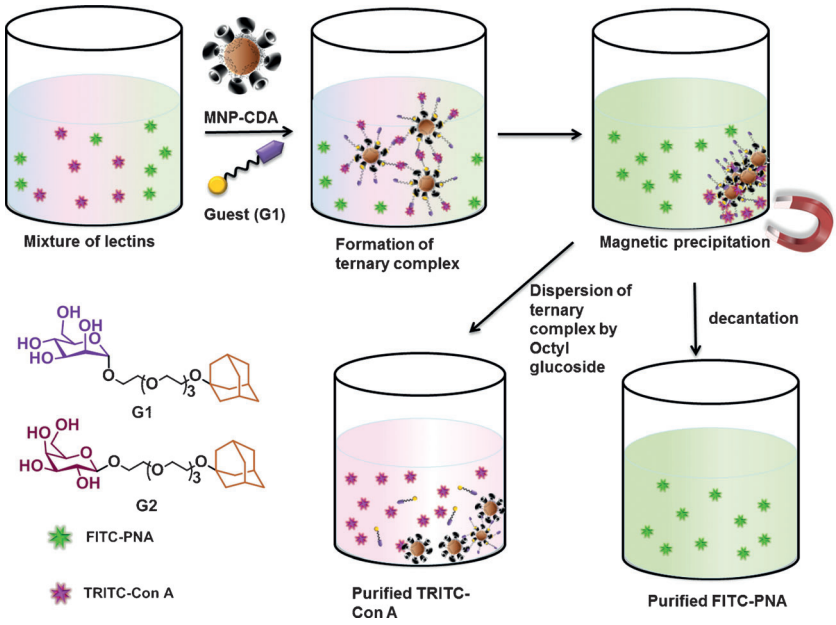

Figure 2. Illustration of the separation of proteins using the cross-linking and magnetic precipitation of MNP-CDA and G1, and molecular structures of G1 and G2..

lectins. To this end, a 25 μM solution of either G1 or G2 was added to a dilute buffered solution of MNP-CDA (0.2 mg mL^{-1}). The optical density (OD_{400}) of a solution of MNP-CDA is less than 0.05, which remains unaltered after addition of G1 (after 2 min, denoted by the first arrow in Figure 3). However, when ConA is added (after 4 min, denoted by second arrow in Figure 3) to the mixture of MNP-CDA and conjugate G1, the OD_{400} value increases from

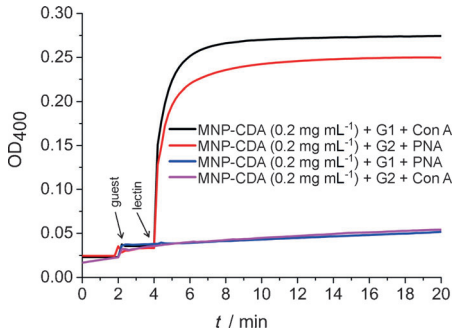

Figure 3. Formation of cross-linked aggregates of MNP-CDA, adamantane–carbohydrate conjugates, and lectins. Time-dependent optical density measurement at $\lambda = 400$ nm. Conditions: [MNP-CDA] = 0.2 mg mL^{-1}, [G1] = [G2] = 25 μM, [ConA] = [P-NA] = 0.05 mg mL^{-1}; 20 mM HEPES buffer (1.0 mM $MnCl_2$, 1.0 mM $CaCl_2$, 0.15 M NaCl, pH 7.4); $T = 23$ °C.

approximately 0.05 to 0.25 within 10 min. This observation indicates the rapid and spontaneous formation of a ternary complex of MNP-CDA, G1, and ConA. As a consequence, MNP-CDA aggregate in microscale clusters owing to multivalent orthogonal cross-linking of MNP-CDA and ConA by linker G1. Aggregation is also observed by DLS (Supporting Information, Figure S5). Identical phenomena are observed in case of MNP-CDA, G2, and PNA (Figure 3, red curve). In case of an incorrect combination of guest and lectin (such as G1 and PNA or G2 and ConA), no aggregation is observed. We conclude that the formation of the ternary complex is highly specific and requires a matching combination of lectin and adamantane–carbohydrate conjugate.

