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Antibody affinity purification using metallic nickel particles☆

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ABSTRACT

Functionalized magnetic particles are emerging as a reliable and convenient technique in the purification of biomacromolecules (proteins and nucleic acids) and cell separation. In this study, we used novel solid nickel ferromagnetic particles coated with Protein A for the affinity purification of antibody. The study demonstrated that IgG can be purified from undiluted mouse serum in as few as 5 min using Protein A-coated nickel particles. Further, protein crosslinking was shown to stabilize the Protein A on the nickel particle surfaces to minimize Protein A leaching during the affinity purification and elution of IgG. The separation procedure is gentle, scalable, automatable, efficient and economical. By modifying the functional groups of amino acids in the protein coating, crosslinked nickel particles can be used not only for protein affinity purification but for other biological sample preparation and chromatographic applications as well. Methods proposed and tested in this study can be easily modified for small and medium scale antibody purification in lab and pre-clinical research.

1. Introduction

Antibody therapy has become the dominant therapeutic class of biotherapeutic molecules and is used to treat many life threatening diseases [1]. However, the production of therapeutic antibodies is quite costly both in terms of capital and variable costs. The downstream processing in antibody purification such as cell culture harvest, Protein A chromatography, and polishing chromatographic steps contributes significantly to the high cost of antibody therapy [2–4]. Among the technologies evaluated to tackle this problem, magnetic adsorbent-based bioseparation is showing promise but has not yet proven economically viable [5,6]. Magnetic-based cell and protein separation has been developed since 1970s [7,8] and is becoming the routine for biological research, immunoassays, and diagnostics [9–11]. Generally, superparamagnetic iron oxide (Fe_3O_4) is used to make magnetic absorbent particles through chemical reactions. These magnetic nanoparticles or microspheres are usually made by encapsulating nano to micro-meter magnetic particles (iron oxide superparamagnetic particles) inside polymers (polystyrene, methylmethacrylate, silica, etc.) followed by covalent modifications of ligands, such as Protein A and antibodies, on the polymer surface [12]. They can also be manufactured by polymer grafting and direct small molecule surface modifications. Protein A affinity purification of antibodies has been routinely practiced in antibody processing [13]. Using Protein A-coupled magnetic sorbents in antibody affinity purification may reduce operational time, and eliminate steps in protein/cell harvest [6]. Pilot research has shown the advantages of magnetic separation over conventional chromatography in preparative purification of antibodies [5,14]. However, the need of fast and strong magnetic response to the applied external magnetic field, the sophisticated manufacturing process, and the high manufacturing cost have limited the industrial application of this approach.

A novel type of magnetic particle, a ferromagnetic nickel particle, was used for the studies in this article. It has been shown that proteins are able to adhere directly to nickel particle surface and form relatively stable complexes without covalent binding of protein to nickel [15,16]. The mechanism by which these complexes form is not fully understood. Antibodies, polypeptide ligands, and enzymes can be coated onto nickel particle surface through simple mixture and incubation. Protein-coated nickel particles then can be used to pull-down antigens, antigen-presenting cells, antigen-labeled molecules, enzyme substrates, etc. In this study, we have shown that Protein A-coated nickel particles can efficiently and quickly purify IgG from mouse serum. This novel magnetic absorbent may be suitable for industry application due to the unique physical properties of nickel.

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These solid nickel particles are very dense (~9 g/cm^3) and strongly magnetic. The significantly higher density than other magnetic particles enhances mixing and thereby product capture in undiluted and viscous solutions. In addition, nickel particles also possess strong magnetic susceptibility. It allows fast and efficient response to an external magnetic field and shortens separation time. The solid core morphology of the particle benefits the rapid equilibrium of association and dissociation during solution incubation and protein elution.

Although coating nickel particles with proteins or peptides is a quick and efficient process, we needed to determine whether the complexes were sufficiently stable to withstand elution conditions to release IgG from the Protein A. Leaching of Protein A during elution of target proteins from nickel particles (using high or low pH buffer) would contaminate the isolated target proteins, and leaching during any of the process steps would slowly decrease the capacity of the particles over time and thereby their half-life [17]. We described a method of crosslinking the nickel-bound protein ligands to prevent leaching during elution of target proteins. Protein A was used as the ligand and bovine serum albumin (BSA) as a blocking protein in experiments described herein. The crosslinking effectively prevented nickel-bound BSA and Protein A leaching (as determined by silver stain) under conditions as harsh as incubation in pH 2.2 and pH 12.8 elution buffer. Moreover, crosslinked and nickel-bound Protein A was able to rapidly and efficiently isolate IgG from mouse serum.

