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Research paper

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

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ABSTRACT

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.

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.

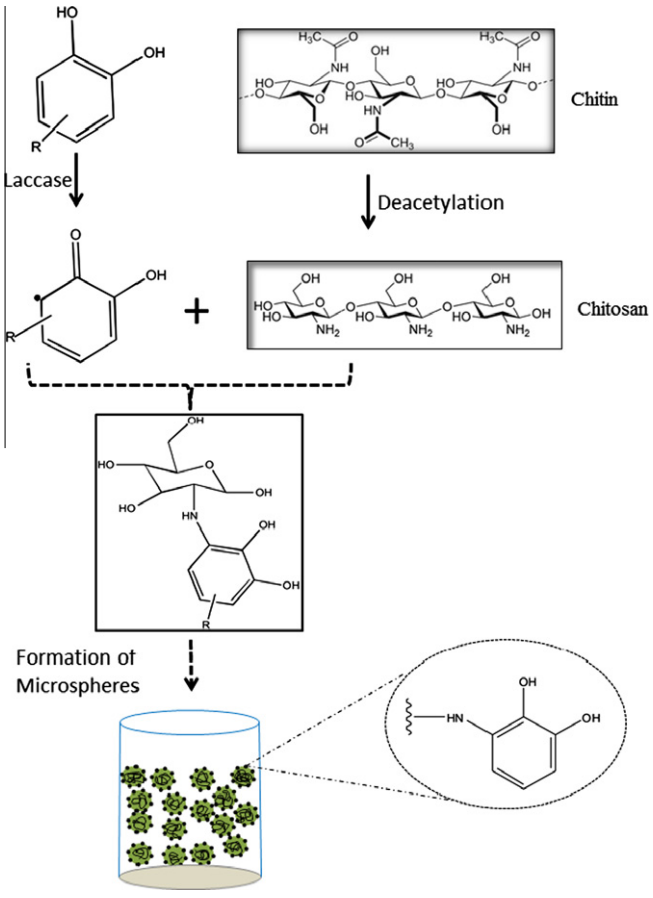

Fig. 1. Envisaged strategy for the enzymatic synthesis of catechol functionalized chitosan microspheres. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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 14,000*g* 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.

2.5. FTIR spectroscopy analysis of reaction products

FTIR spectra were obtained on a Perkin Elmer Spectrum 2000 instrument by the attenuated total reflectance (ATR) technique. The reaction products were frozen in liquid nitrogen followed by lyophilization using Labconco Freeze Dry System/FreeZone 4.5 Liter Benchtop Model 77,500 (Vienna, Austria). The freeze drier was operated at a temperature of −48 °C and at a vacuum pressure of 3×10^{-4} mbar. The lyophilized chitosan/glucosamine-phenolic reacted samples were then analyzed by FTIR. Spectra were recorded in the 4000–600 cm^{-1} range with 16 scans at a resolution of 4.0 cm^{-1} and an interval of 1.0 cm^{-1}.

2.6. Grafting of phenolic molecules onto chitosan

Chitosans of varying molecular weights (15, 50, and 300 kDa) were incubated with each of the phenolic compounds. The grafting of phenolic molecules onto chitosan was performed by first dissolving 1 g chitosan in 20 ml double distilled water supplemented with 2% (v/v) acetic acid. Chitosan (4 ml) solutions were then supplemented with 10 mM final concentration of the respective phenolic molecule. The reaction was then started by adding 50 μl of laccase (20 nkat ml^{-1} in 2 ml 50 mM sodium citrate buffer at pH 4.5 and incubated for 24 h at 30 °C while shaking at 150 rpm. The reaction mixture was then extensively washed (repeatedly washed five times while vigorously shaking) with deionized water in case of catechol and with ethanol in case of 2,5-dihydroxybenzoic acid, to remove any unreacted phenolic molecules. Finally, the resulting insoluble polymer was further rinsed with double distilled water to remove any ethanol.

2.7. Determination of the degree of coupling of different phenolic compounds onto chitosan

The ninhydrin (NHN) assay as described by Mi et al. [28] was used to determine the amount of residual free amino groups remaining in chitosan after cross-linking. The reagents were prepared in two parts. The first solution was prepared by mixing 1.05 g citric acid, 10 ml (1.0 M) NaOH, and 0.04 g $SnCl_2 \cdot 2H_2O$ and adding deionized H_2O until 25 ml. The second solution was prepared by dissolving 1 g ninhydrin in 25 ml ethylene glycol monomethyl ether. The two solutions were then combined and stirred for 45 min before being stored in a dark bottle at 4 °C. Phenolic grafted chitosans (0.5 g) dissolved in 2% (v/v) acetic acid were added to a 1 ml ninhydrin solution and heated to 100 °C in water bath for 20 min. The solution was then cooled down to room temperature, diluted with 5 ml 50% isopropanol, and then the optical absorbance of the solution measured at 570 nm using a UV–Vis spectrometer (Hitachi U-2001). The change in concentration of free NH_2 groups in the chitosan samples was then determined from a standard curve of glycine concentration vs absorbance. The degree of coupling of phenolic monomers was estimated by subtracting the concentration of NH_2 groups remaining after reacting the respective phenolic monomer with chitosan/glucosamine in the presence of laccase from the concentration of NH_2 groups in the reaction containing chitosan/glucosamine and respective phenolic monomers. Laccase was used as the blank in all experiments since it also contains amino groups.

