African Journal of Biotechnology Vol. 10(73), pp. 16602-16609, 21 November, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.2379
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

C@Fe_3O_4/NTA-Ni magnetic nanospheres purify histidine-tagged fetidin: A technical note

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Accepted 21 October, 2011

This study reports synthesis of Ni-nitrilotriacetic acid (Ni-NTA) modified carbon nanospheres containing magnetic Fe_3O_4 particles (C@Fe_3O_4), which can act as a general tool to separate and purify histidine-tagged fetidin. In this experiment, C nanospheres are prepared from glucose using the hydrothermal process, magnetic Fe_3O_4 are packed in the porous C nanospheres and Ni-NTA are conjugated to C@Fe_3O_4. C@Fe_3O_4/NTA-Ni nanospheres are about 400 nm in diameter, providing the nanospheres with excellent magnetic response and dispersity. This study separates recombinant fetidin that are engineered to have six consecutive histidine residues (6×His) by treating with C@Fe_3O_4/NTA-Ni magnetic nanospheres. 10 mg of C@Fe_3O_4/NTA-Ni nanospheres can purify up to 2.103 mg of high-purity 6×His-tagged fetidin from 10 ml of crude *Escherichia coli* lysates. Because of high performance, C@Fe_3O_4/NTA-Ni nanospheres can also be used to separate low concentration 6×His-tagged proteins.

Key words: Magnetic carbon nanosphere, 6×His-tag, fetidin, Ni-chelating.

INTRODUCTION

Recombinant protein expression that obtains the soluble active protein with correct folding has been widely used in pharmaceutics, vaccines and basic scientific research (Salvador et al., 2011). Affinity chromatography is currently the most widely used method for protein separation (Salvador et al., 2011). Prior to separation of interest protein by affinity chromatography, the gene of target protein is cloned into the plasmid or integrated into the genome, in order that host bacterial cells can express the target protein in bulk. Meanwhile, tag sequence is usually inserted into the N-or C-terminal of the target protein so that the tagged fusion protein can be easily and quickly separated.

The currently used tags for affinity chromatography are glutathione transferase (GST) and 6×Histines(His)-tag. 6×His-tag can be identified by specific metal chelates (Fang et al., 2010), by which immobilized metal ion affinity chromatography (IMAC) is developed (Hemdan et al., 1989; Porath, 1992). At present, many biotechnology companies have developed a number of commercial, Ni-NTA mediated, His-tagged protein purification kit. For example, Qiagen Company develops Sepharose® CL-6B Ni-NTA affinity column and Promega Corporation develops magnetic bead Ni-NTA affinity purification kit.

In the past few decades, magnetic microparticles have been widely used in many biological fields, such as magnetic resonance imaging (MRI), high-temperature treatment of cancer and cell sorting (Xu et al., 2004a, b).

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Abbreviations: NTA, Nitrilotriacetic acid; **GST,** glutathione transferase; **IMAC,** immobilized metal ion affinity chromatography; **MRI,** magnetic resonance imaging; **MRSA,** methicillin-resistant *Staphylococcus aureus*; **DMF,** dimethyl formamide; **DMAP,** N,N-dimethyl amino pyridine; **IPTG,** isopropyl thiogalactoside; **TEMED,** tetramethylethylenediamine; **DLS,** dynamic light scattering; **SDS-PAGE,** sodium dodecyl sulfate polyacrylamide gel electrophoresis; **Rh,** hydrodynamic radius; **PDI,** particle dispersion index; **XRD,** X-ray powder diffraction; **ETEM,** electrical myostimulation; **ICP-MS,** inductively coupled plasma mass spectroscopy; **BCA,** bicinchoninic acid; **PMSF,** phenylmethylsulfonyl fluoride.

For the purpose of protein purification with the use of an external magnetic field, the key is to prepare magnetic nanosphere modified in the surface with affinity groups. Xu et al. (2004a, b) has reported FePt/Ni-NTA and Co/Fe_2O_3/Ni-NTA magnetic beads to purify 6×His-tagged recombinant proteins. Lee and Lee (2008) have prepared Fe_2O_3/NTA-Ni magnetic nanosphere, < 10 nm in diameter, modified with Pluronic copolymer in the surface, to purify 6×His-tagged proteins. Due to small diameter, nanosphere moved slowly in the magnetic field. Lee et al. (2006) has prepared Ni/NiO magnetic nano-beads for the purification of His-tagged protein. Fang et al. (2010) has reported Fe_3O_4@SiO_2/P(St-alt-MAn) magnetic nanosphere 220 nm in diameter, to purify 6×His-tagged proteins. Due to a substantial elevation in the size, the nanosphere moved quickly in the magnetic field to enhance the performance for purifying recombinant protein.