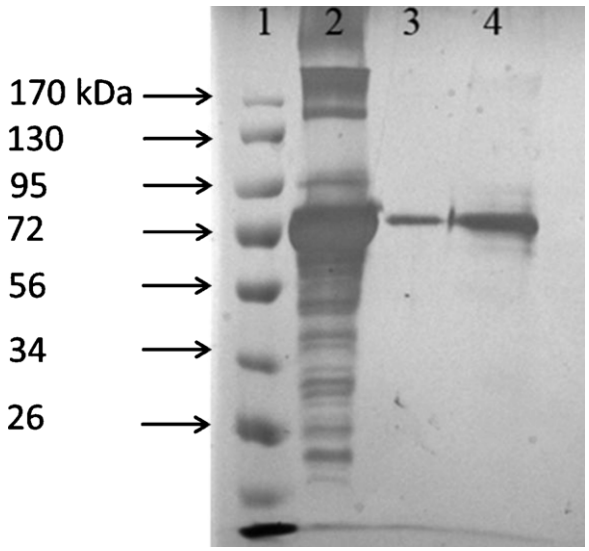

Fig. 1. Leaching of BSA from nickel particles in acid and alkaline elution. Leached BSA was visualized by SDS-PAGE and silver staining. Lane 1, protein marker; lane 2, BSA; lane 3, BSA eluted from 0.1 M citric acid (pH 2.2); lane 4, BSA eluted from 0.1 M triethanolamine (pH 12.8).

2. Materials and methods

2.1. Materials

Nickel particles (~3 μm) were provided by Russell Biotech Inc. (USA). BSA and glutaraldehyde were purchased from Sigma Aldrich (USA). Protein A was purchased from Biovision (USA). Purified mouse IgG and mouse serum were purchased from Jackson ImmunoResearch (USA). Bradford protein assay reagent was purchased from BioRad (USA). BCA protein assay kit was purchased from PIERCE (USA).

2.2. Preparation of protein-coated nickel particles

Nickel particles (1 g), bed volume between 0.4 and 0.5 ml, were washed 3 times for 5 min each with 3 ml PBS. One liter PBS (pH 7.4) contains 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4. The equilibrated particles were then incubated with 2 ml of 0.1% BSA or Protein A (1 mg/ml) at 4 °C overnight. For Protein A coating, the Protein A solution was removed after overnight incubation and followed by blocking with 2 ml 0.1% BSA for 2 h at room temperature. Protein-coated nickel particles were then washed 3 times for 5 min each with 3 ml PBS and stored at 4 °C until use.

2.3. Crosslinking of protein-coated nickel particles

After overnight incubation with BSA or Protein A, 1% glutaraldehyde was added to protein/nickel mixtures at a 1:60 molar ratios between proteins and glutaraldehyde. The mixtures were shaken at 250 RPM for 2 h at 37 °C. The reaction was terminated by adding 1/10 volume of 1 M Tris–HCl (pH 8.0), and the particles were washed 3 times with 3 ml PBS.

2.4. IgG binding and elution

Protein A-coated nickels particles (0.5 g) were incubated with 1 ml of 0.1% mouse IgG or 1 mg IgG/ml mouse serum for 5, 10, 20, 30, 40 and 50 min at room temperature. After incubation, the nickel particles were magnetically removed from the solution. Nickel particles were degaussed and washed 3 times for 5 min each with 1× PBS. Protein A-bound IgG was eluted by adding 500 μl acid buffer (100 mM citric acid, pH 2.2) or alkaline buffer (100 mM triethanolamine, pH 12.8) and rotated at 20 rpm for 5 min at room temperature. After elution, particles were magnetically removed from solution, and supernatants were neutralized by adding 75 μl of 1 M Tris–HCl (pH 8.0). Concentration of eluted IgG was obtained by Bradford and BCA methods. Proteins were visualized by 12.5% SDS-PAGE (BioRad Mini-PROTEAN® 3 Cell, 200 V, and 30–45 min) followed by silver or Coomassie Blue G-250 stain.