2.8. Preparation of hydroxyl-carboxic and catechol functionalized microspheres

Phenolic functionalized chitosan (15 kDa) was prepared as described before in Section 2.6. The purified polymer was dissolved in 2% (v/v) acetic acid as described above enough to form a gel-like material. This was then introduced dropwise via a syringe pump into a solution containing NaOH–methanol bath [22] while stirring solution at 100 rpm. After cross-linking, microspheres were washed three times using 96% (v/v) methanol and rinsed three times with double distilled water and a final rinse with 96% methanol and then dried for 4 h at 40 °C.

2.9. Scanning electron microscopy (SEM) analysis of microspheres

SEM was conducted as previously described by Mistlberger et al. [29]. Briefly after drying, the microspheres were fixed on a conventional SEM specimen holder with a conductive double-sided adhesive carbon tape. Sputter coating was performed, in order to apply a layer of Pt/Pd to the nonconductive samples for the avoidance of charging during SEM investigations. The microspheres were analyzed using a Zeiss Ultra 55 equipped with a Schottky field emitter (SFE) at a voltage of 5 kV. This scanning electron microscope is well established for the morphological characterization of particles in the submicrometer region and even of nanoparticles [29].

2.10. Ferrous metal ions chelating activity

The iron-chelating capacity was estimated by the method described by Dinis et al. [30]. A 25 μl solution of 2 mM $FeCl_2.6H_2O$ was added to the phenolic functionalized chitosan sample. A 5 mM ferrozine (100 μl) solution was then added and total volume adjusted to 2 ml using ethanol. The mixture was then thoroughly mixed and left to stand for 10 min at room temperature. The solution was then measured spectrophotometrically at 615 nm using a Hitachi UV-2001. The concentration of ferrozine–Fe^{2+} complex formation was calculated by subtracting the unreacted chitosan absorbance value from the grafted chitosan.

3. Results and discussion

3.1. Coupling of phenolic compounds to glucosamine

The ability of laccase to mediate the coupling of phenolic compounds onto chitosan was first assessed using glucosamine as a model substrate. Laccase oxidation of phenolic compounds can form reactive species which in turn could reacted non-enzymatically (e.g., Michaels addition) with $–NH_2$ groups of glucosamine [31]. Indeed, the HPLC–MS analysis indicated that both catechol moiety containing molecules (catechol and caffeic acid) and the hydroxyl-carboxic acid containing molecule (2,5-dihydroxybenzoic acid) were readily coupled onto glucosamine in a 1:1 ratio (Fig. 2). In all cases, the observed peaks correlating to the hybrid molecules showed dominant signals as compared with parent compounds (Fig. 2). For example, $[M+H]^+$ ions at m/z = 331.4 correspond to the coupling of 2,5-dihydroxybenzoic acid to glucosamine (Fig. 2) were observed. Similarly, strong signals for the $[M+H]^+$ ions at m/z = 358.1 correspond to the coupling of caffeic acid to glucosamine (Fig. 2). Coupling of catechol onto glucosamine showed strong signals for the $[M+H]^+$ ions at m/z = 288.5 together with a number of other peaks. Considering the HPLC–MS results, structures embedded in the respective MS spectra Figures were proposed (Fig. 2). C–N couplings are known to occur through either Michael addition or radical coupling [31–33]. For example, during enzymatic coupling of chlorogenic acid onto chitosan, Kumar et al. [34] suggested a nucleophilic attack on the chitosan $–NH_2$ on the oxidized chlorogenic acid – a mechanism expected in this study. Here, it is demonstrated for the first time using HPLC–MS the coupling of laccase oxidized phenolic substrates to the monomeric constituent of chitosan namely glucosamine. In previous studies, it was also demonstrated that alkylamines can be coupled onto

