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Immobilization of Proteins on Magnetic Nanoparticles

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Abstract Magnetic nanoparticles prepared from an alkaline solution of divalent and trivalent iron ions could covalently bind protein via the activation of *N*-ethyl-*N'*-(3-dimethylaminopropyl) carbodiimide (EDC). Trypsin and avidin were taken as the model proteins for the formation of protein-nanoparticle conjugates. The immobilized yield of protein increased with molar ratio of EDC/nanoparticle. Higher concentrations of added protein could yield higher immobilized protein densities on the particles. In contrast to EDC, the yields of protein immobilization via the activation of cyanamide were relatively lower. Nanoparticles bound with avidin could attach a single-stranded DNA through the avidin-biotin interaction and hybridize with a DNA probe. The DNA hybridization was confirmed by fluorescence microscopy observations. Immobilized DNA on nanoparticles by this technique may have widespread applicability to the detection of specific nucleic acid sequence and targeting of DNA to particular cells.

Keywords: magnetic nanoparticle, protein immobilization, covalent binding, DNA hybridization

INTRODUCTION

Nanomaterials have recently sparked widespread interest in their use in many biotechnology areas. Some important biomedical and biochemical applications based on complexes and conjugates of nanoparticle and biomolecule have been demonstrated. For example, complexes of DNA and nanoparticle provide carriers for gene transfer and drug delivery. Nanoparticles are capable of translocating drugs across the cell membrane and barriers like epithelial and blood-brain barriers. The conjugates of protein with nanoparticle can be applied for drug delivery, screening of combinatorial library, studying of protein-protein interaction, enzyme immobilization for hydrolysis of polysaccharides, and so on. Magnetic nanoparticles are particularly useful due to the availability of magnetic targeting, *i.e.*, the delivery of drugs can be directed to targeted cells by employing a magnetic field. The magnetic nanoparticles combined with biomolecules are easily controlled and recovered for reuse by the application of an external magnetic field.

Iron oxide nanoparticles have been found to be very potential for applications in the biological and biomedical aspects, due to their strong magnetic property and low toxicity [1,2]. Magnetite (Fe_3O_4) nanoparticles have been coated with poly(glutamic acid, lysine, tyrosine) (PEKY) and then covalently attached a monoclonal antibody. The resultant antibody nanoparticles were characterized as a potential tumor-specific contrast agent for magnetic resonance imaging [3]. Superparamagnetic iron oxide nanoparticles that were coated with dextran and conjugated with a peptide sequence from the HIV-tat protein were internalized into lymphocytes for intracellular magnetic labeling [4]. In addition to the surface coating method, superparamagnetic nanoparticles (γFe_2O_3) can be modified on the surface by using thiol chemistry and then covalently coupled with lectins, enzymes or antibodies for application to the specific targeting of cells [5]. On the other hand, Mehta *et al.* [6] proposed a simple method for the direct binding of proteins on magnetic nanoparticles via carbodiimide activation. The binding of bovine serum albumin (BSA) was confirmed by electron micrograph studies, magnetic measurements and FT-IR spectroscopy. In that study they found that bovine serum albumin could be covalently bound to magnetic particles without loosing its biological properties. Immobilization of other enzymes such as streptokinase was also confirmed by FT-IR spectra [7]. This method was also employed for the immobilization of yeast alcohol dehydrogenase [8,9].

In the aqueous solution, nanoparticles are so small in size that biomolecules attached on particle surface can behavior freely as in the soluble state. Immobilized enzymes on these fine particles are advantageous of diffusing through high-molecular-mass polymeric substrates like polysaccharides, proteins and nucleic acids. Immobilized proteins on nanoparticles have widely applications in drug targeting and highly sensitive immunoassay. Trypsin and avidin are thus taken as model proteins for immobilization study in the present paper.

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MATERIALS AND METHODS

Materials

Cyanamide and trypsin were obtained from Sigma. Avidin and *N*-ethyl-*N*'-(3-dimethylaminopropyl) carbodiimide (EDC) was purchased from Fluka. All other chemicals were of GR or a higher grade and directly used without further purification.

Preparation of Nanoparticles

Magnetic nanoparticles (Fe_3O_4) were synthesized by co-precipitation from an alkaline solution of di and trivalent Fe ions (in the molar ratio of 1:2) followed by a treatment under hydrothermal conditions. A 50 mL 0.3 M $FeCl_2 \cdot 4H_2O$ and a 50 mL 0.6 M $FeCl_3 \cdot 6H_2O$ were thoroughly mixed and then heated to 60°C. A solution of 5 N NaOH was added dropwise to the mixture under continuously stirring at 500 rpm. The addition of NaOH solution was stopped as soon as pH 12 was reached. The pH value was maintained at constant during the reaction process, described by the following reaction scheme:

$$Fe^{2+} + 2Fe^{3+} + 8OH^- \rightleftharpoons Fe_3O_4 + 4H_2O$$

The precipitate (Fe_3O_4) was heated at 60°C for 1 h. After centrifugation and removal of the supernatant, the precipitate was incubated with 100 mL 1 N HCl for 1~2 h. After centrifugation at 4°C the precipitate was suspended in D.I. water. The precipitate was then washed with D.I. water for three times, and finally suspended in D.I. water and stored for more than 3 days. Only the particles that could be well suspended in the D.I. water and pass the 0.22 μm filter were taken for use.

