Preparation and Anticoagulant Property of Phosphorylcholine-Terminated *o*-Benzoylchitosan Derivative

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ABSTRACT: To improve the solubility in organic solvent of chitosan, *o*-benzoylchitosan was synthesized by acylation with benzoyl chloride and then used in the reaction with 2-chloro-1,3,2-dioxaphosphospholane (COP) to prepare phosphorylcholine-terminated *o*-benzoylchitosan (PC-BCS). PC-BCS had a structure with a phospholipid polar group characterized by FTIR and ^{1}H NMR. PC-BCS had been poly-substituted by a PC group, which made PC-BCS viscous liquid. The anticoagulant properties of PC-BCS were evaluated by means of blood-clotting and platelet adhesion assay. The blood-clotting assay indicated that PC-BCS could prolong the blood-clotting process. Platelet adhesion assay showed that PC-BCS could effectively inhibit the platelet adhesion and activation. © 2003 Wiley Periodicals, Inc. J Appl Polym Sci 88: 489–493, 2003

Key words: chitosan; *o*-benzoylchitosan; phosphorycholine; anticoagulant property

INTRODUCTION

Chitosan, (1,4)-2-amine-2-deoxy-β-D-glucan, is a natural polymer generally obtained by extensive deacetylation of chitin isolated from crustacean shells.[1] Due to its special biological, chemical, and physical properties, a number of biomedical applications of chitosan have been studied.[2,3] Chitosan with reactive primary amino and hydroxyl groups has the powerful possibility of chemical derivatization to make itself compatible with blood. For example, the sulfated *N*-hexanoyl and *N*-octanoylchitosan all have the antithrombogenic function.[4,5]

Phosphorylcholine (PC) is a zwitterionic head group that is present in high concentration in the outer leaflet of the lipid bilayer of red blood cell membranes (the major component of the extracellular side of cell membranes) in the form of the phospholipid, phosphatidylcholine.[6] The synthesized materials containing PC groups have been proved to be able to resist the adsorption of proteins and have the blood compatibility. Therefore, PC-based synthesized materials have great potential applications in biomedical fields.[7–10] 2-Methacryloyloxyethyl phosphorylcholine (MPC) is widely selected to prepare PC-based materials.[11–14] Typically, the PC functional group can be incorporated into synthetic polymers either by direct surface grafting or as a component part of a monomer species utilized in copolymer synthesis. Different from the previous methods, the PC group was directly bonded to the *o*-benzoylchtosan, a kind of chitosan derivatives, and a novel *o*-benzoylchtosan with PC groups (PC-BCS) was prepared.

The PC-BCS synthesized is expected to have the anticoagulant property. This article first reports the method that the PC functional group is directly bonded to the reactive hydrogen on *o*-benzoylchitosan molecule to prepare PC-based materials. This article also provides an alternative strategy for the development of blood-compatible chitosan.

EXPERIMENTAL

Chitosan powder was obtained from Lian Yun Gang Biologicals (China). Its viscosity average molecular weight was 5.2×10^5g/mol, while the degree of deacetylation was 90%. Methanesulphonic acid (AR), benzoyl chloride (AR), acetone (AR), tetrahydrofuran (THF), acetonitrile, triethylamine (TEA), and ethyl ether were purified by the conventional way. 2-Chloro-2-oxo-1,3,2-dioxaphospholane (COP) was synthesized according to the Edmundson method of and purified by distillation under reduced pressure, 98°C/1 mm Hg.

Synthesis of *o*-benzoylchitosan

Into a flask were placed 1.0 g chitosan powder and 5.2 ml methanesulphonic acid. The mixture was stirred at

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0°C for 20 min to produce homogeneous solution; 1.1 ml benzoyl chloride was added dropwise and the total mixture was stirred at 0–5°C for 2 h. The obtained gel was stored at −20°C overnight. The thawed product was precipitated by pouring into 150 ml acetone; the precipitate was twice dipped in 150 ml acetone to remove the rudimental reaction reagent. The entire operation was carried out at 0°C to inhibit the product from degradation. The 1.4 g gray–white powder was then received after being filtered off and dried.[15]

Synthesis of PC-BCS

Preparation of COP-BCS

Into a 250 ml three-necked flask equipped with a dropping funnel, a thermometer, and a drying tube, 2.66 g (0.01 mol) benzoylchitosan, 3.03 g (0.03 mol) TEA, 50 ml DMF, and 50 ml dry THF were placed. After the solution was cooled at −20°C, 4.27 g COP in 30 ml dry THF were added dropwise to the stirred solution over a period of 1 h. The temperature of the reaction was maintained at −20 to −30°C for 3 h. Then the precipitate in the reaction mixture, which was triethylammonium chloride, was filtered off. After the filtrate was distilled under reduced pressure, COP-BCS was obtained.