Most significantly, the ternary complex composed of cross-linked MNP-CDA, guest, and lectin shows superparamagnetism owing to the presence of the MNPs and can easily be separated from the solution by magnetic precipitation. As a consequence, lectins that bind to the carbohydrate decorated MNPs can be captured from a mixture by magnetic precipitation of the complex. To quantify the protein separation efficiency, we measured fluorescence intensity of fluorescein isothiocyanate (FITC) and tetramethylrhodamine (TRITC) labeled proteins before and after complexation. The excitation wavelengths (ex = 490 and 540 nm) of these labeled lectins are distinct, and the fluorescence observed (em = 520 and 576 nm) for each protein is independent of the other at the chosen concentration in a mixture (Supporting Information, Figure S6). As expected, the fluorescence spectrum of the mixture of labeled proteins showed emission maxima at 520 nm and 576 nm that correspond to FITC-PNA and TRITC-ConA, respectively. To a mixture of TRITC-ConA and FITC-PNA (0.05 mg mL^{-1} each), a solution of MNP-CDA (0.45 mg mL^{-1}) was added followed by adamantane–mannose conjugate (G1, 40 μM). After 10 min of incubation and magnetic precipitation of the complex, a 88% decrease in intensity was observed at 576 nm, corresponding to the TRITC-ConA, while only a 8% decrease was observed in the intensity at 520 nm for the FITC-PNA (Figure 4A). This observation indicates that the mannose decorated MNP-CDA bind to TRITC-ConA in a multivalent fashion to form a ternary complex, which is precipitated out from the solution. The degree of separation is proportional to the concentration of MNP-CDA as shown in Figure 4A. Similarly, the fluorescence measurements (Figure 4B) show that the magnetic precipitation of the ternary complex after addition of MNP-CDA and adamantane–galactose conjugate (G2) to the protein mixture, a 86% decrease in the intensity was observed at 520 nm corresponding to the FITC-PNA, while only a 6% decrease in the intensity was observed at 576 nm for the TRITC-ConA. The concentration of lectins in every step is calculated from a reference fluorescence calibration plot (Supporting Information, Figure S6 and Table S1). The fluorescence intensities for both labeled lectins remain unaffected after addition of either MNP-CDA or adamantane–carbohydrate conjugate (Supporting Information, Figure S7), which clearly indicates that the fluorescence intensity decreases only because of the capture of the specific lectin from the mixture, and non-