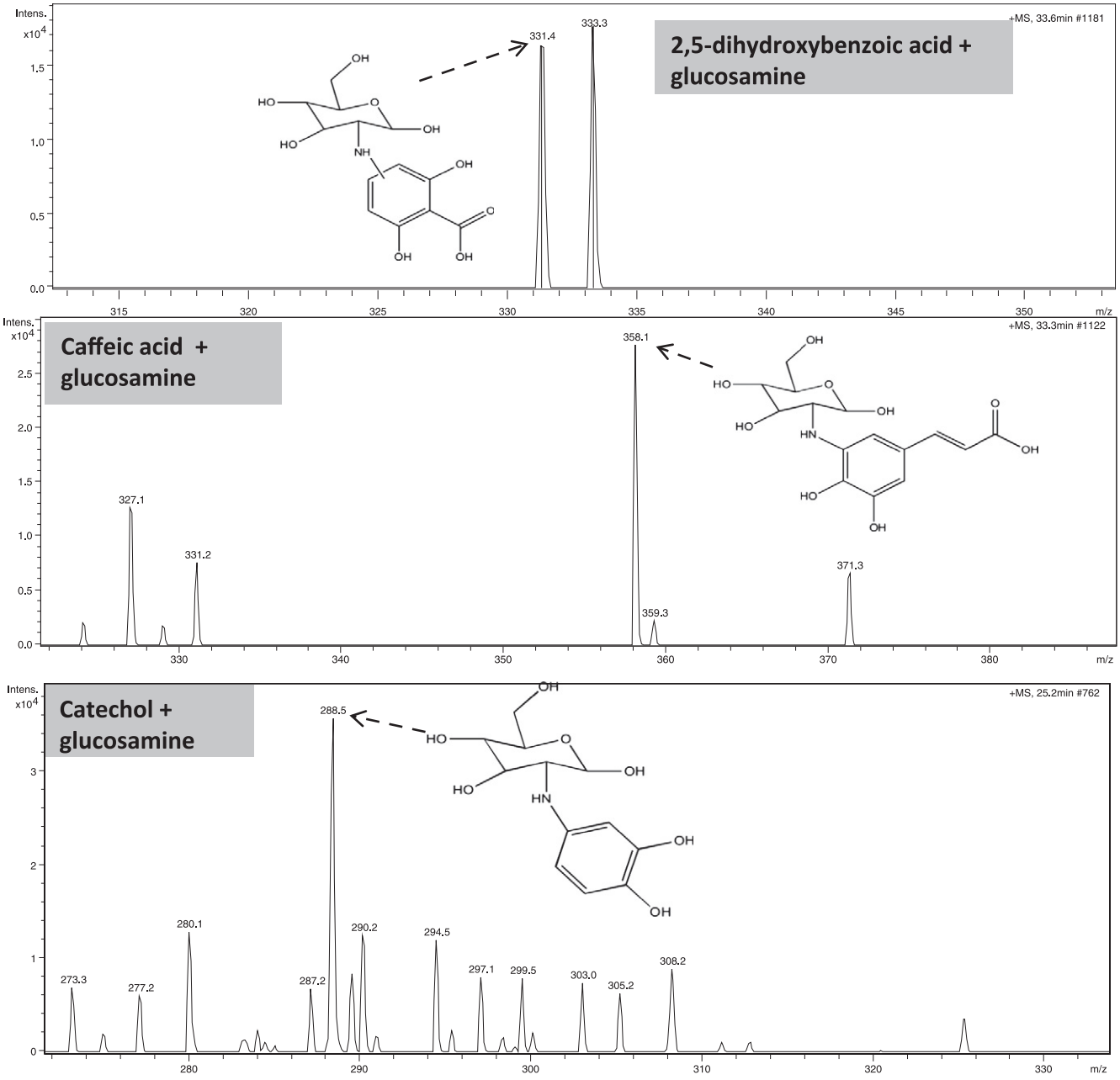

Fig. 2. HPLC–MS chromatograms of the laccase assisted coupling of 2,5-dihydroxybenzoic acid, caffeic acid, and catechol onto glucosamine.

monomeric and oligomeric forms of guaiacol and catechol once they oxidized by laccase [31]. The fact that all phenolic compounds investigated in this study were readily coupled onto glucosamine agrees with previous observations that hydroxyl groups on the benzene ring are *ortho* or *para* directing and facilitates coupling in this way [35–37]. Consequently, molecules with free C-5 position cross-couple through 5–5 linkages due to stability of C–C bonds [38] as speculated for catechol.

FTIR was used to study the coupling of glucosamine to the different phenolic compounds (Fig. 3). The characteristic NH_2 groups were detected within the range 3000–3500 cm^{-1} wavelengths, C–O–H peak around 1060 cm^{-1}, C–O signals between 1000 and 1200 cm^{-1} as well as the strong N–H stretch at 1560–1650 cm^{-1} which is in agreement with previous observations [39,40]. Upon the enzymatic coupling of glucosamine to 2,5-dihydroxybenzoic acid (Fig. 3A), caffeic acid (Fig. 3B), and catechol (Fig. 3C), the intensity of the characteristic NH_2 groups between 3000–3500 cm^{-1} decreased in all reaction mixtures although a characteristic spectra depending on the phenolic compound used was observed. All spectra exhibited broad peaks in the range of 3450–3400 cm^{-1}. The peaks were also assigned to an OH stretching overlapping in the same region with the NH_2 group. The FTIR chromatograms provided further proof that indeed there was a decrease in $-NH_2$ group indicating reaction between the phenolic compounds with glucosamine.

