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One-pot fabrication of chitin-shellac composite microspheres for efficient enzyme immobilization

Shuang Mei[a,b,1], Pingping Han[a,b,1], Hong Wu[a,b,d,*,1], Jiafu Shi[b,c,1], Lei Tang[a,b,1], Zhongyi Jiang[a,b,1]

[a] Key Laboratory for Green Chemical Technology of Ministry of Education, School of Chemical Engineering and Technology, Tianjin University, Tianjin 300072, China
[b] Collaborative Innovation Center of Chemical Science and Engineering (Tianjin), Tianjin 300072, China
[c] School of Environment Science and Engineering, Tianjin University, Tianjin 300072, China
[d] Tianjin Key Laboratory of Membrane Science and Desalination Technology, Tianjin University, Tianjin 300072, China

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ABSTRACT

In this study, all-natural composite microspheres were fabricated through adding shellac into chitin solution followed by self-assembly via thermally-induced phase separation. The pore structure of the composite microspheres was altered into wedge-shape from ink-bottle-shape of the chitin microspheres, whereas, the crystalline structure of these two kinds of microspheres remained unaltered. The as-fabricated chitin-shellac composite microspheres were used for enzyme immobilization through adsorption. And *yeast* alcohol dehydrogenase (YADH) was chosen as the model enzyme, which is a multimer consisting of 4 subunits. The loading capacity of the as-prepared composite microspheres was up to 79.0 mg/g (enzyme/carrier). The immobilized enzyme exhibited a comparable catalytic activity compared to its free counterpart and maintained 49.3% of its initial activity after 54 days' storage at 4 °C while the free enzyme lost all its activity.

1. Introduction

Multimeric biomolecules like proteins, peptides, and nucleic acids, have caused increasing interest in recent years (Webber et al., 2016; Melo et al., 2015; Pieters et al., 2016). But the poor *in vivo* stabilization of said molecules restricts generalized industrial applications (Secundo, 2013). Whereas multimers often display a structure-property relationship, immobilizing them in a moderate way to enhance their structure stabilization or even specificity is desirable (Balcao and Vila, 2015; Mateo et al., 2007; Rodrigues et al., 2013; Fernandez-Lafuente, 2009). The existing immobilization techniques can be divided into two main classes: immobilizing with pre-existing support, and support free cross-linked enzyme aggregates (CLEAS) or crystals (CLECs) (Rodrigues et al., 2013; Garcia-Galan et al., 2011; Cao et al., 2003; Kim et al., 2007; Yamaguchi et al., 2011; Brady et al., 2008). As to the former, immobilizing multimeric enzyme through adsorption is regarded to be good for structure stabilization (Hanefeld et al., 2009; Jesionowski et al., 2014; Sheldon and van Pelt, 2013; Yang et al., 2009; Wu et al., 2015), for the ionic bridges are not strong enough to cause rigidification. But the number of ionic bridges between protein and carrier must be large enough to fix enzyme on the carrier through multipoint progress. To ensure the generation of enzyme-carrier interactions, the physico-chemical parameters, such as surface area, particle size and surface functional groups, of the carrier should be taken into consideration (Jesionowski et al., 2014; de Albuquerque et al., 2016). Notably, using nanomaterials with high surface area as support is of great interest for the accordingly high loading capacity (Cipolatti et al., 2016; Min and Yoo, 2014). With the rapid developments in micro- and nano- technology, e.g. core-shell microspheres, alginate-based microspheres, aerogel microspheres, and nanoparticles, the usage of porous spheres as enzyme immobilization support is becoming more and more popular (Yang et al., 2009; Zhang et al., 2011; Cai et al., 2014; Sohrabi et al., 2014; Ranjbakhsh et al., 2012). Above all, some all-natural materials with nano- or micro- structure open the door for more bio-friendly, green-chemical application.

