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Reduction of hexavalent chromium by *Pannonibacter phragmitetus* LSSE-09 coated with polyethylenimine-functionalized magnetic nanoparticles under alkaline conditions

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ABSTRACT

A novel cell separation and immobilization method for Cr (VI)-reduction under alkaline conditions was developed by using superparamagnetic Fe_3O_4 nanoparticles (NPs). The Fe_3O_4 NPs were synthesized by coprecipitation followed by modification with sodium citrate and polyethyleneimine (PEI). The surface-modified NPs were monodispersed and the particle size was about 15 nm with a saturation magnetization of 62.3 emu/g and an isoelectric point (pI) of 11.5 at room temperature. PEI-modified Fe_3O_4 NPs possess positive zeta potential at pH below 11.5, presumable because of the high density of amine groups in the long chains of PEI molecules on the surface. At initial pH 9.0, *Pannonibacter phragmitetus* LSSE-09 cells were immobilized by PEI-modified NPs via electrostatic attraction and then separated with an external magnetic field. Compared to free cells, the coated cells not only had the same Cr (VI)-reduction activity but could also be easily separated from reaction mixtures by magnetic force. In addition, the magnetically immobilized cells retained high specific Cr (VI)-reduction activity over six batch cycles. The results suggest that the magnetic cell separation technology has potential application for Cr (VI) detoxification in alkaline wastewater.

1. Introduction

Chromium (Cr) is widely used by electroplating, leather tanning, water cooling, metal finishing, etc. [1,2]. Its widespread use has led to large quantities of this element being released into environments [3]. The effluents from these industries contain both hexavalent chromium, Cr (VI), and trivalent chromium, Cr (III). It is important to note that Cr (VI) is toxic and carcinogenic to humans but Cr (III) exhibits only a little toxicity [4]. Hence, Cr (VI) containing wastewaters have become a well recognized bio-hazard in water pollution control [5]. Environmental regulations of Environmental Protection Agency (EPA) on Cr (VI) concentration in drinking water (<50 $\mu g\,L^{-1}$) have prompted extensive research on Cr (VI) removal [6]. Conventional methods for the detoxification of Cr (VI)-contaminated wastewaters include chemical reduction followed by hydroxide precipitation, membrane separation and adsorption technology [1,7]. To overcome the drawbacks such as high cost for Cr (VI) reduction, poor removal efficiency and costly safe disposal of toxic sludge [8], microbial reduction of Cr (VI) to Cr (III) which could be economic and eco-friendly have attracted worldwide attention recently [5,9].

Many researchers have focused their studies on the bioconversion of Cr (VI) by free bacterial cells [10–14]. However, methods using free cells have been considered undesirable because of the loss of activity resulting from Cr (VI) toxicity [15], and difficult solid–liquid separation [16]. Compared to free cells, immobilized cells which have enhanced stability, improved catalytic efficiency and facilitated recovery property have been used in bioconversions, biotransformation and biosynthesis processes [17,18]. Several biopolymers such as alginate, agarose, carrageenan, cellulose and chitosan are widely used as entrapment matrices for cell immobilization as they are non-toxic, efficient and inexpensive [17–19]. On the other hand, magnetic immobilization and separation technology which show lower diffusional limitations and steric hindrance provide a quick, easy and convenient alternative over traditional cell-immobilizing methods [18,20]. Fe_3O_4 nanoparticles (NPs) are often modified by functional chemicals including

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chitosan [19,21], ammonium oleateon [18,22] and carbohydrates [23] to possess specific properties such as optimal particle surface charge and functional groups. Cell immobilization is then achieved by the magnetic nanoparticles under neutral conditions via electrostatic attraction [21] and hydrophobic interaction [18,22]. It is convenient to concentrate and reuse the dispersed coated-cells from a suspension by a magnetic method. However, until recently the information about the utilization of Fe_3O_4 NPs for cell immobilization is still insufficient, especially for those specific cases under alkaline conditions.