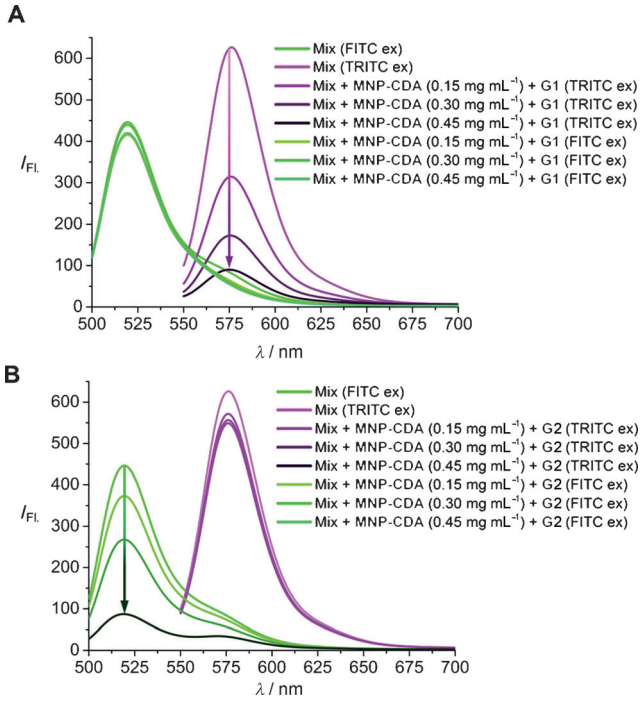

Figure 4. Fluorescence spectra showing the emission intensity before and after magnetic precipitation from the mixture (Mix) of lectins. A) Stepwise decrease in emission intensity at 576 nm after precipitation of the complex of MNP-CDA, G1, and TRITC-ConA (pink to violet traces), where the emission at 520 nm remains unchanged; light green traces). B) Stepwise decrease in emission intensity at 520 nm after precipitation of the complex composed of MNP-CDA, G2, and FITC-PNA (light green to dark green traces), where the emission at 576 nm remains unaffected (pink traces). Conditions: $[TRITC\text{-}ConA]_0 = [FITC\text{-}PNA]_0 = 0.05\ mg\,mL^{-1}$, [G1] = [G2] = 40 μM, [Ad-teg] = 10 μM, [MNP-CDA] = 0.15–0.45 $mg\,mL^{-1}$; 20 mM HEPES buffer (1.0 mM $MnCl_2$, 1.0 mM $CaCl_2$, 0.15 M NaCl, pH 7.4); $T = 23\,°C$. ex = 490 and 540 nm.

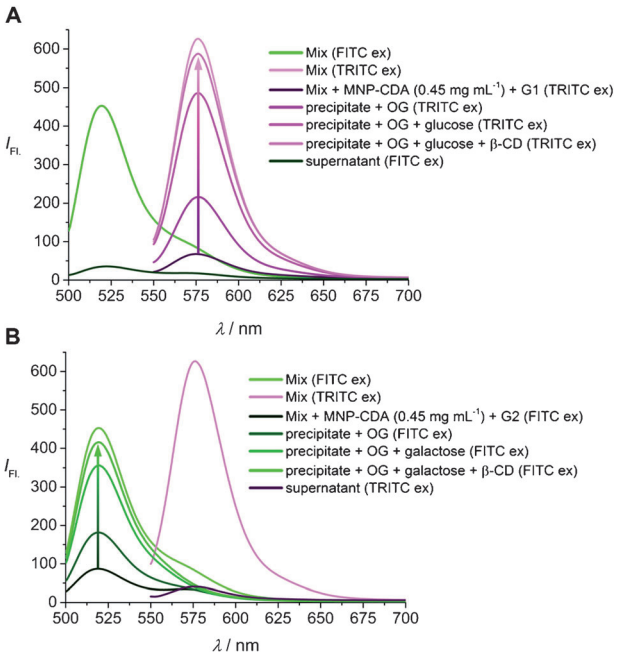

Figure 5. Fluorescence spectra showing the emission intensity before and after dispersion of precipitated lectins. A) Increase in emission intensity at 576 nm after dissolving the complex of MNP-CDA, G1, and TRITC-ConA (violet to pink traces). The intensity at 520 nm indicates residual FITC-PNA. B) Increase in emission intensity at 520 nm after dissolving the complex of MNP-CDA, G2, and FITC-PNA (dark green to light green traces). The intensity at 576 nm indicates the residual TRITC-ConA. Conditions: [OG] = 50 mM, [D-glucose] = [D-galactose] = 100 mM, [β-CD] = 100 μM; 20 mM HEPES buffer (1.0 mM $MnCl_2$, 1.0 mM $CaCl_2$, 0.15 M NaCl, pH 7.4); $T = 23\,°C$. ex = 490 and 540 nm.

specific capture is negligible. Note that to minimize unspecific binding, 10 μM of adamantyl tetra(ethylene glycol) (Ad-teg) was added in all of the experiments (Supporting Information, Figure S8).

After magnetic precipitation of the ternary complex, predominantly one lectin remained in the supernatant solution. To recover captured lectins from the ternary complex, we used a solution of β-octyl glucoside (OG) along with β-CD and carbohydrate ligand, as shown in Figure 2. OG, a nonionic surfactant, was chosen owing to its ability to solubilize proteins without denaturation.[17] The surfactant concentration was set above the critical micelle concentration (ca. 25 mM) at 50 mM to ensure that the MNPs are solvated by a protective micelle. The supernatant solution (FITC-PNA: 0.046 $mg\,mL^{-1}$) was decanted after the magnetic separation of the complex, as shown in Figure 2. The precipitate contains MNP-CDA, G1, Ad-teg, and most of the TRITC-ConA. The fluorescence intensity at 576 nm increases 36% after the incubation of residue with 50 mM of OG solution which indicates a limited dispersion of ternary complex and release of TRITC-ConA by OG alone. A 85% increase was observed when the precipitate was treated with a mixture of 50 mM OG, 100 mM D-glucose, and 100 μM β-CD (Figure 5A). Therefore, the formation of the ternary complex can be completely reversed by the addition of excess competitive binding agents D-glucose and β-CD. From the fluorescence intensity at 520 nm, it can be estimated that only 6% of FITC-PNA is present in the solution (Figure 5A; Supporting Information, Table S1). Similarly, 77% of FITC-PNA was released from the ternary complex composed of MNP-CDAs, G2 and FITC-PNA by incubating the precipitate with a mixture of OG, D-galactose, and β-CD (Figure 5B; Supporting Information, Table S1). These results indicate that the separation of two lectins by first precipitating a supramolecular magnetic complex and subsequently dispersing it by competitive binding agents is highly efficient and selective.