3. Results and discussion

3.1. Leaching of BSA adsorbed onto nickel particles in alkaline and acid buffer

Although the mechanism is unclear, proteins adhere to the nickel particles and form relatively stable complexes [15,16] without being covalently conjugated to the nickel surface. Protein-coated nickel particles can be used to pull-down and separate target molecules binding to the proteins attached to nickel. Since leaching of proteins from nickel would contaminate the target molecules and limit chromatographic utility, we tested the amount of protein released from the particles under different incubation conditions. BSA was used as a model protein to evaluate the leaching in alkaline and acid buffer (Fig. 1). BSA-bound nickel particles were incubated with acid buffer (lane 3, 100 mM citric acid, pH 2.2) and alkaline buffer (lane 4, 100 mM triethylamine, pH 12.8), respectively. Supernatants were collected, and eluted BSA was identified by SDS-PAGE and silver staining. BSA bound to the nickel particles was calculated by the difference of total amount of BSA in solution before and after incubating with nickel particles. BSA leached into elution buffers was measured by BCA assay. These measurements indicated that less than 2% of nickel-bound BSA eluted in acid buffer (eluted BSA/nickel-bound BSA X 100), and about 2–5% eluted in alkaline buffer.

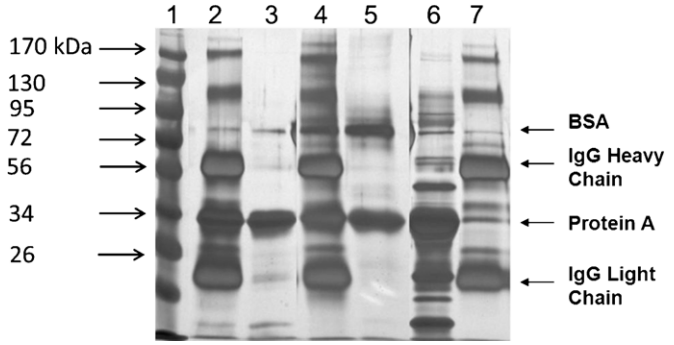

Fig. 2. Protein A-coated nickel particles are able to capture antibody. Leached Protein A and eluted antibody were visualized by SDS-PAGE followed by silver stain. Lane 1, protein marker; lane 2, Protein A and IgG eluted by citric acid; lane 3, Protein A only eluted by citric acid; lane 4, Protein A and IgG eluted by triethanolamine; lane 5, Protein A only eluted by triethanolamine; lane 6, Protein A as control; lane 7, IgG as control.

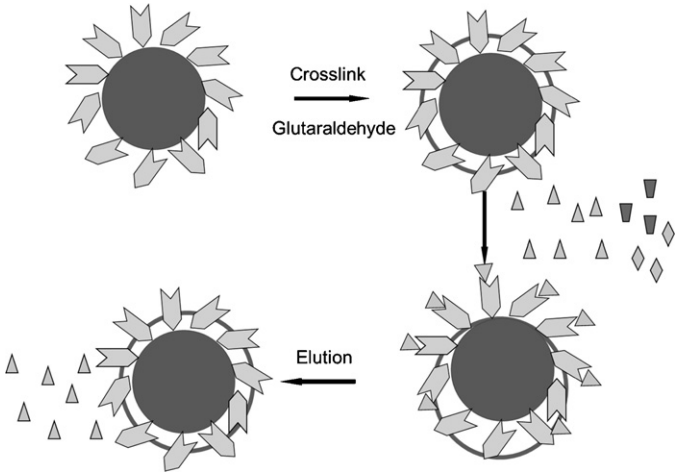

Fig. 3. Flowchart of affinity purification using crosslinked protein-coated Nickel particles.

3.2. Leaching of Protein A during antibody elution using Protein A-coated nickel particles

Protein A, like BSA, forms relatively stable and non-covalent complexes on the surface of nickel particles. Nickel-bound Protein A particles would be useful for antibody purification if leaching of Protein A was minimal. Protein A leaching was tested under acid and alkaline elution conditions. Protein A-coated nickel particles were incubated with 1 mg/ml of mouse IgG for 20 min. Nickel particles containing the Protein A-IgG complexes were magnetically removed from the solution. Data in Fig. 2 demonstrate that Protein A-bound nickel particles were able to capture purified IgG. However, during IgG elution (lanes 2 and 4) significant amounts of Protein A leached from the particles. Furthermore, Protein A eluted from the nickel surface more readily than BSA in both acid and alkaline conditions (lanes 3 and 5).

