Analytical Biochemistry 540–541 (2018) 45–51

Contents lists available at ScienceDirect

Analytical Biochemistry

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

Functionalization of paramagnetic nanoparticles for protein immobilization and purification

Lara A.B.C. Carneiro[a], Richard J. Ward[b,*]

[a] Departamento de Bioquímica e Imunologia, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo-USP, Ribeirão Preto, SP CEP 14049-900, Brazil
[b] Departamento de Química, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP CEP 14049-901, Brazil

ARTICLE INFO

Keywords:
Chitosan-NTA coating
Nanocomposites
Protein purification

ABSTRACT

A paramagnetic nanocomposite coated with chitosan and N-(5-Amino-1-carboxy-pentyl) iminodiacetic acid (NTA) that is suitable for protein immobilization applications has been prepared and characterized. The nanoparticle core was synthesized by controlled aggregation of Fe_3O_4 under alkaline conditions, and Transmission Electron Microscopy revealed a size distribution of 10–50 nm. The nanoparticle core was coated with chitosan and derivatized with glutaraldehyde and NTA, as confirmed by Fourier Transform Infrared Spectroscopy. The final nanoparticles were used as a metal affinity matrix to separate a recombinant polyhistidine-tagged β-galactosidase from *Bacillus subtilis* directly from *E. coli* cell lysates with high purity (> 95%). After loading with Ni^{2+}, nanoparticles demonstrated a binding capacity of 250 μg of a polyhistidine-tagged β-galactosidase per milligram of support. The immobilized enzyme retained 80% activity after 9 cycles of washing, and the immobilized recombinant protein could be eluted with high purity with imidazole. The applications for these nanomagnetic composites extend beyond protein purification, and can also be used for immobilizing enzymes, where the β-galactosidase immobilized on the nanomagnetic support was used in multiple cycles of catalytic reactions with no significant loss of catalytic activity.

Introduction

Nanomaterials are at the leading edge of the rapidly developing field of nanotechnology [1]. Magnetic nanoparticles have a broad range of uses not only in drug and DNA delivery and in tumour suppression through localized heating (hyperthermia), but also for the separation and purification of biological molecules or cells, and for protein detection [2]. These diverse applications can be ascribed to a series of properties. Magnetic nanoparticles have controlled sizes ranging from 1 to 100 nm, which can be functionalized with biological molecules to specifically interact with the biological material of interest. Furthermore, their magnetic properties allow manipulation by an external magnetic field [2].

Paramagnetic nanoparticle supports are commonly based on magnetite (Fe3O4), and are readily synthesized from commonly available iron salts under benchtop laboratory conditions [3]. After synthesis, the surface of Fe_3O_4 nanoparticles may be further modified by coating or by the introduction of diverse chemical groups. Chitosan is an unbranched polysaccharide composed of β-1,4-linked 2-acetamino-2-deoxy-D-glucopyranose and 2-amino-2-deoxy glucopyranose subunits that has been used to coat Fe_3O_4 nanoparticles [4,5]. Chitosan coating modifies the nanoparticle surface, introducing properties including hydrophilicity, non-toxicity and improved biocompatibility [6].

Hritcu and co-workers [7] have synthetized colloidal magnetite nanoparticles (Fe_3O_4) by co-precipitation and stabilized the suspension with the non-ionic surfactant Pluronic F127. The nanoparticles were subsequently coated with a layer of chitosan prepared by ionotropic gelation using sodium tripolyphosphate as a crosslinking agent. Coupling agents such as glutaraldehyde are also frequently utilized to covalently crosslink surface groups of coated magnetic nanoparticles. The aldehyde group can react with primary amine groups on the surface of the modified magnetic nanoparticle and the molecule of interest to be immobilized [8]. The frequent use of glutaraldehyde for improving biocompatibility and durability of natural scaffold materials reflects its safety and reliability as a crosslinking agent [9].