One functional polymer that has widely attracted attention for Fe_3O_4 NPs-coating is polyethyleneimine (PEI) [24,25]. PEI is a water-soluble polycation consisting of primary, secondary, and tertiary amine functional groups [26]. It has been used for the design of DNA delivery vehicles [27,28] and as a functional coating to enhance the performance of heavy metals adsorption [29–32]. It is known that electrostatic interaction between adsorbent and adsorbate plays an important role in the process of adsorption [29]. Since the surface charge of bacteria is negative in aqueous systems [33], adsorbents with positive surface charges are favorable for bacteria sorption. PEI is the organic macromolecule with the highest cationic-charge density potential, and has a high buffer capacity over a very broad pH as well [34]. Several researchers have reported that PEI-modified adsorbents exhibited positive zeta potential under alkaline conditions [29,35,36]. From the electrostatic interaction point of view, it is possible to use PEI-modified NPs as a tool for negative-charged bacterial cells immobilization under alkaline conditions.

As we previously reported, *Pannonibacter phragmitetus* LSSE-09 was isolated from the industrial sludge. This Gram-negative bacteria showed a strong potential to reduce Cr (VI) to Cr (III) aerobically and anaerobically under alkaline conditions [10]. We have evaluated the capability of strain LSSE-09 encapsulated in alginate capsules for reducing Cr (VI) along with organo-Cr (III) removal [37]. However, due to the lower diffusional limitations and steric hindrance, the Cr (VI)-reduction rate of encapsulated cells was lower than that of free cells. Herein, we attempted to develop a simple and effective technique for Cr (VI) reduction under alkaline conditions, by integrating the advantages of magnetic separation and cell immobilization. To the best of our knowledge, there have not been any reports about the utilization of Fe_3O_4 NPs-immobilized cells under alkaline conditions, especially for Cr (VI) reduction. In the present work, Fe_3O_4 NPs were modified with PEI to produce polycationic magnetic NPs, which successfully recovered the cells of strain LSSE-09 from the biological systems. The magnetically immobilized cells exhibited good catalytic activity under alkaline conditions over several batch cycles.

2. Materials and methods

2.1. Materials

Iron (II) chloride ($FeCl_2{\cdot}4H_2O$, analytical reagent grade), iron (III) chloride ($FeCl_3{\cdot}6H_2O$, analytical reagent grade), and concentrated ammonium hydroxide (25%) were purchased from Sinopharm Chemical Regent Beijing Co., Ltd. Poly (ethyleneimine) (PEI, 10172 Da), sodium citrate ($C_6H_5O_7Na_3{\cdot}2H_2O$, analytical reagent grade) were purchased from Sigma–Aldrich.

2.2. Preparation of surface modified magnetic NPs

The PEI-modified magnetic Fe_3O_4 NPs were prepared by a co-precipitation method with minor modification [38]. Firstly, 8.67 mmol of $FeCl_3{\cdot}6H_2O$ and 4.34 mmol of $FeCl_2{\cdot}4H_2O$ were dissolved in 100 mL deionized water. The mixture was stirred for 30 min under a nitrogen gas atmosphere at 85 °C. Then 25 mL of concentrated ammonium hydroxide (25%) was added to the solution. After the solution turned black, 0.26 mmol of sodium citrate was quickly added into the system. After 5 min stirring, 50 mL of the prepared PEI aqueous solution containing 0.26 mmol PEI was added, and the reactions continued for 2 h. Finally, the precipitate was separated by magnet and washed three times with de-ionized water. The resulting nanoparticles were suspended in water at 4 °C for storage. The concentration of magnetic nanoparticles was expressed in terms of dry weight per unit volume of suspension medium.

2.3. Preparation NPs-coated bacterial strain

P. phragmitetus LSSE-09 cells were inoculated into 100 mL LB medium, cultured aerobically at 37 °C and 150 rpm for 12 h. The cells were harvested by centrifugation (4000 × *g*) for 15 min at 4 °C, washed twice with de-ionized water and then suspended in 50 mM pH 9.0 Tris–HCl buffers. The dry weight of cells was approximate 1.95 g L^{-1}. A volume of 0.5 mL of PEI-modified NPs (10 g L^{-1}) was added and mixed thoroughly. The microbial cells were coated by adsorbing the magnetic NPs. For magnetic separation, a permanent magnet was placed at the side of the vessel. After several minutes (1–2 min), the coated cells were concentrated and separated from the suspension medium by decantation (Fig. 8).

