A chitosan–arginine conjugate as a novel anticoagulation biomaterial

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Chitosan (CS) was modified with arginine using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) as coupling agents. FTIR and ^{13}C NMR spectra showed that arginine was chemically coupled to CS to form a chitosan–arginine conjugate (CS–ArgC). The substitution degree of arginine in CS estimated from elemental analysis was 20.1%. The circular dichroism spectra indicated that the incorporation of arginine significantly altered the conformation of thrombin; while no obvious variation in the conformation of thrombin was observed with the addition of CS. The anticoagulation activity of glucose aldehyde crosslinked CS–ArgC and CS membranes was evaluated by assaying prothrombin time (PT), thrombin time (TT) and activated partial thromboplastin time (APTT). The APTT of CS–ArgC membrane was prolonged two times as that of CS counterpart, suggesting that the CS–ArgC is a promising candidate as an anticoagulation biomaterial.
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1. Introduction

The past decades witnessed a remarkable increase in the application of chitosan (CS) as drug delivery carriers [1, 2], tissue engineering scaffolds [3, 4], cell microcapsules [5, 6] due to its low production costs, biodegradability and biocompatibility. Although CS is a promising biomedical material, it tends to induce thrombosis and embolization upon implantation *in vivo*, and this needs to be addressed prior to ultimate clinical applications [7]. In view of the above facts, researchers have made an effort to improve the blood compatibility of CS by chemical modifications. From the point of view of imitating heparin, researchers have prepared sulfated CSs and carboxybutyrylated hydroxyethyl chitosan sulfates. These heparin-like derivatives were found to exhibit an improved anticoagulation activity [8–10]. Partially *N*-acylated CS derivatives synthesized by Lee *et al.* were shown to be blood compatible [11]. In addition, surface modification methods, such as coupling with polyethylene glycol [12], complexing with anionic polysaccharide were also adopted to enhance the anticoagulation activity of CS [13]. It has been verified that almost all blood coagulation enzymes are serine proteases, and the activities of these proteases are controlled by plasmatic serine protease inhibitors (serpins) whose inhibitory mechanism involves the formation of an initial non-covalent complex followed by intermediate substrate-like cleavage of the serpin reactive center loop and the formation of a tight serpin-protease covalent complex [14, 15]. The serine protease recognizes a specific region $(P_1\text{–}P'_1)$ in the serpin molecule, and arginine is just located at the P_1 position. Furthermore, the metabolized product of arginine, NO, was demonstrated to inhibit platelet activation by increasing the concentration of platelet cyclic 3,5-guanosine [16, 17]. Therefore, arginine could be an interesting new pharmacological anticoagulant.

Inspired by the aforementioned research work we, for the first time, have prepared a chitosan–arginine conjugate (CS–ArgC) by chemically coupling arginine to CS to explore the possibility of CS–ArgC as an anticoagulation biomaterial.

2. Experimental procedure

2.1. Materials

Chitosan (Mv = 5000, degree of deacetylation = 80%) was supplied by Haihui Bioengineering Com. (Qingdao, China). Arginine was purchased from Sigma Chemical Company (St. Louis. MO) USA. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), *N*-hydroxy-succinimide (NHS) and *N,N,N′,N′*-tetramethylethylenediamine (TEMED) were purchased from Aldrich Com. PT, TT and APTT kits were supplied by Dade Behring Com. Glucose aldehyde was prepared in our laboratory as reported in our previous publication [18].

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2.2. Preparation of CS–ArgC

Chitosan was modified with arginine using EDC and NHS as coupling agents. 0.3 g of arginine and 1 g of CS were dissolved in 50 ml of *N*,*N*,*N'*,*N'*-tetramethylethylenediamine (TEMED)/HCl buffer solution (pH 4.7). NHS (0.14 g) and EDC (0.6 g) were added to this solution and magnetically stirred for 24 h at room temperature. The resultant product was purified using a dialysis tube (2000 MWCO) against distilled water for five days, followed by lyophilization. The substitution degree of arginine in CS was estimated by elemental analysis (C: 45.06%, N: 12.47%, H: 6.83%) to be 20.1%.

2.3. FTIR analysis

FTIR spectra of the samples were measured on a Bio-Rad FTS 135 spectrophotometer.

2.4. NMR determination

100 mg of CS–ArgC was dissolved in 1 ml of D_2O/H_2O (1 : 1 v/v) solution, and ^{13}C NMR was performed on a 400 MHz (Varian UNITY plus 400) spectrometer. The NMR spectra of CS and arginine were recorded in the same manner.

