Research paper

Enzymatic synthesis of catechol and hydroxyl-carboxic acid functionalized chitosan microspheres for iron overload therapy

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A B S T R A C T

Excess "free" iron which occurs under certain physiological conditions participates in the formation of toxic reactive oxygen species via the "fenton" chemistry. The reactive oxygen species oxidize biomolecules and have been implicated in many oxidative stress-related diseases. However, the ideal therapy for treating iron overload problems in humans has not yet been developed. In this study, the phenolic molecules catechol, caffeic acid, and 2,5-dihydroxybenzoic acid were successfully coupled to glucosamine as model substrate in a 1:1 ratio using laccase. Furthermore, coupling of these molecules onto chitosans of different sizes was demonstrated, resulting in decrease in –NH_2 groups as quantified via derivatization. A concomitant increase in iron-chelating capacity from below 3% to up to 70% upon phenolic functionalization was measured for the chitosans based on reduced ferrozine/Fe^{2+} complex formation. Interesting these phenolic compounds seems to also participate as cross-linkers in producing characteristic microspheres. This work therefore opens-up new strategies aimed at developing a new generation of iron-chelating biomedical polymers.

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1. Introduction

Although iron is essential for many metabolic functions (oxygen transport, DNA synthesis, electron transport, as a cofactor in enzymes, etc.), it is also potentially toxic because of its role in the generation of free radicals [1] which have been implicated in many oxidative stress-related diseases. Iron metabolism is therefore tightly regulated, with 80% bound to hemoglobin and myoglobin while the remaining 20% is distributed between storage proteins, hemosiderin, and ferritin (each molecule able to accommodate >4500 iron atoms) [2–4]. A small amount of iron is found in enzymes and the rest is bound to transferrin in plasma [5]. The presence of iron transporting and storage proteins demonstrates the need to tightly regulate iron metabolism. However, under certain conditions, for example, increased iron absorption from the diet or due to genetic disorders like hemochromatosis or parenteral administration of iron in transfusion-dependent anaemias, iron overload or the presence of "free" iron occurs [6,7]. The "free" iron participates in the formation of toxic reactive oxygen species via the "fenton" chemistry [8]. The generated reactive oxygen species oxidize biomolecules (lipids, proteins, and nucleic acids) thereby damaging cells, tissues, and organs alike. Consequently, iron overload has been attributed to diseases such as β-thalassemia, neurodegenrative diseases, cancer, and arteriosclerosis [1,9–11].

To treat or protect patients from the consequences of iron toxicity, iron-chelating drugs have been introduced in clinical practice. The first generations of drugs were based on siderophores, iron-chelating molecules produced by nearly all microorganisms [12]. Of the 500 characterized siderophores [13], only desferrioxamine introduced in 1962 and produced by Streptomyces pilosus is the current drug of choice [14]. Nevertheless, desferrioxamine is associated with several drawbacks including narrow therapeutic window and lacks bioavailability orally [15]. In addition, the vast array of chelators that have been artificially designed and synthesized [6,16] have been reported to be clinically ineffective [1]. Therefore, the ideal chelator for treating iron overload problems in humans has not yet been found [7]. The development of orally effective iron chelators is urgent and forms the basis of this work. This study is therefore aimed at enzymatically incorporating catechol and hydroxyl-carboxic acid moieties into chitosan to produce active iron-chelating polymers. Previous studies on sidephores demonstrated that hydroxycarboxylates and catechols [17] are selective for tribasic metal cations including iron(III). Most of the other tribasic cations are not essential for living cells therefore their removal is of no consequence, which makes

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hydroxycarboxylates and catechols very attractive as compared with the other iron chelators which bind iron(II) because such ligands have affinity for other biologically important bivalent metals such as copper(II) and zinc(II) ions. An additional observed advantage of high-affinity iron(III) chelators is that, under aerobic conditions, they will chelate iron(II) cations and rapidly autoxidise it to the corresponding iron(III) species [18]. Thus, high-affinity iron(III)-selective ligands bind both iron(III) and iron(II) under most physiological conditions. Therefore, novel chitosan-based microspheres (Fig. 1), functionalized with catechol or hydroxyl-carboxic acid could have high iron-chelating abilities while the formation of microspheres will provide the necessary stability for the transport through the changing gastrointestinal tract environment. Apart from enhancing the already inherent iron-chelating properties of chitosan [19], the presence of –NH$_2$ reactive groups on chitosan (Fig. 1) provides sites for enzymatic incorporation of catechol and hydroxyl-carboxic acid functional groups. In addition, chitosan has many remarkable properties among them, mucoadhesive properties, biocompatilibity, non-toxic, antioxidant, antimicrobial, none immunogenicity [20–24], and above all chitin (parent compound) is the second most abundant renewable polymer after cellulose [25]. Further [5] advantages of using chitosan is that it can be designed in many different forms including highly functionalized microspheres (Fig. 1) as envisaged in this study. Microspheres will be made from the phenolic functionalized chitosans as illustrated in Fig. 1. These microspheres can potentially be used either prophylactically or therapeutically while the inherent mucoadhesive properties of chitosan will make it ideal for increasing the residence time of iron chelators.
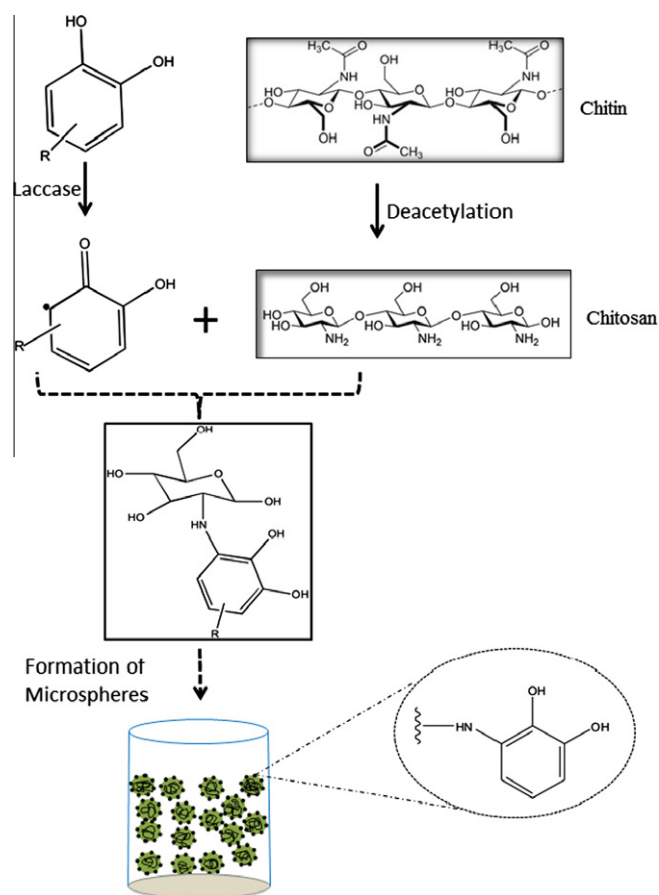