2.4. Characterization

2.4.1. TEM measurement

The PEI-modified NPs and the NPs-coated cells were characterized by JEM-2010 transmission electron microscopy coupled with an EDS (Oxford) system. Samples were prepared by placing a drop of aqueous solution containing NPs and NPs-coated cells onto a Formvar-covered copper grid, and dried in the air.

2.4.2. FTIR measurement

FTIR spectra of PEI, bare Fe_3O_4 nanoparticles, and PEI conjugated Fe_3O_4 nanoparticles were recorded on a Bruker Vecter 22 FTIR spectrometer. Lyophilized nanoparticles was dispersed in KBr and then pelletized before measurements.

2.4.3. TGA measurement

The number of PEI incorporated into magnetite nanoparticles was determined by thermogravimetric analysis (Netzsch STA 449 C). Samples were heated from 30 to 800 °C at a heating rate of 10 °C min^{-1} in air.

2.4.4. Magnetization measurement

The magnetization measurement of magnetic nanoparticles was carried out with a vibrating sample magnetometer at room temperature (VSM, Model 4 HF VSM of ADE Technologies, Inc.).

2.4.5. Zeta potential measurement

The zeta potential of PEI-modified NPs and NPs-coated cells at different pH values was observed using Zeta PALS (Brookhaven Instruments Co., USA). The pH of the solution was adjusted with 0.01 M NaOH or 0.01 M HCl. After 1 h of stabilization with self-sealing rubber septum, the final solution pH was recorded, and the supernatant with small PEI-modified NPs or NPs-coated cells was then used to conduct zeta potential measurements.

2.5. Microbial reduction of Cr (VI)

2.5.1. Cr (VI) reduction

Cr (VI)-reduction experiments were performed anaerobically at 37 °C and 150 rpm under alkaline conditions (initial pH 9.0).

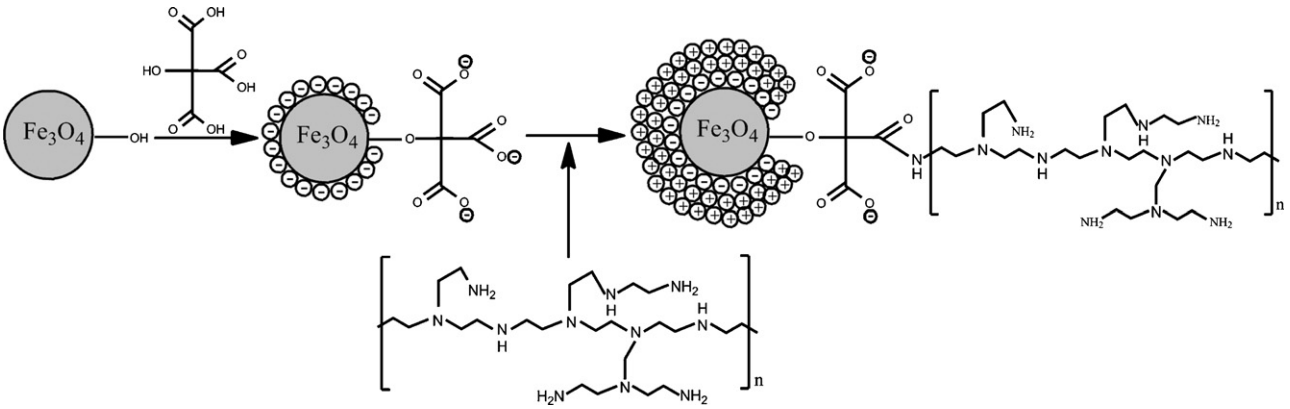

Fig. 1. Schematic illustration of the fabrication strategy for PEI-modified Fe_3O_4 nanoparticles [38].