Liver transplantation has become an important treatment of advanced liver diseases. However, liver transplantation is often complicated by bacterial infection, especially the infection with methicillin-resistant Staphylococcus aureus (MRSA) (Tacconelli et al., 2009). The earthworm 40 kDa fetidin is an antibacterial peptide with good resistance to *Bacillus megaterium*, *Staphytococcus aureus* and *Spiroplasma citri* (Lassegues et al., 1997; Procházková et al., 2006). Expression and purification of recombinant 40 kDa fetidin based on fusion protein technology and study on the *in vitro* resistance to *S. aureus* can verify an auxiliary therapeutic method for preventing the involved infection with *S. aureus* after liver transplantation. Our pre-experiments (internal data) have shown that only the recombinant earthworm 40 kDa feditin purified through the metal Ni chelation column can keep its biological activity via protein renaturation.

To improve the performance for purification of His-tagged protein, this study prepared Carbon@Fe_3O_4 (C@Fe_3O_4) nanosphere, about 400 nm in diameter. These large nanospheres can rapidly move in a magnetic field, thereby increase the performance of purification.

MATERIALS AND METHODS

Reagents and Instruments

E. coli DH5α, E. coli BL21 and PET-21b expression plasmids containing 6×His-tagged earthworm 40 kDa fetidin with N-terminal fusion were kept in laboratory. Glucose, p-nitrophenyl chloroformate, dimethyl formamide (DMF), pyridine, nitrilotriacetate (NTA), N,N-dimethyl amino pyridine (DMAP), isopropyl thiogalactoside (IPTG), acrylamide, methylene bisacrylamide, Tris base, tetramethylethylenediamine (TEMED) and ammonium persulfate were purchased from the Sigma Company; Marker proteins purchased from the GE Company; bicinchoninic acid (BCA) protein Assay Kit for protein concentration determination purchased from the Pierce Company; other reagents made in China were analytical pure.

Vertical sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) apparatus were purchased from the Bio-rad Company, low-temperature high-speed refrigerated centrifuge purchased from the Beckman Company, ultrasonic cell disruption apparatus purchased from Xinzhi Institute of Scientific Instruments (Ningbo, China).

Preparation of C@Fe_3O_4 magnetic nanosphere

Carbon (C) nanosphere was prepared from glucose by the hydrothermal method (Deng et al., 2005). Briefly, 7.2 g of glucose was dissolved in 80 ml of triple distilled water. The solution was placed in a tight hydro-thermal reactor, followed with incubation in 180°C for 3.5 h. The produced C nanosphere was placed for natural cooling, followed with centrifugations and rinses by triple distilled water for two times.

811 mg of $FeCl_3$ and 338 mg of $FeCl_2$ were separately dissolved in 20 ml of triple distilled water cooled in ice bath and then were mixed to 40 ml. 300 mg of C nanosphere and 40 ml of Fe^{2+}/Fe^{3+} mixture were added into tri-neck flask, followed with evacuation and incubation in ice bath for 20 min to allow the diffusion of Fe ions into the pores of C nanosphere. 10 ml of aqueous ammonia precooled in ice bath was added. The reaction system was incubated in 80°C oil bath for 30 min. After magnetic separation, the produced C@Fe_3O_4 nanosphere was immersed in 20 ml of 0.1 M hydrochloric acid, followed with ultrasonic cleaning for 20 min. The cleaning procedure was repeated five times to remove free and exterior magnetic nanoparticles.

