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A novel method for immobilization of proteins via entrapment of magnetic nanoparticles through epoxy cross-linking

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ABSTRACT

A method for immobilization of functional proteins by chemical cross-linking of the protein of interest and uncoated iron oxide nanoparticles in the presence of Epichlorohydrin is described. As a result of the cross-linking, the proteins form a matrix in which the particles get entrapped. The optimum concentration of Epichlorohydrin that facilitates immobilization of protein without affecting the functional properties of the protein was determined. This method was used to immobilize several functional proteins and the development and functional activity of Protein A-magnetic nanoparticles (MNPs) is described here in detail. The Protein A-MNPs possess high binding capacity due to the increased surface area of uncoated nanoparticles and robust magnetic separation due to the absence of polymeric coating materials. Protein A-MNPs were successfully used for purification of antibodies and also for immunoprecipitation. We also immobilized enzymes such as horse radish peroxidase and esterase and found that by providing the optimum incubation time, temperature and protein to nanoparticle ratio, we can retain the activity and improve the stability of the enzyme. This study is the first demonstration that Epichlorohydrin can be used to entrap nanoparticles in a cross-linked matrix of protein without impairing the activity of immobilized protein.

1. Introduction

Biomolecules immobilized on magnetic particles have several applications in biotechnology. Advanced methods for synthesis and characterization of magnetic materials especially nanoparticles, have aided in enhanced production of magnetic biomaterials. Magnetic particle based products are used in purification of biomolecules, cell separation, immunoassay, magnetic resonance imaging, drug delivery etc. [1]. Immobilized proteins are extensively used for preparation of affinity chromatography resins for antigen and antibody purification, isolation and expansion of cells using antibody-coated particles, production of immobilized enzymes for industrial applications etc. [2,3]. Though different types of magnetic materials based on metals such as Cu, Co, Fe and Ni with diverse physical and biochemical properties are available, iron oxide (Fe_3O_4) nanoparticles have demonstrated greater potential for research applications [4–6]. Due to properties such as superparamagnetism, low toxicity, high surface area, biocompatibility and easy separation under external magnetic field, iron oxide nanoparticles have attracted much attention in the past decade [2].

Common methods of immobilization favor pre-coating of magnetic particles with a polymer followed by cross-linking of the protein to the coating material. The process involves either incorporation of magnetic particles during synthesis of the supporting polymer or direct coating of the particles with support materials

Abbreviations: BSA, Bovine Serum Albumin; ECH, Epichlorohydrin; Fab, Fragment-antigen binding; HRP, Horse Radish Peroxidase; IgG, Immunoglobulin; MNP, Magnetic Nanoparticle; SDS-PAGE, Sodium dodecyl sulphate-poly acrylamide gel electrophoresis.

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such as dextran or agarose [7,8]. Earlier reports have documented the use of proteins immobilized on coated magnetic beads like the heparin-stabilized colloidal magnetite used by Lauva et al. for binding of cells from whole blood [9] and dextran-coated magnetite, used as a drug carrier by Rusetski and Ruuge [10]. In the present study we developed a novel method to immobilize proteins on uncoated magnetic nanoparticles to take advantage of the many desirable attributes of uncoated particles. In the absence of polymeric coating materials the size of the nanoparticles does not increase and hence the high binding capacity and magnetic properties will remain unaffected. It has been reported that in larger coated magnetic beads or aggregates non-specific entrapment of contaminants occur; but in smaller uncoated particles non-specific binding is quite low [11]. Thus uncoated magnetic nanoparticles provide a more potent solid support system to immobilize proteins while ensuring a simple and inexpensive method of preparation. Previously Koneracka et al. and Mehta et al. had demonstrated the immobilization of proteins on uncoated magnetic particles in the presence of Carbodiimide [11,12].

The objective of the present study was to develop a robust method for immobilization of proteins which takes advantage of the properties of uncoated nanoparticles. The authors designed a novel strategy wherein the magnetic nanoparticles (MNPs) get entrapped in a cross-linked matrix of the protein of interest using an epoxide Epichlorohydrin (ECH) as the cross-linking agent. In our method, proteins and iron oxide (Fe_3O_4) nanoparticles were incubated with ECH at specific molar ratios in the pH range of 6–9. This results in the formation of a cross-linked matrix of protein and nanoparticles since the −NH2, -SH and −OH groups present in the protein and −OH groups present on the surface of the nanoparticles bind to the epoxy group [13]. The extent of cross-linking was regulated by adjusting the concentration of ECH so that extensive cross-linking does not inhibit the activity of proteins. This type of cross-linking facilitates the entrapment of nanoparticles in the cross-linked protein matrix and in turn accomplishes the immobilization of the protein. The immobilized protein and nanoparticles remain in direct association due to lack of polymeric coating. We have immobilized several functional proteins including Protein A, enzymes and antibodies and have found that these proteins maintained their functional activity after immobilization and exhibited more stability during long term storage.