3.3. Crosslinking of nickel-bound proteins to prevent protein leaching during elution

Because both Protein A and BSA were found to leach from the nickel particle surface during elution of mouse IgG, a more stable complex of these proteins to the nickel surface was required. Since there is no obvious functional group for covalent binding on the nickel surface, it is not possible to covalently conjugate Protein A to nickel particles. We tested whether crosslinking nickel-bound proteins could stabilize protein binding and prevent or, at least, minimize leaching. The flowchart of this approach is illustrated in Fig. 3. If crosslinking nickel-bound proteins/polymers could be demonstrated to provide stable matrices for further chemical modification on chemical functional groups presented by proteins/polymers, it would dramatically expand the application of nickel particles in protein purification.

Glutaraldehyde was used to crosslink nickel-bound BSA and Protein A to the nickel particle surface (Fig. 4). Both BSA (lanes 2–7) and Protein A (lanes 9–14) were bound to nickel particles. Protein A particles were then blocked for 2 hr with BSA. The glutaraldehyde crosslinking was performed in the presence of 0.1% BSA (lanes 2 and 5) or Protein A (lanes 9 and 12) or in PBS alone (lane 3, 6, 10, and 13). The crosslinking in the presence of proteins in solution showed the least leaching in elution buffer (lanes 2, 5, 9, and 12). Alkaline elution (lanes 5, 6, 12 and 13) caused more leaching than acid elution (lanes 2, 3, 9 and 10). Non-crosslinked BSA (lanes 4 and 7) and Protein A (lanes 11 and 14) showed the most leaching in both acid and alkaline elution. As presented in previous sections, under the same conditions, leaching of BSA from the nickel particles was much less than that of Protein A.

3.4. Antibody purification using Protein A-coated and glutaraldehyde-crosslinked nickel particles

Although data in Fig. 2 demonstrate that Protein A-coated nickel particles can capture IgG in solution and data in Fig. 4 demonstrate that protein leaching from nickel particles can be dramatically reduced by crosslinking, it is also necessary to demonstrate that the crosslinking does not interfere with the antibody binding activity of the Protein A. Thus, Protein A-coated and glutaraldehyde-crosslinked nickel particles were tested for antibody capture (Fig. 5). The data show that glutaraldehyde-crosslinked Protein A still possessed mouse IgG binding activity (lanes 3 and 8). The crosslinked Protein A (lanes 3 and 8) showed almost undetectable leaching by silver stain during low and high pH buffer elution compared with non-crosslinked Protein A (lanes 2 and 7). The glutaraldehyde modification reduced the IgG binding capacity of Protein A by <10% (Table 1). The binding capacity might be opti-

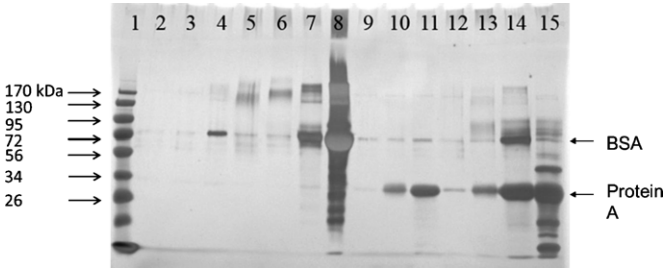

Fig. 4. Crosslinking of Nickel-bound BSA and Protein A can minimize protein leaching from particles during acid or alkaline elution. Lane 1, marker; lane 2, particles crosslinked in 0.1% BSA solution followed by 0.1 M citric acid elution; lane 3, BSA bound particles crosslinked in 1× PBS buffer followed by acid elution; lane 4, non-crosslinked BSA bound particles followed by incubation in acid elution buffer; lane 5, BSA bound particles crosslinked in 0.1% BSA solution followed by 0.1 M triethanolamine elution; lane 6, BSA bound particles crosslinked in 1× PBS buffer followed by alkaline elution; lane 7, non-crosslinked BSA bound particles incubated in alkaline elution buffer; lane 8, BSA as control; lanes 9–14 correspond to lanes 2–7 but with Protein A bound to the nickel particles, e.g. lane 9, crosslinked in the presence of Protein A followed by citric acid elution; lane 10, crosslinked in PBS followed by acid elution; lane 11, non-crosslinked followed by acid elution; lane 12, crosslinked in the presence of Protein A followed by triethanolamine elution; lane 13, crosslinked in PBS followed by alkaline elution; lane 14, non-crosslinked followed by alkaline elution; lane 15, Protein A as control.

Table 1
The capacity of BSA and Protein A binding to nickel particles and the IgG binding capacity to crosslinked and non-crosslinked Protein A.