Nickel chelating to C@Fe_3O_4 nanosphere

44 mg of C@Fe_3O_4 nanosphere and 2.2 g of p-nitrophenyl chloroformate was added to flask with vacuum, followed with nitrogen ventilation. 10 ml of anhydrous DMF and 0.931 ml of pyridine was added for neutralizing the produced acid. The system was reacted in ice bath for 24 h, followed with magnetic separation and washing by DMF for three times. 0.1 g of NTA and 1.2 g of DMAP were used in reaction for 24 h, followed with magnetic separation and washings. 1.0 g of NiCl was used in reaction for 5 h, followed with magnetic separation, washing in six times by triple distilled water and lyophilization. The products were characterized by inductively coupled plasma mass spectrometry (ELAN 9000/DRC-e ICP-MS, Shanghai Perkinelmer), environmental transmission electron microscopy (Titan ETEM, Hongkong FEI) with an accelerating voltage of 80 kV and X-ray diffraction (Shimadzu XRD-7000S/L XRD, Japan), respectively. Size and size distribution were determined by dynamic light scattering (DLS) with a vertically polarized He-Ne laser (DAWNEOS, Wyatt Technology, USA). The scattering angle was fixed at 908° and the measurement was carried out at 25°C.

Inducible expression of His-tagged proteins

BL21 *E. coli* containing PET-21b expression plasmid with 6×His-tagged fetidin was cultured in 100 ml of 2×YTA medium until OD_{600} reached 0.6. IPTG was used as inductive agent with a final concentration of 1.0 mM at 37°C for 16 h to induce the expression of His-tagged fetidin. Bacteria were precipitated by centrifugation at 15,000×*g* for 2 min. Induced fetidin were identified by routine 10% SDS-PAGE as follows (Wang and Liu, 2007).

Purification of fusion protein

Bacteria were suspended in 10 ml of Lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH = 8.0) containing 5 mM phenylmethylsulfonyl fluoride(PMSF) as protease inhibitor and 1

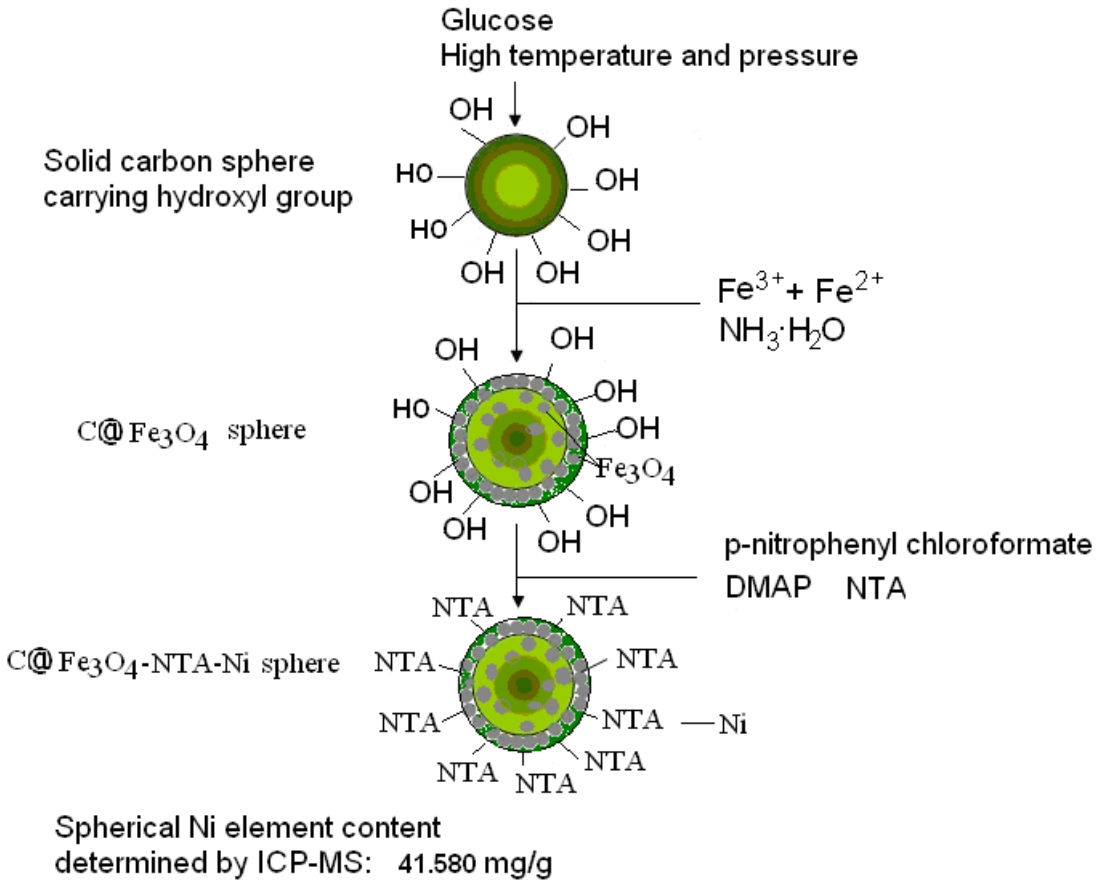

Figure 1. Schematic synthesis of C@Fe_3O_4/NTA-Ni nanospheres.