2. Materials and methods

2.1. Synthesis and characterization of MNPs

The method to synthesize magnetic nanoparticles involves co-precipitation of Fe^{+2} and Fe^{+3} ions in the molar ratio of 1:2 which precipitate in alkaline conditions to form Fe_3O_4. The nanoparticles were synthesized using 1 M $FeCl_2 \cdot 4H_2O$ and 2 M $FeCl_3 \cdot 6H_2O$ according to a previously published protocol with modifications [12]. Alkaline condition was maintained using 8 M NaOH and was added drop wise with constant stirring at 80 °C for maturation. To remove impurities, the nanoparticles were washed several times with hot distilled water and the suspension was dried overnight at 60 °C. Finally the nanoparticles were dispersed in 5 mM sodium phosphate buffer, pH 8. For Transmission Electron Microscopy (TEM) analysis, a drop of the samples, both free and protein bound MNPs were spotted on the grid and allowed to dry in air. The TEM analysis was performed at the Sophisticated Test and Instrumentation Center (STIC), Cochin University of Science and Technology, Kochi, Kerala, India. The size of the nanoparticles prepared by this method was in the range of 10–20 nm.

2.2. Immobilization of proteins and enzymes on MNPs

Protein A-MNPs were prepared by cross-linking 10 mg of MNPs in 1 ml sodium phosphate buffer (5 mM, pH 8) with 4 mg of Protein A (MicroProtein Technologies, KS, USA) in the presence of 0.6 M Epichlorohydrin (Sigma Aldrich, St. Louise, MO, USA). The cross-linking was performed at 4 °C for 24 h with rotation on a Roto-spin tube rotator (Tarsons, Kolkata, India) at 20 rpm. Non-specific binding to the MNPs was reduced by blocking in 1 M Tris-HCl, pH 9.5 by mixing on a tube rotator for 24 h at 4 °C. Horseradish peroxidase (HRP) and bovine serum albumin (BSA) (both from Sigma Aldrich) were also immobilized. To immobilize BSA, 10 mg/ml of BSA was mixed with an equal amount of MNPs along with 0.6 M ECH with continuous rotation for 24 h at room temperature. The HRP enzyme was immobilized by incubating 1 mg of MNP with 0.025–0.5 mg of HRP and 0.6 M ECH at 4 °C for 24 h with continuous rotation. After immobilization of any protein or enzyme, the particles were washed at least three times on a magnetic stand and finally stored in 1 ml of sodium phosphate buffer (5 mM, pH8). The binding efficiency was found to be 85–90% in all cases.

2.3. Purification of antibodies using protein A-MNPs

Purification of IgG from blood plasma was accomplished using a modified protocol [14]. Human blood was collected from healthy adult volunteers following the institutional review committee guidelines. Briefly, 50 μl of plasma was diluted with 850 μl of phosphate buffer and mixed with 100 μl of Protein A-MNPs (0.4 mg Protein A immobilized per mg MNP) followed by gentle rotation for 2 h at room temperature. The supernatant was discarded and the pellet was washed thrice with phosphate buffer. The bound antibody was eluted with 0.2 M L-Arginine (pH 3) and neutralized using 1 M Tris buffer, pH 9. The presence of IgG was visualized by sodium dodecyl sulphate-poly acrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions. The protocol for purification of IgG from cell culture media was modified from a published protocol [15]. 1 ml of Protein A-MNPs (4 mg Protein A per 10 mg MNP) was mixed overnight with 125 ml of cell culture medium (Expi293 expression medium, Invitrogen) which contains IgGs secreted by mammalian cells (Expi293F™ cells, Invitrogen) transiently co-transfected with modified pSC-derived proprietary expression vectors carrying sequences which encode the light and heavy chains of IgG. After incubation, the spent medium was removed and the MNPs were washed with 1X PBS three times and finally eluted the IgGs with 0.2 M L-Arginine, pH 3.0. After neutralization, IgGs were analyzed by SDS-PAGE under non-reducing conditions. Similarly, a recombinant Fab (antigen binding fragment of IgG) was also purified from BL-21 bacterial cells which were transfected with a proprietary expression vector which carries a pTac promoter. Briefly 1 ml of Protein A-MNPs (4 mg Protein A per 10 mg MNP) was mixed overnight with 20 ml of bacterial cell lysate which contains the Fab. After incubation, the spent lysate was removed and the MNPs were washed with 1X PBS three times and finally eluted the Fab with phosphate buffer, pH 2.8. The eluted Fab was immediately neutralized with 1 M Tris pH 10. The presence of Fab was visualized by performing SDS-PAGE under non-reducing conditions.

2.4. Immunoprecipitation using protein A-MNPs

The antibody binding experiment was carried out using 25 μl of Protein A-MNPs and 25 μl of Protein A-Sepharose (GE Healthcare Life Sciences) equilibrated in RIPA buffer. The Balb/c mice used in the experiment were bred and reared in the animal house of CCMB, Hyderabad in accordance with the guidelines from Indian Science