Preparation of PC-BCS

TEA was used in the ring opening reaction to prepare the PC-BCS, which could simplify the synthesis process compared with the traditional method, in which trimethylamine was used in the ring opening reaction to prepare the phosphorylcholine group.[12]

The preparation process was as follows: into a 100 ml flask were placed 5.0 g of the above COP-BCS, 2.6 g TEA, 50 ml DMF, and 50 ml dry acetonitrile. The mixture was stirred at 60°C for 24 h. The reaction mixture obtained was distilled under reduced pressure to remove acetonitrile. To obtain the pure PC-BCS, the crude PC-BCS was washed with dry THF and dry ethyl ether, respectively, for more than three times and the solvent was removed under reduced pressure until no impurity was present in the ^{1}H NMR spectra. The pure PC-BCS showed viscous liquid and could be soluble in water.

Characterization and measurements

FTIR analyses were performed on a Nicolet 170 SX spectrometer. ^{1}H NMR spectra were obtained by a Bruker DPX 300 instrument. Elemental analyses were carried on a CHN—O—RAPID. The surface topography was analyzed by scanning electron spectroscopy (SEM) on an X-650 Scanning Electron Microanalyzer.

Blood-clotting assay

A blood-clotting assay was developed in order to assess to the effectiveness of the polymer coatings in delaying the blood-clotting process. When platelets are contacted to the glass surface, factor XII will adhere to the surface and activate, thereby initiating the clotting cascade via the intrinsic pathway. For this assay, 75 mm × 12 mm glass tubes were used, sonicated in DCM and dried prior to use.

The concentration of PC-BCS solution was 10 mg/ml. In order to coat a glass tube, the tube was filled with the PC-BCS solution, left for 1 min before the solution was poured out of the tube, while rotating the tube between the fingers. The tube was then placed on a spiramix to allow the polymer coating to dry evenly within the tube for several hours. Ten samples of each sample were prepared in each case and compared to 10 uncoated controls.

Fresh-frozen platelet-rich plasma (100 μl) was added to each sample tube and allowed to stand for 2–3 min at 37°C before the addition of 100 μl 0.025 M $CaCl_2$ solution, also at 37°C, at which point a stopwatch was started. The mouth of the tube was then sealed tightly with parafilm and the tube was tilted backward and forward while still partially submerged in the water bath to maintain the temperature at 37°C. The time at the point when a fibrin clot was visible was noted.

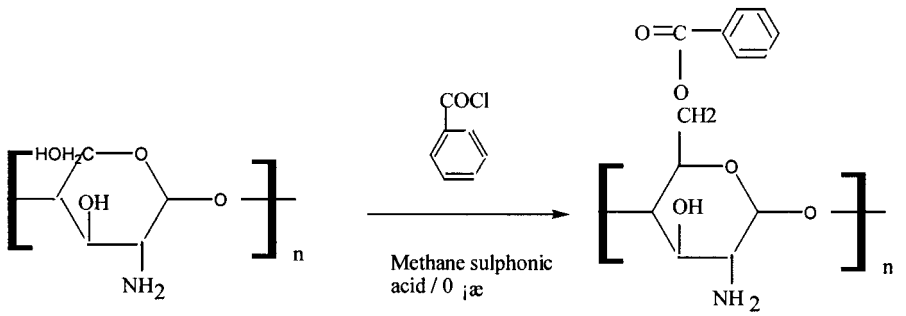
Platelet adhesion

Platelet contact SEM studies were also carried out on the samples in order to check the activation of the platelets, fibrin and clots, etc. Polyurethane (PU) sheets of 10 mm diameter were cleaned thoroughly by wiping with DCM and then dipped into the PC-BCS solution at a concentration of 10 mg/ml. These were allowed to dry overnight at room temperature. The PU sheets were contacted with 4 ml of platelet-rich plasma for 3 h. After washing in PBS, they are fixed using 2% gluteraldehyde solution for 30 min, washed again, then sequentially immersed into 50%, 60%, 70%, 80%, 90%, and 100% ethanol solution and dried in a desiccator. It was sputter-coated with gold before being imaged by SEM.