3.2. Laccase mediated grafting of phenolic molecules onto chitosan

Laccase-catalyzed oxidation of phenolic compounds and their subsequent non-enzymatic reaction with the primary amino groups of various chitosans with different molecular weights (15, 50, and 300 kDa) and glucosamine as a model substrate was

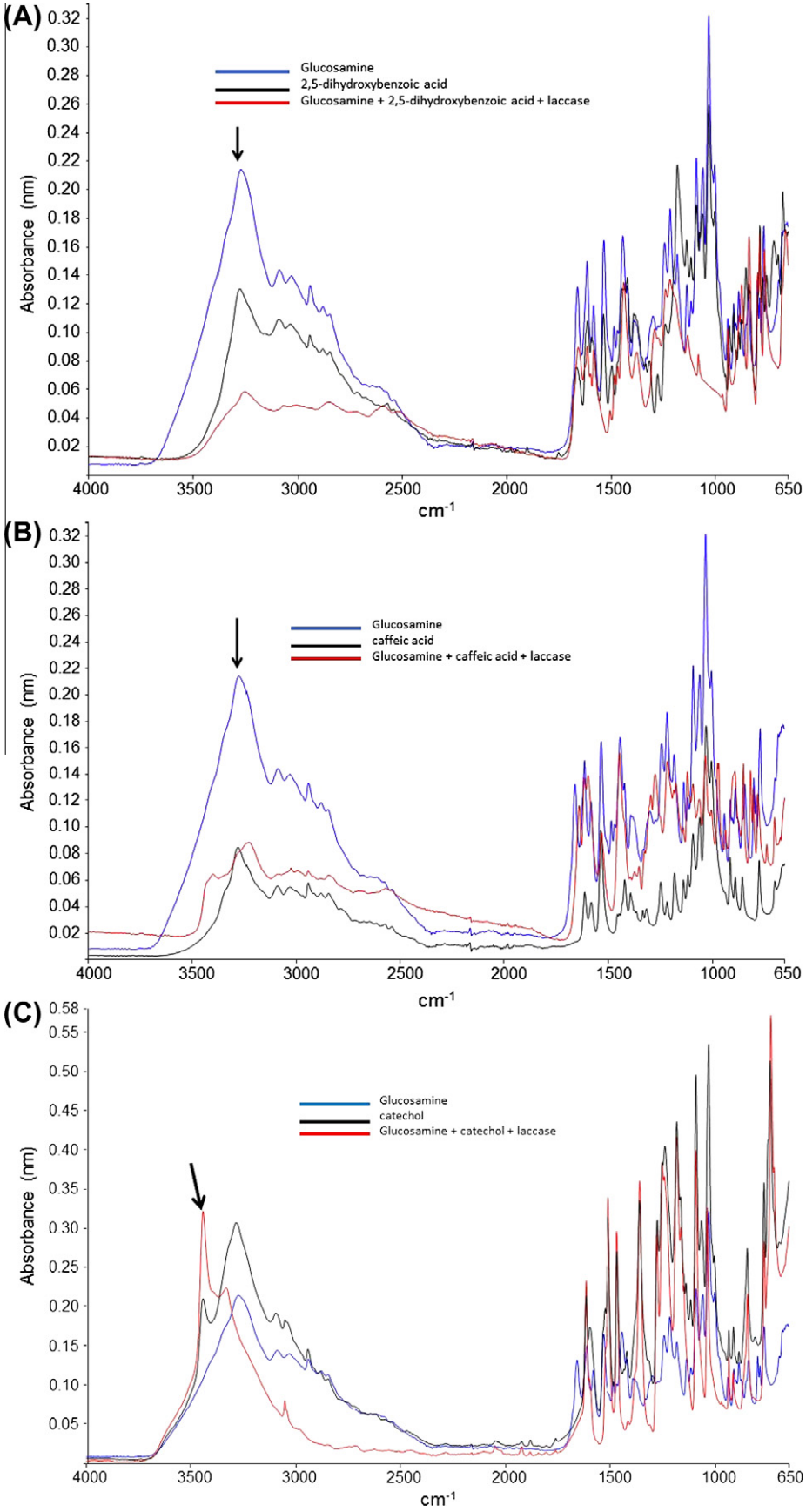

Fig. 3. FTIR spectra of different coupling of (A) 2,5-dihydroxybenzoic acid, (B) caffeic acid, and (C) catechol onto glucosamine. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