Herein, the natural chitin is chosen as the polymer material for the construction of enzyme carrier matrix. Chitin is widely distributed in the extracellular matrix of animals' connective tissues, which can form nanoporous structure with various morphologies (Rinaudo, 2006). Both chitin (Fig. 1a) and its most important derivative chitosan (Fig. 1b) have been widely used in biocatalysis for their fibrous structure, biocompatibility, easy availability, et al. (Rinaudo, 2006; Krajewska, 2004; Shang et al., 2014; Wang et al., 2015; Pillai et al., 2009; Chang et al., 2011). In some case, chitin/chitosan can co-immobilize cofactors and

* Corresponding author at: Key Laboratory for Green Chemical Technology of Ministry of Education, School of Chemical Engineering and Technology, Tianjin University, Tianjin, 300072, China.
E-mail address: wuhong@tju.edu.cn (H. Wu).
[1] Present address: Tianjin University, School of Chemical Engineering and Technology.

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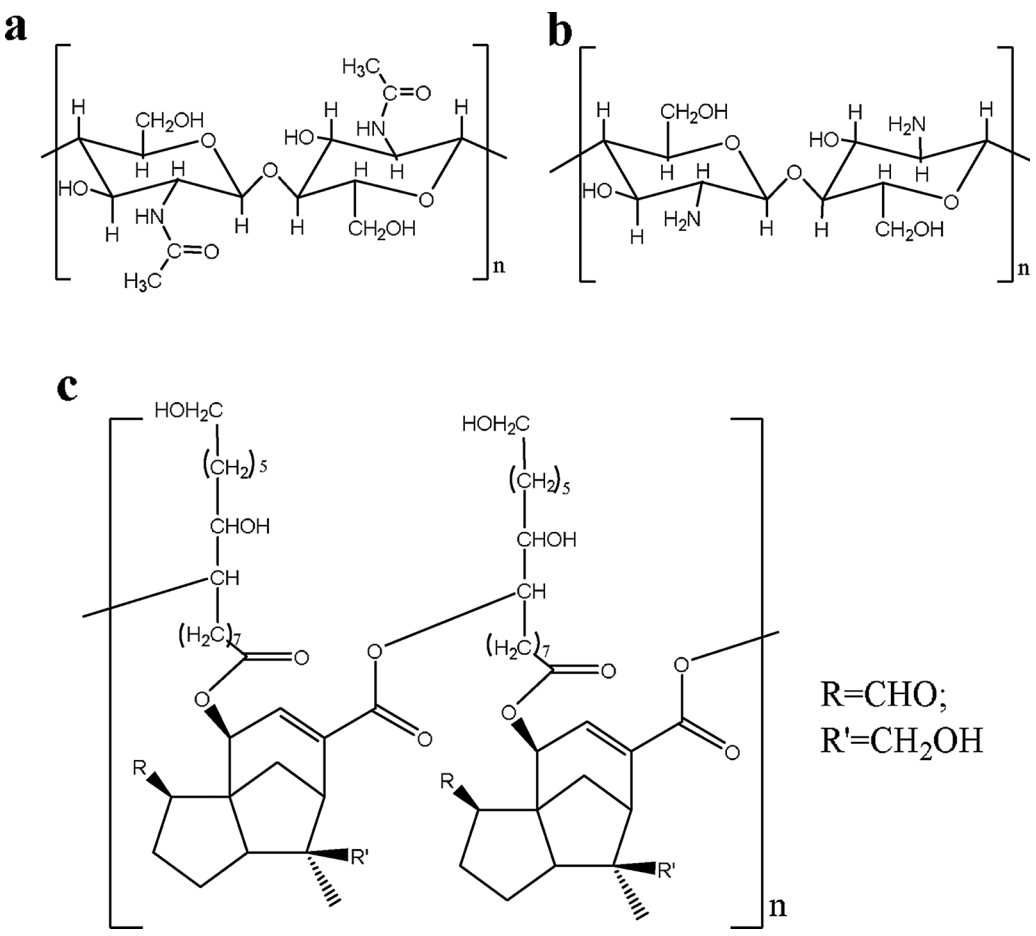