2.5. Circular dichroism (CD)

CD spectra were recorded on a J-7150 spectropolarimeter in a range of wavelengths of 320–180 nm at 25 °C. Measurements were performed with a speed of 20 nm/min and a resolution of 0.5 nm. The spectra were corrected by subtraction of PBS buffer spectrum, and three spectra were accumulated and averaged for each sample. The final concentration of thrombin in solution was 1.11×10^{-5} M. The CD signal was converted to molar ellipticity [θ], deg · $cm^2/dmol$.

2.6. Preparation of crosslinked CS–ArgC and CS membranes

About 200 mg of purified CS–ArgC or CS was dissolved in 20 ml of double distilled water to form a homogenous solution, followed by the addition of 10 mg of glucose aldehyde. The mixture was poured into a freshly cleaned plastic plate and maintained in a vacuum oven at 50 °C overnight for membrane formation. The obtained membranes were cut into rectangular shape (5 mm × 10 mm), and immersed in 0.9% sodium chloride for 24 h. The treated samples were ready for anticoagulation assay.

2.7. *In vitro* anticoagulation assay [19]

Human whole blood (20 ml) collected from a healthy volunteer was mixed with an aqueous solution of anhydrous D-glucose (0.13 M), sodium citrate trihydrate (75 mM) and citric acid monohydrate (0.4 mM). Then the mixture was centrifuged for 10 min to separate the blood corpuscles, and the platelet poor plasma (PPP) obtained was used for anticoagulation test. For each assay, the crosslinked CS–ArgC membranes were placed into test tubes containing 100 μl of PPP.

Prothrombin time (PT) assay: The above test tubes were incubated at 37 °C for 1 min, and then 200 μl of Thromborel[R] S was added. Meanwhile, the starting time at which the fibrous substance appeared was recorded and the clotting time was calculated.

Thrombin time (TT) assay: In a similar way, the test tubes were incubated at 37 °C for 1 min, followed by addition of 200 μl of Test-Thromborel[R] reagent, and the clotting time was recorded. Activated partial thromboplastin time (APTT) assay: an actin-activated cephaloplastin reagent (100 μl) was added into the test tube, and incubated at 37 °C for 5 min. Then 0.025 M $CaCl_2$ solution (100 μl) was added, and the clotting time was recorded. All the experiments were repeated in triplicate and a mean value was calculated.

3. Results and discussion

Fig. 1 shows the FTIR spectra of arginine, chitosan and CS–ArgC. For arginine, the absorption band at 1630 cm^{-1} is assigned to the guanido group, and the band at 1419 cm^{-1} is attributed to COO^- symmetric bending. The C–C–N asymmetric bending and COO^- scissioning modes are found at 1139 and 770 cm^{-1}, respectively [20, 21]. Chitosan exhibits the characteristic bands of NH_2 scissioning vibration at 1657 cm^{-1}, carbonyl asymmetric stretching vibration at 1564 cm^{-1}, and C–O stretching vibrations of the pyranose ring at 1071–1029 cm^{-1} [22]. Comparing the bands of CS–ArgC (Fig. 1(c)) with those of chitosan and arginine, one can see that there appear characteristic bands of arginine at 1630 and 1413 cm^{-1}, and also an ether bond stretching vibrations of the pyranose of CS in conjugate. The new band at 1526 cm^{-1} is most likely due to an amide bond linking chitosan and arginine. To further verify the formation of CS–ArgC, ^{13}C NMR spectra of chitosan, arginine and CS–ArgC were

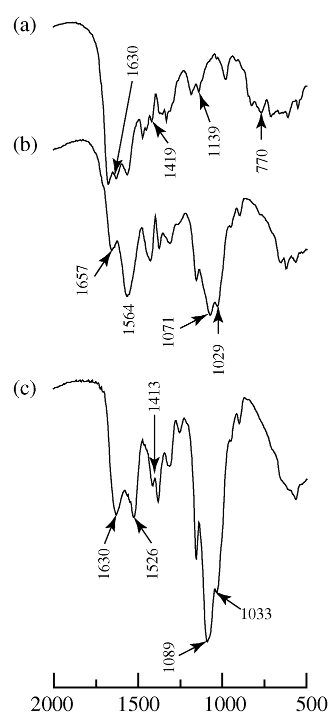

Figure 1 FTIR spectra of arginine (a), CS (b) and CS–ArgC (c).