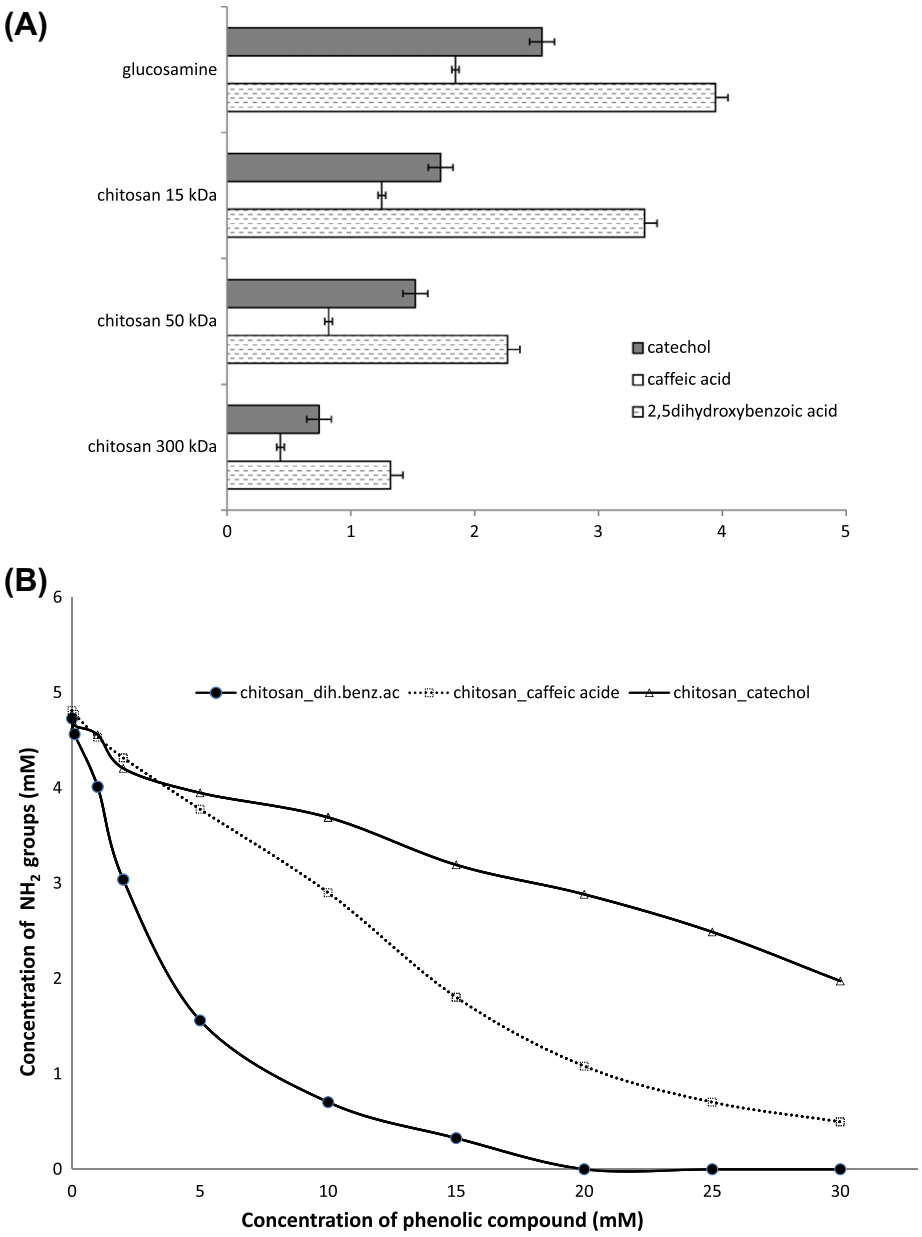

Fig. 4. Consumption of $-NH_2$ groups during enzymatic coupling of phenolic molecules to glucosamine and chitosan with different molecular weights (A) and the effect of the concentration of the phenolic molecules in coupling to 15 kDa chitosan (B). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

investigated. Quantification of $-NH_2$ groups on chitosan was used to determine coupling efficiencies (Fig. 4A). The number of $-NH_2$ groups consumed during grafting decreased with increasing MW of chitosans. The ability to enzymatically graft functional molecules onto chitosan is quite interesting since current chemical based methods employ multistep organic synthesis processes [41] and initiator systems (i.e., azo compounds and peroxides), which require relatively high reaction temperature [42,43]. Therefore, the enzymatic functionalization of chitosan represents a new mild approach for grafting functional molecules onto chitosan for many biomedical applications. Some studies have previously employed tyrosinases [44–49] or peroxidases [50–52] for the coupling of phenolic compounds onto chitosan. However, there is hardly any study using laccase for this purpose or has shown any mechanistic insights into the coupling of phenolics onto glucosamine.