mM lysozyme, and was stirred in 4℃ for 30 min. Bacterial solution was treated with ultrasonication in ice bath until the solution was clear. The ultrasonic parameters included 500 Watt power, alternately working/stop time for 3s/7s, 5 min for each cycle and total four cycles. Lysate was precipitated by centrifugation at 15,000×*g* for 30 min. Supernatant (cleared lysate) and cell debris was detected by routine 10% SDS-PAGE.

10 ml of cleared lysate was transferred to a new tube and then 10 mg of C@Fe_3O_4/NTA-Ni nanospheres were added with vortex, incubating in 4℃ for 2 h. C@Fe_3O_4/NTA-Ni nanospheres were attracted by magnet until the solution (flow through) was clear, followed with washing by separate 2 ml of buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH = 8.0) for three times, 5 min for each time. Elution with separate 1 ml of buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH = 8.0) was performed for four times, 3 min for each time, combined with magnetic separation. Routine 10% SDS-PAGE (Wang and Liu, 2007) and the BCA method (according to the kit manufacture's instructions) were performed to identify fetidin and determine the protein concentration.

RESULTS AND DISCUSSION

Characterization of C@Fe_3O_4/NTA-Ni nanosphere

The procedure reported by Deng et al. (2005) for preparing Fe_3O_4 nanospheres was improved in the present study. Deng et al. (2005) first synthesized Fe_2O_3 or Fe_3O_4 separately and then carbon nanospheres encapsulated these magnetic particles. In the present study, Fe2+/Fe3+ mixture was used directly to be packed in carbon nanospheres and then Ferric ions were oxidized in the nanospheres after they entered the pores of carbon nanospheres. C@Fe_3O_4 nanosphere reacted with p-nitrophenyl chloroformate in anhydrous oxygen-free conditions under nitrogen protection, in which, chloroformic acid reacts with hydroxyl of C nanosphere. The left nitrophenyl group of p-nitrophenyl chloroformate is spontaneously removed when it meet amino group of NTA. This reaction is widely used in the coupling reaction of hydroxyl and amino groups. Figure 1 illustrates the preparation and reaction process of C@Fe_3O_4/NTA-Ni nanosphere. In Figure 1, relatively transparent C nanosphere, with massive hydroxyl groups distributed on surface, was prepared from glucose; magnetic Fe_3O_4 was carried in the random pores of C nanospheres, in which C layer protected Fe_3O_4 layer from oxidation and was facile to functionalization. Massive hydroxyl groups distributed on C layer surface were sequentially reacted with p-nitrophenyl chloroformate and NTA. Then NTA-Ni