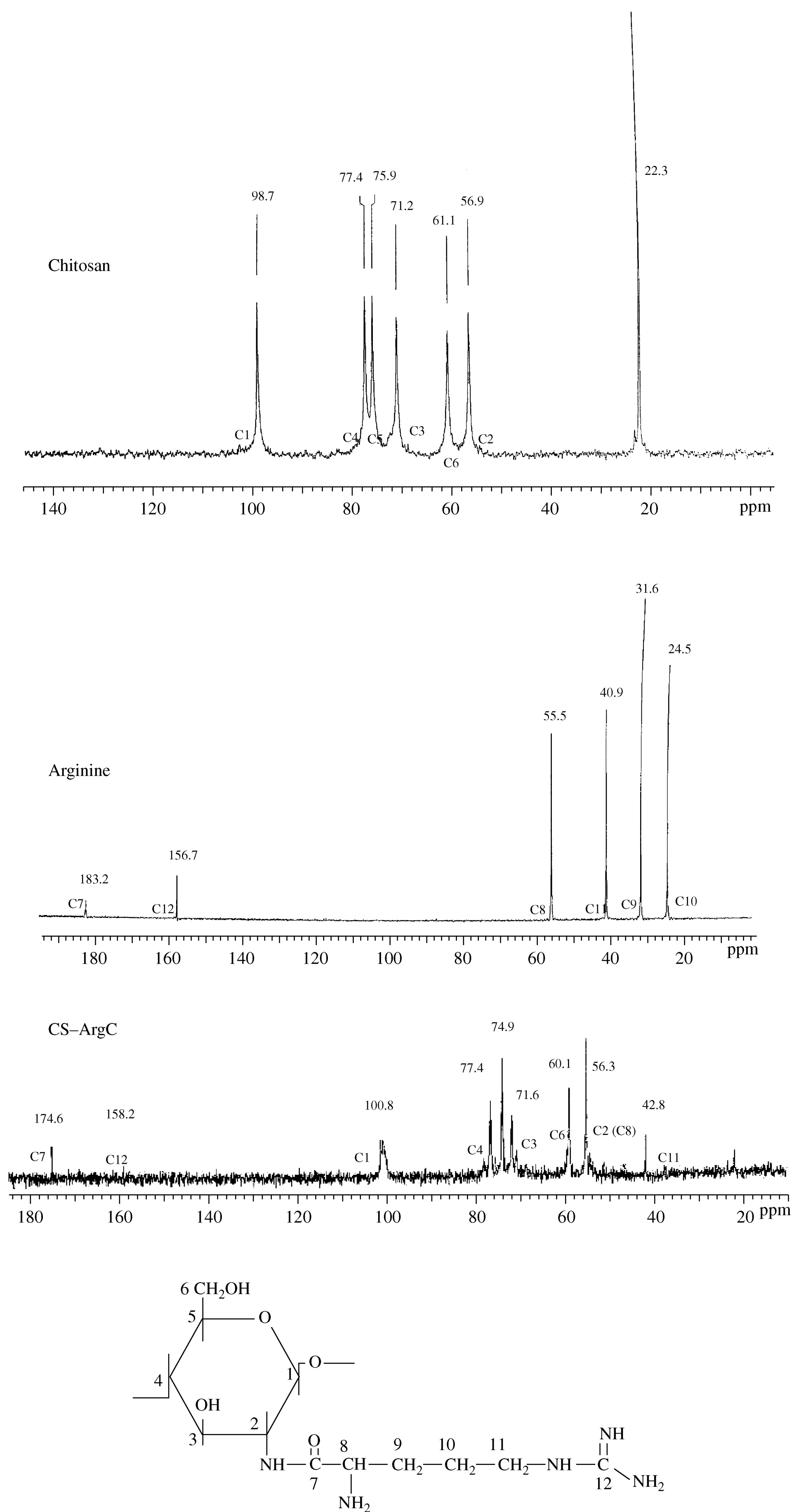

Figure 2 ^{13}C NMR spectra of chitosan, arginine and CS–ArgC.

recorded (Fig. 2). As shown in the figure, some characteristic peaks of arginine overlap with those of CS in CS–ArgC. Nonetheless, there indeed exist intense methylene (δ 42.8 ppm) and weak guanido (158.2 ppm) bands of arginine in CS–ArgC, indicating the generation of CS–Agrc.

