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A novel method for immobilization of chitosan onto nonporous glass beads through a 1,3-thiazolidine linker

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Abstract

A new method for the surface modification of nonporous glass beads (average diameter, 6 μm), which characterized by formation of a 1,3-thiazolidine ring between L-cysteine linkers on the glass bead and reducing ends of chitosan, has been developed. γ-Aminopropyltriethoxysilane (APES)-treated glass bead was first subjected to condensation with an L-cysteine derivative, L-4-carboxy-3-formyl-2,2-dimethylthiazolidine (CFMT), in the presence of water-soluble carbodiimide hydrochloride (WSC) and HOBt. After deprotection by diluted hydrochloric acid, the glass beads with activated cysteine linkers on the surface were treated with reducing chitosan in aqueous acetic acid solution at room temperature. The maximum content of chitosan immobilized on the glass beads estimated by acid-hydrolysis and subsequent glucosamine analysis by Svennerholm method after was 0.73% (w/w). This was obtained by using chitosan having an average molecular weight of 14 kD. Model reactions of the cysteine derivatives with reducing chitosan were also performed and the product was examined by IR and NMR spectroscopy to verify the linkage between cysteine and chitosan.

Keywords: Chitosan; Surface modification; Glass beads

1. Introduction

Chitosan is a β-(1,4)-linked polysaccharide of D-glucosamine derived from chitin, one of the most abundant polysaccharides. Although chitosan is a polymer with unique chemical and biological properties, it seems to be lack of mechanical strength. In order to reinforce chitosan polymer, various composite materials have been manufactured and reported to exhibit novel or unique properties of their biological and physical functions [1,2]. Such successful findings stimulated us to develop new composite materials on the basis from a different standpoint. Our attention focused on the surface modification with chitosan, because modification of surface of materials does not change their mechanical properties. We chose glass beads, which are well known to be preferable as a supporting material, owing to having narrow size dispersion, stronger mechanical strength, and low cost [3]. Although a number of new useful materials have been prepared through grafting synthetic polymers onto the surface of glass beads [4–6], little are known to fix chitosan onto the surface of glass beads.

Since chitosan is not soluble in ordinary solvents but aqueous acetic acid, reactions that achievable in an aqueous media such as the Schiff's reaction and N-acylation are usually utilized for binding chitosan on the surface of solids. Indeed, we have successfully immobilized chitosan onto cotton fibers and nonporous glass beads through Schiff's base formation [7,8]. However, the presence of a lot of the amino groups in the chitosan chain resulted in limited amount of the introductions. Because 1,2-amino thiol group of cysteine is more reactive nucleophile to aldehyde derivatives than amino group itself, cysteine has been used recently in the orthogonal ligation of unprotected peptide segments through a specific thiazolidine formation [9,10], and in the synthesis of a 2-substituted-4-thiazolidinecarboxylic acid type of prodrugs in combination with various sugars [11–13] or others [14,15]. On the other hand, it has been reported that a chemical degradation of chitosan with nitrous acid gives chitosan derivatives having aldehyde groups at their reducing ends [16–21]. On the basis of these

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fundamental researches, we undertook to examine the immobilization of chitosan through cysteine linkers present on the surface of glass beads. In this paper, we describe the results in detail.

2. Experimental

2.1. Materials

Chitosan (deacetylation degree of 0.80, average molecular weight of 350 kD) was purchased from Aldrich Chemical Co., Inc. (Milwaukee Wi, USA) and treated twice with 40% aqueous sodium hydroxide at 121 °C before use. The degree of deacetylation of chitosan was determined to be 98% from the NMR data recorded by Bruker ASX-300. Nonporous glass beads EMB-10 (2–10 μm diameters) obtained from Toshiba Barotynee Co., Ltd. (Tokyo, Japan) were fractionated in water by a sedimentation method to remove particles with diameter sizes over 8 μm or below 3 μm. The sizes of the selected particles were confirmed by scanning electron microscopy (SEM). L-4-Carboxy-3-formyl-2,2-dimethylthiazolidine (CFMT) was prepared by a reported method [22]. γ-Aminopropyltriethoxysilane (APES) was purchased from Shin-Etsu Chemical Industries, Co., Ltd. (Tokyo, Japan). 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride, water-soluble carbodiimide hydrochloride (WSC), and 1-hydroxy-1*H*-benzotriazole, monohydrate (HOBt) were purchased from Peptide Institute, Inc. (Osaka, Japan) and Wako Pure Chemical Industries Ltd. (Tokyo, Japan), respectively.