The reaction kinetics and cell activity were investigated by measuring the retained Cr (VI) and total Cr (III) in the supernatant. Free cells and NPs-coated cells were separately introduced into a 50 mL glass bottle containing 20 mL of 350 mg L^{-1} Cr (VI) and 3000 mg L^{-1} acetate, both of which were dissolved in Tris–HCl buffer (50 mM, pH 9.0). Acetate was used as external electron donors and was essential to improve the reduction rate. Each bottle was sparged with nitrogen for 3 min, introduced by a syringe needle through the self-sealing rubber septum. Air displacement was achieved by inserting another syringe needle as the outlet. Cr (VI)-reduction experiments were performed at 37 °C with shaking at the speed of 150 rpm. At different time intervals, aliquots of solution were withdrawn using a syringe and centrifuged at 12,000 × *g* for 5 min. The supernatants were analyzed for residual Cr (VI) and total Cr (III) concentrations. Blank experiments were conducted to investigate the Cr (VI) adsorption capacities of PEI-modified Fe_3O_4 NPs. A volume of 0.5 mL PEI-modified Fe_3O_4 NPs (10 g L^{-1}) was added into a 20 mL reaction mixture, but without adding cells. Thus, the concentration of Fe_3O_4 NPs in the reaction mixture was 0.25 g L^{-1}.

The analysis of Cr (VI) in aqueous samples was performed using a UV-VIS spectrophotometer (UNICO7200, USA) at 540 nm after complexation with 1,5-diphenylcarbazide [39]. Total Cr (III) concentration in the supernatant after reduction was measured by an inductively coupled plasma atomic emission spectrometer (ICP-OES, Perkin Elmer Optima 7000DV, USA).

2.5.2. Recycling stability

Free and NPs-coated LSSE-09 cells were tested repeatedly in a reaction mixture containing 350 mg L^{-1} Cr (VI) and 3000 mg L^{-1} acetate under anaerobic condition, both of which were dissolved in 20 mL Tris–HCl buffer (50 mM, pH 9.0). At the end of each batch, the free and NPs-coated cells were collected by centrifugation and magnetic field, respectively, rinsed with Tris–HCl buffers and then added to the next reaction cycle. The recycling stabilities were explored by measuring the reduction rate in each successive reaction cycle.

The Cr (VI)-reduction rate (TCR) is given by

$$\mathrm{TCR} = \frac{C_0 - C_f}{1.95t} \quad (1)$$

where TCR is the Cr (VI)-reduction rate (mg $g^{-1}_{(\text{dry weight})}$ min^{-1}), C_0 is the initial Cr (VI) concentration (mg L^{-1}), 1.95 is the dry cell weight (g L^{-1}), C_f is the finial Cr (VI) concentration (mg L^{-1}), and t is the Cr (VI)-reduction time (min).

3. Results and discussion

3.1. Characteristics of magnetic nanoparticles

The surface modification of Fe_3O_4 magnetic nanoparticles by PEI is schematically illustrated in Fig. 1. The hydroxyl groups on the surface of Fe_3O_4 NPs were exchanged with citrate ligands to provide excess carboxylate groups, which bring negative charges on particle surface to prevent their aggregation [40,41] and induce the formation of surface complexes between Fe_3O_4 and compounds [42]. PEI molecules were conjugated to the anionic surface of magnetite nanoparticles through strong ionic interactions [38], leading to a significantly enhanced stable suspension [31]. Fig. 2 shows that the monodisperse particles were approximately spherical in shape, with a mean magnetite core diameter of 15 nm. To confirm the binding of PEI, the FTIR spectra of bare Fe_3O_4 NPs, and PEI-modified NPs were investigated (Fig. 3). The peak at 584 cm^{-1} corresponds to the Fe–O vibration, and it is the characteristic peak of bare Fe_3O_4 NPs. When Fe_3O_4 NPs were coated with PEI molecular, the spectrum exhibited some changes. The bands around 3425 cm^{-1} are assigned to the stretching vibration of N–H bonds of PEI, and the peak at 1628 cm^{-1} corresponds to the amino groups [43,44]. The peaks at 1452 and 1083 cm^{-1} are attributed to the C–H bending and C–N stretching, respectively [32]. The spectrum reveals the binding of PEI onto the surface of Fe_3O_4 NPs. The conclusions were further verified by thermogravimeric analysis (TGA) result.

As shown in Fig. 4, PEI-modified NPs shows a mass loss of about 11.6 wt.% after heating to 600 °C. In contrast, only about a 1.5 wt.% of mass loss was observed for the bare Fe_3O_4 NPs. The content of PEI-citrate in PEI-modified Fe_3O_4 NPs was determined to be about 10.1 wt.% with respect to the total formulation weight.

