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Preparation and characterization of Fe_3O_4-NH_2@4-arm-PEG-NH_2, a novel magnetic four-arm polymer-nanoparticle composite for cellulase immobilization

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ABSTRACT

A geometrically symmetric four – arm dendritic polymer 4-arm-PEG-NH_2 was employed to modify on the surface of Fe_3O_4 as support for cellulase immobilization. Glutaraldehyde was used as a coupling agent of this magnetic support to covalently immobilize cellulase. The morphology and properties of the synthesized magnetic material were characterized by Fourier transform infrared (FT-IR) spectroscopy, vibrating-sample magnetometer (VSM), thermogravimetric analysis (TGA), transmission electron microscopy (TEM) and X-ray diffraction (XRD). The properties of immobilized enzyme such as the optimum temperature and pH, Michaelis constant, thermal stability, reusability and storage stability were investigated. The loading capacity of cellulase was 132 mg/g. Compared with free cellulase, immobilized cellulase showed wider pH and temperature ranges, higher operational stability and good storage stability. Besides, the results of hydrolysis filter paper and microcrystalline cellulose showed that the catalytic effect of immobilized cellulase on the free cellulase was increased by about 76%.

1. Introduction

Fiber raw materials, as the most abundant renewable resources in nature, have not been fully exploited but are discarded or burned at will, which not only cause a huge waste of resources, but also pollute the environment. The cellulose was degraded into oligosaccharides such as glucose and cellobiose by cellulase, which is a strategy that is considered to be of great potential for efficient use of cellulose [1–3]. However, at present, there are still some technical obstacles such as low cellulase activity, sensibility to inactivation as well as low enzymolysis efficiency [4–6]. It can be said that the low efficiency of cellulase has led to the waste of cellulosic resources [7]. The most effective way to solve these problems is to use an immobilized form of cellulase.

Although the use of magnetic nanoparticles as an immobilized enzyme carrier has a large number of advantages such as, it can realize magnetic separation and recycling [8–13], the magnetic nanoparticles having a single modification in the application of immobilized enzyme will appear some striking problems such as low load capacity, poor recycling effect and poor stability, making it in the practical application of a considerable degree of restrictions. At present, it is an important means to address these problems by preparing of functional composite magnetic nanoparticles to enhance its activity and stability [14,15].

Dendrimers with a large number of amino, carboxyl, hydroxyl and other functional groups show high solubility and low viscosity and can be adjusted in the nano-scale, which have been successfully applied in biology, medicine and catalysis and other related fields [16–19]. Compared to conventional linear macromolecular polymers, dendrimers also have a high degree of geometric symmetry, in the molecular center and molecular ends can be imported a large number of reactive and functional groups [20–24]. Based on this, the modification of polyethylene glycol dendrimers on magnetic nanomaterials can effectively increase the loading enzyme capacity and enhance the water solubility of the carrier, so as to further enhance the catalytic effect and stability of immobilized enzyme. Among that, polyethylene glycol dendrimer is a kind of polymer with good water solubility, innocuity and biocompatibility. It is modified on the surface of magnetic nanomaterials to further enhance the biocompatibility of the carrier and to protect the carrier structure. Therefore, polyethylene glycol is often used for modification of materials [25–28].

In this work, a four-arm polyethylene glycol dendrimer (4-arm-PEG-NH_2) was modified on the surface of Fe_3O_4 nanoparticles

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to construct a novel polymer composite magnetic nanomaterial (Fe_3O_4-NH_2@4-arm-PEG-NH_2) and applied to immobilization of cellulase. The morphology and properties of the synthesized magnetic materials were characterized by TEM, VSM, XRD, FT-IR and TGA. Subsequently, the factors affecting immobilization were investigated and the optimum conditions of immobilized cellulase were obtained. The enzymatic properties of thermal stability, operational stability and reusability of free and immobilized cellulase were also explored. Finally, Filter paper and microcrystalline cellulose were used as substrate to investigate the catalytic effect of immobilized cellulase.

2. Material and methods

2.1. Materials

Cellulase from *Trichoderma viride* (BR, +4 °C), anhydrous sodium acetate (CH_3COONa), 1, 6-Diaminohexane ($C_6H_{16}N_2$), anhydrous methanol (CH_3OH), anhydrous ethanol (C_2H_5OH), ethylene glycol (EG), 3,5-Dinitrosalicylic acid (DNS) and Glutaraldehyde (GDA) aqueous solution of 25% (BR) were purchased from Sinopharm Chemical Reagent Co., Ltd. Sodium cyanoborohydride ($NaBH_3CN$) was obtained from Sigma. Iron (III) chloride hexahydrate ($FeCl_3{\cdot}6H_2O$) was purchased from Aladdin. 4-arm-PEG-NH_2 (10 K) was procured from Shanghai Jinpan Biotech Co., Ltd. All other chemicals and solvents used were analytical reagent grade and deionized water was used go through the whole experiments.

