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ORIGINAL CONTRIBUTION

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Preparation and characterization of a novel Si-containing crosslinkable *O*-butyrylchitosan

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Abstract *O*-Butyrylchitosan is a water-soluble chitosan derivative which is not sufficiently durable for use as an antithrombogenic coating. A crosslinkable*O*-butyrylchitosan was studied to improve its stability and durability. The process included preparation of methoxysilyl-terminated butyrylchitosan (MOS-OCS) by Michael addition of (3-acryloxypropyl)trimethoxymethylsilane to the amino groups of *O*-butyrylchitosan and Si-containing crosslinkable butyrylchitosan was obtained by hydrolysis and condensation of methoxysilyl groups in the MOS-OCS. The mechanical properties of crosslinkable butyrylchitosan films were investigated by mechanical testing. The film with 20 mol% (3-acryloxypropyl)trimethoxymethylsilane as crosslinking agent had good mechanical characteristics. All the samples made were colorless, flexible films, with hydrogel structure. Blood-clotting and platelet adhesion assay confirmed that Si-containing crosslinkable butyrylchitosan had good antithrombogenic properties.

Keywords *O*-Butyrylchitosan · (3-Acryloxypropyl)trimethoxy methylsilane · Crosslinking · Antithrombogenic properties

Introduction

With the basics of non-thrombogenic material now defined, the challenge is to further modify the architecture of the polymer to bring about specific properties relevant to a wider range of applications. Surface coating on polymeric materials is an important method for changing surface properties [1, 2, 3, 4, 5], because biocompatibility mainly depends on surface properties [6, 7, 8]. Coating an excellent antithrombogenic biomaterial on the device surface is an effective means of enhancing blood-compatibility.

Chitosan (2-amino-2-deoxy-D-glucan) is a unique polysaccharide derived from chitin [9, 10]. Its hemostatic property has limited its use in blood-contacting material. The chitosan molecule has many reactive groups, which give it great potential to be changed into excellent antithrombogenic biomaterial. For example *N*-hexanoylchitosan and *N*-octanoylchitosan have the blood-compatibility [11, 12].

According to the relationship between material structure and anticoagulant property, we supposed that *O*-butyrylchitosan might have anticoagulant properties. The water-solubility of *O*-butyrylchitosan makes it unsuitable for anticoagulant coating of a substrate, so formation of a three-dimensional cross-linked structure is indispensable. A crosslinkable coating would have additional advantages over existing systems in terms of film stability and the possibility of anchoring the polymer to the substrate [13].

In this paper, a novel crosslinkable *O*-butyrylchitosan was fabricated to improve the stability of the *O*-butyrylchitosan coating. The amount of crosslinking agent was balanced to produce significant improvement in the mechanical characteristics of the *O*-butyrylchitosan, while preventing embrittlement. Blood-clotting and

platelet adhesion experiments were conducted as a preliminary test to verify the blood-compatibility

Experimental

Materials and reagents

Chitosan (food grade) purchased from Lian Yun Gang Biologicals (China). The degree of deacetylation was found to be 90%. The viscosity-average weight was 5.3×10^5. Methanol (AR), methanesulfonic acid (AR) (purchased from Sigma), acetone (AR), butyric anhydride (AR) (purchased from Sigma), (3-acryloxypropyl)trimethoxymethylsilane (AR) (purchased from Sigma) were of analytical grade and were used without further purification.

Preparation of *O*-butyrylchitosan

The *O*-butyrylchitosan was synthesized according to the method reported by Grant et al. [14]. In brief, chitosan powder (2.1 g) was added to methanesulfonic acid (11 mL) and the mixture was stirred at 0 °C for 15 min to furnish a homogeneous solution. Butyric anhydride (20 mL) was added drop-wise and the total mixture stirred at a temperature between 0 °C and 5 °C for 2 h. The obtained gel was stored at −15 °C overnight. The thawed product was precipitated by pouring into acetone (300 mL), filtered, extracted for 18 h with acetone, and the *O*-butyrylchitosan was obtained after vacuum drying.