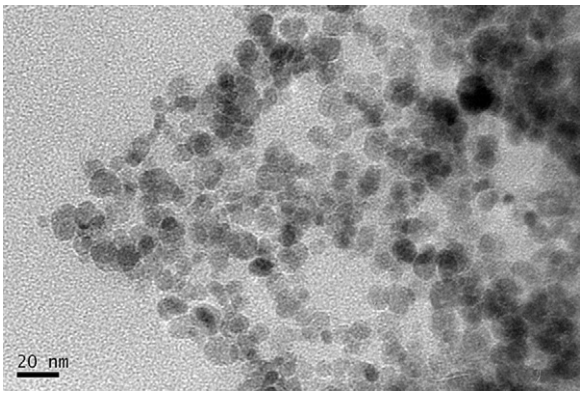

Fig. 2. TEM image of PEI-modified Fe_3O_4 NPs (scale bar: 20 nm).

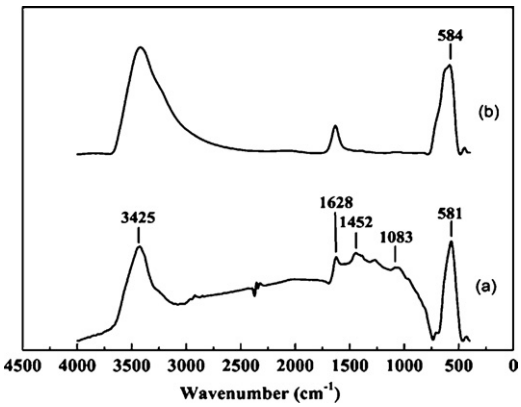

Fig. 3. FTIR spectra of PEI-modified and bare Fe_3O_4 NPs: (a) PEI-modified Fe_3O_4 NPs; (b) bare Fe_3O_4 NPs.

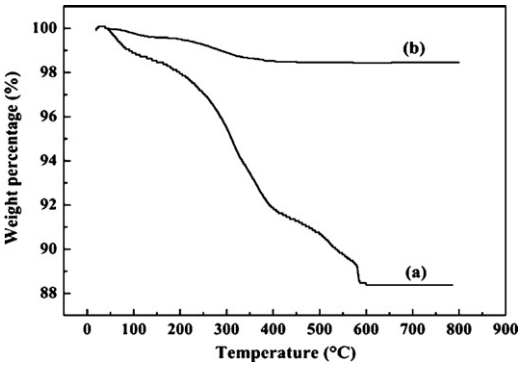

Fig. 4. TG analysis of PEI-modified and bare Fe_3O_4 NPs: (a) PEI-modified Fe_3O_4 NPs; (b) bare Fe_3O_4 NPs.

The magnetic properties were also obtained by VSM, as illustrated in Fig. 5. The PEI-modified NPs exhibited typical superparamagnetic behavior, and the saturation magnetization value (σ_s) was 62.3 emu/g. In addition, as shown in Fig. 6, the zeta potential of the PEI-modified Fe_3O_4 NPs in the pH range of 7–12 (adjusted by NaOH or HCl) was measured to calculate the isoelectric point (pI ≈ 11.5). The result reveals that the PEI-modified Fe_3O_4 NPs exhibited positive zeta potential at pH < 11.5, which can be attributed to the protonation of amine groups in PEI molecules on the particle surface [29]. Goon and his co-workers also reported that the PEI-modified Fe_3O_4 NPs exhibited positive zeta potential under alkaline conditions, and the pI shifted toward a higher pH as more PEI was attached [31].

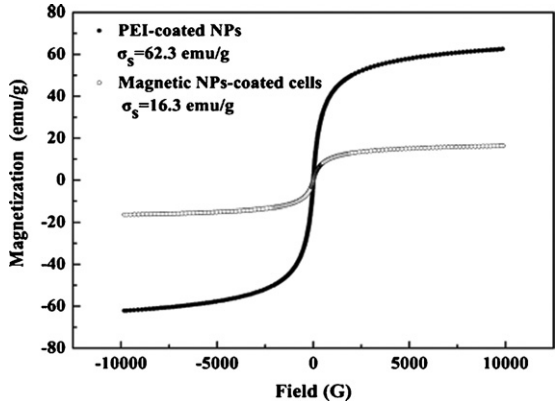

Fig. 5. Magnetization curves of PEI-modified magnetite and magnetic coated LSSE-09 cells.

