Hemocompatibility of 2-N-3,6-O-sulfated chitosan films

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ABSTRACT: Sulfated chitosan (SC) can be used for such coating because of its similarity with heparin. The position of sulfate group incorporation in the chitosan chain is important for the improvement of its biological mechanism. The substitution of each reactive group of chitosan is required to increase the electrostatic repulsion between the sulfated films and blood proteins or platelets, and, thereby, reduce the thrombus formation. Moreover, the polymeric hydroxyl groups can activate the complement system through the alternative pathway. In this study, we developed SC films, with larger distribution of sulfate groups by substitution of three reactive groups in the chitosan chain to improve its hemocompatibility. Successful chemical modification confirmed the partially 2-N-3,6-O-SC. The hemocompatibility results showed decrease in bovine serum albumin and fibrinogen adsorption, platelet adhesion and an increase in the intrinsic pathway anticoagulant activity. Based on these results, partially tri-SC can be considered suitable candidate for coating blood-contacting medical devices. © 2018 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 2019, 136, 47128.

KEYWORDS: anticoagulant films; hemocompatibility; sulfated chitosan

INTRODUCTION

Cardiovascular diseases (CVDs) are one of the leading causes of death, annually (17.5 million cases), around the world.1 The most common treatment used for these diseases is the percutaneous transluminal angioplasty (PTA) involving the implantation of stents. This device unblocks diseased blood vessels that had been blocked by atheroma and contributes to efficient blood transfer to other organs.2 However, these implants can cause clinical failures, leading to the requirement of special care or medical therapy for the patient, or repair or replacement of the implant. One of the most common failures in this device is the in-stent restenosis, which manifests through a series of events: (1) the contact of the implant with vessel intima causing the activation of the inflammatory and thrombogenic cascade, (2) activation of abnormal vascular smooth muscle cell proliferation and neointimal hyperplasia, and (3) subsequent luminal narrowing and vessel reocclusion.3

Previous studies have shown evidence of reduction in restenosis by blocking the binding site of glycoprotein IIb/IIIa, a receptor in the platelet membrane that binds certain proteins to promote the platelet activation and aggregation resulting in the thrombus formation.4 Therefore, the long-term prevention of thrombus formation and the activation of the immune system can be the determinants of success for the cardiovascular implants. Researchers from several areas are therefore motivated and wanted to develop strategies to improve the hemocompatibility of materials, like chitosan, which can be suitable for coating implants.

Chitosan, a linear co-polymer obtained from partial or total N-deacetylation of chitin (mainly present in crustacean shells), is composed of N-acetyl-2-amino-2-deoxy-D-glucopyranose and 2-amino-2-deoxy-D-glucopyranose monomers linked by (1,4)-β-glycosidic bonds. Studies in the biomedical fields have focused on this polymer because of its favorable properties, such as biocompatibility, biodegradability, nontoxicity, and antimicrobial activity, which make it suitable for application in biomaterials.5–8 The presence of the highly reactive hydroxyl and amino groups in the chain makes it amenable to a variety of chemical modifications for several biological applications.9,10

The introduction of sulfate group ($SO_4^{2-}$) on the chitosan chain is of primary interest due to its similarity to heparin, a natural blood anticoagulant, and anticoagulant and antiviral activity and...
However, the position of sulfate group substitution in the chitosan chain influences its biological effects. This fact can be explained by the finding that an unsubstituted amino group (NH$_3^+$) in chitosan can interact electrostatically with the negative charge of proteins, followed by a series of biological events, such as platelet activation and aggregation, and finally resulting in thrombus formation and consequently embolism.

The introduction of negative charge (SO$_4^{2-}$) in place of the amino groups in the chitosan leads to an electrostatic repulsion between proteins and the surface, avoiding these side effects. Conversely, the presence of unsubstituted hydroxyl groups in the chitosan can activate the complement system, as these groups resemble natural initiators. Therefore, the substitution of hydroxyl groups with sulfate groups can inhibit immune system activation. Another noteworthy point is that chitosan can be obtained from the residues generated by the commercial industries manufacturing fish products, making it inexpensive compared to heparin.