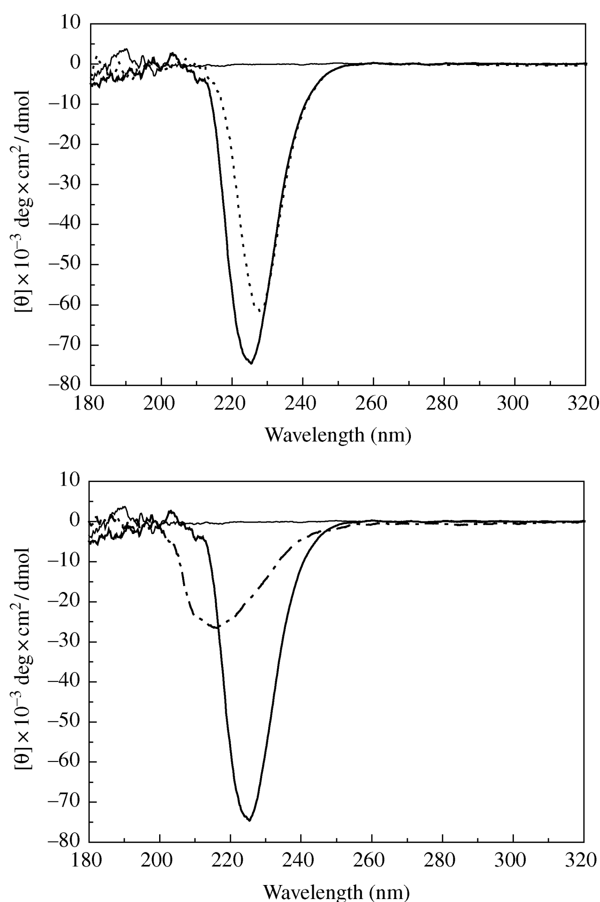

Figure 3 CD spectra of thrombin (solid line), mixtures of thrombin/chitosan (dot line) and CS–ArgC/thrombin (dash dot line) in PBS solution.

Thrombin is a multifunctional serine protease, and plays a central role in thrombosis and hemostasis by regulating the blood coagulation cascade and platelet activation processes. One anticoagulant is capable of specially recognizing and binding to thrombin, and consequently inhibits the occurrence of coagulation. This interaction will be reflected by the variation in the conformation of thrombin. Fig. 3 demonstrates the CD spectra of thrombin, chitosan/thrombin and CS–ArgC/thrombin mixtures in PBS. For pristine thrombin, a negative CD signal around 226 nm is observed, an indication of β-sheet conformation. The band is merely slightly altered with the addition of CS, implying a weak interaction between chitosan and thrombin. In contrast, an obvious alteration of CD spectrum occurs after mixing CS–ArgC with thrombin. The conformation has been transformed from β-sheet to random coil, which suggests that there exists a strong interaction between CS–ArgC and thrombin.

Herein, what we are concerned about is the anticoagulation activity of CS–ArgC membranes. So CS–ArgC was crosslinked with glucose aldehyde taking into account the non-cytotoxicity of this crosslinker [18]. The anticoagulation activity of crosslinked CS and CS–ArgC membranes is listed in Table I. APTT increases up to 66.0 s, twice that of CS, indicating the enhancement of anticoagulation activity. While the TT and PT for CS–ArgC are only moderately prolonged when compared to CS, implying that the variations in TT and PT are not sensitive to the incorporation of arginine with 20.1% substitution degree. But an evident increase of APTT demonstrates that the CS–ArgC is of good blood compatibility. Studies on the effect of CS molecular weight and substitution degree on anticoagulation activity are ongoing in our laboratory.

TABLE I Anticoagulation activity of CS and CS–ArgC

Sample	TT (s)	PT (s)	APTT (s)
CS–Arg	19.0 ± 2.3	24.6 ± 2.1	66.0 ± 3.0
CS	17.7 ± 1.3	15.1 ± 1.9	33.1 ± 2.4

The values obtained are the results of three measurements, and are denoted as the mean values ± SD.

In summary, this work demonstrated that a CS–Argc could be synthesized using EDC and NHS as coupling agents. Coupling arginine to chitosan can significantly prolong APTT, which was considered to be as a result of the strong interaction between CS–ArgC and thrombin. The CS–ArgC has potential as a new anticoagulation biomaterial.

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