Preparation of MOS-OCS

MOS-OCS was prepared by Michael addition of (3-acryloxypropyl)trimethoxymethylsilane to amino groups of *O*-butyrylchitosan. In a 100-mL flask materials were added in the order and quantities listed in Table 1, the flask was sealed, and the mixture was stirred with a magnetic stirrer at 30 °C for 20 h. The reaction mixture was concentrated; the thawed product was precipitated by pouring into acetone, filtered, extracted with acetone in a Soxhlet apparatus for 24 h, then dried under vacuum.

Preparation of Si-containing crosslinkable *O*-butyrylchitosan films

O-butyrylchitosan crosslinkable films can be conveniently prepared by casting 10% (*w*/*w*) of MOS-OCS in methanol solution on 10 cm diameter Petri dishes at room temperature. The Petri dishes were weighed accurately and placed at room temperature until their temperature was constant. Adjustment of the concentration and thickness of the casting solution can predetermine film thickness.

Table 1 Raw material and formulae

MOS-OCS S-X	*O*-Butyrylchitosan (g)	(3-Acryloxypropyl) trimethoxymethylsilane (g)	Methanol (mL)
S-10	1.5	0.124	25
S-20	1.5	0.248	25
S-30	1.5	0.372	25

X: (3-Acryloxypropyl)trimethoxymethylsilane content (mol)
S: Silica-containing *O*-butyrylchitosan

Swelling of the hydrogels

To determine the effect of pH on hydrogel swelling, McIlvaine buffer [15] with the same ionic strength, $I = 0.5$ mol L^{-1}, at different pH was used (citric acid–Na_2HPO_4 (pH 2.3–7.4) and 0.5 mol L^{-1} NaOH–KCl (pH 12.0)). The samples (approximately 0.05 g) were immersed into 200 mL buffer solution; the weight of solution absorbed by the gels was calculated from the weights of the gel before and after vacuum drying at room temperature. The degree of swelling of each film was calculated by use of the equation:

$$\omega = (W_s - W_d)/W_d$$

where W_d and W_s are the weights of the samples in the dry and swollen states, respectively.

Characterization and measurements

Fourier transform infra-red (FTIR) analyses were recorded on a Nexus 970 FTIR spectrometer. Elemental analyses were performed on CHN-O-Rapid. Thermogravimetric analysis was carried out with an American TA Instrument (SDT 2960 Simultaneous DTA–TGA). Samples (3–9 mg) were heated to 800 °C at 10 ° min^{-1}. The surface topography was analyzed by SEM on an X-650 scanning electron micro analyzer. Rectangular test samples (0.5 cm×2.5 cm) were cut from circles of crosslinkable *O*-butyrychitosan films and strained to failure on an Instron-4466 materials-testing instrument.

Blood-clotting assay

A blood-clotting assay was developed in order to assess the effectiveness of the polymer coatings in delaying the blood-clotting process. When platelets were brought into contact with the glass surface, factor XII would adhere to the surface and be activated, thereby initiating the clotting cascade via the intrinsic pathway. For this assay, 75 mm×12 mm glass tubes were used; they were sonicated in 25% ethanol solution and dried before use.

MOS-OCS was used as a 10 mg mL^{-1} solution in methanol. In order to coat a glass tube the tube was filled with the polymer solution and 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 days. Ten samples were prepared in each case and compared with ten uncoated controls.

Fresh frozen platelet-rich plasma (PRP; 100 μL) was added to each sample tube and allowed to stand for 2–3 min at 37 °C before addition of $CaCl_2$ solution (0.025 mol L^{-1}, 100 μL; 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 backwards and forwards whilst still partially submerged in the water both to maintain the temperature at 37 °C. At the point when a fibrin clot was visible, the stopwatch was stopped and the time was noted.

Platelet adhesion

Platelet adhesion assay was also carried out on the samples in order to check for activated platelets, fibrin clots, etc. Polyester (PET) strips (30 mm×9 mm) were cleaned thoroughly by wiping with 25% ethanol solution and then dipped into 10 mg mL^{-1} MOS-OCS solution . They were allowed to dry at room temperature for several days and then treated at 80 °C for 2 h to obtain PET strips coated with crosslinkable *O*-butyrylchitosan. The PET strips were contacted with 4 mL PRP for 3 h. After washing in PBS they were fixed using 2% glutaraldehyde solution for 30 min, washed again,

and then subsequently immersed into 55%, 70%, 80%, 90%, 95%, and 100% ethanol solution and dried in a desiccator. They were sputter-coated with gold before imaging by SEM.