2.2. General methods

^{13}C NMR spectra were recorded for solutions in D_2O with a Bruker ASX-300 spectrometer at 75.48 MHz. Infrared spectra (IR) were recorded on a HORIBA FT-210 spectrophotometer using a potassium bromide pellet. UV–visible spectra were recorded with a HITACHI U-2000A spectrometer. Gel permeation chromatography (GPC) was carried out in a column (27 mm × 1500 mm) of Sephadex G-10 using water as the eluent. The molecular weight of chitosan was estimated by viscometry using the Mark–Houwink equation [23].

2.3. Depolymerization of chitosan by sodium nitrite

2.3.1. Water insoluble chitosan

According to our previous report [20], aqueous solutions (40 ml) that contained $NaNO_2$, amounts ranging from 0.18 to 0.83 mmol, were added dropwise to the solutions of chitosan (2.0 g, 12.5 mmol in glucosamine units) in 2% aqueous acetic acid (100 ml) at 0 °C with stirring. The solutions were stirred for 5 h at room temperature and neutralized with 2.5% aqueous ammonia. The precipitates were collected by centrifugation at 10,000 rpm for 10 min and the residues were washed sufficiently with distilled water. The resulting chitosan were subjected to the determinations of molecular weight and used for preparations of chitosan-modified glass beads (B-5).

2.3.2. Water soluble chitosan

Similar depolymerization of chitosan (2.0 g, 12.5 mmol in glucosamine units) was carried out by using $NaNO_2$ (0.7 g, 10.1 mmol). The resulting solution was concentrated and dried for 15 min. Ethanol (20 ml) was added to the residue, and the insoluble material was collected by filtration to give water soluble chitosan (0.8 g). The product was redissolved in distilled water (10 ml) and subjected to the coupling reaction with L-cysteine freshly.

2.4. Preparation of chitosan-modified glass beads (B-5)

Nonporous glass beads B (50 g) with average diameter of 6 μm were dispersed in distilled water (100 ml) with stirring and then heated to 50 °C. An aqueous solution of NaOH (1 M, 100 ml) was added to the suspension and kept at 50 °C for 50 min. The suspension was poured into distilled water (800 ml) and the glass beads were filtered, washed with distilled water until neutral, and dried in a vacuum oven at 120 °C for 24 h. The activated glass beads B-1 were treated with APES in anhydrous toluene under reflux in the same way as our previous method [8], giving APES-treated glass beads B-2. The presence of amino groups on the surface of B-2 was confirmed by a purple color changing after treatment with ninhydrin reagent.

A suspension containing HOBt (0.43 g, 2.8 mmol), CFMT (0.30 g, 1.73 mmol), the APES-treated glass beads B-2 (5.0 g), dry 1-methyl-2-pyrrolidone (NMP, 6 ml), and *N*,*N*-dimethylformamide (DMF, 24 ml) was stirred at −20 °C. Then a solution of WSC (0.5 g, 2.6 mmol) in chloroform (5 ml) was added dropwise into the suspension with continuous stirring at −20 °C for 30 min and then stirred at room temperature for 12 h. The precipitate was filtered, washed sequentially four times with DMF (50 ml), twice with chloroform (50 ml), and twice with methanol (50 ml), giving dimethylthiazolidine-treated glass beads B-3.

The resulting glass beads B-3 (5.0 g) were immersed in 0.5 M hydrochloric acid (100 ml) with gently stirring for 24 h, filtered, washed with distilled water to neutral, and dried in vacuum at 50 °C for 24 h, giving cysteine-treated glass beads B-4. This step was monitored by Ellman method [24] using aqueous 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB).