Oshige and co-workers [10] have prepared glutaraldehyde

Abbreviations: AB-NTA, N-(5-Amino-1-carboxy-pentyl) iminodiacetic acid; TEM, Transmission electron microscopy; SEM, scanning electron microscopy; FTIR, Fourier transform infrared; YesZ, β-galactosidase from *Bacillus subtilis*; GFPuv, Green Fluorescent Protein excited at UV wavelengths; pNPβGal, *p*-nitrophenyl-β-D-galactopyranoside; IMAC, immobilized metal affinity chromatography

* Corresponding author. Departamento de Química – Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Avenida Bandeirantes 3900, Ribeirão Preto, SP CEP 14049-901. Brazil.
E-mail addresses: larabuffoni@gmail.com (L.A.B.C. Carneiro), rjward@ffclrp.usp.br (R.J. Ward).

https://doi.org/10.1016/j.ab.2017.11.005
Received 3 August 2017; Received in revised form 8 November 2017; Accepted 13 November 2017

crosslinked chitosan derivatized with nitrilotriacetic acid to coat a planar silica surface for use in biosensors and bioelectrodes. However, the planar geometry results in extensive enzyme/support contacts that limits the access of substrates to the immobilized enzyme active site that restricts the use of this system in catalysis with immobilized enzymes. The use of spherical nanoparticles reduces the contact area between the immobilized enzyme and the support and facilitates access of substrate molecules. Although the synthesis of chitosan-stabilized Fe_3O_4 nanoparticles for protein purification has previously been described [11], the approach uses a complex and costly method in which the paramagnetic particles are further decorated with gold nanoparticles functionalized with lipoic acid and multidentate iminodiacetic acid complexed with Cu^{2+}.

Here we report improvements in the methodology for the simple preparation of Fe_3O_4 nanoparticles that are subsequently derivatized with chitosan/glutaraldehyde/N-(5-Amino-1-carboxy-pentyl) iminodiacetic acid (AB-NTA). Apart from the potential uses of this nanomaterial for immobilized enzyme catalysis, targeted delivery agents and other biotechnological applications, the nanoparticles were successfully applied for purification of histidine-tagged proteins from bacterial lysates with superior binding capacity when compared to commercial related supports. The functionalized nanoparticles were also a suitable support for protein immobilization in multiple cycles of enzyme use and washing with minimal loss of catalytic activity.

Material and methods

Preparation of functionalized paramagnetic particles

Magnetite (Fe_3O_4) nanoparticles were prepared by co-precipitation of Fe^{2+} and Fe^{3+} ions under alkaline conditions [12,13]. Iron (III) chloride hexahydrate (0.0551 mol) was dissolved in 84 mL of distilled water and 36 mL of a 2% aqueous solution of Pluronic F127. Iron (II) sulfate heptahydrate (0.0275 mol) was dissolved in 84 mL of distilled water and 36 mL of a 2% aqueous solution of Pluronic F127. These two solutions were mixed and 120 mL of NaOH 2.7 M was added at a flow rate of 10 mL/min and maintained at 65 °C for 30 min with stirring. The resulting Fe_3O_4 nanoparticles were washed repeatedly by separation with a neodymium magnet and resuspension in distilled water until neutral pH was reached. A solids content of 10% (w/w) was determined for the final particle suspension by drying [14].

Chitosan 0.1% dissolved in acetic acid 83 mM (20 mL) was added to 2 mL of magnetic nanoparticle suspension (0.2 g) and the mixture was stirred for 30 min at room temperature. The magnetic nanoparticles coated with chitosan were treated with the crosslinking agent glutaraldehyde. The chitosan-coated magnetic suspension was added to 20 mL of glutaraldehyde 1.25% in 50 mM Tris-HCl pH 8.5 containing 100 mM NaCl. The initial reaction was performed for 1 h at room temperature and the particle suspension was subsequently incubated at 4 °C for 16 h. To remove excess glutaraldehyde, the nanoparticles were separated with a neodymium magnet and washed with distilled water 8 times with 100 mL for each wash cycle.

The glutaraldehyde-treated magnetic nanoparticles were incubated overnight at 37 °C and 180 rpm in a 0.05% (w/v) solution of N-(5-Amino-1-carboxy-pentyl) iminodiacetic acid (AB-NTA) in 0.1 M HEPES buffer pH 8.0, then rinsed 3 times with milli-Q water. The nanoparticles were then incubated in blocking solution (1% (w/v) glycine pH 9.8) for 1 h at 37 °C and 180 rpm, and washed 3 times with milli-Q water, 3 times with 0.5 M $NiCl_2$, and 3 times with milli-Q water to produce Ni-NTA immobilized functionalized magnetic nanoparticles [10].