Immobilization of Proteins

Proteins were bound to nanoparticles in the presence of carbodiimide. Two kinds of carbodiimide were employed: cyanamide and *N*-ethyl-*N*'-(3-dimethylaminopropyl) carbodiimide (EDC). The latter is also known as 1-ethyl-3(3-dimethylaminopropyl) carbodiimide. For the immobilization of proteins via the activation of EDC, 1 mL nanoparticle solution (8.9 g/L, *i.e.*, 38.4 mM) was mixed with 150 μL protein solution (1 g/L avidin or trypsin) and a small aliquot of EDC solution (57 mM). The volume of EDC solution was such controlled that the molar ratio of EDC to nanoparticle was at a specified value. This EDC/nanoparticle ratio changed from 1:2 to 1:8 for different runs. The total volume of the mixture was 1.3 mL and protein concentration was 117 mg/L in the average. The mixture was shaken at 4°C for 24 h. Magnetic nanoparticles were then collected by using an NdFeB sintered magnet (Hing Mag Technology Co., Taiwan), washed with D.I. water, and then resuspended in D.I. water. The amount of protein that bound on the nanoparticles was determined by measuring the loss of protein in the solution. Protein concentration was determined by the colorimetric method at 595 nm using the Bio-Rad protein assay kit (Bio-Rad) with bovine serum albumin as the standard.

The procedure for protein immobilization on magnetic nanoparticles via the activation of cyanamide was similar to the above-mentioned method via EDC. The concentration of cyanamide solution added to the mixture was 100 mM. For different runs, the molar ratio of cyanamide to nanoparticle varied from 1:1 to 1:6. The average concentrations of avidin and trypsin in the mixture were 115 and 116 mg/L, respectively.

Binding of Biotinylated DNA Fragment on Avidin-immobilized Nanoparticles and Its Application for DNA Hybridization

A 912 bp-long double-stranded DNA fragment that was amplified from the gene of *N*-acetyl-D-neuraminic acid aldolase in *E. coli* K12 had a biotinylated 5' end in one strand. A 20 μL of this DNA solution (37.1 mg/L in 0.15 mM Tris-HCL, pH 8) was incubated with the avidin-immobilized nanoparticles solution (100 μL) at the room temperature for 8 h for coupling. After an extensive wash, the DNA immobilized nanoparticles were incubated with 0.4 N NaOH for 3 h to denature the bound DNA. The non-biotinlyated strand was removal from the particle while the biotinylated strand was still bound on the particles. A fluorescently labeled oligonucleotide (probe) having a sequence of FAM-TTGAT GCAAGAGCGCGGGTGAGAA was then incubated with the single-stranded DNA (ssDNA) bound on the magnetic particles at the dark conditions for 3 h for the hybridization. The DNA hybridized nanoparticles were observed under a fluorescence microscopy (Zeiss Axioskop 2). The fluorescent group FAM has an excited wavelength of 494 nm and radiated wavelength of 522 nm.

RESULTS AND DISCUSSION

The iron oxide particles obtained is magnetite (Fe_3O_4). The average particle diameter was about 10 nm, based on the observation by TEM. After acidification the particles were well dispersed into water. Therefore, the immobilization of protein onto the nanoparticles could be carried out in an aqueous solution. The binding of protein on nanoparticles was confirmed by IR spectra. Fig. 1 shows the FT-IR spectral characteristics of protein (avidin) bound to magnetic particles. It is evident that two characteristic bands of avidin in the interval between 1600 and 1650 cm^{-1} are present in pure avidin and in avidin bound to magnetic particles. The agreement of characteristic bands between the spectrum for avidin and bound avidin via the activation of EDC is much more conclusive.