Lima et al. studied protein adsorption (BSA and Fibrinogen from bovine plasma) on sulfonated chitosan films, with the amino groups substituted, and they observed a reduction in the protein adsorption when compared with that on natural chitosan (NC). Campelo et al. studied the platelet adhesion on the same kind of sulfonated chitosan film, and they observed a reduction in platelet adhesion on the film surface compared to that of NC film. Karthik et al. studied the anticoagulant and antiviral activity of sulfated chitosan (SC) (low molecular weight). The amino and primary hydroxyl groups were substituted with 2-N-6-0-bisulfate, and the authors concluded that this polymer showed anticoagulant and antiviral activity in vitro. Yuan et al. studied the interaction between regioselectively SC and protein mixtures (lysozyme, BSA, and globulin). In the study involving regioselectivity, either only the primary hydroxyl group (6-O-unisulfated), the primary and secondary hydroxyl groups (3,6-O-bisulfated), or the only amino and primary hydroxyl groups (2-N-6-O-bisulfated) was substituted with the sulfate group. They observed that the SC with uni-substitution of the primary hydroxyl group improved interaction and increased its affinity with lysozyme than the SC with bi-substitution on the amino and primary hydroxyl group or the primary and secondary hydroxyl group.

Based on these studies, we, through this study, suggest that the substitution of the amino and the primary and secondary hydroxyl groups with sulfate groups in the chitosan chain may improve the hemocompatibility of chitosan due to (1) the higher hydrophilicity caused by the increase of sulfate groups; (2) the increased surface negative charge leading to a greater electrostatic repulsion between proteins and surface; and (3) the partially tri-SC structure, which is similar to that of heparin. Thus, these properties presented by tri-SC can decrease the thrombus formation and the immune system activation, indirectly reducing clinical failures.

To avoid clinical failures postimplantation of blood-contacting medical devices (stents), we conducted this study to synthesize partially tri-SC (2-N-3,6-O-tri-sulfated) films, with improved hemocompatibility, for stent coating. The polymer modification was confirmed and characterized by elemental analysis and infrared spectroscopy, and the position of sulfate group substitution was observed by $^{13}$C nuclear magnetic resonance (NMR). The molecular weight ($M_w$) was investigated by viscometric measurements. The hydrophilicity was investigated by X-ray diffraction (XRD) and thermogravimetric analyses (TGA). The morphology of the films was observed by scanning electron microscopy (SEM). To evaluate the hemocompatibility of the SC, certain assays to assess protein adsorption on surfaces (BSA and BFG), platelet adhesion on surfaces, anticoagulant activity, and cytotoxicity were performed.

### EXPERIMENTAL

#### Materials

Chitosan (48165), fibrinogen from bovine plasma (F8630-16), bovine serum albumin (BSA-A2153), and phosphate buffered saline (PBS, prepared from powder dissolved in 18 MΩ deionized water [pH 7.4]) were obtained from Sigma-Aldrich, Saint Louis, MO, USA. Chlorosulfonic acid (HCISO$_3$) was obtained from Merck. N,N-dimethylformamide (DMF) was purchased from Labsynth, and glutaraldehyde was obtained from VETEC Química Fina (Brazil). Other chemical reagents were all of analytical grade. The dialysis membranes with an approximate molar mass cut-off of 12,000–16,000 g mol$^{-1}$ and porosity of 25 Å were purchased from INLAB.

#### NC Film Preparation

NC solution (2%, w/v) was added to 10 vol % acetic acid solution and mechanically stirred for 24 h. Subsequently, 18 g from this solution was dispersed on Petri dish and dried in an oven at 60 °C for 5 h or until the film remained constant. The film obtained was immersed in NaOH solution (2 mol L$^{-1}$) for 24 h, washed with distilled water until the wash water reaches pH 7, and dried under vacuum desiccator.

#### SC Film Preparation

The sulfation of chitosan was performed following the method described by Carreón et al. with some modifications. First, NC solution (2%, w/v) was prepared as described in “NC film preparation” section and precipitated with NaOH solution (2 mol L$^{-1}$). The gel obtained was washed with Milli-Q water until the wash water reached a pH of 7, and subsequently subjected to serial solvent exchange procedure by incubating it at different concentrations (20, 40, 60, 80, and 100 vol %) of methanol and DMF with each incubation time being 30 min. Next, the chitosan gel impregnated with DMF was subjected to a sulfation reaction by sulfating complex prepared with DMF and chlorosulfonic acid (60:6) at 40–45 °C for a period of 5 h. The obtained solution was reacted with NaOH solution (2 mol L$^{-1}$) until the mixture reached a pH of 12 and precipitation occurred. The product was filtered, washed with methanol, and dried at room temperature (RT). It was dissolved in distilled water and dialyzed against distilled water for 48 h, with the water being changed twice per day. Finally, the solution was solidified using liquid nitrogen and lyophilized to obtain the SC powder samples (SC). A SC solution (2%, w/v) was prepared and dispersed on Petri dishes, in a similar method as that followed to obtain NC films.