The observed general decrease in consumed NH_2 groups (Fig. 4A) with increasing chitosan molecular weight [although initial concentration of NH_2 groups was the same (4.0 mM) in all samples] may be attributed to the increasing difficulty in accessing NH_2 groups inside the chitosan polymer by the oxidized phenolics. Restricted diffusion of molecules through the polymer network has been reported by Ruiz et al. [53] and may also be responsible for lowering effective functionalization of high molecular weight chitosan in this study. Since the highest consumption of NH_2 groups was obtained with the 15 kDa chitosan, a dose–response curve to determine the concentration of phenolic compound required to couple phenolic compound onto chitosan was investigated. As shown in Fig. 4B, 2,5-dihydroxybenzoic acid showed the most effective coupling effect to chitosan as compared with catechol and caffeic acid. A concentration of 20 mM 2,5-dihydroxybenzoic acid was required to completely occupy all the available NH_2 groups while both catechol and caffeic acid required more than 30 mM (Fig. 4B) for the same concentration of chitosan. Interestingly, FTIR analysis showed the decrease in signals

corresponding to NH_2 group on the chitosan (Fig. 5A and B). This confirms the spectra obtained with glucosamine. In a similar study, Pak et al. [54] also demonstrated that by increasing the degree of deacetylation of chitosan also significantly increased its radical reactivity. However, this study also shows that for successful coupling an excess of phenolic compounds is needed, probably to compensate for the intra-molecular coupling of some of the oxidized phenolic compounds.

3.3. Iron-chelating ability of phenolic functionalized chitosan

The iron binding capacity of functionalized chitosan was estimated by measuring the inhibition of complex formation between ferrozine and Fe^{2+}. This reaction can be monitored based on a change in color of blue dye-chrome azurol sulphonate solution to red. The iron binding capacity of all chitosans alone was generally lower than 3% from a 5 mM solution. However, all the functionalized chitosan (15, 50, and 300 kDa) showed a remarkable increase in their respective iron sequestrating capacity to up to 70% of the iron from the solution as compared with chitosan alone (Fig. 6). Previous iron chelation studies with phenolic compounds found varying iron binding capacity of phenolics bearing catechol and galloyl moiety and no iron binding capacity with vanillic acid, syringic acid, and ferulic acid [54]. The functionalized 15 kDa chitosan carrying hydroxy and carboxyl groups showed highest iron binding capacity of 70% (Fig. 6). Although generally the iron binding capacity of chitosan functionalized with catechol was lower than that obtained with 2,5-dyhydroxybenonzoic (Fig. 6), the observed 15-fold increase in the iron-chelating capacity as compared with chitosan only is still remarkable. The observed lower iron binding capacity of catechol as compared with hydroxyl–carboxyl functionalized chitosans may also be attributed to previous observed steric obstruction of the 3′,4′-catechol structure for flavonoids substituted with carbohydrate moieties [55,56] and the cross-linking effects of catechol. Nevertheless, catechol containing polyphenols like catechin, an abundant catecholate-type polyphenol present in green tea, have been shown to effectively chelate iron [57,58]. Although different mechanisms implying interaction of iron with amine, carbonyl, and hydroxyl groups of chitosans have been proposed [59] which may also apply to this study, grafting of catechol and hydroxybenzoic acids functional groups onto chitosan dramatically increased iron chelation. The observed general increase in iron binding capacity for both catechol and 2,5-dihydroxybenzoic acid functionalized further demonstrates that functionalized chitosan can play a very important role in metal chelating processes. This study therefore demonstrates the ability synthesizing hydroxy–carboxy chitosan for enhanced iron chelation.