RESULTS AND DISCUSSION

Synthesis of *o*-benzoylchitosan

Compared to the FTIR spectrum of chitosan, four new absorption bands at 1,718, 781, 714, and 1,202 cm^{-1} appear in the spectrum of benzoylchitosan; 1,718 cm^{-1} is attributed to the flex libration of carbonyl group in benzoyl group, 781 and 714 cm^{-1} are attributed to the swing libration of benzene, and 1,202 cm^{-1} belongs to ν(C–O). These indicate that benzoylation takes place



Scheme 1 Synthesis reaction of o-benzoylchitosan.

on the hydroxyl group of chitosan and *o*-benzoylchitosan is obtained (ref. 15 reported the same result). The reaction scheme is shown in Scheme 1.

Chitosan is initially treated with benzoyl chloride to improve its solubility in organic solvent. Chitosan is soluble in DMF and DMSO by introducing hydrophobic benzoyl group. *o*-Benzoylchitosan is soluble in water, which is attributed to the decrease of intermolecular interaction such as hydrogen bond.

Synthesis of PC-BCS

There are three reactive hydrogen atoms in benzoylchitosan, as can be seen in Scheme 1. These reactive hydrogen atoms are supposed to be able to react with COP as shown in Scheme 2.

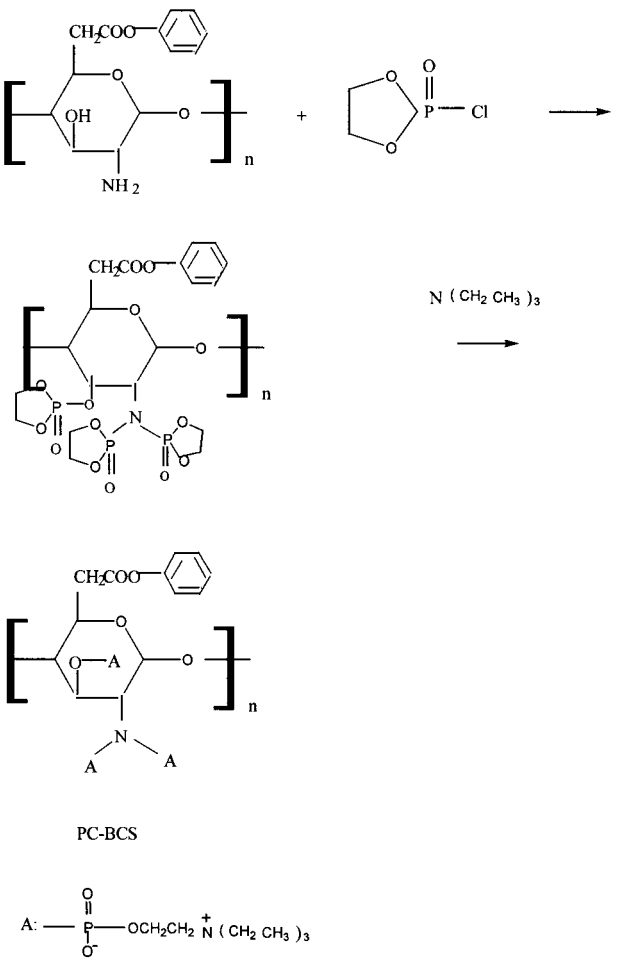
In the FTIR spectrum of PC-BCS (Fig. 1), the absorptions at 1,718, 781, and 714 cm^{-1} are characteristic absorptions of the benzoyl group; the absorptions at 1,298, 1,175, and 1,080 cm^{-1} are attributed to the P—O—CH_2 stretch; the absorption at 967 cm^{-1} is a characteristic absorption of —$N^+(C_2H_5)_3$; and the peak at 1,460 cm^{-1} is a characteristic absorption of N—P. FTIF analysis indicates that the PC group does bond to the benzoylchitosan.