The high solubility of SC film in PBS solution, as a consequence of the hydrophilic features of SC, allowed a crosslinking reaction.
between the film and glutaraldehyde (2.5 vol %) to be performed. SC Crosslinked films (SCCs) (Scheme 1) were obtained as the product of this reaction, and the chemical stability of SC films in aqueous solution was improved through this process. These improved features allowed us to study the protein adsorption and platelet adhesion on the polymeric surface.

**Film Characterizations**

**Elemental Analysis.** Elemental analysis was performed using Elemental Analyzer LECO CHNS 932 to measure the carbon (C), nitrogen (N), hydrogen (H), and sulfur (S) composition of NC and SC samples. Degree of sulfation (DS) was calculated following the method described by Zhang et al.\textsuperscript{16}:

\[
\text{DS} = \frac{\%S/32}{\%N/14}
\]  

(1)

**Fourier Transform Infrared Spectroscopy.** The IR spectrum analysis was used to confirm the presence of the functional groups and the occurrence of chemical modification. Infrared spectra were obtained for the chitosan samples. Degree of sulfation (DS) was calculated following the method described by Zhang et al.\textsuperscript{16}:

\[
\text{DS} = \frac{\%S/32}{\%N/14}
\]  

(1)

**Nuclear Magnetic Resonance**

**Proton Nuclear Magnetic Resonance.** The NMR spectrum of NC powder was obtained in a 600 MHz Agilent DD2 spectrometer equipped with a 5-mm One Probe (2H-19F/15N-31P) with z-gradient coils at 70 °C. The sample was prepared by dissolving approximately 10 mg of NC or SC powdered sample in 1 mL of D₂O/HCl (1 vol %). For the complete solubilization process, the sample was stirred for 24 h and then transferred to 5-mm NMR tubes. The ¹H NMR spectrum was acquired with 16 transients, acquisition time and delay of 1 s, 16 ppm of spectral window, and 32 k of data points.

The ¹H NMR signals was used to obtain the degree of deacetylation (DD) of the chitosan as reported by Lavertu et al.\textsuperscript{17} For this, the methyl proton in acetyl-glucosamine (GlcNAc) (at δ 2.0 ppm) and the anomeric proton of the glucosamine (at 4.9 ppm) were manually integrated (using Varian VNMRJ 4).

**Thermogravimetric Analysis.** Thermogravimetric analyses were performed on ~14.0 mg of sample using a STA 449F3 (Jupiter/Netzsch) equipment, with a heating rate of 10 °C min⁻¹, range of 25–700 °C, dynamic N₂ atmosphere, and air flow rate maintained at 60 mL min⁻¹.

**Protein Adsorption.** NC and SCC films (1 cm² area) were immersed in PBS solution (pH 7.4) at 37 °C for 1 h, with soft stirring to promote the moisture equilibrium on films prior to contact with protein solutions. The PBS was removed and 3 mL of BSA and fibrinogen solution (1.0 mg mL⁻¹) was added, followed by incubation at 37 °C with soft stirring for 2 h. The absorbance of protein solutions was evaluated using a Biomate 3S spectrophotometer at 280 nm. The amount of adsorbed protein on chitosan films was calculated using the following equation:

\[
Q = \frac{[(C_a - C_f) V]}{A}
\]
where $C_0$ and $C_f$ are the initial and equilibrium protein solutions concentrations, respectively, $V$ is the total volume of the solution (mL), and $A$ is the film area ($cm^2$).

**Platelet Adhesion Assay.** The protocol followed was in accordance with the one proposed by Xue et al., with some modifications. Blood was collected from healthy volunteers in Vacutainer tubes, suitable for this purpose, followed by centrifugation at 1000 rpm at 25 °C for 15 min to obtain platelet-rich plasma (PRP). The SCC films were equilibrated in PBS at 37 °C for 1 h. Next, the PBS was removed and 2 mL of PRP was added on each film, followed by incubation at 37 °C for 1 h with soft agitation. The PRP was removed and the samples were washed thrice with PBS (pH 7.4). Subsequently, the films were treated with 2.5% glutaraldehyde in PBS at 4 °C for 24 h to fix adherent platelets on the surface. Finally, the samples were washed with PBS and subjected to a drying process by passing them through a series of graded acetone/PBS solutions (30, 50, 70, 80, 90, 95, and 100 vol %) with pauses of 30 min between each treatment. The critical point drying of the samples was performed using hexamethyldisilazane (HMDS). The platelet adhesion was observed using a Quanta-FEG FEI scanning electron microscope.