The identical separation method was applied to a mixture of TRITC-ConA and FITC-labeled horseradish peroxidase (FITC-HRP). HRP does not have any affinity towards either of the carbohydrates (D-mannose or D-galactose). It was possible to capture and precipitate selectively 93% of TRITC-ConA from the mixture by using MNP-CDA and G1 without affecting the concentration of FITC-HRP (Supporting Information, Figure S9). After decanting the supernatant containing FITC-HRP, 74% of captured TRITC-ConA is released by using OG, D-glucose and β-CD mixture. Furthermore, to demonstrate the potential of our approach

under near-physiological conditions, we extracted TRITC-ConA from 5% fetal bovine serum (Supporting Information, Figure S10). Also in this case, the extraction was highly efficient.

In conclusion, we have presented a straightforward method for the functionalization of MNPs with CD and we demonstrate an effective magnetic protein separation by combining two orthogonal supramolecular interactions, that is, adamantane-β-CD inclusion and carbohydrate–lectin binding. The carbohydrate decorated MNPs provide a very large effective surface area for selective adsorption of protein and the magnetic core allows the particles to be separated from the solution by applying an external magnetic field. The efficiency of the selective capturing and releasing proteins is 85% in a single extraction. To the best of our knowledge, a dynamic and multivalent supramolecular system which can capture a specific protein from a protein mixture is unprecedented.

Received: June 2, 2014
Revised: July 18, 2014
Published online: September 22, 2014

Keywords: cyclodextrins · lectins · magnetic separation · nanoparticles · self-assembly

[1] A. H. Lu, E. L. Salabas, F. Schüth, *Angew. Chem. Int. Ed.* **2007**, *46*, 1222 – 1244; *Angew. Chem.* **2007**, *119*, 1242 – 1266.

[2] a) Y. R. Chemla, H. L. Crossman, Y. Poon, R. McDermott, M. D. Alper, J. Clarke, *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 14268 – 14272; b) J. W. Bulte, D. L. Kraitchman, *NMR Biomed.* **2004**, *17*, 484 – 499; c) W. J. M. Mulder, G. J. Strijkers, G. A. F. van Tilborg, A. W. Griffioen, K. Nicolay, *NMR Biomed.* **2006**, *19*, 142 – 164; d) L. Yao, A. C. Jamison, S. Xu, *Angew. Chem. Int. Ed.* **2010**, *49*, 7493 – 7496; *Angew. Chem.* **2010**, *122*, 7655 – 7658.

[3] a) H. W. Gu, P. L. Ho, K. W. T. Tsang, C. W. Yu, B. Xu, *Chem. Commun.* **2003**, 1966 – 1967; b) H. W. Gu, P. L. Ho, K. W. T. Tsang, L. Wang, B. Xu, *J. Am. Chem. Soc.* **2003**, *125*, 15702 – 15703.

[4] A. K. Gupta, M. Gupta, *Biomaterials* **2005**, *26*, 3995 – 4021.

[5] a) M. G. Harisinghani, J. Barentsz, P. F. Hahn, W. M. Deserno, S. Tabatabaei, C. H. van de Kaa, J. de La Rosette, R. Weissleder, *N. Engl. J. Med.* **2003**, *348*, 2491 – 2495; b) H. T. Song, J. S. Choi, Y. M. Huh, S. Kim, Y. W. Jun, J. S. Suh, J. Cheon, *J. Am. Chem. Soc.* **2005**, *127*, 9992 – 9993; c) M. K. So, C. J. Xu, A. M. Loening, S. S. Gambhir, J. H. Rao, *Nat. Biotechnol.* **2006**, *24*, 339 – 343; d) N. Lee, H. Kim, S. H. Choi, M. Park, D. Kim, H.-C. Kim, Y. Choi, S. Lin, B. H. Kim, H. S. Jung, H. Kim, K. S. Park, W. K. Moon, T. Hyeon, *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 2662 – 2667.