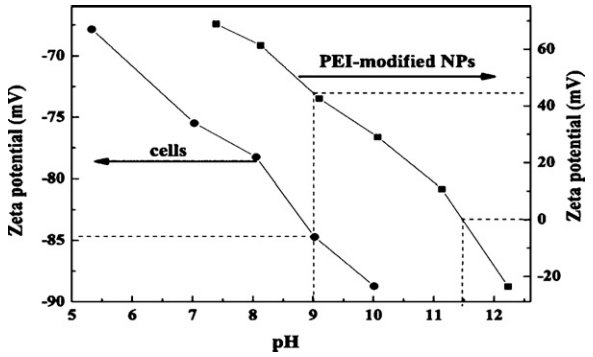

Fig. 6. Zeta potentials of LSSE-09 cells and PEI-modified Fe_3O_4 NPs.

It is known that the highly charged nature of lipopolysaccharides confers an overall negative charge on the Gram-negative cell wall [33]. As shown in Fig. 6, the Zeta potentials of LSSE-09 cells and PEI-modified Fe_3O_4 NPs at pH 9.0 are −84.7 and 42.7 mV, respectively. Therefore, the positive-charged PEI-modified NPs may be strongly adsorbed on the surface of negative-charged LSSE-09 cells via electrostatic attraction.

3.2. Characteristics of magnetic NPs-coated cells

Fig. 7 shows the TEM image of *P. phragmitetus* LSSE-09 cell coated with Fe_3O_4 NPs. In this experiment, only a few nanoparticles were added into the medium. Fe_3O_4 nanoparticles were strongly adsorbed on the surface of microbial cells and thus coated the cells. EDS analysis showed that the precipitates on cell surface contained Fe (Fig. 7b). It can be deduced that some Fe_3O_4 NPs were coated on

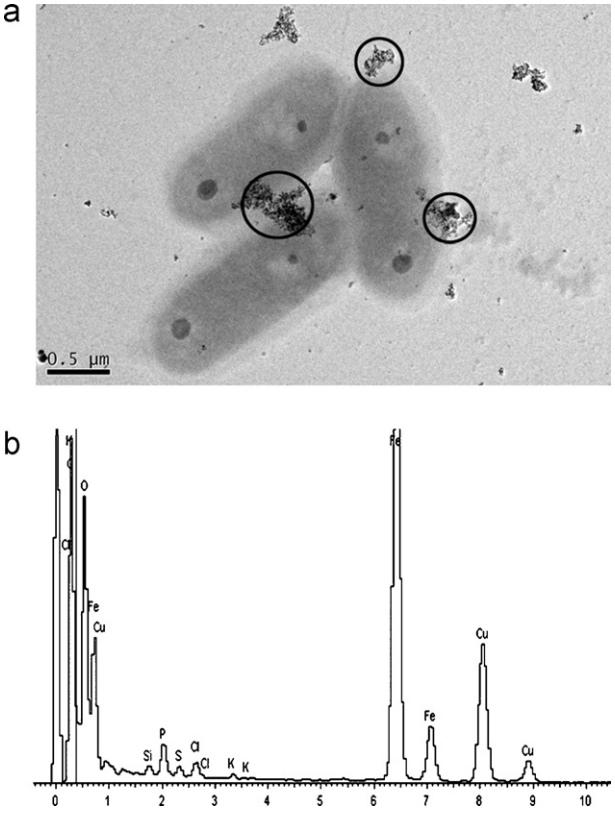

Fig. 7. Conventional TEM images of *P. phragmitetus* LSSE-09 cell coated with Fe_3O_4 NPs: (a) TEM results of precipitates coated on bacteria surface; (b) EDS spectrum of these precipitates (black circular portion).