Fig. 1. Molecular structure of chitin (a), chitosan (b) and shellac (c).

enzyme, which may facilitate enhanced catalytic activity and reusability (Velasco-Lozano et al., 2017). The special advantage of chitin/chitosan is that when dissolved in NaOH/urea solutions, the NH^{3+} cation offers possibilities for later ion exchange between support and enzyme (Hu et al., 2007), resulting in multipoint immobilization *via* electrostatic adsorption (Fernandez-Lafuente, 2009).

However, the lack of surface activity restricts direct applications of pure chitin/chitosan microspheres. Thus, post modifications of chitin microspheres through chemical or physical methods were usually performed (Shang et al., 2014; Wang et al., 2015). In nature, biomaterials are mostly functionalized simultaneously during their formation, for example, the cell membrane. In situ modification during the formation of chitin microspheres is desirable but finding a simultaneous soluble modifier is challenging. Shellac (Fig. 1c), a kind of resin dissoluble in alkali solutions, is a natural biomolecule secreted by *lac* insect. It's a polymer with one carboxyl groups, three ester bonds, five hydroxyl groups and one aldehyde groups every unit, on average (Limmatvapirat et al., 2004). These functional groups, especially the aldehyde groups can react with the amino groups on enzyme, thus provide oriented multipoint immobilization (Hernandez and Fernandez-Lafuente, 2011). In addition, shellac has good antibacterial property, which may contribute to the storage stability of all-natural carriers.

In this study, we presented an approach to prepare all-natural shellac-modified chitin composite microspheres by adding shellac into chitin solution followed by self-assembly via thermally-induced phase separation. The morphology and structure of the composite microspheres were investigated by scanning electron microscope (SEM), Brurauer Emmerr Teller (BET), X-ray diffraction (XRD), Fourier Transform Infrared Spectroscopy (FTIR), different scanning calorimeter (DSC) and X-ray photo electron spectroscopy (XPS) characterizations. The properties of microspheres used as enzyme immobilization carrier were tested through YADH, which can convert various alcohols to aldehydes or ketones with nicotinamide adenine dinucleotide (NADH) as a coenzyme (Dreifke et al., 2017). Hitherto, YADH has been immobilized by entrapment, covalent binding, or adsorption in/on various supports (Soni et al., 2001; Trivedi et al., 2005). The main problem was the activity recovery for the multipoint immobilization may cause protein denaturation. The redox reaction is accompanied with the oxidation of NADH to nicotinamide adenine dinucleotide phosphate (NAD^+), where the recycling of coenzyme is another crucial issue (Velasco-Lozano et al., 2017). Herein, we were aimed at the first problem and tried to offer moderate interactions between YADH and carrier to avoid unwished allosteric effect. The loading capacity, catalytic activity and stability of YADH immobilized in the chitin-shellac microspheres were systematically explored.

2. Experimental section

2.1. Materials

Chitin powder (chemically pure) was purchased from Gold-Shell Biochemical Co.Ltd (Zhejiang, China). Shellac (chemically pure), *yeast* alcohol dehydrogenase (YADH, EC1.1.1.1, from *saccharomyces cerevisiae*, M_w 14–15 kDa, pI 5.2–5.6), nicotinamide adenine dinucleotide (NADH, grade I, 98%), Tris (hydroxymethyl) aminomethane (Tris, analytical grade) and coomassie brilliant blue (analytical grade) were purchased from Sigma-Alrich. Sodium hydroxide (NaOH, chemically pure), urea (chemically pure), isooctane (analytical grade), Span 80 (chemically pure) and Tween 80 (chemically pure) were purchased from Tianjin Guangfu Fine Chemical Research Institute (Tianjin, China). Formaldehyde (analytical grade) was purchased from Tianjin University Kewei Company (Tianjin China).