Beads (3 μm) 1 g (~400 μl bed volume)	Protein binding	IgG binding to Protein A W/O crosslinking	IgG binding to Protein A with crosslinking
Protein A (μg)	384	612	570
BSA (μg)	700	N/A	N/A

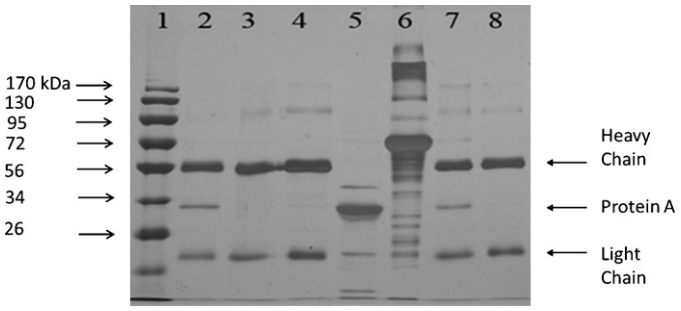

Fig. 5. Crosslinked Protein A bound to nickel particles can be used in IgG affinity purification with barely detectable Protein A leaching (SDS-PAGE and silver stain). Lane 1, protein marker; lane 2, non-crosslinked Protein A bound IgG and eluted by acid buffer; lane 3, crosslinked Protein A bound IgG eluted by acid buffer; lane 4, IgG control; lane 5, Protein A as control, lane 6, BSA as control; lane 7, non-crosslinked Protein A bound IgG eluted by alkaline buffer; lane 8, crosslinked Protein A bound IgG and eluted by alkaline buffer.

mized and improved by using nickel particles smaller than 3 μm to increase surface area and/or by other crosslinkers.

3.5. Antibody isolation from mouse serum

Protein A-coated nickel particles have very rapid mixing and capture kinetics due to the unique physical properties of nickel. Therefore, they can also be used to isolate antibody from undiluted mouse serum rapidly. Mouse serum was incubated with crosslinked Protein A-coated nickel particles for the times indicated in Fig. 6. In as few as 5 min (lane 8), the Protein A-bound nickel particles efficiently isolated antibodies from the serum. Very little additional antibody was captured by incubation times up to 50 min (lane 13). The purity and yield of antibody were not compromised because of the short incubation time. In addition, the magnetic removal of antibody-bound particles from the serum occurred in less than 1 min. This process is significantly more rapid than that of other Protein A-based modified chromatographic substrates, such as agarose beads. This increased rate of reaction may be due to the non-porous nickel surface, or it may be due to the rapid mixing of the dense particles in the viscous solution.

Excellent purity, simple procedures and high yield are attractive characteristics for scaling up antibody purification from crude serum or cell extracts using Protein A-coated nickel particles. In addition, short incubation times may significantly decrease contamination during antibody purification.

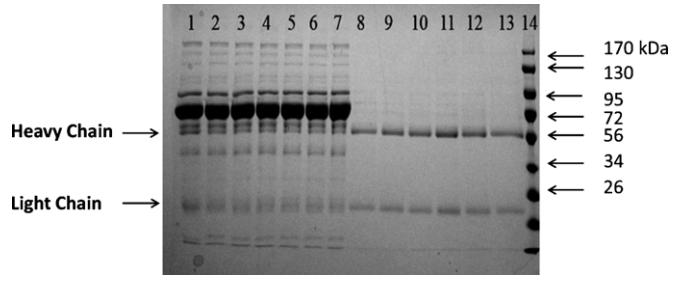

Fig. 6. Protein A-coated nickel particles can quickly isolate antibody from serum visualized by SDS-PAGE and silver stain. Lane 1, mouse serum; lanes 2–7, mouse serum left after 5, 10, 20, 30, 40 and 50 min of Protein A-coated nickel particles depletion; lanes 8–13, antibody isolated from mouse serum after 5, 10, 20, 30, 40 and 50 min incubation; lane 14, protein marker.

3.6. Discussion

Proteins adhere to nickel particles forming complexes that are sufficiently stable to provide a simple and efficient matrix for protein purification that takes advantage of the ferromagnetic properties of nickel particles. However, the chemical bonds between protein and nickel are not understood [15]. Antibody-coated nickel particles have been successfully used for cell capture. Antibody and ligand-coated nickel particles may also be useful in protein purification. Protein A affinity chromatography has been routinely applied in antibody purification [13]. The Protein A-coated nickel particles have been utilized in this study to explore the feasibility of applying protein-coated nickel particles in protein purification. Acid or alkaline conditions are often found in the process cycles of Protein A affinity media for either antibody elution or viral inactivation. Because proteins attach to nickel particles through non-covalent bonds, leaching of proteins from the nickel surface under either acid or alkaline conditions (Figs. 1 and 2) has limited the potential of applying nickel particles in protein purification and ligand detection. A simple approach (Fig. 3) has been proposed and confirmed in this study to crosslink and stabilize nickel-bound Protein A.