Fig. 8. Magnetic separation and immobilization of *P. phragmitetus* LSSE-09 cells: (a) appearance of the cell suspension immediately after adding magnetite nanoparticles; (b) free cells in Tris–HCl buffer (50 mM, pH 9.0); (c) after 2 min, the coated cells concentrated and collected by an external magnet.

cell surface. Meanwhile, the nanoparticles on cell surface could not be washed off by deionized water, ethanol, saline water (0.85 wt.%), or Tris–HCl buffer (50 mM, pH 9.0). Thus, there is little cell loss during the reduction process. Other researchers also reported that some bacteria could be immobilized by magnetic nanoparticles which were modified with functional chemicals. Honda and his co-workers prepared a chitosan-conjugated magnetite to separate a recombinant *Escherichia coli* harboring the β-galactosidase gene [21]. Bacterial cells coated with superparamagnetic Fe_3O_4 nanoparticles were developed for biodesulfurization [18,22]. In another study, EI-Boubbou and co-workers have developed magnetic glyco-nanoparticles as a unique tool for rapid pathogen detection, decontamination, and strain differentiation [23].

Additionally, the cells coated with Fe_3O_4 NPs were superparamagnetic with zero remanence and coercivity (Fig. 5). The magnetization (σ_s) of magnetic coated LSSE-09 cells was as high as 16.3 emu/g. Thus, magnetic separation was found to be applicable to separate cells, even though only a few nanoparticles were coated on cell surface (Fig. 7). The cell-nanoparticles aggregating in the aqueous suspension could be easily separated with an externally magnetic field and re-dispersed by gentle shaking after the magnetic field was removed, as shown in Fig. 8.

3.3. Microbial reduction of Cr (VI)

3.3.1. Cr (VI) reduction by free and magnetic NPs-coated cells

LSSE-09 cells were immobilized by PEI-modified NPs under alkaline conditions. It is known that PEI shows not only an effective nonviral vector for DNA transfection, but also cytotoxicity in a range of cell lines [45,46]. In order to investigate the Cr (VI)-reduction activity of the magnetic separated/immobilized cells, experiments were performed anaerobically at initial pH 9.0, with 3000 mg L^{-1} acetate as external electron donors. Fig. 9a shows the Cr (VI)-reduction capacity by free and Fe_3O_4 NPs-coated cells. The coated cells exhibited similar reduction activities to free cells, and both of them completely reduced 350 mg L^{-1} Cr (VI) after incubating 20 min. In addition, a small number of nanoparticles coated on cell surface did not have negative effect on Cr (VI)-reduction activity. To our knowledge, this is the first report to describe the immobilization of living bacterial cells with Fe_3O_4 NPs under alkaline conditions, especially for Cr (VI) reduction.

It is necessary to note that some researchers developed PEI-modified biosorbents with enhanced Cr (VI) adsorption capacities [29,30]. Blank experiments were conducted to investigate the Cr (VI) adsorption capacities of PEI-modified Fe_3O_4 NPs. Because of

