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Research paper

An engineered platform based on chitin-affinity immobilization for producing low molecular weight heparin

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ABSTRACT

Using chitin-affinity interaction between triple-functional heparinase I (Hep I) and chitin, an engineered platform was prepared to produce controllable low molecular weight heparin (LMWH). Chitin microspheres with well-defined nanofibrils were fabricated through a "bottom up" pathway. An enhanced soluble protein, ChBD-SUMO-Hep I (CSH-I), was expressed in 3 L batch fermentation with a high bioactivity of 2.5×10^3 IU/L. Chitin binding domain (ChBD) can specifically bind to chitin in noncovalent way, which leads to the immobilization and purification of enzyme in a single step. The immobilized CSH-I was preferred over its free counterpart due to its higher tolerance to heat and pH, as well as improved shelf-life. The restraint enzyme could be reused up to 8 times to achieve a conversion yield exceeding 90%. By using the bioinspired conjugates, the qualified LMWH fractions were obtained by monitoring the degradation process with an absorbance range of 44.5–68.3 at 232 nm.

1. Introduction

Heparin as a highly sulfated glycosaminoglycan is present on mucosal tissues such as bovine lungs or porcine intestines, which has been the most widely used natural anticoagulant in clinics for over half a century (Liu & Linhardt, 2014). The heterogeneous chains of heparin contain some specific pentasaccharide sequence with a very well defined structure (GlcNAc6S-GlcA-GlcNS3S6S-IdoA2S-GlcNS6S, GlcNAc is α-D-*N*-acetylglucosamine, IdoA is α-L-iduronic acid, GlcA is β-D-glucuronic acid, and S is sulfo group), which is responsible for the specific binding to the inhibitor antithrombin (AT), resulting in its conformational activation and leading to the inhibition of major coagulation cascade proteases, including thrombin (factor IIa) and factor Xa (Li, Johnson, Esmon, & Huntington, 2004; Yin, Wessler, & Stoll, 1971). Heparin is divided into 3 categories based on the average molecular weight (M_w): unfractionated heparin (UFH, M_w ~14000), low molecular weight heparin (LMWH, M_w ~6000), and the ultralow molecular weight heparin (ULMWH, M_w ~1508.3) which is more difficult to obtain (Xu et al., 2011). LMWH is derived from the animal-sourced UFH by chemical or enzymatic depolymerization to yield fragments approximately 1/3 the size of the heparin (Liu et al., 2017). There are several important LMWHs widely used in clinic including Enoxaparin, Tinzaparin and Dalteparin. Despite they share a similar active mechanism, but the varied molecular weight distribution results in their different pharmacologic entities (Racine, 2001). Enoxaparin was initially approved for medical use in 1993, which is prepared based on the benzylation followed by alkaline depolymerization (Liu et al., 2017). Tinzaparin is produced by enzymatic depolymerization by heparinases (Friedel & Balfour, 1994), while Dalteparin is produced by nitrous acid (Racine, 2001). Compared with UFH, LMWHs have similar antithrombotic activities, a longer half-life, a more predictable anticoagulant response and can reduce hemorrhagic complications, which enables its subcutaneous administration for home therapy without the routine laboratory monitoring of the anticoagulant response (Liu et al., 2017; Xu et al., 2011). Hence, it is beneficial for the market and public health to explore new technologies for preparing LMWHs.

Compared with the chemical degradation, enzyme catalyzed degradation is more attractive due to its selectivity, efficiency, and sustainability (Liu et al., 2012). Commercial heparinase from *Flavobacterium heparinum* (*F. heparinum*) mainly consists of 3 isoforms, heparinase I, II, and III (Hep I–III), which varied in their specific position on the heparin backbone (Dietrich et al., 1999; Wu et al., 2014). However, the free enzymes have drawbacks, such as a short lifetime and low stability, thus hampering their practical application (Zare-Eelanjegh et al., 2016). Enzyme immobilization displays attracted benefits including improved operational stability and re-use of enzymes (DiCosimo, McAuliffe, Poulose, & Bohlmann, 2013). Three mainstream strategies for immobilization have been developed: physical adsorption

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(Huang, Wu, Li, & Li, 2014), cross-linking (Dettin et al., 2011), and enzyme entrapment (Qiu, Zhong, Du, Lv, & Xu, 2016). Cross-linking requires chemical modifications on the matrix or enzyme, while enzyme entrapment hampers the efficient recovery of products. Affinity absorption is achieved by genetically engineering the protein to carry a binding domain that specifically binds to the affinity cognate supports (Wang et al., 2015).