The results shown here demonstrate that crosslinking stabilizes Protein A and/or BSA bound to nickel particles (Fig. 4). This modification is simple and efficient and minimizes leaching of proteins from nickel surfaces in both low and high pH (pH 2.2 and 12.8) conditions. The glutaraldehyde-crosslinked Protein A nickel particles showed comparable IgG binding capacity with unmodified Protein A bound particles (Fig. 5). These particles efficiently isolated antibodies directly from mouse serum in as few as 5 min without compromising purity or yield (Fig. 6). These particles could also isolate antibodies directly from fetal bovine serum (data not shown). More important, Protein A-coated nickel particles are quite stable. These particles still possess robust antibody binding properties after storing in PBS at 4 °C for two years.

Protein A nickel particles are novel and possess several features that enhance the solid phase magnetic separation of proteins over other magnetic separation technologies available today. They are very dense (~9 g/cm^3) which allows them to move through and mix well in undiluted viscous solutions. They are highly magnetic and can be removed from solutions much faster than other types of magnetic beads. Also because of their density, they can simply settle out of solution. Most likely because of their efficient mixing properties, these particles exhibit rapid binding and capture kinetics. In addition, the irregular surfaces of the particles increase their surface area and likely their binding capacity as well. All of these properties make metallic nickel particles ideal for use in the magnetic purification of proteins. The coating of proteins onto nickel particles is fast and gentle and the crosslinking reaction is simple and efficient.

This study has demonstrated the feasibility of using Protein A-bound nickel particles in magnetic antibody affinity purification. The procedure to produce crosslinked Protein A-bound nickel particles is simple, fast, efficient and economical. In addition to its potential application in small to large scale antibody/antigen purification, this procedure can be easily modified for all

antibody-based affinity purification and analysis, such as c-myc tag, T7 tag, S-tag, and Flag-tag affinity purification, in routine biological and medical research. This crosslinking procedure can also be used to stabilize other proteins or nucleic acids on nickel surface for quick and efficient purification of cells, subcellular organelles, biotinylated proteins, DNA and RNA. Streptavidin-coated nickel particles plus biotynylated antibodies have been used in capturing cells directly from undiluted blood. It has also been tested that streptavidin-coated nickel particles can isolate biotinylated albumins and lysozymes (data not shown).

Antibodies are routinely used in biomedical research for affinity protein purification, diagnosis, drug targeting and delivery, and imaging analysis. In fact, hundreds of antibody drugs are under development for the treatment of various diseases. Molecular engineering approaches have dramatically improved the production of monoclonal antibodies in mammalian cell culture from low milligrams to grams per liter [2]. Protein-A-based affinity purification of antibody is a critical step in the production of antibody drugs and one of the most costly. Tremendous efforts have been made to match the needs for cost efficient production processes, especially on the affinity purification step using Protein A chromatography. Magnetic separation is a promising technique and has emerged as a standard method for biological research, immunoassays, and diagnostics [18]. Solid nickel-based protein affinity purification is different from currently available superparamagnetic particles due to its superior magnetic properties and relatively high density. Therefore, nickel particles can be directly added into cell culture or other raw materials (serum or tissue extract) to capture expressed antibodies or target proteins eliminating pre-cleaning steps such as centrifugation and filtration. This procedure also allows the passing of solid phase protein-coated particles through the solution rather than passing the solution through the porous packed chromatographic bed as in conventional chromatography. It saves buffer for column equilibration and wash, and avoids the dilution of proteins during elution.

4. Conclusions

In this study, we have developed a novel method to coat and crosslink Protein A on the surface of solid nickel ferromagnetic particles for the affinity purification of antibody. Our data have demonstrated that the binding between IgG antibody and nickel-bound Protein A is rapid and specific, and the leaching of crosslinked Proetin A during purification is mininal. Functionalized metallic nickel particle-based protein purification has the potential to accelerate the processing time, improve yield and purity, and minimize chances of contamination. This bioseparation approach combines the pre-cleaning step and the affinity capture into one single step, thereby reducing both the time and cost of production of proteins.

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