Cysteine-treated glass beads B-4 (2.0 g) were added into a solution of water insoluble chitosan prepared as mentioned above (1.0 g) in 2% aqueous acetic acid (50 ml) and slowly stirred under nitrogen at room temperature for 3 h, filtered, washed with distilled water, and dried, giving chitosan-modified glass beads B-5. The chitosan-modified glass beads were treated with 4 M hydrochloric acid at 100 °C for

16 h to hydrolyze chitosan into glucosamine, and then the amount of glucosamine was determined by a modified Svennerholm method [25].

2.5. Coupling reaction of water soluble chitosan with L-cysteine

L-Cysteine hydrochloride monohydrate (2.5 g, 14.2 mmol) was added to the solution of water soluble chitosan under nitrogen, and the mixture was stirred for 7 h at room temperature. Ethanol (10 ml) and pyridine (2 ml) were added to the mixture and allowed to stand at 4 °C overnight, giving two separated liquid phases. The lower phase with green color was subjected to GPC to remove small molecular materials, giving cysteine coupled-chitosan Cys-C; ν_{max} (KBr): 3348, 3278, 2972, 1589, 1405, 1315, 1072, 1045, 883, 804 cm^{-1}; ^{13}C NMR (D_2O) δ 172.7, 98.1, 97.9, 75.1, 72.1, 70.5, 70.0, 60.7, 57.8, 56.1, 39.0, 25.0, 17.2.

3. Results and discussion

3.1. Depolymerization of chitosan by sodium nitrite

Our strategy for immobilization of chitosan on to glass beads through L-cysteine linker required a chitosan derivative with an aldehyde functional group. It is well known that degradation of D-glucosamine derivatives by using nitrous acid results in a rearrangement at the C-2 position to give 2,5-anhydro-D-mannose derivatives having an aldehyde group at C-1 [16–21]. Application of this reaction to chitosan, a polymer of D-glucosamine, results in a depolymerization of chitosan to give new reducing terminals as shown in Scheme 1. The reducing chitosan derivative (**a**) with an aldehyde group on its end and inactive chitosan (**b**) were produced in the mixture. In the present study, two precipitators, ammonia and ethanol were used to precipitate the products. 2.5% aqueous ammonia was used as a precipitator of the mixture of sodium nitrite degradation gave the water insoluble chitosan. Such chitosan was redissolved in aqueous acetic acid and subjected to the determinations of molecular weight. As summarized in the Fig. 1, the average molecule weights of chitosan were

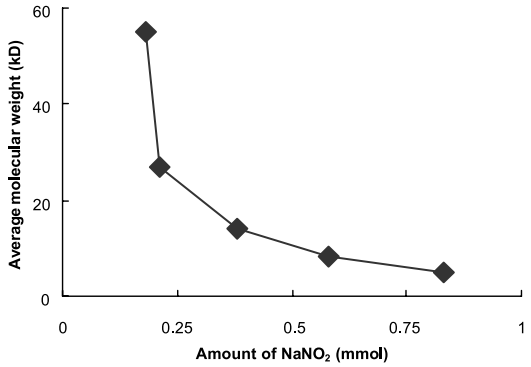
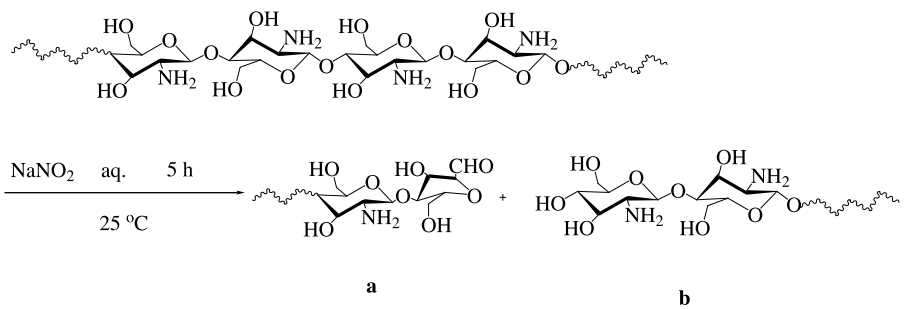


Fig. 1. The average molecular weights of chitosan and the amounts of sodium nitrite used to depolymerization of chitosan (12.5 mmol glucosamine units).