Cyanamide (CH_2N_2) is also called carbodiimide. When it was used to activate the particle surface for protein immobilization. Results as shown in Fig. 2 indicates that the yield of protein immobilization on nanoparticles was lower for either avidin or trypsin. With the concentration of avidin kept at the averaging values of 115 mg/L and that of nanoparticles at 6.8 g/L, the cyanamide to

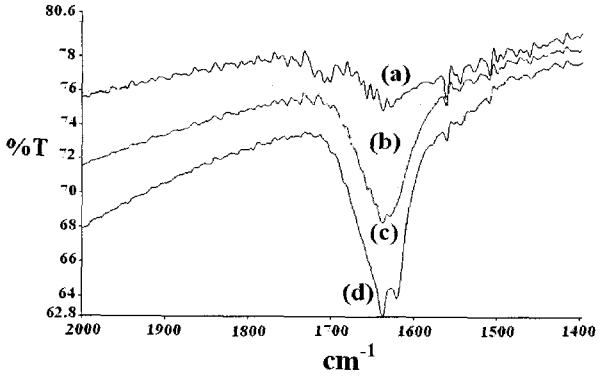

Fig. 1. FT-IR spectra of avidin bound to magnetic nanoparticles via the activation of cyanamide (c) and EDC (d). Spectra of nanoparticles (a) and avidin alone (b) are also shown.

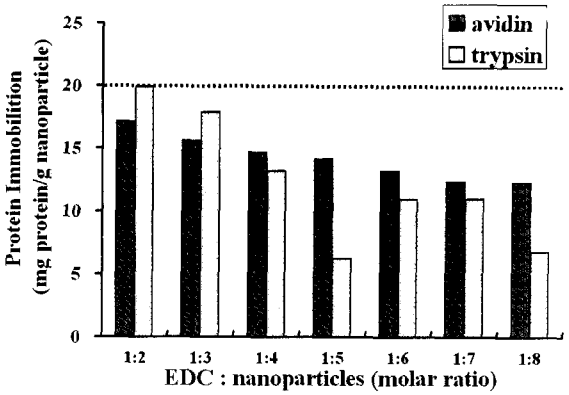

Fig. 3. Protein immobilization on magnetic nanoparticles via the activation of EDC. The dashed line indicates a 100 % protein immobilization.

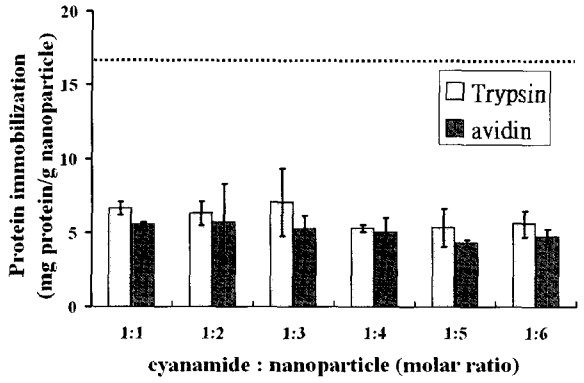

Fig. 2. Protein immobilization on magnetic nanoparticles via the activation of cyanamide. The dashed line indicates a 100 % protein immobilization.

nanoparticle molar ratio present in the reaction mixture varying from 1:1 to 1:6 led to a density of protein immobilization scattering from 4.3 to 5.7 mg protein/g nanoparticle, with an average value of 5.1 mg protein/g nanoparticle that corresponds to 30% of protein immobilization yield. For the immobilization of trypsin, the immobilized protein density scattered from 5.3 to 7.1 mg protein/g nanoparticle with an average value of 6.1 mg protein/g nanoparticle (corresponding to 36% of protein immobilization) as the molar ratio of cyanamide to nanoparticle varied from 1:1 to 1:6. Although increasing the amount of cyanamide in the reaction mixture (increasing the cyanamide/nanoparticle ratio) was used intentionally to increase the coupling yield of protein, the trend of yield increasing with cyanamide amount was not evident from the results as shown in Fig. 2. The coupling yield was such low that the use of cyanamide for the activation of nanoparticles was found to be impractical.

In contrast to cyanamide, EDC was found to well activate the nanoparticle surface for protein immobilization. The data shown in Fig. 3 were resulted from the change of EDC/nanoparticle molar ratio from 1:2 to 1:8, using constant amounts of nanoparticle and protein in the coupling reaction mixture. It is clear that the yield of protein immobilization could be increased by raising the EDC/nanoparticle molar ratio, *i.e.*, increasing the relative concentration of EDC. When the EDC/nanoparticle ratio was at 1:2, the immobilized yields were highest for both avidin and trypsin. In these conditions, 86% of avidin and 100% of trypsin were immobilized on the nanoparticles via the activation of EDC. The amounts of avidin and trypsin on the particles were thus calculated to be 17.1 and 19.9 mg/g, respectively. The protein binding yields are close to that reported for the immobilization of bovine serum albumin by Mehta *et al.* [6]. They showed that 86% of added protein was bound to the particle surface under the optimum concentrations of EDC, nanoparticle and protein. The present study suggests that raising the concentration of protein for immobilization could increase the immobilized protein density. In other runs of avidin immobilization with a fixed molar ratio of EDC/nanoparticle at 1:3, the immobilized protein density increased linearly with the incubated protein concentration. As the protein concentration increased from 0.038 to 0.23 g/L, a protein density up to 34.5 mg protein/g nanoparticle could be achieved.