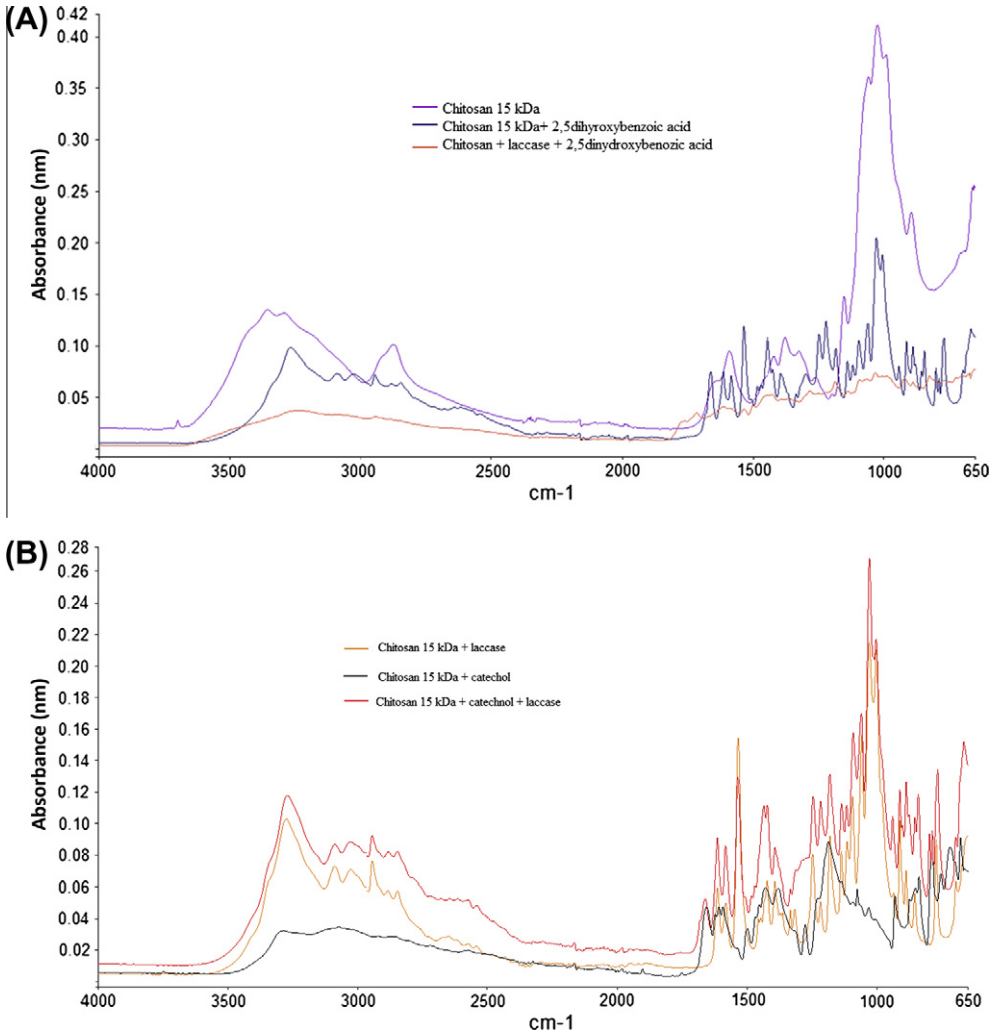

Fig. 5. FTIR spectra of 2,5-dihydroxybenzoic acid and catechol functionalized chitosan. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

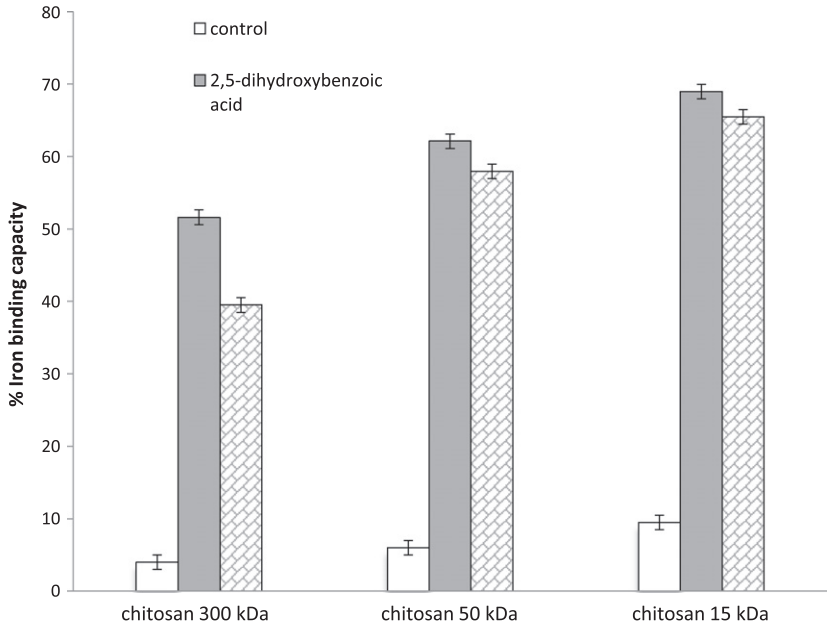

Fig. 6. Chelation of Fe^{2+} by various chitosans and their phenolic functionalized derivatives. Data are an average of three independent reactions.

3.4. Synthesis of phenolic functionalized chitosan microspheres

Many different strategies for the synthesis of chitosan-based microspheres have been developed as summarized by Agnihotri et al. [20]. These chitosan-based microspheres are currently being extensively investigated for drug delivery vehicles for controlled release and targeting studies of almost all class of bioactive molecules in medicine. In this study, a simple drop method into an alkaline solution while stirring was adopted. The formation of microspheres is based on the insoluble property of chitosan in alkaline pH and determined by the size of chitosan drop. Different characteristic structures were obtained using a 15 kDa chitosan catechol and hydroxy–carboxic functionalized polymer as shown in Fig. 6. The microspheres obtained had an average size between 0.8 and 2 μM. Interestingly, the catechol and hydroxy–carboxic seem to also participate as cross-linkers producing characteristic microspheres (Fig. 7). However, further design studies are needed since some of the formed microspheres collapsed during the drying phase.