**Cytotoxicity In Vitro.** The cytotoxicity tests were performed in vitro by assessing the lactate dehydrogenase (LDH) activity, which was determined using a commercially available kit (LDH LiquiForm of Labtest Diagnosis) according to the manufacturer’s instructions. In this method, the consumption of NADH was monitored by conversion of pyruvate to lactate at 340 nm. Human neutrophils ($2.5 \times 10^6$ cells mL$^{-1}$) were incubated at 37 °C for 15 min with Hank’s balanced salt solution (HBSS) (negative control), acetic acid solution (0.5 vol %) neutralized with NaOH (2 M) (vehicle control), Triton X-100 (0.2 vol %) (cytotoxic control), and with the NC and SC samples (10, 50, and 100 μg mL$^{-1}$).

**RESULTS AND DISCUSSION**

**Characterization of NC and SC Films**

**Elemental Analyses.** The elemental analyses of NC and SC were performed to evaluate the composition and DS. Table I report the results obtained. DS greater than 1 indicates that grafting may be occurred through a complex mechanism, with di- or tri-substitution. A particular amount of acid limits the substitution and an excess amount can contribute to partial degradation of the polymer, in turn interfering with the film formation.

The DD of chitosan due to its precision. Thus, the DD was obtained for NC and SC films. The three most significant changes in the signals observed postsulfation can be attributed to the substitution of primary and secondary hydroxyl groups and amine groups. The appearance of two new bands at 1206 cm$^{-1}$ and 794 cm$^{-1}$ corresponds to the asymmetric $\tilde{S}$=O and C–O–S bond stretching, respectively, and these bands are characteristic for the grafting of sulfate groups in the chitosan chain. The signal at 794 cm$^{-1}$ (C–O–S) indicates the substitution of the $\tilde{O}$H groups, however only a small part of the secondary $\tilde{O}$H might be substituted due to steric hindrance. In the NC spectrum, the bands corresponding to amide group I and amine group deformation shift from 1654 cm$^{-1}$ and 1590 cm$^{-1}$ to 1625 cm$^{-1}$ and 1524 cm$^{-1}$, respectively, and become weak signals after chemical modification. These changes suggest that amine groups were partially involved in sulfate grafting. In addition, the stretching-vibration reduction in the region of $\tilde{N}$H (3200 cm$^{-1}$) observed in the spectrum for SC films indicates substitution of the amine groups. However, the precise substitution position can be confirmed by 2D $^1$H/$^13$C HSQC analysis in the following item.

**Nuclear Magnetic Resonance**

**DD of chitosan.** DD helps to differentiate the chitin from the chitosan as the chitosan is obtained by partial (above 60%) or total N-deacetylation of chitin. This parameter implies different physical, chemical, and biological properties of the material. In this regard, $^1$H NMR has been widely used to determine the DD of chitosan due to its precision. Thus, the DD was calculated using the integral of the methyl proton of the N-acetylglucosamine (GlicNAc) monomer and H1 of the N-glucosamine (GlicN) monomer (Figure 2), in accordance with the method described by Lavertu et al. The DD for NC was calculated to be 77%.

**Table I.** Elemental Analysis and Degree of Substitution (DS) of NC and SC.

<table>
<thead>
<tr>
<th>Samples</th>
<th>% C</th>
<th>% H</th>
<th>% N</th>
<th>% S</th>
<th>DS</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>38.52</td>
<td>7.443</td>
<td>7.142</td>
<td>0.059</td>
<td>-</td>
</tr>
<tr>
<td>SC</td>
<td>21.620</td>
<td>4.528</td>
<td>4.134</td>
<td>12.91</td>
<td>1.37</td>
</tr>
</tbody>
</table>

**Figure 1.** FTIR-ATR of NC and SC films.
Position of incorporated sulfate groups on chitosan chain. The information regarding the distribution of sulfate groups in the chitosan chain is important for evaluation of the biological activity of the polymer. For this purpose, the 2D 1H/13C HSQC spectra of the polymer were obtained and are shown in Figure 3. The characteristic resonance of the sulfate groups are highlighted in the figure. Table II summarizes the signals of carbon-13 at different resonances for both NC and SC.

