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Short communication

Reversible immobilization of glucoamylase onto magnetic polystyrene beads with multifunctional groups

Jianzhi Wang[a], Duoming Wu[b], Guanghui Zhao[a], Minrui Li[c], Yanfeng Li[a,*], Yuan Han[c], Aixiao He[a], Yu Jiang[a]

[a] State Key Laboratory of Applied Organic Chemistry, Key Laboratory of Nonferrous Metal Chemistry and Resources Utilization of Gansu Province, College of Chemistry and Chemical Engineering, Institute of Biochemical Engineering & Environmental Technology, Lanzhou University, Lanzhou 730000, China
[b] The First Affiliated Hospital of Lanzhou University, Lanzhou University, Lanzhou 730000, China
[c] School of Petrochemical Engineering, Lanzhou University of Technology, Lanzhou 730050, China

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ABSTRACT

A novel and simple process for the surface functionalization of micron-sized monodisperse magnetic polystyrene (PS) microbeads was reported. The polystyrene seed particles were prepared prior to the dispersion polymerization method. Afterwards, series of surface chemical modifications on polystyrene microspheres were conducted, and three end-functional microspheres with carboxyl, imidazolyl and sulphydryl groups were obtained. The functional magnetic polystyrene microspheres were prepared by impregnation and subsequent precipitation of ferric and ferrous ions into the polystyrene particles. Finally, the functional magnetic polystyrene was used for the reversible immobilization of glucoamylase via metal-affinity adsorption. The results indicated that the obtained immobilized glucoamylase presented excellent reusability, applicability, magnetic response and regeneration of supports. The magnetic PS microspheres retained >65% of its initial activity at 65 °C over 6 h; and the lowest residual activity of immobilized glucoamylase prepared by regenerated supports still remained about 50% of the initial activity after the 10th cycles.

1. Introduction

Enzyme is a popular biocatalyst with high selectivity, specificity under mild reaction conditions [1,2]. However, the industrial applications of enzyme are limited due to its low thermal stability and difficulty in recovery and recycle, resulting in very high cost. The technology of enzyme immobilization on solid support can overcome these drawbacks by enabling the separation, recovery and efficient reuse of enzyme, preventing product contamination, and improving enzyme properties as biocatalyst [3]. One current problem of the immobilized enzyme is the complete lose of its activity after several times of reuse. One very efficient technique to solve this problem, as reported by our previous work [4,5] is the regeneration of solid support to obtain reversible enzyme immobilization. The simple and fast metal-chelation of enzyme on the supports is a good option among various reversible methodologies. The inactivated enzyme can be desorbed away from the support and this support can be reused many times after enzyme inactivations, which help reduce the final price and generate fewer residues [6,7].

Until now, a variety of supports with a range of functionality, morphology, and physical properties have been studied for the immobilization of enzymes such as CNT [8], graphene [9], silica [10], polysaccharide [11], polymer nanofibers [12] and hydrogels [13]. Functional magnetic polymer microspheres represent an important category of promising supports for reversible enzyme immobilization due to their highly selective, efficient and easily regenerable relative to other types of supports materials. Currently, many approaches have been reported to prepare magnetic polymer microspheres including miniemulsion polymerization [14], suspension polymerization [15], dispersion polymerization [16], and swelling and thermolysis technique [17]. However, these methods need further improvement for poor monodisperse, low magnetite content, or density of surface functional groups [18]. In addition, it is very difficult to conduct amino groups, hydroxyl groups, or other groups that respond to reversible immobilization of enzyme, since

* Corresponding author. Tel.: +86 931 8912113; fax: +86 931 8912113.
E-mail addresses: liyf@lzu.edu.cn, wangjz10@lzu.edu.cn (Y. Li).

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the reaction is generally carried out under a strong acidic circumstance, which causes the rapid losing of magnetic property within the magnetic polystyrene microspheres.

In this study, a novel and simple method to prepare the functional and monodisperse magnetic polymer microspheres was explored. First, micron-size magnetic polymeric microspheres were synthesized by the dispersion polymerization method. Second, chemical modification with EDA was employed to introduce internal anchor groups (–OH). Third, the terminal –OH groups were reacted with differently functional groups, subsequent precipitation of iron ions impregnating within the particles to form magnetite. Three end-functional microspheres with carboxyl, imidazolyl and sulphydryl groups were obtained, named as PS-DEA-IDA, PS-DEA-imidazole and PS-DEA-TSC. Glucoamylase was immobilized onto the three kinds of functional magnetic supports by metal-ion affinity interactions. The factors affecting the activity recovery and properties of the immobilized glucoamylase were investigated. The main novelty of our functional magnetic polymer microspheres, compared with the previous reports, mainly includes the following points: (1) it is also widely accepted that nitrogen/oxygen-containing functional groups act as adsorption sites for heavy metals ions. Therefore, the functional magnetic polymer microspheres can be binded with different groups (e.g. amino, hydroxyl, or thiol moieties) on the protein surface by metal-ion affinity interactions to form strong linkages, which can prove the stabilities of enzymes [4]. Second, functional magnetic polymer microspheres was selected for this study because it has proven to be an efficient means to achieve rapid separation and recover from the reaction system to enhance the reusability of enzyme. Third, the main novelty of the strategies is that the method allows for the regeneration of metal chelate support after inactivation of immobilized enzyme.