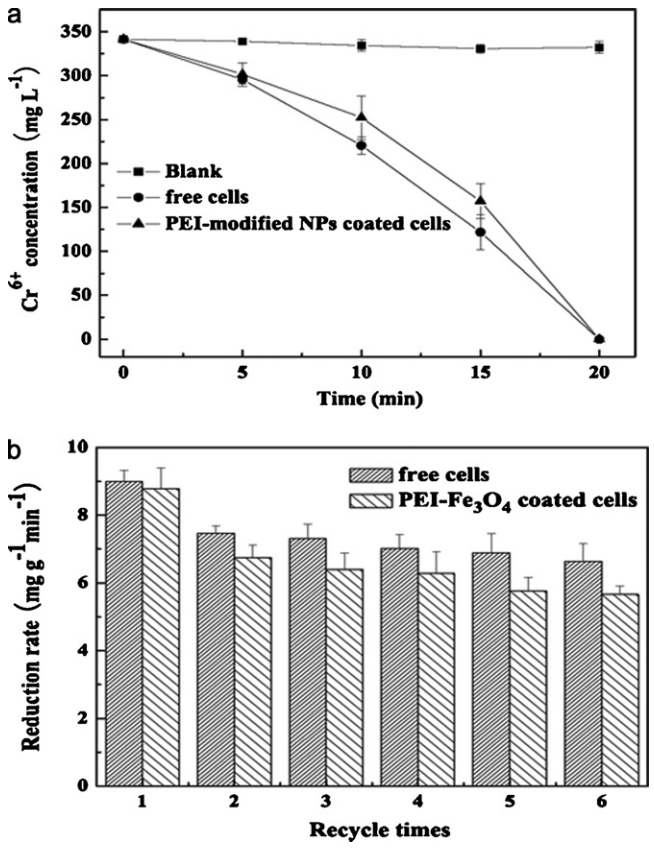

Fig. 9. Typical Cr (VI)-reduction by free and Fe_3O_4 NPs-coated cells: (a) Cr (VI)-reduction capacity; (b) recycling stability of strain LSSE-09 over six batch cycles.

the decreased protonation of amine groups at pH 9.0, PEI could adsorb only a little anionic Cr (VI) (CrO_4^{2-}) via electrostatic attraction [29,30]. Thus, in blank experiments, no obvious adsorption of Cr (VI) by PEI-modified Fe_3O_4 NPs occurred within 20 min (Fig. 9a).

Several PEI-grafted materials reported were also applied in adsorption of inorganic Cr (III) under acidic conditions [47,48]. As we previously reported, most Cr (III) did not precipitate and formed considerable soluble organo-Cr (III) end-products after reduction [10]. The characteristics of the supernatant rising from Fe_3O_4 NPs-coated cells after reduction were compared with those of free cells. As shown in Table 1, there was no obvious difference between free and coated cells. Zeta potential of coated cells was a little lower than that of free cells, resulting from the small amounts of positive-charged nanoparticles retained in the supernatant after magnetic separation. Besides, more than 80% of the Cr (III) retained in a soluble form in the culture supernatant after reduction by both free and coated cells.

3.3.2. Recycling stability of free and magnetic NPs-coated cells

The free and coated cells of strain LSSE-09 were tested repeatedly in a reaction mixture containing 350 mg L^{-1} Cr (VI) and 3000 mg L^{-1} acetate. At the end of each batch, the coated cells were collected by applying a magnetic field and then reused in another test. Cr (VI) was completely reduced by strain LSSE-09 in each batch. The reduction rate was calculated by Eq. (1). As shown in Fig. 9b, the original reduction rate of coated and free cells was 8.78 mg g^{-1} min^{-1} and 8.99 mg g^{-1} min^{-1}, respectively. It is evident that the activity of coated LSSE-09 which was lower than that of free cells decreased during the Cr (VI)-reduction process, indicating that few coated cells appeared to have been lost during cell-separation and Cr (VI)-reduction processes. Six batch cycles later, the reduction rate of coated cells was 5.66 mg g^{-1} min^{-1}, comparing with that

Table 1
Characteristics of the supernatant after reduction.

Parameters	Final pH	Zeta potential (mV)	Total Cr concentration (mg L^{-1})
Free cells	9.37 ± 0.03	−40.80 ± 2.21	271.02 ± 8.15
Fe_3O_4 NPs-coated cells	9.38 ± 0.06	−35.97 ± 3.67	275.31 ± 13.11

of free cells, 6.63 mg g^{-1} min^{-1}. The results suggested that magnetic NPs-coated cells of LSSE-09 could be repeatedly used and retain high specific Cr (VI)-reduction activity. Moreover, the distinct superparamagnetic property of coated cells presents further options in a magnetically stabilized fluidized bed bioreactor, and further investigation is being undertaken to evaluate its application for Cr (VI)-reduction.

4. Conclusions

A novel cell separation and immobilization method for Cr (VI)-reduction was developed by using superparamagnetic nanoparticles via electrostatic attraction under alkaline conditions. At initial pH 9.0, the positive-charged PEI-modified Fe_3O_4 nanoparticles were found applicable for separation and immobilization of negative-charged *P. phragmitetus* LSSE-09 cells from reaction mixtures within minutes. The magnetically immobilized cells exhibited high catalytic activity and repeated-batch Cr (VI)-reduction operational stability. The results suggested that the magnetic cell separation technology which was rather convenient and easy to perform showed potential biotechnological application for the detoxification of Cr (VI)-contaminated wastewater.

Acknowledgment

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