Result and discussion

Crosslinkable *O*-butyrylchitosan preparation

Preparation of MOS-OCS

The reaction used for synthesis of MOS-OCS occurs readily in methanol at room temperature. The reaction is shown in Scheme 1. Methoxysilyl-groups were introduced into *O*-butyrylchitosan by Michael addition of (3-acryloxypropyl)trimethoxymethylsilane.

From MOS-OCS, crosslinkable *O*-butyrylchitosan can be obtained by hydrolysis and condensation of methoxysilyl groups in the MOS-OCS. The silanol condensation reaction can be easily accomplished either by exposure of the coating to atmospheric moisture, or by controlled addition of water, either in the form of vapor (for example in a controlled humidity chamber), or as a liquid in the MOS-OCS solution.

FTIR

Figure 1 shows the FTIR spectra of *O*-butyrylchitosan, MOS-OCS and crosslinkable *O*-butyrylchitosan film. In the FTIR spectrum of *O*-butyrylchitosan (Fig. 1,*1*), three new absorption bands at 1746.3 cm^{-1}, 779 cm^{-1} and 1207 cm^{-1} were attributed to $\nu(C=O)$, $\omega(CH_2)$, and $\nu(C-O)$ respectively, which were characteristic absorptions of the butyrylate group. The absorption intensity of amino group band at (1546.1 cm^{-1}) was still intense. This result indicated that acylation took place on the hydroxyl group of chitosan, which confirmed *O*-acylation [14].

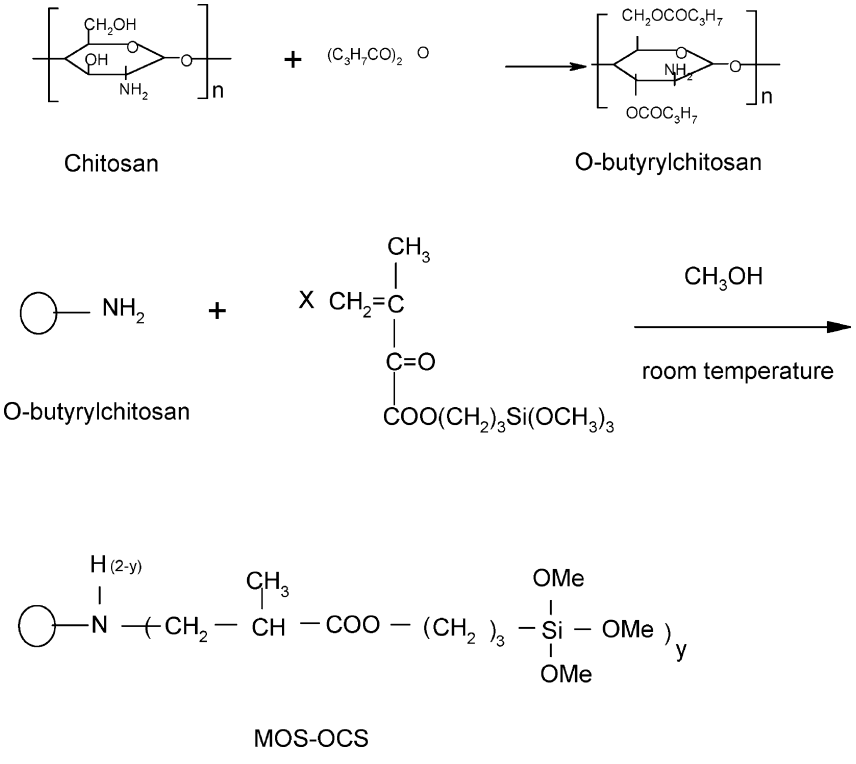
In the FTIR spectrum of MOS-OCS (Fig. 1, *2*), it could be seen that there was a decrease in the intensity of the amino groups compared with that of *O*-butyrylchitosan, while the intensity of the ester carbonyl group (1736 cm^{-1}) increased, because of the bonding of (3-acryloxypropyl) trimethoxymethylsilane to the amino groups of *O*-butyrylchitosan.