It was observed that signals for SC were shifted due to sulfation reaction and new signals were distinct due to sulfate group introduction in the chain, compared to those for NC. The signals, C3S and C6S, displayed a higher deviation in the chemical shift, showing that the grafting of sulfate groups occurred preferentially on hydroxyl groups. A new and weak signal, C2S, was observed that indicated that few amine groups were affected. This confirmed that the chitosan was partially tri-sulfated, and these results were in agreement with those of the FTIR study. Table II shows a comparison between these results and those reported in previous studies.

\( M_v \) of NC and SC was determined by a viscometric method and the results revealed a low \( M_v \) for both samples (50,000–190,000 Da). The \( M_v \) for NC was 78,093 Da and that for SC was 5,050 Da. The reduction in the \( M_v \) for SC indicates that there was a degradation of the polymer chain during sulfation. This reduction was observed, especially after the sulfation of chitosan, by several researchers in their respective studies.21,27–29 Although, in this study, SC has presented a lower \( M_v \) due to the chemical degradation, this did not affect its ability to form films. Studies have shown a better anticoagulant activity for low \( M_v \) SC due to its similarity to heparin.27 Heparin, a sulfated polymer, is a natural anticoagulant with low \( M_v \) (5000–25,000 Da), and it is used in prophylaxis and treatment of thrombosis. Similarly, a SC with a high DS and low \( M_v \) might present improved hemocompatibility.

Scanning Electron Microscopy. SEM images obtained for NC and the SCC films, as shown in Figure 4(a,b), revealed the smooth and nonporous surface, characteristics required for blood-contacting medical devices, of the films. In addition, they helped to confirm that the crosslinking performed on SC film did not modify its structure and suggest that the biological properties were retained.

X-ray Diffraction. XRD diagrams for NC and SC were obtained and are shown in Figure 5. The diagram for NC showed reflections at 10°–20 and 20°–20, which are characteristic of the semi crystalline structure of this polymer.19 However, for SC, the reflections at 10°–20 and 20°–20 disappeared, indicating an amorphous structure. These results indicate that the sulfation of chitosan caused a change in its structure, more specifically a reduction in its ability to form intermolecular hydrogen bonds due to

<table>
<thead>
<tr>
<th></th>
<th>NC (90 °C)</th>
<th>SC (90 °C)</th>
<th>SC2a (70 °C)</th>
<th>SC3,6b (80 °C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1-S'</td>
<td>-</td>
<td>105.3</td>
<td>105</td>
<td>98.1</td>
</tr>
<tr>
<td>C1</td>
<td>100.2</td>
<td>102.8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C2</td>
<td>58.6</td>
<td>59.5</td>
<td>-</td>
<td>56.7</td>
</tr>
<tr>
<td>C2-S</td>
<td>-</td>
<td>58.1</td>
<td>63.5</td>
<td>-</td>
</tr>
<tr>
<td>C3</td>
<td>77.5</td>
<td>75.5; 75.7</td>
<td>76.5</td>
<td>-</td>
</tr>
<tr>
<td>C3-S</td>
<td>-</td>
<td>83.7</td>
<td>-</td>
<td>77.3</td>
</tr>
<tr>
<td>C4</td>
<td>79.9</td>
<td>81.9; 82.0</td>
<td>82</td>
<td>75.1</td>
</tr>
<tr>
<td>C5</td>
<td>72.7</td>
<td>76.0</td>
<td>78</td>
<td>74.3</td>
</tr>
<tr>
<td>C6</td>
<td>63.0</td>
<td>-</td>
<td>64</td>
<td>-</td>
</tr>
<tr>
<td>C6-S</td>
<td>-</td>
<td>69.5; 69.8</td>
<td>-</td>
<td>68.2</td>
</tr>
</tbody>
</table>

a N-sulfated chitosan.30
b Chitosan 3,6-disulfated (SC-1).31
substitution of the –OH or –NH₂ groups with sulfate groups. These changes enhanced the hydrophilicity of SC, which is corroborated by the results of the FTIR and ¹³C-NMR experiments. Chaudhari and Murthy³⁰ also observed an amorphous structure of SC, and their results suggested that the incorporation of sulfate groups in the polymer chain reduced the intermolecular hydrogen bond-forming ability of the polymers, leading to the development of an amorphous structure.