Fig. 1. Envisaged strategy for the enzymatic synthesis of catechol functionalized chitosan microspheres. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2. Materials and methods

2.1. Materials

All chemicals used were of analytical grade. The phenolic compounds (caffeic acid, 2,5-dihydroxybenzoic acid, catechol) and glucosamine were purchased from Sigma–Aldrich. Laccase from *Trametes hirsuta* was produced and purified as previously reported by Almansa et al. [26]. Chitosan samples were kindly provided by Dr. Guillermo Rocasalbas of the University of Catalunya, Spain. All other chemicals were purchased from Merck.

2.2. Laccase activity assay

The activity of laccase was determined spectrophotometrically by monitoring the oxidation of 2,2′-azinobis-(3-ethylbenzothiazoline)-6-sulphonate (ABTS) ($\varepsilon 436 = 29,300 \, M^{-1} \, cm^{-1}$) as substrate at 436 nm in 50 mM succinate buffer at pH 4.5 and 37 °C as described by Niku-Paavola, et al. [27] with some modifications. Briefly, the reaction mixture contained 30 μl laccase, 350 μl ABTS (1 mM), and 50 mM succinate buffer, pH 4.5, to make a final volume of 1.5 ml. A blank was set in the same way as the sample experiment except that the laccase was initially heat denatured at 100 °C for 10 min. The spectrometric measurements were done by recording the absorbance in the time scan mode for 2 min.

2.3. In vitro coupling reactions of phenolic molecules onto glucosamine

To investigate if laccase was able to mediate the coupling of phenolic compounds onto chitosan, glucosamine was used as a model substrate representing monomeric unit of chitosan. The reaction mixture contained one of the phenolic compounds (catechol, caffeic acid or 2,5-dihydroxibenzoic acid) and glucosamine (200 mM) in the molar ratio of 1:1 and 13.4 nkat ml^{-1} laccase in 50 mM succinate buffer (pH 4.5). Reactions were carried out at 37 °C while shaking at 650 rpm using a thermomixer (Thermomixer Comfort Eppendorf AG, Hamburg, Germany) for 24 h and the coupling products analyzed by HPLC–MS.

2.4. HPLC–MS analysis of reaction products

An equal volume of ice cold methanol was added to the reaction mixtures above to precipitate protein. The mixture was allowed to stand on ice for 30 min before centrifuging at 0 °C for 15 min at 14,000g and 650 μl aliquots were transferred into clean vials. HPLC analysis was performed using a system from Dionex equipped with a P580 pump, an ASI-100 autosampler, and a PDA-100 photodiode array detector while monitoring elution at 254 nm. Separation of coupling products was achieved by reversed phase HPLC RP-C18 column (Eurospher 100-5, C18, 150 × 4.6 mm with pre-column, Knauer GmbH, Berlin, Germany) using a linear gradient of formic acid (solvent A) and acetonitrile (solvent B) as solvent at a flow rate of 1 ml min^{-1}, an injection volume of 10 μl, and an oven temperature of 30 °C. Initially, the gradient was set at 100% for 30 min, later at 50% for 20 min, then at 5% for 20 min, and finally 0% formic acid for 30 min. The MS spectra were acquired with an Agilent Ion Trap SL (Palo Alto, CA, USA) equipped with electrospray ionization coupled to the Dionex HPLC-UVD-system described above. The coupling products were measured in positive ion mode and the electrospray voltage was set to +3500 V. Dry gas flow was set to 12 l min^{-1} with a temperature of 350 °C, nebulizer to 70 psi. Maximal accumulation time was fixed to 300 ms and the loading of the trap was controlled by the instrument with an ICC of 30,000.