From Fig. 1, 3, the FTIR spectrum of the film of crosslinkable o-butyrylchitosan, it could be seen that a new wide absorption band at 1079.4 cm^{-1} appeared, which was the characteristic of Si–O–Si. This result confirmed that crosslinkable network structure had been formed by hydrolysis and condensation of methoxysilyl end-groups in MOS-OCS.

Elemental analysis

Elemental analysis of MOS-OCS was used to estimate the percentages of carbon, nitrogen, hydrogen, and silicon. From the elemental analysis result the amount of amino remaining in chitosan could be calculated (Table 2).

Scheme 1 Synthesis of *O*-butyrylchitosan and MOS-OCS



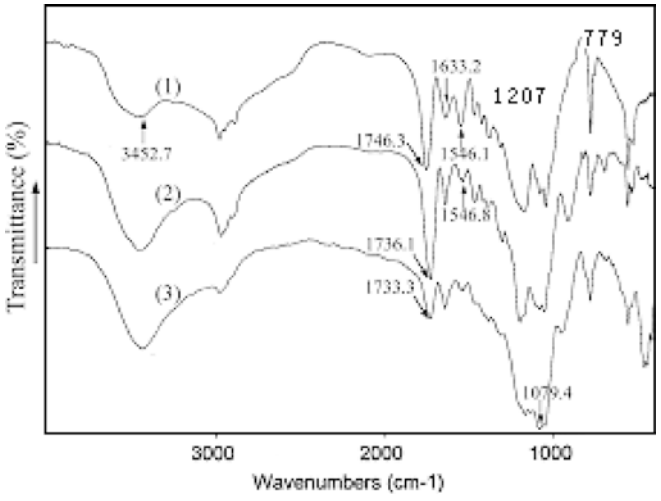


Fig. 1 FTIR spectra of *O*-butyrylchitosan (*1*), MOS-OCS (*2*), and crosslinkable *O*-butyrylchitosan film (*3*)

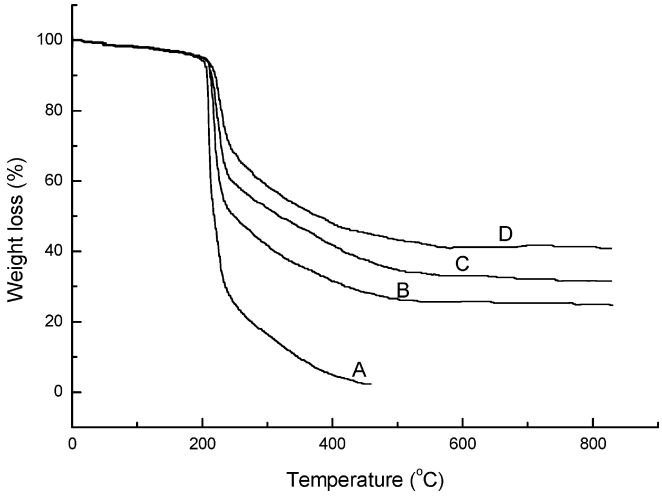


Fig. 2 TGA curves of *O*-butyrylchitosan (*A*) and its crosslinkable films F-10 (*B*), F-20 (*C*), and F-30 (*D*)

TGA analysis

TGA and differential thermogravimetric (DTG) curves for *O*-butyrylchitosan and its crosslinkable films are shown in Figs. 2 and 3 respectively, from which it could be seen that the onset of the thermal decomposition of crosslinkable *O*-butyrylchitosan film was shifted significantly toward higher temperature than that of *O*-butyrylchitosan and the decomposition velocity of crosslinkable *O*-butyrylchitosan was much lower than that of *O*-butyrylchitosan. These results indicated indirectly that crosslinked networks had been formed by hydrolysis and condensation of methoxysilyl groups in the MOS-OCS. It was the formation of the crosslinking network that imposed restrictions on the molecular motion of *O*-butyrylchitosan and made the thermal decomposition temperature of *O*-butyrylchitosan increase.

