Biochemical Engineering Journal 98 (2015) 75–83

Contents lists available at ScienceDirect

Biochemical Engineering Journal

journal homepage: www.elsevier.com/locate/bej

Regular article

Facile self-assembly of magnetite nanoparticles on three-dimensional graphene oxide–chitosan composite for lipase immobilization

Jianzhi Wang, Guanghui Zhao, Lingyun Jing, Xiaomen Peng, Yanfeng Li*

State Key Laboratory of Applied Organic Chemistry, Institute of Biochemical Engineering & Environmental Technology,
College of Chemistry and Chemical Engineering, Lanzhou University, Lanzhou 730000, China

ARTICLE INFO

Article history:
Received 17 July 2014
Received in revised form 12 October 2014
Accepted 18 November 2014
Available online 26 November 2014

Keywords:
Graphene oxide–chitosan
Fe_3O_4 nanoparticles
Immobilised enzymes
Biocatalysis
Lipase
Enzyme activity

ABSTRACT

Three dimensional (3D) magnetic graphene oxide–chitosan (GO–CS) composites with orderly self-assembled magnetite (Fe_3O_4) nanoparticles (GO–CS–Fe3O4) were successfully fabricated. First, the covalent functionalization of graphene oxide (GO) using chitosan (CS) was successfully accomplished via a facile amidation process, forming a 3D network. In the as-fabricated GO–CS composites, GO sheets remained less aggregated, exhibiting a large surface area, and the interconnected pores in the hydrogels allowed object molecules to diffuse easily into the composites. Subsequently, the GO–CS–Fe_3O_4 composites were synthesized using $FeCl_3{\cdot}6H_2O$ and GO–CS composite as the starting materials through a solvothermal reaction. The resulting composite combined the features of the high surface area of GO, abundant amino, and hydroxyl functional groups of CS, and the magnetic response of magnetic nanoparticles. For further application, these composites were utilized to immobilize *Candida rugosa* lipase via different routes, and they showed excellent immobilization capacities.

1. Introduction

Enzymes are incredibly efficient biological catalysts possessing some excellent unique properties such as high effectiveness, high specificity, and they function under mild reaction conditions of temperature, pH, and salt. Therefore, they are extensively used in the pharmaceutical, chemical, food, and textile industries to prepare biochemical products, biosensors, and drugs [1]. Lipase is a type of ubiquitous enzyme possessing an intrinsic capacity to catalyze enantioselective hydrolysis and esterification, chiral resolution, synthesis of enantioenriched monomers and macromolecules for polymerization, and other enzymatic reactions [2]. Nevertheless, despite its appreciable intrinsic stability, the industrial applications of enzyme catalyzed reactions have numerous problems, e.g., low catalytic efficiency and stability of enzyme, recovery of enzyme from the reaction system and separation, recyclability and reuse of enzyme. An intriguing approach to solve the above mentioned problems is to immobilize the enzymes on various supports. Moreover, with the development of various supports, immobilized enzymes have become more affordable over a broader range of applications such as bioconversion, bioremediation, biodetection, and as biosensors [3].

Graphene, a fascinating new member of the carbon materials, with honeycomb and one-atom-thick structure, has attracted world-wide attention due to its excellent electronic, mechanical, and thermal properties [4–6]. Moreover, graphene can be used as valuable substrate to interact with various species because of its large specific surface area, good biocompatibility and chemical stability, and high adsorption capacity [7]. However, despite all the promising results obtained so far, pure graphene sheets are limited for many applications and scientists face challenges to induce increasingly controlled functionality [8]. Graphene oxide (GO), a novel two-dimensional (2D) nanomaterial prepared from natural graphite, has recently attracted significant attention. GO, with many oxygen-containing functional groups on its basal planes and edges [9,10], is more water soluble and easily undergoes chemical modification [11]. To expand and optimize the use of GO in diverse applications in energy and biotechnology, integration of GO with other materials, such as polymer and inorganic nanoparticles, to fabricate composites or hybrids is always highly desirable. In particular, covalently grafted polymers onto GO sheets is an efficient method to improve properties of GO such as solubility, interfacial interactivity with a target matrix, and electronic properties [12]. In recent years, there has been a surge of interest in exploring water-soluble polymer-grafted graphene composites for biocompatible materials, catalysis, supercapacitors, and lithium ion batteries due to their biocompatibility, unique conjugated structure, large surface area, and relatively low cost [13].