Transmission electron microscopy (TEM)

Transmission electron microscopy (TEM) studies were performed on a Jeol JEM-100 CXII electron microscope. Nanoparticle samples were applied to Carbon-coated Pioloform™ support films and dried at room temperature before analysis.

Scanning electron microscopy (SEM)

The characterization of the surface and shape characteristics of the magnetic nanoparticles were performed by scanning electron microscopy (SEM) using a JEOL model JSM 5200 instrument (JEOL, Akishima, Tokio, Japan), at 50×10^3 and 100×10^3 magnification. A 5 μL drop of the particle suspension at 0.5% (w/v) was applied to a glass microscope slide and dried at room temperature for 12 h before analysis.

Fourier Transform Infrared Spectroscopy (FTIR)

Fourier transform infrared (FTIR) spectroscopy was used to analyze the nanoparticles on a Perkin–Elmer Spectrum Two IR Spectrometer (Perkin–Elmer Inc., Waltham, MA, USA). The samples were mixed with KBr pellets and the FTIR spectra were recorded in the middle infrared (4400 cm^{-1} to 400 cm^{-1}).

Cloning of β-galactosidase and GFPuv

The gene encoding β-galactosidase from *Bacillus subtilis* (YesZ) was amplified by PCR from genomic *B. subtilis* DNA (ATCC® Number: 23857D-5) using the primers P-forward (TTA TAT CAT ATG AGA AAA CTG TAT CAT GGC GCT TGC) and P-reverse (TTA TAT CTC GAG GCT GTG ATT GTC AAA TTG AAT CAC ACG) containing NdeI and XhoI restriction sites (underlined), respectively. The PCR conditions were: 94 °C/2 min (1 cycle); 94 °C/1 min, 55°C/30 s, 72 °C/4 min (30 cycles); 72 °C/10 min (1 cycle), using Pfu polymerase. The resulting fragment was cloned into pJET (Thermo Fischer Scientific, Waltham-MA, USA), and subcloned into expression vector pET22b using standard molecular cloning protocols [15]. The resulting expression plasmid was designated as pET22b-YesZ, and encodes the recombinant β-galactosidase fused with a C-terminal 6 × Histidine tag. The correct construct was confirmed by DNA nucleotide sequence analysis. The cloning of the coding sequence of the Green Fluorescent Protein (GFPuv) in the pET28 expression vector carrying the coding sequence has been previously described [16].

Overexpression and purification of the β-galactosidase and GFPuv in E. coli

E. coli Rosetta (DE3) pLysS carrying the pET22b-YesZ plasmid was grown in HDM medium (tryptone 1.5 g, yeast extract 2.5 g, 100 mL H_2O, pH 7.5) containing 100 μg/mL ampicillin and 34 μg/mL chloramphenicol at 37 °C for 12 h. This culture (500 μL) was used to inoculate 50 mL of HDM medium containing 100 μg/mL ampicillin and 34 μg/mL chloramphenicol at 37 °C. The cells were grown at 37 °C to A_{600} of 0.6, when protein expression was induced by adding 0.15 mM isopropyl-D-thiogalactopyranoside. Expression was performed at 30 °C and 130 rpm for 4 h. Cells were harvested at 4 °C for 15 min at 6000 *x*, and subsequently resuspended in 10 mL of lysis buffer pH 8.0 (100 mM NaH_2PO_4, 500 mM NaCl, 20 mM imidazole, 1 mM PMSF, 1%, Triton X-100). The suspension was sonicated (*Q sonica*) for 6 min (10s on/10s off pulse, 50% amplitude) and cell debris was removed by centrifugation (7000 × *g*, 4 °C, 20 min) to obtain a cell lysate. The GFPuv expression was performed as previously described [16]. The β-galactosidase and GFPuv were purified from the cell lysate by IMAC using a Ni-Silica column (Promega, Fitchburg, WI, USA) pre-equilibrated with the buffer A (100 mM NaH_2PO_4, 500 mM NaCl and 20 mM imidazole, pH 8.0). The incubation time of the cell lysate with the Ni-Silica resin was 1 h. Elution was performed by increasing the imidazole concentration in buffer A initially to 40 mM and subsequently to 250 mM. Fractions eluted at the highest imidazole concentration were dialyzed against 80 mM Mcilvaine's buffer, pH 6.5.

The purification of the β-galactosidase using the synthetized Fe_3O_4