For the coupling of protein to nanoparticles, water-soluble EDC was used to activate the electrophilic groups, probable surface hydroxyls due to adsorption of water or surface state, on the Fe_3O_4 surface and then allow them to interact with amino groups on protein. The mechanism may be similar to the use of EDC for activating the carboxyl group of ligand or phosphate group of DNA in order to form a covalent linkage with amino group on the solid support. In the case of DNA immobilization, the electrophilic phosphate group of DNA on its terminus is activated by EDC to become phosphorimidazolide derivatives in imidazole buffer and then react with primary [10] or secondary amine [11].

Although the yield of protein coupling could be increased by raising the EDC/nanoparticle ratio (Fig. 3), EDC/nanoparticle ratios higher than 1:2 were impractical. If the molar ratio were increased to 1:1, for example,

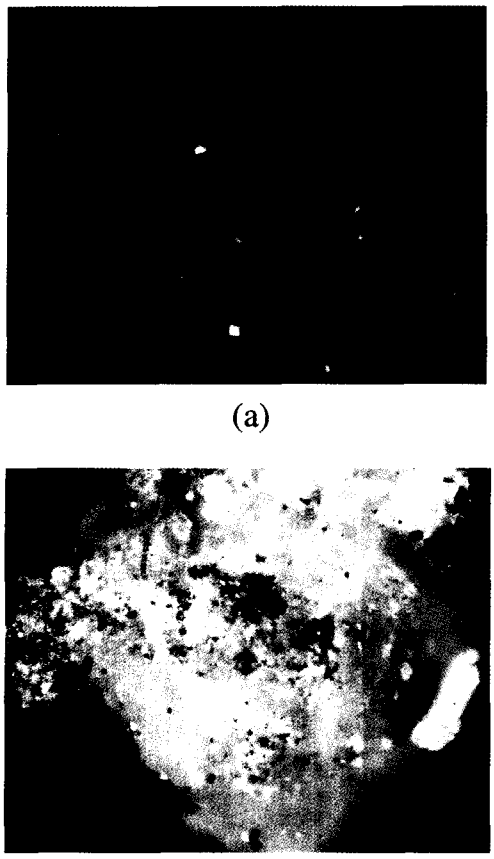

Fig. 4. Fluorescence microscopy of fluorescence-labeled oligonucleotide hybridized to the single-stranded DNA that has been bound on magnetic nanoparticles. DNA-hybridized nanoparticles were suspended in an aqueous solution (a) and collected by a magnet (b).

the nanoparticles would coagulate and then precipitate. Re-suspension of this protein-nanoparticle conjugate was impossible, so that its application will be limited. This coagulation behavior occurred even in the absence of protein, suggesting that the higher concentration of EDC in the aqueous solution might lead to a crosslinking between nanoparticles. When the EDC/nanoparticle ratio was 0.5 (*i.e.*, 1:2) or lower, the resultant suspension of protein-immobilized particles was clear and translucent with a color of dark-brown. The protein-immobilized nanoparticles resulted from the recipe using an EDC/nanoparticle ratio of 0.5 could be stable at 4°C for about one week, before starting to precipitate. The precipitate could easily to resuspended in the water after shaking for about 10 min. However, EDC/nanoparticle ratios lower than 0.5 could lead to a long-term stable suspension of protein-immobilized particles.

The avidin-immobilized magnetic nanoparticles is potential for biomedical and biochemical applications. As a demonstration, the avidin-bound particles (resulted form an EDC/nanoparticle molar ratio of 1:3) were first coupled with single-stranded biotinylated DNA via the strong affinity between avidin and biotin and then hybridized with a fluorescently labeled oligonucleotide. The sequence of this fluorescent oligonucleotide (24-mer) was complementary to that of immobilized ssDNA at its 3'end such that the hybridization could occur. No significant fluorescence was observed in the solution after hybridization, implying that almost of the fluorescently labeled oligonucleotides were hybridized on the nanoparticles. As shown in Fig. 4, fluorescence was observed on the DNA hybridized nanoparticles, either being suspended in the aqueous solution or precipitated due to the collection of a magnet.

The single-stranded DNA target on the particle was obtained by NaOH denaturation of immobilized double-stranded DNA. Fluorescently labeled oligonucleotide was hybridized to the target on the nanoparticle and then detected. Results indicated that the conjugation between biotinylated ssDNA target and avidin-bound nanoparticles was achieved and stable over the hybridization process. Polynucleotides immobilized on nanoparticles by the present technique are ideally suit to the specific detection of nucleic acid sequence and cellular targeting of DNA

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