The microspheres with a large surface-to-volume ratio can reduce the possible auto-digestion of enzyme, as well as improved stability and efficiency (Ansari & Husain, 2012). Chitin is a resource-rich natural polymer, which mainly exists in the skeletons of crustaceans, algae, and fungi (Chang et al., 2013). Researchers have focused on the fabrics made of chitin derivatives including chitosan and N-acylchitosan (Verlee, Mincke, & Stevens, 2017). Very limited solvents including LiCl/Dimethylacetamide (DMAc) mixtures can dissolve this intractable polysaccharide (Wang, Lu, & Zhang, 2016). Zhang et al. discovered a novel solvent system in which chitin is dissolved in an 11 wt% NaOH/4 wt% urea aqueous solution and then subjected to freezing (–30 °C)–thawing cycles to prepare chitin carriers (Duan, Liu, He, & Zhang, 2014; Cai et al., 2008). Meanwhile, short peptides consisting of 45 amino acids, the chitin binding domain (ChBD), can only specifically bind to the solid chitin surface, but shows no affinity to chitosan or other chitin derivatives (Hashimoto et al., 2000; Kikkawa et al., 2014). The high selectivity and small size could make ChBD a popular choice in protein engineering. However, compared with the commercial tags including poly-His and maltose-binding protein (MBP) (Pagliano et al., 2017; Wu et al., 2014), ChBD-tag was less widely applied in enzyme purification. One possible reason was due to the lack of efficient techniques for fabricating fine chitin microspheres. Most of researches employed the chitin resins contained in an IMPACT™ Kit sold by New England Biolabs (Wang et al., 2015). Some indirect approaches were explored for the ChBD binding, such as acetylated chitosan or colloidal chitin suspension (Bernard, Cao, Myers, & Moyle, 2004).

Here, we modified Hep I with dual functional tags at designated sites, and used ChBD as an affinity tag and small ubiquitin-related modifier (SUMO) as a solvation enhancer (Sabate, Espargaro, Graña-Montes, Reverter, & Ventura, 2012). In our previous work, 4 kinds of ChBD-fused Hep I were constructed and expressed in the *E. coli* system, and ChBD-SUMO-Hep I (CSH-I) was found to have the optimal enzymatic properties (Xu, Qiu, Zang, & Chen, 2017). The idea about this research was displayed in Scheme 1. We constructed the retrievable chitin microspheres without modification through the sol-gel transition in a low-cost and energy-effective pathway. The recombinant Hep I was then incorporated into chitin microspheres to realize the purification and immobilization of the enzyme in a single step. Subsequently, the LMWH fractions were obtained by eluting the UFH-solution through the enzyme-immobilized chitin column. Using the protein Hep I as a model, the ChBD-tag was explored to immobilize and purify the target proteins in a simple and environmentally-friendly way.

2. Materials and methods

The experimental description was provided as Supporting Information (SI).

3. Results and discussion

3.1. Preparation of the chitin microspheres

As stated in the methods, the concentrated chitin solution was achieved by dissolving chitin in a NaOH/urea aqueous solution via freezing/thawing cycles. Fig. 1a shows the apparent hydrodynamic radius distributions $f(R_h)$ of chitin in the 11 wt% NaOH/4% wt urea/water aqueous solution with a concentration of 0.07 mg/mL at a scattering angle (θ) of 30°. There was a single peak with a symmetrical and narrow distribution, indicating that only isolated chitin chains existed in the extremely dilute solution. The hydrodynamic radius (R_h) of individual molecules was 26 nm, which was close to the reported R_h of 23 nm (Fang et al., 2015). Zhang et al. hypothesized that the chitin complexes provided a water-soluble shell consisting of NaOH and urea, which surround the chitin chain (Cai et al., 2008).