---

* Corresponding author. Tel.: +86 931 8912528; fax: +86 931 8912113.
E-mail address: liyf@lzu.edu.cn (Y. Li).

http://dx.doi.org/10.1016/j.bej.2014.11.013
1369-703X/© 2015 Published by Elsevier B.V.

The biopolymer chitosan is a natural polysaccharide, and is extensively distributed in the exoskeleton of crustaceans, fungal cell wall, and other biological materials [14]. It is an inexpensive and renewable material with applications in cosmetics, pharmaceuticals, food science, waste water treatment, and biotechnology. It exhibits excellent biocompatibility, biodegradability, non-toxicity, adsorption properties, multiple functional groups, and film-forming ability [15,16]. CS is a stimulus-responsive polymer with a solubility that can be adjusted reversibly by changing the pH value. This is attributed to the presence of primary amino groups in the polymer chain. The natural polymer CS is often selected as an effective biosorbent for the recovery of hazardous dyes, proteins, and heavy metals because of its intrinsic characteristics. However, despite numerous advantages and unique properties of CS, its mechanical properties are not good enough to satisfy a wide range of applications [16]. Therefore, to overcome the above mentioned problems, GO–CS composite hydrogels have been prepared by the introduction of a covalent conjugation between GO and CS. However, there are some disadvantages of GO–CS composite hydrogels in their practical applications, such as difficult separation and poor re-dispersibility of dry gels.

Magnetic nanomaterials with controlled sizes, shapes, and functionalities are of considerable interest in materials chemistry because of their unique physical properties and nontoxicity, making them attractive in a wide range of application fields [17]. Importantly, Fe_3O_4 is an environmental friendly material and could be used as biosorbent for recovery of heavy metals and proteins. However, the lack of surface functional groups has restricted Fe_3O_4 in practical applications. Therefore, functionalized magnetite nanomaterials are promising materials for biological and medical fields, such as the immobilization of proteins, peptides, and enzymes; bioseparation; immunoassays; drug or gene delivery; and magnetic resonance imaging [18].

Based on the above mentioned considerations, herein, we report a rapid and facile strategy for aminated Fe_3O_4 nanoparticles self-assembled on GO–CS composite hydrogels. GO–CS composite hydrogels can be prepared by the conjugation of GO with CS via a covalent mechanism. These composite hydrogels exhibit a loose 3D network structure consisting of 2D GO sheets with pore sizes of several micrometers to tens of micrometers. In this study, the amine-functionalized magnetic GO–CS composites were synthesized using ferric chloride hexahydrate ($FeCl_3{\cdot}6H_2O$) as a single iron source and 1,6-hexadiamine as ligand by a solvothermal reaction. The composites revealed good distribution of Fe_3O_4, rich surface functional groups, and a considerable saturation magnetization. Moreover, the magnetic composite was synthesized by a one-step in situ hydrothermal method, which has operational simplicity and capability for large-scale production. Such a novel composite with high specific surface area and strong magnetic sensitivity could act as promising candidate for significant applications in various fields. To further investigate the potential application of these composites in biotechnology, the GO–CS–Fe_3O_4 composites were used as supports for lipase immobilization via different routes. Further, the optimal conditions for immobilization of lipase were determined and the properties (thermal stability, reusability, and storage ability) of the immobilized lipase were investigated.