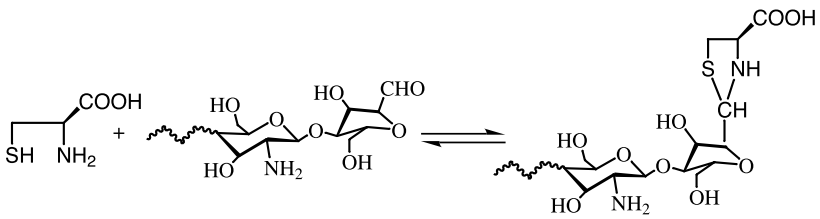
significantly decreased by using small amount of sodium nitrite at first, and were trended to approaching a limit about 5.0 kD subsequently when more sodium nitrite was used. These results showed that it was possible to control the average molecule weights of chitosan by using different concentrations of sodium nitrite. The chitosan derivatives with average molecular weight from 5.0 to 55.0 kD, thus obtained, and those were used for the next immobilization onto glass beads. Furthermore, water soluble chitosan with smaller molecular weight was collected by an ethanol precipitation and used for the next structural elucidation of coupling reaction between cysteine and depolymerized chitosan.

3.2. Coupling reaction between cysteine and the water soluble chitosan

Because some thiazolidine prodrugs of L-cysteine possess radioprotective properties and protective effects against hepatotoxicity, many reports about condensation of L-cysteine with aldose saccharides, such as lactose, maltose, glucose, and mannose, have been published recently [11–13]. Thus, we next examined the possibility that reducing chitosan can be linked to the cysteine residues present on the surface of glass beads. At first, a model experiment was conducted as summarized in Scheme 2. The water soluble chitosan having reducing aldehyde terminals (**a**) were reacted with L-cysteine hydrochloride at room temperature, and then the products were separated by



Scheme 1.



water soluble chitosan (a) **Cys-C**

Scheme 2.

ethanol precipitation and preparative GPC method, giving cysteine-linked chitosan, Cys-C.

Fig. 2 showed FTIR spectra of the Cys-C and the coupling product (Cys-G) between L-cysteine and a reducing derivative of D-Glucosamine.[1] In contrast to the FTIR spectrum of chitosan (Fig. 2(A)), the FTIR spectra of Cys-C (Fig. 2(B)) and Cys-G (Fig. 2(C)) showed strong characteristic absorptions at 1405 cm^{-1} and 1045 cm^{-1}, suggesting that the chitosan with aldehyde group end was reacted with cysteine. The peaks at 1405 cm^{-1} and 1045 cm^{-1} were assignable to the C–OH bending (in plane) and –C–O stretching absorption of carboxyl groups, respectively. The Cys-C (Fig. 2(B)) also showed a characteristic peak at 1072 cm^{-1}, assignable to –C–O stretching adsorption of hydroxyl groups on sugar rings in the chitosan chain [26–28]. These spectral data strongly indicated that almost cysteine residues were coupled with the aldehyde groups on the chitosan ends. In the ^{13}C NMR spectrum of Cys-C (Fig. 3), peaks ranging between 60 and 110 ppm were arisen from sugar carbons [29,30] and peaks at 172, 56, and 39 ppm were attributed to cysteine residues.

3.3. Preparation of chitosan-modified glass beads B-5

On the basis of the results mentioned above, we attempted to immobilize chitosan onto the surface of glass beads using cysteine as the linker. The immobilization of chitosan was carried out in five steps, as summarized in Scheme 3. Similar to the our previous research [8], nonporous glass beads were first etched with 1 M aqueous sodium hydroxide to activate the surface, and then treated with a silane-coupling reagent (APES), to aminated the surface. The introduction of L-cysteine was performed through a condensation of CFMT with amino groups on the glass beads B-2. CFMT was described to be an unusual protective system of L-cysteine in an earlier report [22]. This protective group can be removed by 0.5 M hydrochloric acid to avoid damage on the structure of glass beads that bring by the acids in higher concentration. Therefore, CFMT was prepared in our laboratory and used for the condensation of cysteine and silane-coupled glass

[1] Cys-G was the product compound of the coupling reaction between cysteine and the reducing derivative of glucosamine after treatment with $NaNO_2$. The coupling reaction was performed according to the previous reports [11–13].