2. Materials and methods

2.1. Materials

Styrene was purchased from Tianjin Chemicals Co. Ltd., (China) and dried over CaH_2 and distilled under reduced pressure. Divinylbenzene (DVB) was purchased from Sigma–Aldrich and distilled under reduced pressure to remove inhibitors; 2,2′-Azobisisobutyronitrile (AIBN) and poly-vinylpyrrolidone (PVP) were purchased from Sigma–Aldrich and used as initiator and stabilizer, respectively. Glucoamylase (EC 3.2.1.3, from *Aspergillus niger*) was purchased from Yixing Enzyme Preparation Company (China); all other chemicals were with analytical grade of purity. Deionized water was used in the experiment.

2.2. Glucoamylase immobilization

Glucoamylase was immobilized onto the three kinds of supports (Fe_3O_4@PS-DEA-IDA, Fe_3O_4@PS-DEA-imidazole and Fe_3O_4@PS-DEA-TSC) by metal-ion affinity interactions. Briefly, 25 mg of supports and 5 mL enzyme solution (1.5 mg/mL) were mixed uniformly. The immobilization process was carried out at 30 °C in a shaking air bath for 8 h. After this, the immobilized glucoamylase was recovered by magnetic separation and thoroughly rinsed with acetate buffer solution (50 mM, pH 3.5) two times to remove unbound glucoamylase. The washed solution was collected to assay the amount of residual enzyme. The chemical process to fabricate the supporting material and enzyme immobilization is schematically represented in Fig. 1. The resulting immobilized glucoamylase was stored at 4 °C prior to use. The enzymatic activities of free and immobilized glucoamylase were measured from the amount of liberated reducing sugars by the dinitrosalicylic acid (DNS) method [19]. Finally, the pH value endurance, temperature endurance of the three immobilized glucoamylase was investigated.

2.3. The regeneration of supports for enzyme repeated immobilization

The immobilized enzyme also could loss its activity completely after several times of reuse. Therefore, the regeneration of supports is very essential in industry applications. The regeneration of supports is carried out in three steps; firstly, the immobilized enzyme preparations were placed within the 1.0 M EDTA solution at room temperature for 5 h to remove the metal ions and the enzyme. Subsequently, the regenerated supports were washed several times with deionized water and pH 3.5 buffer solution in sequence. After that, the regenerated supports were used to adsorb again for a fresh glucoamylase described above and were then repeatedly used in adsorption/desorption cycle of glucoamylase. To determine the reusability of immobilized enzymes, adsorption–desorption cycle of glucoamylase was repeated ten times by using the same supports.

3. Results and discussion

3.1. Preparation and characterization of magnetic polystyrene nanospheres with functional groups

First, polystyrene (PS) microspheres were synthesized by dispersion polymerization. Subsequently, series of surface functionalization of polystyrene microspheres were conducted, obtaining three kinds of modified microspheres: PS-DEA-IDA, PS-DEA-imidazole and PS-DEA-TSC. Upon the particles were impregnated with iron ions under N_2 protection, the magnetite nanoparticles were irreversibly entrapped within the functional polystyrene particles by a modified coprecipitation method. Then the resulting magnetic polystyrene particles were used to immobilize glucoamylase by metal-ion affinity interactions. The chemical process to fabricate the material and the glucoamylase immobilization is described in the experimental section in details, and it is visually summarized schematically in Fig. 1.

Fig. 2a shows the morphology and structure of the polystyrene micro-beads, the particles had a smooth surface and uniform shape, where most of the particles are quasispherical with an average diameter of 2 μm. Fig. 2b–d are TEM images of polystyrene particles coated with magnetite. It can be seen that the size of the magnetic polystyrene particles is 1.8 μm, which could be attributed to the fact that the reaction only occurred on the surface of particles. It is clear that the particle shape did not exhibit noticeable change upon incorporation of magnetite nanoparticles compared with the parent polystyrene particles, though the colors turned black. Dispersion polymerization is a simple and effective method for preparing monodisperse spherical particles in the micron size range in a single step. Amino groups, hydroxyl groups, or other groups were introduced onto the surface of the magnetic polystyrene microspheres by the method of surface modification. However, because these surface functionalization reaction are carried out under a strong acidic circumstance, it is very difficult to introduce the functional groups onto the surface of the magnetic polystyrene microspheres without destroying the magnetic property. Therefore, in our work, polystyrene particles were prepared in advance; the chloride groups were introduced onto the surface of polystyrene microspheres by surface Friedel–Crafts acylation reaction. In order to lengthen the carbon chain and increase the amount of surface functional groups, the chloride groups (Cl) on the acylated polystyrene microbeads were replaced via an ammonolysis reaction between the diethanolameine (DEA) and the chloride groups on the surface of the acylated magnetic polystyrene microbeads. Subsequently, PS-DEA-Cl particles were prepared by a second acylation. Because the chloride groups are very reactive, the polystyrene microbeads with surface chloride groups were activated with IDA, imidazole and TSC. Upon addition of iron ions (Fe^{2+} and Fe^{3+}) into the suspension of the functional polystyrene particles, the particles were impregnated with iron ions with nitrogen protection. The functional magnetic polystyrene particles were prepared when concentrated ammonia was added into the suspension.

The saturation magnetization of Fe_3O_4@PS-DEA-IDA, Fe_3O_4@PS-DEA-TSC and Fe_3O_4@PS-DEA-imidazole are found to be 15.7 emu/g, 11.7 emu/g, 15.7 emu/g, respectively. With such high magnetization and superparamagnetic behaviors, the magnetic polymer particles could be easily and rapidly separated from the solution, and easily redispersed by gentle shaking, enabling the target to meet the need of immobilized enzyme.

Taking advantage of metal-chelate properties, Cu^{2+} ions was coordinated to the iminodiacetic acid (IDA), imidazole and