Mechanical properties

Mechanical properties of*O*-butyrylchitosan and its crosslinking films are shown in Table 3, from which it could be seen that the tensile elongations of crosslinking *O*-butyrylchitosan films were higher than those of *O*-butyrylchitosan while the modulus of crosslinking films was lower than that of *O*-butyrylchitosan. The modulus of crosslinkable films increased with increasing crosslinking agent, while tensile elongation decreased with increasing crosslinking agent. Mechanical testing showed that films with 20 mol% (3-acryloxypropyl)trimethoxymethylsilane crosslinking agent had comprehensive mechanical characteristics.

Table 2 Elemental analysis result of MOS-OCS and the amount of amino remaining

MOS-OCS S-X	C	N	H	Si	Amount of chitosan amino remaining (%)
S-10	55.34	4.12	7.78	0.82	90.05
S-20	54.89	3.65	7.43	1.54	78.9
S-30	54.01	3.55	7.34	2.18	69.3

O-Butyrylchitosan hydrogels

Si-containing crosslinkable *O*-butyrylchitosan film formed a hydrogel structure in aqueous solutions because of interaction of hydrophilic *O*-butyrylchitosan

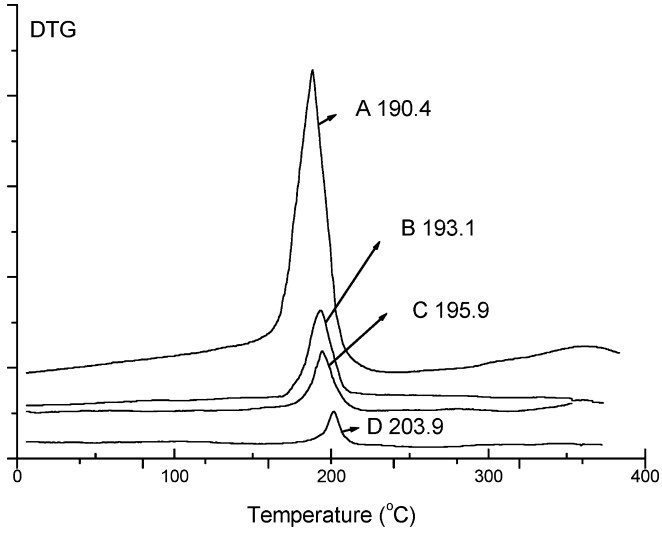


Fig. 3 DTG curves of *O*-butyrylchitosan (*A*) and its crosslinkable films F-10 (*B*), F-20 (*C*), and F-30 (*D*)

Table 3 Mechanical properties of butyrylchitosan and its crosslinking films

Sample F-X	Strain at break (%)	Modulus (Aut Young) (MPa)
F-0 (butyrylchitosan)	13.344	984.14
F-10	38.669	201.493
F-20	30.317	381.642
F-30	29.086	453.957

X: (3-Acryloxypropyl)trimethoxymethylsilane content of the MOS-OCS (mol)
F: Cross-linked *O*-butyrylchitosan film

and the hydrophobic siloxane interdendrimer. The degree of swelling of crosslinkable films is shown in Fig. 4. The highest water uptake was obtained in the pH 2.3 buffer in all cases; all samples had similar values in the pH range 4.0–7.5.

Anticoagulant property

From the data in Table 4, it could be seen that the clotting time was significantly extended for the samples coated with *O*-butyrylchitosan and crosslinkable *O*-butyrylchitosan. This result indicated that *O*-butyrylchitosan did have antithrombogenic properties as we had supposed. The crosslinkable version produced better results. This might be because the crosslinkable *O*-butyrylchitosan contained silyl groups and there was a possibility of interaction between these groups and the substrate surface, producing a more coherent and durable coating on this substrate compared with the linear *O*-butyrylchitosan when in contact with the PRP.