Thermogravimetric Analysis. Figure 6 shows thermogravimetric curves (TGA and DTG) for NC and SC and Table III presents the parameters obtained from these curves. NC thermograms indicated degradation at two moments. The first moment begins at 52 °C and involves a weight loss due to dehydrogenation of chitosan. The second and more important moment begins at 249 °C, involving a weight loss due to thermal decomposition of the GlcNAc groups of the polymer chain. The thermal stability of SC was lower when compared with that of NC. SC thermograms indicated degradation at three moments. The first moment begins at 50 °C and involves a weight loss due to the hydrophilic property of SC.²¹ The second moment begins at 213 °C and continues with the third moment, which begins at 494 °C and involves a weight loss due to thermal degradation of GlcNAc and –SO₃Na groups, respectively.

The higher hydrophilicity resulting from the sulfation can be explained by the higher moisture content and higher weight loss for SC (Table III), leading to a lower thermal stability compared to that of NC.²³ These results corroborate with those of the XRD experiment and were similar to those reported in the previous studies.²¹,²³

Hemocompatibility Study

Protein Adsorption. Protein adsorption is known as the first event to occur post blood–surface interaction, and it is a complex phenomenon. Hydrophobic interaction, hydrogen bonding, and electrostatic interaction simultaneously control this process. Thus, a single-protein study is necessary to better understand the correlation between the physicochemical properties of the proteins and its surface interaction. The protein present in higher concentration in the blood plasma is albumin; therefore BSA was selected for this study due to its chemical similarity with the human albumin. Fibrinogen also was selected as it is an important protein in the activation of coagulation cascade, platelet adhesion, and platelet aggregation leading to thrombus formation.

Figure 7 presents the amount of adsorbed protein for each chitosan film. A 36.84% reduction in the amount of adsorbed BSA onto the modified surface of SCC film, compared with that onto the NC film, was observed. This can be explained by the conversion of carboxylic acid to negatively charged carboxylate ion in BSA at pH 7.4,³¹ leading to electrostatic repulsion between the protein and film surface. Fibrinogen adsorption was more adsorbed (either on NC or SC films) compared to BSA (60 kDa), probably because fibrinogen has a higher Mᵣ and, therefore, a greater ability to be adsorbed on surfaces compared to smaller protein as explained by Vroman effect.³²
A greater reduction in protein adsorption of partially tri-SC obtained in this study was observed when compared with the results of previous studies on chitosan sulfated only in the place of the amino groups. In that study, BSA and fibrinogen adsorption on NC and SC films (DS 0.32) was investigated, and a reduction of about 19% and 3.85% for SC, respectively, was observed, compared to that for NC. In this study, we showed that the increase in the DS (1.37) was efficient to reduce protein adsorption onto modified polymer (36.8% and 20%, respectively). Moreover, a larger distribution of the negative surface charge in the polymeric chain may have improved these results.

In addition, the increase in hydrophilicity of SC, due to substitution of hydroxyl and amino groups with sulfate groups, was an important property influencing protein-adsorption reduction for SC. Hydrophilic surface is strongly bounded by water molecules that are not easily displaced by proteins, which get adsorbed, present at a low concentration in the solution. This phenomenon is known as “water hydration effect,” therefore making the surface biocompatible. Thus, the electrostatic repulsion of the negative charge and increase of hydrophilicity were the key factors to surface resistance to protein adsorption.

Platelet Adhesion. The platelet adhesion assay is important to study the extent of platelet adhesion and activation induced by the surface. It helps to assess the thrombogenic potential, an important property for hemocompatibility, of blood-contacting biomaterials. Chitosan and SC film-induced platelet activation were analyzed qualitatively for the spreading behavior of adherent platelets by SEM images as shown in Figure 8. Few aggregates and many adhered platelets were found on the NC surface, with spreading occurring in flattened or irregular patterns [Figure 8(a)]. The number of adhered platelets on the SC surface was reduced substantially compared with that on NC surface [Figure 8(b)]. When activated, platelets extend their pseudopods and release granular contents. However, the appearance of pseudopodia was not observed for both surfaces.