Figure 2 shows the spectrum of ^1H NMR (D_2O) of PC-BCS. The analysis result of ^1H NMR is shown in Table I. From this result, it can be seen that there is obviously a PC group in PC-BCS, but it is surprising to find that only few characteristic peaks of *o*-benzoylchitosan appear in the spectrum of ^1H NMR of PC-BCS, while there are obviously characteristic absorptions of benzoylchitosan in FTIR spectrum of PC-BCS. This phenomenon is probably attributed to the result of the intercoincidence of hydrogen in *o*-benzoylchitosan macromolecule.

From the above discussion, we can confirm that the PC group has been bonded to the *o*-benzoylchitosan and PC-BCS has the same structure as shown in Scheme 2. Different from the form of *o*-benzoylchitosan, PC-BCS shows viscous liquid. This may be due to the reactive hydrogens in *o*-benzoylchitosan having been polysubstituted (substitution degree is about 2.5) by PC groups characterized by element analysis result (data not shown).

Anticoagulant property

Although PC-BCS can be soluble in water, the dissolution of PC-BCS is much slower compared with that of low molecules. The usual blood-clotting assay is used to evaluate the blood compatibility of PC-BCS. The data in Table II clearly shows that the clotting



Scheme 2 Synthesis reaction of PC-BCS.

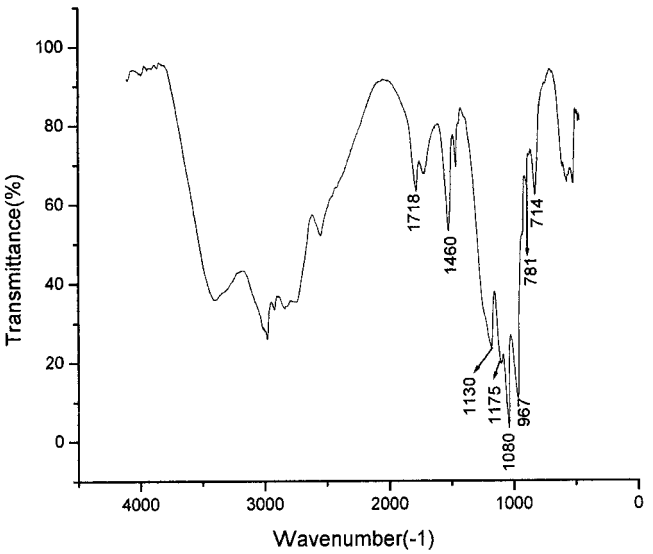


Figure 1 FTIR spectrum of PC-BCS.

time extends for the samples coated with PC-BCS and indicates that PC-BCS has antithrobogenicity.

Scanning electron micrographs of sheets after being contacted with platelet-rich plasma are shown in Figure 3, which obviously shows that the uncoated PU sheet surface is covered with cellar matter, but there is almost no sign of any cellular matter on the PU sheet coated with PC-BCS. This collection of *in vitro* data shows that PC-BCS can inhibit platelet adhesion and activation.

When the blood encounters foreign materials, the contact without exception leads to a non–self-reaction with blood coagulation and inflammatory response. Thus, it is of considerable interest to surface technology to improve the biocompatibility of materials. The present result indicates that a significant control over

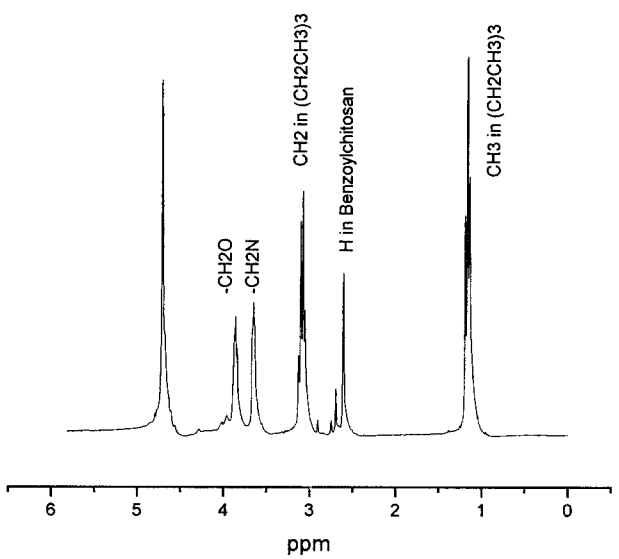


Figure 2 ^{1}H NMR spectrum of PC-BCS.