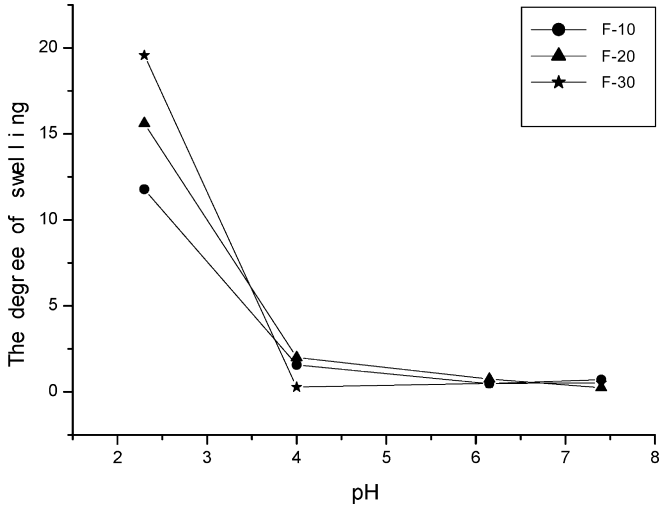


Fig. 4 Degree of swelling of hydrogels as a function of buffer pH

Table 4 Clotting times for uncoated and coated sample tubes

Sample coating	Clotting time (s)
Uncoated	205.3 ± 5
O-Butyrylchitosan	402.3 ± 8
F-20	456.7 ± 11

Figure 5 shows SEM images of PET after contact with platelet-rich plasma. The uncoated surface was covered with cellular matter whereas there was almost no sign of any cellular matter on the coated PET strip. This collection of in-vitro data suggested that crosslinkable coatings of *O*-butyrylchitosan could effectively inhibit platelet adhesion.

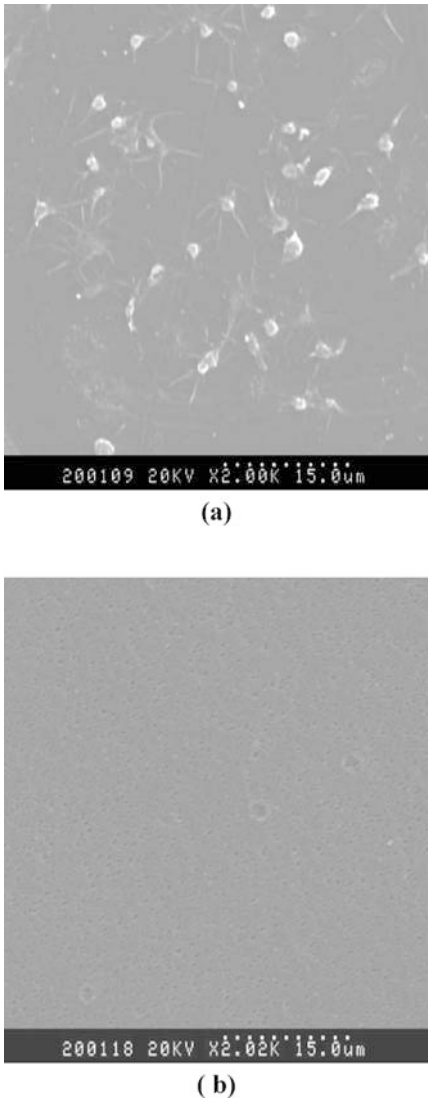


Fig. 5 SEM of PET strip contacted with platelets: (**a**) uncoated PET strip; (**b**) PET strip coated with F-20

The anticoagulant properties of *O*-butyrylchitosan might be because of its special structure—not only having hydrophilic amino groups and hydroxyl groups but also having hydrophobic butyryl groups, because it has been reported that materials with a suitable ratio of hydrophilic and hydrophobic groups have excellent compatibility. The Si-containing cross-linked and hydrogel structure of crosslinkable *O*-butyrylchitosan might also be relevant to its excellent antithrombogenicity, because:

- it was recognized that a crosslinkable coating would have additional advantages over the existing systems in terms of stability of the film and the possibility of anchoring the polymer to the substrate;
- silyl groups had blood compatibility [13]; and
- hydrogel was believed to have good biocompatibility [16].

Mechanical property analysis showed that crosslinkable *O*-butyrylchitosan coating with 20 mol% silyl crosslinking agent not only had excellent antithrombogenicity but also ideal mechanical properties.

Conclusions

A novel Si-containing crosslinkable type of *O*-butyrylchitosan was prepared to improve the durability of *O*-butyrylchitosan. The crosslinkable *O*-butyrylchitosan films obtained were clear and colorless with hydrogel structure. *O*-Butyrylchitosan was antithrombogenic and Si-containing crosslinkable *O*-butyrylchitosan coatings were shown to have good mechanical properties and excellent hemocompatibility.

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