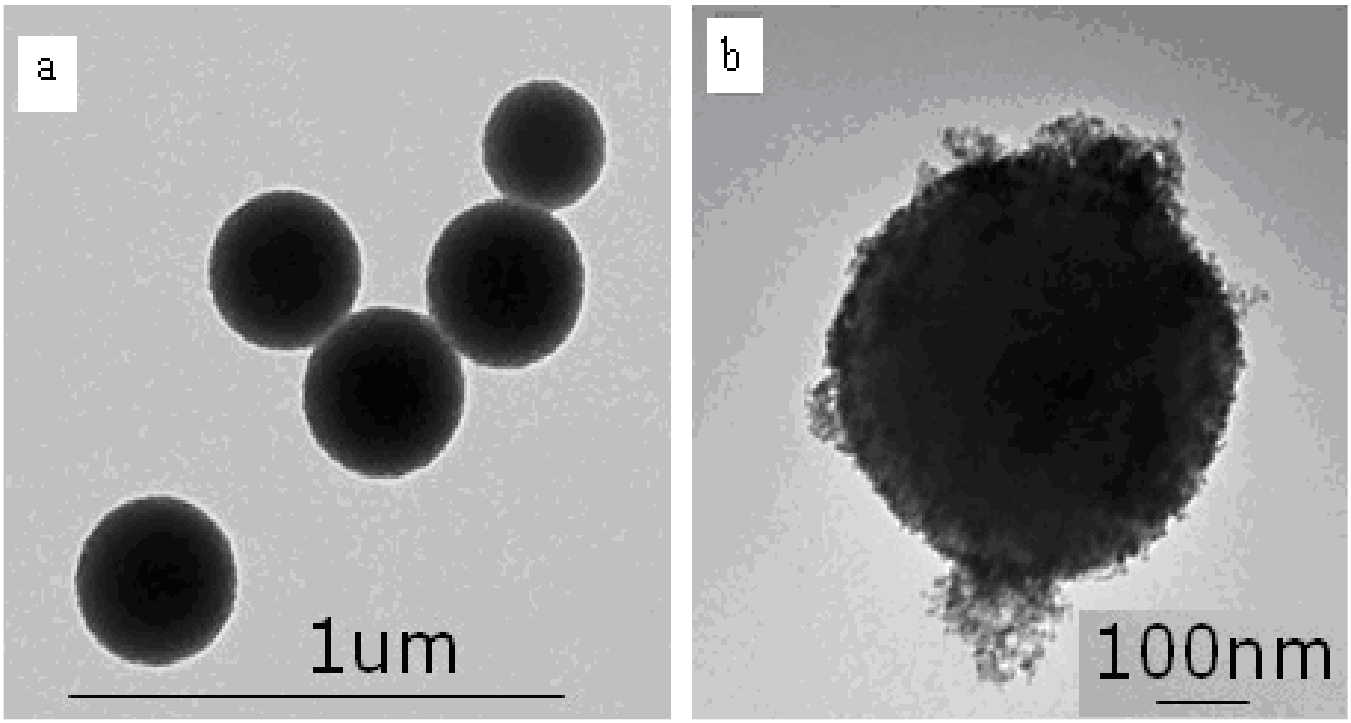

Figure 2. ETEM images of C nanospheres (a) and C@Fe_3O_4/NTA-Ni nanospheres (b).

chelates were used to modify magnetic nanosphere for affinity adsorption of 6×His-tagged fetidin. Ni elemental content on the surface of C@Fe_3O_4/NTA-Ni nanosphere was 41.580 mg/g determined by inductively coupled plasma mass spectroscopy (ICP-MS).

On ETEM results, C nanosphere with smooth surface was about 300 nm in diameter (Figure 2a); C@Fe_3O_4/NTA-Ni nanosphere was approximate 400 nm in diameter (Figure 2b). The size was larger than previous magnetic spheres and conduced to nanospherical fast movement in magnetic fields to improve the purification efficiency of C@Fe_3O_4/NTA-Ni nanospheres (Fang et al., 2010). On DLS results, the average hydrodynamic radius (Rh) of C nanospheres and C@Fe_3O_4/NTA-Ni nanospheres were 164 nm (328 nm in diameter, Figure 3a) and 201 nm (402 nm in diameter, Figure 3b), respectively; the particle dispersion index (PDI) was 0.16/0.21 respectively indicating the size distribution was narrow, relatively uniform. The results were consistent with ETEM results. X-ray powder diffraction (XRD) spectrum of C@Fe_3O_4/NTA-Ni nanosphere was consistent with the standard pattern of Fe_3O_4 (JCPDF: 01-075-0033), indicating good crystal formation in magnetic nanosphere (Figure 4).

Figure 5 demonstrates a process that C@Fe_3O_4/NTA-Ni nanosphere in ultrasonic dispersion was attracted by the magnet and the nanosphere dispersion was clarified 15 min later (The light brown in water belongs to some non-magnetic particles or small size particles). This phenomenon indicated that synthetic C@Fe_3O_4/NTA-Ni nanosphere could be well dispersed in solution and move quickly in a magnetic field to achieve satisfactory magnetic separation.

Characterization by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins from noninduced cells, induced cells, cleared lysate and induced cell debris were separated on routine 10% SDS-PAGE. In Figure 6, cleared lysate contained bulk of protein that conduces to downstream magnetic separation of 6×His-tagged protein. Cleared lysate, flow through, washing and eluates were analyzed with routine 10% SDS-PAGE. The concentration of IPTG (1.0 mM), the temperature (37°C) and the induction time (16 h) used for inducible expression of fusion protein have been optimized in pre-experiments. Under these conditions, fusion proteins were soluble with a large quantity (Figure 6).