TABLE I
^{1}H NMR Assignment of PC-BCS

δ	Assignment
1.15–1.25	$-CH_3$, 9H in $N^+(C_2H_5)_3$
3.06–3.13	$-CH_2$, 6H in $N^+(C_2H_5)_3$
3.61–3.66	$-CH_2N$, 2H
3.80–3.85	$-CH_2O$, 2H
2.4–2.8, 3.61, 4.2–4.5	H in *o*-benzoylchitosan

platelet adhesion and activation has been achieved in PU substrate by the surface coated with PC-BCS, a nontoxic and biocompatible polymer.

Both blood-clotting and platelet adhesion assay make sure that PC-BCS has excellent antithrombogenicity. From the above discussion, it can be concluded that PC-BCS has obviously antithrombogenic function and excellent blood compatibility.

The function of PC groups bonded on *o*-benzoylchitosan on reduction of platelet adhesion is somewhat similar to the synthetic polymer incorporated with the PC functional group.[11–14] These antithrombogenic characteristics of the PC-BCS may be due to the phosphorylcholine group, a typical phospholipid polar group located at the outer cell membrane, and the water structure surrounding the polar group maintaining a similar structure to that of bulk water.[16] It may be the above reason that results in good antithrombogenic effect of PC-BCS.

Although it is inevitable that coated PC-BCS will be eluted from specimens for the slow water solubility of PC-BCS, the antithrombogenic effect is still observed, which further indicates that PC-BCS has blood compatibility.

CONCLUSIONS

o-Benzoylchitosan was synthesized by acylation with benzoyl chloride. Benzoylchitosan was soluble in organic solvent, then used in the reaction with 2-chloro-1,3,2-dioxaphosphospholane to prepare phosphorylcholine-terminated *o*-benzoylchitosan. PC-BCS has a structure with a phospholipid polar group and has been polysubstituted by PC groups, which makes PC-BCS show viscous liquid. The blood-clotting assay shows that PC-BCS prolongs the blood-clotting process. Platelet adhesion assay indicates that PC-BCS can effectively inhibit the platelet adhesion and activation.

TABLE II
Clotting Times for Uncoated and Coated Sample Tubes

Sample (n = 10)	Clotting time (s)
Uncoated PU	205.3
PU (coated with PC-BCS)	406.1

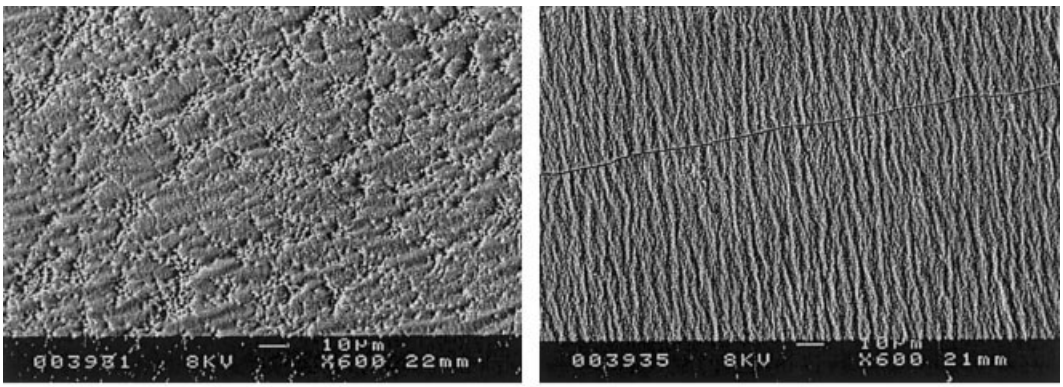


Figure 3 SEM images of sheets after contact with platelet-rich plasma: (a) uncoated PU sheet; (b) PU sheet coated with PC-BCS.

This article is the first to report that the reactive hydrogen in *o*-benzoylchitosan can be used to prepare phosphorylcholine-based materials. This article provides an alternative strategy for the development of nonthrombogenic property of chitosan and a novel method to prepare polymer with PC functional group. PC-BCS with excellent antithrombogenic property has potential applications in several diverse biomedicine fields.

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