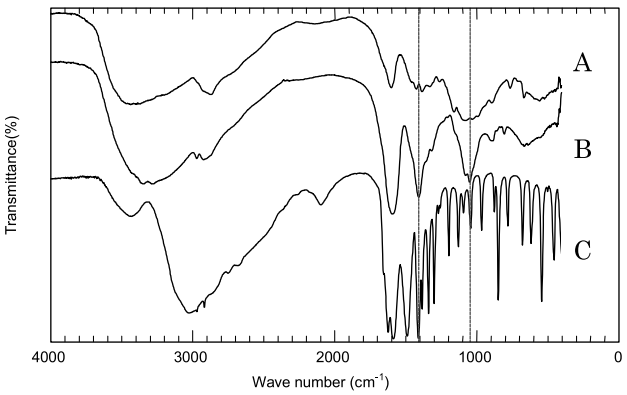


Fig. 2. IR spectra of samples: (A) chitosan; (B) Cys-C; (C) Cys-G.

beads B-2. Hydrochloric acid (0.5 M) was used for the deprotection of the modified beads B-3, giving activated cysteine-linked glass beads B-4. The presence of the cysteine groups introduced on the surface of glass beads B-4 was confirmed by a reaction with DTNB solution, showing the color change to yellow. Thereafter, chitosan-modified glass beads B-5 were obtained by coupling cysteine linker on the glass beads B-4 with reducing termini of chitosan at room temperature. Various preparations of chitosan with average molecule weights ranging from 2.5 to 55 kD were applied. The respective chitosan content of the chitosan-modified glass beads B-5 was determined by glucosamine analysis after

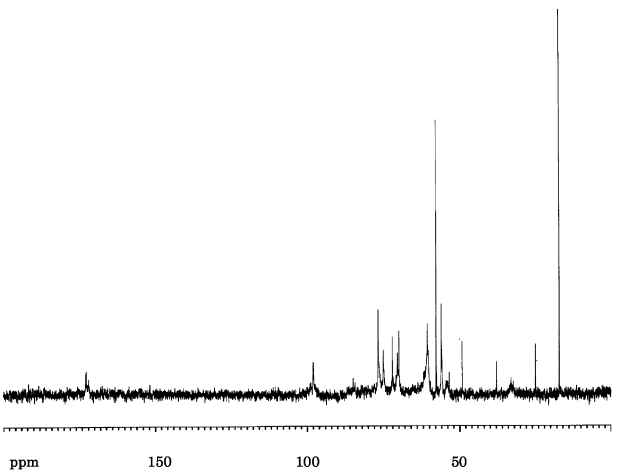
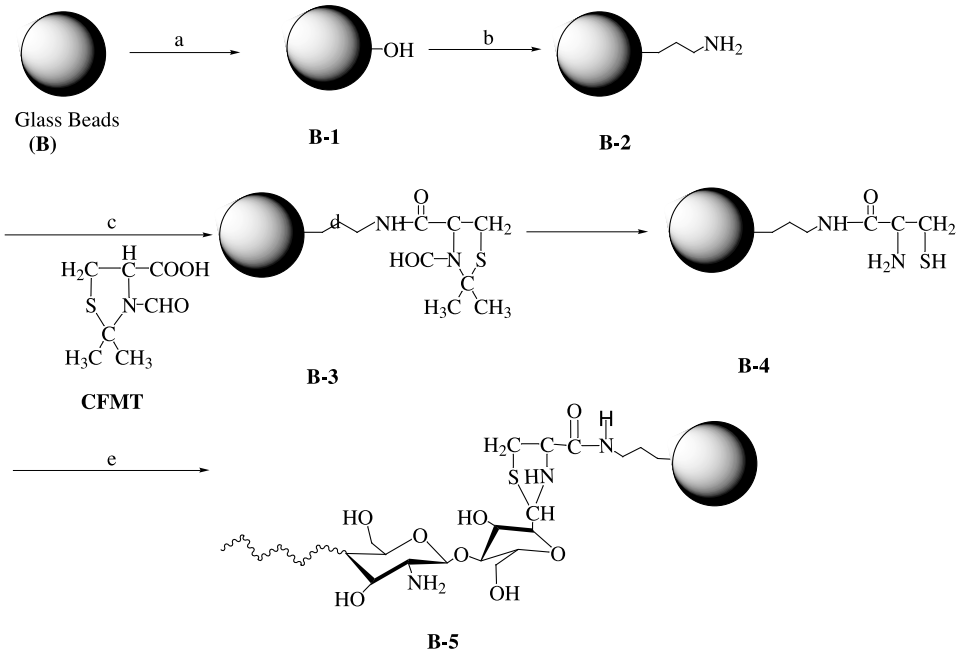