3.5. Iron-chelating ability of synthesized microspheres

The ability to chelate iron of catechol and 2,5-dihydroxybenzoic acid functionalized microspheres was investigated. Since the gastrointestinal pH fluctuates between 1.8 (stomach pH) and 7.4, the ability of the polymers to chelate iron was assessed in the respective pH range. Both catechol and 2,5-dihydroxybenzoic acid functionalized microspheres had a marginal loss at pH 7.4 (<4.5%)

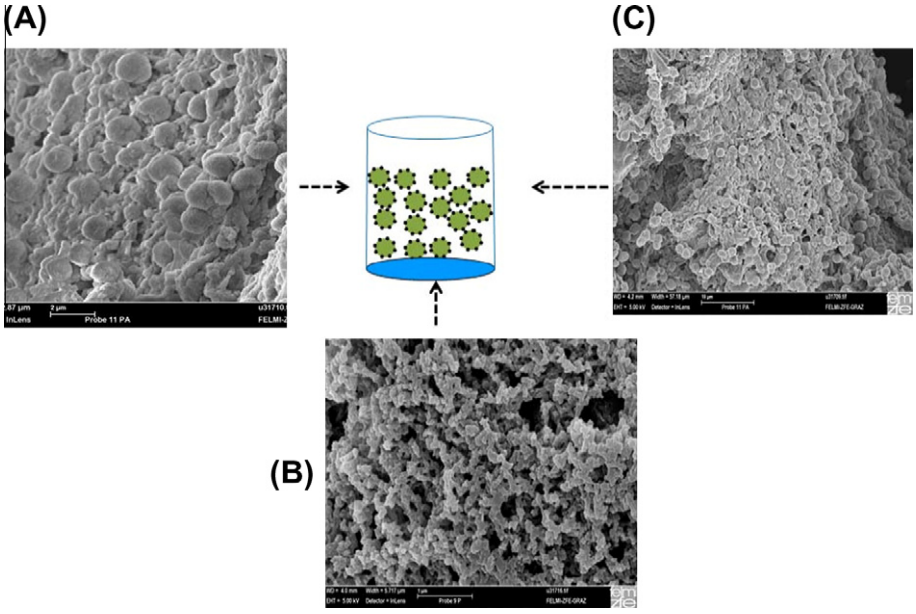

Fig. 7. SEM of microspheres formed from 15 kDa chitosan functionalized with 2,5-dihydroxybenzoic acid (A), cafeic acid (B), and catechol (C) (micrometer bar: 2 μm for A, 10 μm for B, 1 μm for C). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

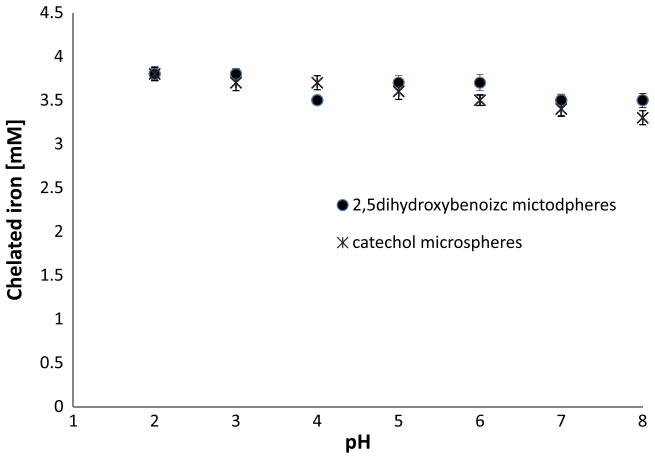

Fig. 8. Chelation of Fe^{2+} (3.8 mM initial concentration) by 2,5-dihydroxybenzoic acid and catechol functionalized microspheres. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of the 3.8 mM originally chelated iron at pH 2 (Fig. 8). This study therefore shows the great potential of using catechol and hydroxy–carboxic functionalized chitosan microspheres aimed at increasing both its iron-chelating ability and stabilizing the complex along the fluctuating gastrointestinal pH environment. This is because it is known that although ligands containing the catechol moieties possess a high affinity for iron, the binding of cations by catechol is pH sensitivity. Functionalization of chitosan with carboxylated molecules was shown to overcome its pH-sensitive [34] which may be particularly true for these catechol and hydroxy–carboxic functionalized chitosans.

4. Conclusions

Catechol and hydroxyl–carboxyl group containing phenolics were successfully grafted onto glucosamine as confirmed by HPLC–MS. Further incubation of the respective phenolic compounds in the presence of laccase with the different chitosans led to a decrease in NH_2 groups and subsequent exponential increase in iron-chelating ability. Interestingly, the use of these phenolic compounds required no additional cross-linker as they seem to participate as cross-linkers producing characteristic microspheres. This work opens a new strategy which can be explored for the development of a new generation of chitosan-based iron-chelating therapeutic polymers.

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