**Table III.** Data Obtained from the Thermogravimetric Curves for NC and SC

<table>
<thead>
<tr>
<th>Samples</th>
<th>$T_i$ (°C)</th>
<th>$T_{\text{max}}$ (°C)</th>
<th>Residual mass (700 °C) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>249</td>
<td>303</td>
<td>-</td>
</tr>
<tr>
<td>SC</td>
<td>213</td>
<td>234</td>
<td>564</td>
</tr>
</tbody>
</table>

$^a$ Initial decomposition temperature.

$^b$ Maximum decomposition temperature from DTG.
The number of platelets on the surfaces was estimated using the SEM images showing a platelet-adhered surface area of 2799.81 μm² and the software ImageJ. The results are shown in the histograms of Figure 9. Platelets were defined as the structures with a diameter of 1–5 μm.37,38 As expected, NC film was highly thrombogenic, allowing an average of 3.49 × 10⁻² μm² platelets to be adhered. This is mainly due to the presence of reactive amino groups on NC that induce protein adsorption leading to platelet adhesion on the surface. On SCC film, an average of 0.22 × 10⁻² μm² platelets was observed. It was reported that proteins, mainly fibrinogen, can induce platelet adhesion when pre-adsorbed on the surface of a medical device. This is because it can bind to the platelet GP IIb/IIIa receptor and induce platelet activation and platelet aggregation, to form fibrin network, followed by thrombus stabilization on the surface.34–39 Thus, the reduction in platelet adhesion for SCC can be attributed to the negative charge of the sulfate groups that led to electrostatic repulsion between fibrinogen and film surface. In addition, the hydrophilic nature of the sulfated surface can decrease the protein adsorption and platelet adhesion on the film surface, improving hemocompatibility of chitosan blood-contacting device.

Campelo et al.12 observed a platelet-adhesion reduction of 53.71% for SC film, when the chemical modification took place by substituting only the amino groups, compared to that for NC films. In this study, with SCC films (substitution of hydroxyl and amino groups), we observed a reduction of 93.7% in the number of platelets adhered to the surface compared to that of NC surface. This result confirms that a high DS and higher distribution of sulfate groups in chitosan chain can contribute to reduction in platelet adhesion on polymer surface.

**Anticoagulant Activity.** Anticoagulant activity assay results of NC and SC are shown in Figure 10. NC did not present any anticoagulant activity when compared with that of control group for all concentrations tested. Conversely, an increasing concentration of SC in plasma showed an increase in anticoagulant activity, preventing coagulation for about 72.1 s, when compared with the control group (27.00 s), which indicate a dose-dependent occurrence. For TP test, both samples had low anticoagulant activity. Heparin and other sulfated polysaccharides did not have a strong influence on this coagulation pathway,29,40 as normal prothrombin time is between 12 and 15 seconds.41 Similar results were found by other authors.29,42

The prolonged anticoagulant effect of SC in the intrinsic pathway might be attributed to the high level of sulfate groups in the chitosan chain; the larger distribution of sulfate groups in the chain, the greater the hydrophilicity, and lower the protein adsorption.

**Cytotoxicity.** The cytotoxicity of the chitosan and partially tri-SC in human neutrophils were studied by measuring LDH activity (Figure 11). LDH is an enzyme present in the cell cytoplasm and is released after cell membrane damage. Therefore, the amount of LDH released is an indicator of cytotoxicity. The results obtained showed no increase in LDH release for the sample concentrations tested (10, 50, and 100 μg mL⁻¹) when compared to the control group. This indicated that the polymers studied were not cytotoxic to human neutrophils.

The cell viability is inversely proportional to the amount of LDH released and based on these findings, we suggested that the polymers did not affect cell viability. These results corroborate with previously reported findings that affirm the low cytotoxicity of chitosan.43,44

**CONCLUSION**

Sulfation of chitosan realized in this study resulted in a partially 2-N-3,6-O-SC with DS of 1.37, which improved its
hemocompatibility. This material provides desirable features that should be incorporated in biomaterials for future applications. The structure and low Mₜ of the polymer were similar to heparin, and it exhibited similar hemocompatible properties, such as reduction of BSA and fibrinogen adsorption by 36.8% and 20%, respectively; reduction of platelet adhesion by 93.7%; anticoagulant activity in the intrinsic pathway for 72 s (200 mg mL⁻¹); and no cytotoxicity against human neutrophils (innate immune system). Therefore, SC emerged as a potential substitute for heparin for coating blood-contacting medical devices.

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