In Figure 7, cleared lysate contained bulk of target protein, which was significantly reduced in flow though. Washing 1 contained a large quantity of nonspecific proteins and washing 3 contained little. Eluate 1 contained little target protein, because C@Fe_3O_4/NTA-Ni nanosphere had a small amount of residual washing buffer, resulting in a diluted Eluate 1. Then Eluate 2 contained peak target protein that decreased in Eluate 3 and 4. There was no nonspecific protein in Eluate 1~ 4, indicating that C@Fe_3O_4/NTA-Ni nanosphere successfully

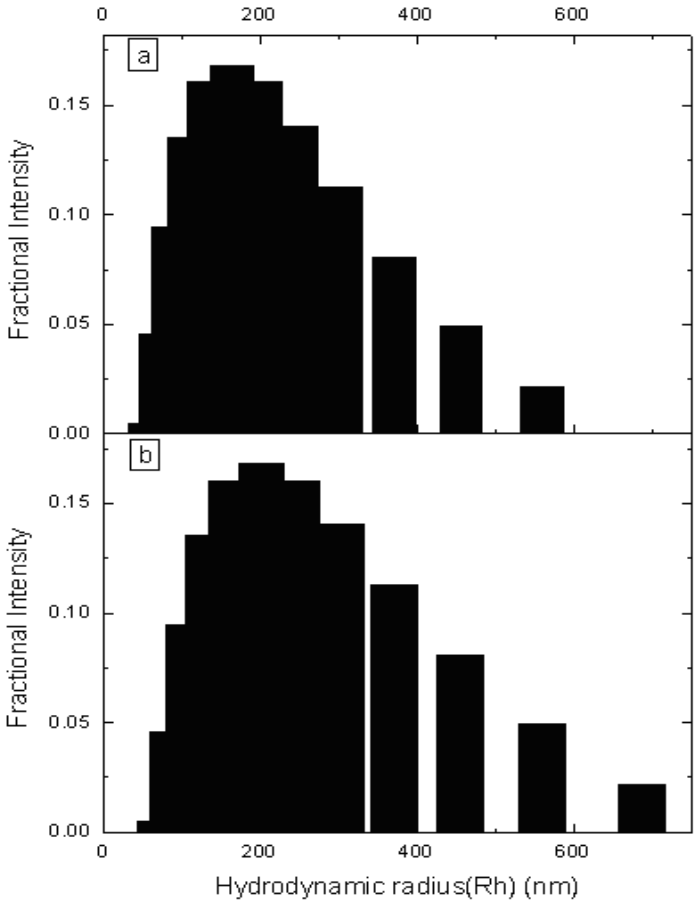

Figure 3. Size and size distribution of C nanospheres; a, C@Fe_3O_4/NTA-Ni nanospheres; b, determined by DLS.

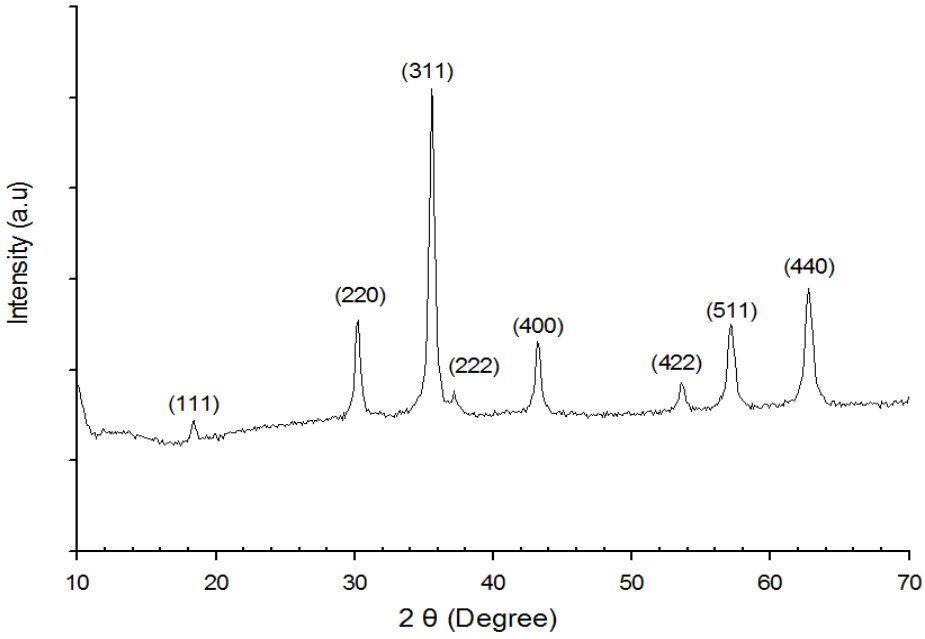

Figure 4. XRD spectrum of C@Fe_3O_4/NTA-Ni nanospheres.