The structures of purified chitin powder and chitin microspheres were confirmed by their infrared (FT-IR) spectra (Fig. 1b). The bands at 3260 and 3110 cm^{-1} belong to the asymmetric and symmetric stretching vibrations of N—H for the chitin powder, which shifted slightly to higher wavenumbers for the chitin microspheres. The strong inter- and intra-molecular hydrogen bonds exiting in the native chitin molecules may have been broken in the process of dissolution (Chang et al., 2013). The degree of acetylation (DA) of chitin microsphere was calculated to be 96.3% of its native one based on the equation $A_{1560}/A_{2875} = 0.0125 \times DA + 0.2$. Here, A_{1560}/A_{2875} is the ratio of the absorption tendency at 1560 and 2875 cm^{-1} (Duan et al., 2014). Similar DA suggested that it was a physical process to fabricate the chitin microsphere from the NaOH/urea aqueous solution through non-covalence, and few chitin derivatives was produced. The X-ray diffraction patterns of the purified chitin powder and chitin microspheres are shown in Fig. 1c. The chitin powder showed 6 diffraction peaks at 2θ = 9.3°, 12.8°, 19.2°, 20.7°, 23.4°, and 26.4°, which were ascribed as (020), (021), (110), (120), (130), and (013), respectively, suggesting the characteristic crystallinity of α-chitin. The diffraction peaks of the regenerated chitin microspheres were much broader and weaker than that of the chitin powder, suggesting a decrease in the crystallinity and the crystallite size (Chang et al., 2013). It indicated that the order structure of native chitin was dissociated into disordered state.

The morphology of chitin microspheres was characterized by SEM in Fig. 2. The pristine chitin microspheres with various sizes displayed a well-defined spherical shape with a porous architecture. Fig. 2a4–2d4 shows the size distributions for the particles prepared from the chitin with different M_w. It resulted in the major fractions with average diameters of 91, 31, 24, and 13 μm for 15.2×10^4, 23.7×10^4, 28.3×10^4, and 8.6×10^4, respectively, and displayed a narrow size distribution which fitted the Gaussian distribution as a whole. All the chitin microspheres with various sizes exhibited homogenous micro-porous structures, which favored of the enzyme fixation and stability (Hartmann & Jung, 2010). The high magnification image shown in Fig. 2 indicated that the average pore sizes were 120–300 nm. A bunch of nanofibers were aligned in parallel and self-assembled into endless fibrils that made up the scaffold of microspheres. We observed the well-designed nanofibers with a mean diameter of about 23 ± 7 nm, and the larger chitin microspheres in Fig. 2a3 and 2b3 were especially clear (Duan et al., 2014). However, the microspheres with a size smaller than 25 μm exhibited short and imperfect nanofibers. The microspheres of ~10 μm exhibited a rough morphology. The chitin with a moderate M_w was prone to self-assemble into intact nanofibrils (Chang et al., 2013; Verlee et al., 2017). In this way, size-controllable chitin microspheres with a homogenous micro-porous structure were successively fabricated from the novel solvent in an easy and economical approach.

3.2. CSH-I immobilized chitin microsphere

The specific conformation is important for most proteins to display their functions. The proteins with correct folding confer the solubility in the cellular environment, as well as the selective binding with their targets (Pan et al., 2014). Presently, we can combine different protein segments with desired functions via genetic engineering. However, only the fusion constructs with proper folding and a high solubility can exhibit multiple joined bioactivities (Wang et al., 2015). SUMO, as one of the Ubiquitin-like (Ubl) protein family, is a post-translation modifier with sizes around 12 kDa. SUMO conjugation favors protein solubility and aggregate stability, which has made SUMO peptides a popular tag for improving the expression and solubility of fusion proteins in eukaryotic or prokaryotic system (Sabate et al., 2012). In order to achieve