2. Experimental

2.1. Materials

Graphite powder (320 mesh, spectrographic grade) was purchased from Sinopharm Chemical Reagent Co., Ltd.; Chitosan with viscosity-average molecular weight of 6.0×10^5 and *N*-deacetylation degree of 90% was obtained from Yuhuan Ocean Biochemical Co., Ltd. (China); *Candida rugosa* lipase (CRL, Type VII, 1180 units mg^{-1}, solid), bovine serum albumin (BSA), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC·HCl, 99%), and *N*-hydroxysuccinimide (NHS, 97%) were purchased from Sigma Chemical Co.; other chemicals were all supplied by the Tianjing Chemical Reagent Company (China).

2.2. Preparation of magnetic graphene oxide–chitosan (GO–CS–Fe_3O_4) composites

2.2.1. Preparation and purification of graphite oxide (GO)

GO was prepared from natural graphite by modified Hummers method [19,20]. The detailed process is described as follows: graphite (1 g) and sodium nitrate (1 g) were mixed with sulfuric acid (46 mL, 98%) in an ice bath. The mixture was stirred for 0.5 h. Potassium permanganate (3 g) was slowly added with stirring, and the rate of addition was controlled to prevent the mixture temperature from exceeding 20 °C. The mixture was then kept in water bath maintained at 50 °C and stirred for about 12 h which resulted in the formation of thick paste. When the reaction was completed, the contents were cooled down to room temperature. Subsequently, de-ionized water (46 mL) was added gradually, and the temperature was maintained at 98 °C for 2 h. Then, the temperature was reduced to 60 °C, hydrogen peroxide (10%, 10 mL) was added dropwise into the mixture, and the reaction was further stirred for 2 h. The mixture was centrifuged and washed with de-ionized water until the pH was 7. The as-obtained sample was dispersed in deionized water by ultrasonication for 2 h.

2.2.2. Preparation of graphene oxide–chitosan (GO–CS) composite

Conjugation of GO with CS was accomplished via a covalent mechanism. The GO–CS composite was prepared by the following method: first, carboxyl (—COOH) groups of GO were activated using EDC and NHS mixture; GO (0.2 g) in phosphate buffer solution (20 mL, 50 mM, pH 7.0) was sonicated for 3 h to obtain a homogeneous dispersion. Next, a solution of EDC (0.05 M) and NHS (0.05 M) were added to GO dispersion with continuous stirring for 2 h to activate the—COOH groups of GO. CS solution (1%, w/v) was prepared by dissolving CS (0.2 g) into aqueous solution of acetic acid (20 mL, 2.0%) under ultrasonic stirring for 2 h at room temperature. The CS solution was then added dropwise to the activated GO solution within 20 min. The reaction was performed at room temperature under sonication for 6 h, and the contents were allowed to shake gently for 8 h. The mixture was centrifuged and washed with acetic acid solution (0.1 M) and de-ionized water to remove unreacted CS. The obtained product was denoted as GO–CS. The as-obtained sample was dispersed in ethylene glycol by ultrasonication for 2 h.

2.2.3. Preparation of magnetic graphene oxide–chitosan composites (GO–CS–Fe_3O_4)

In a typical procedure, the surface amine-functionalized GO–CS–Fe_3O_4 composites were synthesized by modified solvothermal reactions according to the following procedure [18,21]. In general, $FeCl_3{\cdot}6H_2O$ (0.8 g) was dissolved in ethylene glycol (24 mL) to form a clear solution. Then, sodium acetate (NaAc, 1.6 g) and GO–CS (2 mL, 30 mg/mL) in ethylene glycol were added, followed by stirring for another 30 min. Later, 1,6-hexanediamine (2 mL) was ultrasonically dispersed in the resulting dispersion for 10 min. The mixture was sealed in a teflon-lined stainless steel autoclave (50 mL capacity) and maintained at 200 °C for 8 h. The black product so-obtained was washed several times with ethanol by centrifugation and was dried at 50 °C in a vacuum oven. The obtained product was denoted as GO–CS–Fe_3O_4.