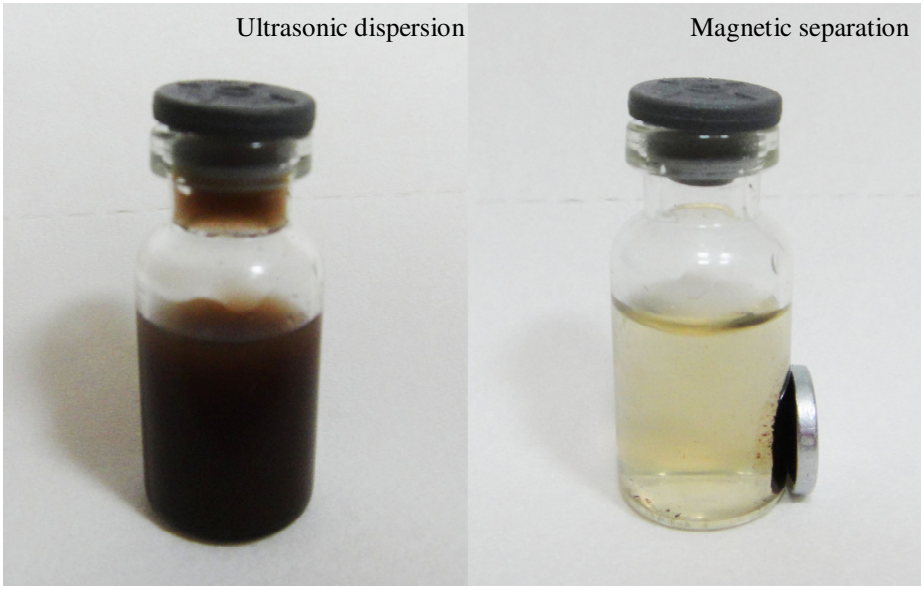

Figure 5. Pictured presentation with magnetic attraction of C@Fe3O4/NTA-Ni nanospheres.

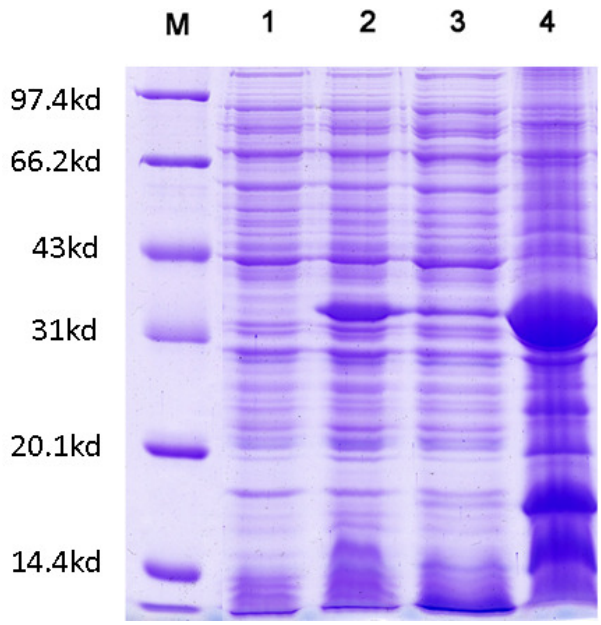

Figure 6. Coomassie blue stained fetidin expressed in *E. coli* cells induced with IPTG (1.0 mM and 16 h) 37℃. M: Low molecular marker; lane 1, noninduced cells; lane 2, cells induced with IPTG; lane 3, cleared lysate; lane 4, cell debris.

separated high-purity 6×His-tagged proteins from cleared lysate.