Fig. 3. ^{13}C NMR spectrum of Cys-C for solution in D_2O.



Scheme 3. Regents and conditions: (a) 1 M NaOH, 50 °C, 50 min; (b) APES, toluene, 80 °C, 24 h; (c) DMF, WSC, HOBt, room temperature, 12 h; (d) 0.5 M HCl, room temperature, 24 h; (e) water insoluble chitosan, 2% (v/v) aqueous acetic acid solution, room temperature, 3 h.

acidhydrolysis. As showed in the Table 1, the amount of chitosan immobilized on the surface of glass beads B-5 was distinctly dependent on the molecular weights of used chitosan. The use of chitosan with an average molecular weight of 14.0 kD gave a maximum content of 0.73%, whereas, the use of larger chitosan with average molecule weight, 55 kD gave a limited chitosan content of 0.04%. The lower coupling efficiency in this study is interpreted as the lower concentration of the reactive aldehyde group in the reaction mixture and/or steric hindrance by the chitosan chain. That is, some active ends of the larger chitosan chains may be sequestered inside of the polymer molecules in higher probability than the smaller chitosan chains.

4. Conclusions

L-cysteine linker can be introduced on the aminated surface of glass beads through a condensation using L-4-carboxy-3-formyl-2,2-dimethylthiazolidine (CFMT) as a protection substituent of 1,2-amino thiol groups on L-cysteine. Chitosan with a reducing end that has aldehyde group obtained by depolymerization with sodium nitrite can be efficiently immobilized on the chemically modified surface of glass beads through a coupling reaction between the reducing end of chitosan chain and L-cysteine linker on the surface of glass beads. The amount of chitosan introduced on beads was influenced by the average molecular weight of used chitosan. A largest chitosan content of 0.73% (w/w) was given when the chitosan used having 14.0 kD of average molecular weight was used. Moreover, a model experiment using L-cysteine hydrochloride instead of L-cysteine linker on the glass beads were investigated. The IR and NMR spectral data indicated that the couple between cysteine and the reducing end of the chitosan derivative, which arose from the treatment with sodium nitrite, was clearly obtained.

Table 1
The amount of chitosan immobilized on the surface of glass beads and the average molecular weight of chitosan

Amount of sodium nitrite vs. chitosan (mol/mol glucosamine units)	Average molecular weight[a] (kD)	Yield of the water insoluble chitosan (%)	content of chitosan on the beads[b] % (w/w)
0.014	55.0	72	0.04
0.017	27.0	66	0.66
0.030	14.0	57	0.73
0.046	8.2	51	0.68
0.066	5.0	45	0.12

[a] The molecular weight of chitosan was determinated by the Mark–Houwink equation as: $[\eta] = 8.93 \times 10^{-4} \times M_v^{0.71}$.
[b] The content of chitosan on the beads was determined by the modification from the Svennerholm method.

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