[6] a) N. Pamme, C. Wilhelm, *Lab Chip* **2006**, *6*, 974 – 980; b) J. Chatterjee, Y. Haik, C. J. Chen, *J. Magn. Magn. Mater.* **2001**, *225*, 21 – 29.

[7] a) H. Gu, K. Xu, C. Xu, B. Xu, *Chem. Commun.* **2006**, 941 – 949; b) S. G. Penn, L. He, M. J. Natan, *Curr. Opin. Chem. Biol.* **2003**, *7*, 609 – 615; c) A. Hultgren, M. Tanase, C. S. Chen, G. J. Meyer, D. H. Reich, *J. Appl. Phys.* **2003**, *93*, 7554 – 7556.

[8] a) C. Xu, K. Xu, H. Gu, X. Zhong, Z. Guo, R. Zheng, X. Zhang, B. Xu, *J. Am. Chem. Soc.* **2004**, *126*, 3392 – 3393; b) C. Xu, K. Xu, H. Gu, R. Zheng, H. Liu, X. Zhang, Z. Guo, B. Xu, *J. Am. Chem. Soc.* **2004**, *126*, 9938 – 9939.

[9] K. B. Lee, S. Park, C. A. Mirkin, *Angew. Chem. Int. Ed.* **2004**, *43*, 3048 – 3050; *Angew. Chem.* **2004**, *116*, 3110 – 3112.

[10] a) T. Viegers, E. Pelssers, E. Lenders, *Med. Device Technol.* **2008**, *19*, 23 – 25; b) D. M. Bruls, T. H. Evers, J. A. H. Kahlman, P. J. W. van Lankvelt, M. Ovsyanko, E. G. M. Pelssers, J. J. H. B. Schleipen, F. K. de Theije, C. A. Verschuren, T. van der Wijk, J. B. A. van Zon, W. U. Dittmer, A. H. J. Immink, J. H. Nieuwenhuis, M. W. J. Prins, *Lab Chip* **2009**, *9*, 3504 – 3510.

[11] a) A. Fragoso, J. Caballero, E. Almirall, R. Villalonga, R. Cao, *Langmuir* **2002**, *18*, 5051 – 5054; b) M. J. W. Ludden, A. Mulder, R. Tampe, D. N. Reinhoudt, J. Huskens, *Angew. Chem. Int. Ed.* **2007**, *46*, 4104 – 4107; *Angew. Chem.* **2007**, *119*, 4182 – 4185; c) M. J. W. Ludden, X. Li, J. Greve, A. van Amerongen, M. Escalante, V. Subramaniam, D. N. Reinhoudt, J. Huskens, *J. Am. Chem. Soc.* **2008**, *130*, 6964 – 6973.

[12] A. Samanta, M. C. A. Stuart, B. J. Ravoo, *J. Am. Chem. Soc.* **2012**, *134*, 19909 – 19914.

[13] A. Mulder, J. Huskens, D. N. Reinhoudt, *Org. Biomol. Chem.* **2004**, *2*, 3409 – 3424.

[14] Y. Sahoo, H. Pizem, T. Fried, D. Golodnitsky, L. Burstein, C. N. Sukenik, G. Markovich, *Langmuir* **2001**, *17*, 7907 – 7911.

[15] H. Lis, N. Sharon, *Chem. Rev.* **1998**, *98*, 637 – 674.

[16] J. Voskuhl, M. C. A. Stuart, B. J. Ravoo, *Chem. Eur. J.* **2010**, *16*, 2790 – 2796.

[17] a) M. T. Paternostre, M. Roux, J. L. Rigaud, *Biochemistry* **1988**, *27*, 2668 – 2677; b) L. T. Mimms, G. Zampighi, Y. Nozaki, C. Tanford, J. A. Reynolds, *Biochemistry* **1981**, *20*, 833 – 840.