Purification efficiency of C@Fe_3O_4/NTA-Ni

Cleared lysate, flow through, washing and eluates were detected with the BCA method for determination of protein concentration. In Table 1, 10 mg of nanosphere separated 2.103 mg of fetidin from 10 ml of cleared lysate containing10.041 mg of total protein. The capacity of affinity nanosphere was 210.3 mg/g nanosphere and the recovery rate was up to 20.94%. GE Healthcare (website: http://www.gelifesciences.com) produced affinity column

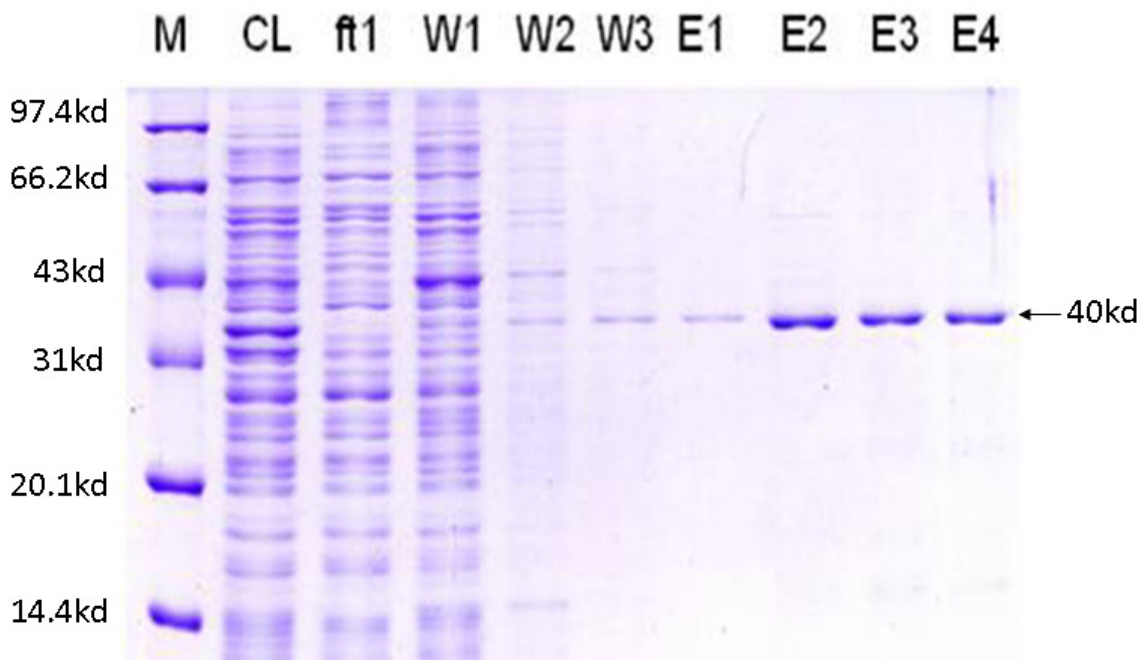

Figure 7. Coomassie blue stained His-tagged fetidin derived from affinity purification with Ni-NTA magnetic nanosphere under denaturing condition. M, low molecular marker; CL, cleared lysate; CD, cell debris; ft1, flow-through; W1~W3, washing; E1~E4, eluates.

Table 1. Recovery rate of His-tagged protein purified by C@Fe_3O_4/NTA-Ni.

Group	Concentration (µg/ml)	Volume (ml)	Content (µg)
Cleared lysate	1,004.10	10	10,041.00
Flow through	701.97	10	7019.68
Wash 1	356.57	2	713.14
Wash 2	56.21	2	112. 42
Wash 3	18.90	2	37.80
Elute 1	22.18	1	22.18
Elute 2	1337.23	1	1337.23
Elute 3	707.12	1	707.12
Elute 4	90.41	1	90.41
Recovery rates	Elute1+2+3+4/Cleared lysate		20.94%

with the capacity of 100~120 mg/g resin. The capacity of affinity nanosphere studied was superior to commercial affinity column, implying that magnetic C@Fe_3O_4/NTA-Ni nanosphere was promising for affinity purification of His-tagged protein by magnetic separation.

In addition, these large C@Fe_3O_4 nanospheres, rather than the nanosphere with small size, can also be used to purify His-tagged protein by centrifugation. The low centrifugal force can be very good in precipitation, but does not affect the combination between His-tagged recombinant protein and Ni. Therefore, when the large volume of C@Fe_3O_4 nanosphere is used in the purification of His-tagged proteins, both magnetic and centrifugal separation methods can be used.

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