2.2. Preparation of Fe_3O_4-NH_2 nanoparticle

The Fe_3O_4-NH_2 nanoparticle was prepared via a coprecipitating method with some modifications [29]. Briefly, $FeCl_3{\cdot}6H_2O$ (2.0 g, 0.007 mol) was dissolved in 60 mL of ethylene glycol under ultrasonic dispersion to obtain a clear and homogeneous orange-yellow solution. Next, anhydrous sodium acetate (4.0 g, 0.049 mol) was added into the above solution slowly under magnetic stirring for 30 min. The resulting uniform yellow suspension was transferred to a 100 mL beaker. Then, 1, 6-Diaminohexane (13.0 g, 0.1 mol) was added to the above 100 mL beaker and stirred continuously with a glass rod for 5 min to get a clear red solution. Then, the resulting solution was transferred into a 100 mL Teflon-lined stainless-steel autoclave. The autoclave was heated to 200 °C and maintained for 6 h. After cooling to room temperature, the resulting black product was washed with anhydrous ethanol and deionized water in sequence by the aid of a magnet, and then dried in a vacuum oven at 45 °C for 3 h (yield, 89%).

2.3. Preparation of Fe_3O_4-NH_2@4-arm-PEG-NH_2 nanoparticle

The Fe_3O_4-NH_2@4-arm-PEG-NH_2 nanoparticle was prepared according to the method of Tingting Xia, et al. with some modifications [30]. Specifically, 0.2 mg of Fe_3O_4-NH_2 was dispersed in a mixed solution of 2 mL (0.008 mol) of glutaraldehyde and 38 mL (0.65 mol) of anhydrous methanol and kept in a 25 °C water bath under mechanical agitation for 12 h. The obtained product was washed with anhydrous methanol three times and was redispersed into 30 mL of anhydrous methanol for further use (yield, 86.5%).

The resulting black suspension as described above was removed into a three-necked flask containing 0.5 g (0.05 mmol) of 4-arm-PEG-NH_2 and was stirred mechanically at room temperature for 12 h. In the meantime, 100 mg of 1% (w/w) (0.016 mmol) of $NaBH_3CN$ was added to the three-necked flask every four hours. After that, the obtained product was washed with anhydrous ethanol and deionized water several times and dried in vacuum oven at 50 °C for 12 h prior to use (yield, 78%).

2.4. Immobilization of cellulase

Cellulase was immobilized on Fe_3O_4-NH_2@4-arm-PEG-NH_2 through a modified GDA activation method [31]. 3 mg of Fe_3O_4-NH_2@4-arm-PEG-NH_2 was dispersed in 10 mL of 0.1 M citrate buffer, then 2 mL of GDA (25% v/v) was added into the above solution, which activated at 50 °C for 6 h. The resulting product Fe_3O_4-NH_2@4-arm-PEG-NH_2@GDA was washed 7 times with citrate buffer (pH = 5.5) containing 0.1 M NaCl and dried in a vacuum oven. The Fe_3O_4-NH_2@4-arm-PEG-NH_2@GDA nanoparticles were prepared as a carrier to immobilize cellulase. Concretely, 4.0 mg of Fe_3O_4-NH_2@4-arm-PEG-NH_2@GDA nanoparticles were dispersed in the 10 mL of 0.1 M citrate buffer (pH = 5.5) containing 2 mg of cellulase. Then, the mixture was placed in a thermostatic shaker at 30 °C for 30 min. The supernatant after magnetic separation was used to calculate the loading of cellulase by Bradford method, and the final immobilized cellulase product was washed two times with citrate buffer to remove the weakly bounded enzyme.

2.5. Enzyme assay

The activity of cellulase was assayed according to the method of International Union of Pure and Applied Chemistry (IUPAC) with some modifications [32,33]. The assay of cellulase was carried out using a reaction mixture containing 0.2 mL of cellulase (1 mg/mL) and 1.8 mL of 1% CMC dissolved in 0.1 M of citrate buffer and maintained at 50 °C for 30 min with shaking, the reaction was stopped by addition of 2 mL of DNS reagent and kept for 10 min in a boiling water bath. After rapid cooling, 16 mL of citrate buffer was added into the above mixture. Blank experiment was performed by replacing the 1.8 mL of 1% CMC with 1.8 mL of citrate buffer while everything else stayed the same. The amount of generated glucose was measured at 510 nm by a spectrophotometer. All experiments were carried out in triplicate and took the average. One unit of enzyme activity is defined as the amount of cellulase that hydrolyzes CMC to generate 1 μmol glucose per minute.

2.6. Application of free and immobilized cellulase for saccharification of filter paper and microcrystalline cellulose

The filter paper (or microcrystalline cellulose) weighed 1 mg was employed as substrate of free and immobilized cellulase preparations, and the saccharification was performed by adding 0.2 mL of 1 mg/mL free cellulase (or immobilized cellulase) and 1.8 mL of citrate buffer to this substrate keeping for 30 min with shaking. After termination of the reaction by addition of 2 mL of DNS maintaining in violent boiling water, 16 mL of citrate buffer was added into the mixture when cooling. The amount of hydrolytic product glucose was quantified using a spectrophotometer at 510 nm.

2.7. Characterization

The crystal structure of the microspheres were detected by X-ray diffraction (XRD; Shimadzu, XRD-6100, Japan). The morphology and size of nanoparticles were observed by Transmission electron microscope (TEM; Tecnai G2 F30 S-TWIN, USA). Thermogravimetric analysis (TGA) measurements were carried out on a Netzsch STA 499C TGA instrument. The temperature was increased from room temperature to 800 °C at the rate of 10 °C/min under nitrogen atmosphere. The structures of nanoparticles were analyzed by Fourier transform infrared (FT-IR, Nicolet Nexus 470, USA) spectroscopy. The magnetization curves were obtained by a vibrating samples