Immobilization of heparin onto the novel cellulose: chitosan vascular grafts. A preliminary study

Imobilização de heparina na superfície de enxertos vasculares à base de celulose e quitosana. Um estudo preliminar

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Research performed at MSc program in Biotechnology – Potiguar University (UnP), Natal, Rio Grande do Norte, Brasil and Department of Pharmaceutical Sciences and Experimental Therapeutics, The University of Iowa, Iowa City, IA, USA.
Financial support: none.
Conflict of interest: none
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Submitted: 31 Jul 2014. Accepted, after review: 10 Aug 2014.

ABSTRACT

Purpose: The aim of this study was to prepare a compliant cellulose:chitosan hollow tube (with 6 mm in internal diameter), to be used as a coronary bypass graft, and to investigate its ability to covalently immobilize heparin onto its surface. **Method**: Cellulose and chitosan solutions were mixed in equal proportions, spread in an appropriate mold and allowed to solidify for 24 hours, where a hollow tube was obtained. Heparin was covalently immobilized onto these hollow tubes, where the results were compared with that of a plain cellulose one. In addition, a correlation between the heparin concentration and the immobilization efficiency was investigated. **Results**: The presence of chitosan was essential for the heparin immobilization onto the cellulose: chitosan hollow tubes as no heparin was immobilized onto the plain cellulose ones. The immobilization density showed to be linearly dependent upon the concentration of the activated heparin, where a maximum concentration of 4 mg/mL was reached. Higher concentrations of activated heparin did not provide a greater immobilization density, which indicates that the amine groups of chitosan have been saturated. **Conclusion**: The cellulose: chitosan hollow tubes were successfully prepared, where the presence of chitosan was required for an efficient immobilization of heparin. The obtained heparin-immobilized cellulose:chitosan hollow tube, with its mechanically strong, reasonably compliant and antithrombogenic properties seems to be a good candidate to be used as a vascular bypass graft.

Keywords: *Vascular graft; Cellulose; Chitosan; Heparin*.

RESUMO

Objetivo: O objetivo deste estudo foi produzir tubos à base de celulose e quitosana (com 6 mm de diâmetro interno), para serem usados como enxertos vasculares, e investigar a capacidade desses tubos em imobilizar covalentemente a heparina em suas superfícies. **Método**: As soluções de celulose e quitosana foram misturadas em proporções iguais, espalhadas num molde adequado e deixadas em repouso durante 24 horas até a total solidificação e obtenção do tubo (enxerto). A heparina foi covalentemente imobilizada sobre estes enxertos de celulose: quitosana, onde os resultados foram comparados com o de um enxerto preparado apenas com celulose. Além disso, uma correlação entre a concentração de heparina e a eficiência de imobilização foi investigada. **Resultados**: A presença da quitosana se mostrou essencial para a imobilização da heparina nos enxertos, já não houve imobilização da heparina sobre os tubos obtidos apenas com celulose. A densidade de imobilização da heparina mostrou ser linearmente dependente da concentração da heparina ativada, onde uma concentração máxima de 4 mg/mL foi atingida. Concentrações mais elevadas de heparina ativada não proporcionaram uma maior densidade de imobilização, o que indica que os grupos amina da quitosana foram saturados. **Conclusão**: Tubos de celulose: quitosana foram preparados com sucesso, onde a presença da quitosana foi necessária para uma imobilização eficaz da heparina na superfície dos tubos. A rigidez mecânica, a complacência adequada e a capacidade antitrombogênica deste tubo de celulose: quitosana mostra que o mesmo possui potencial de ser utilizado como enxerto vascular.

Descritores: Enxertos vasculares; Celulose; Quitosana; Heparina.

INTRODUCTION

Heart attack, also known as myocardial infarction, occurs when the blood supply to part of the heart is interrupted, mainly due to blockage or stenosis of the coronary artery[1]. The most common therapy available consists of bypassing the coronary artery that became stenosed. Coronary artery bypass graft (CABG) surgery is performed in more than a million patients every year at a cost exceeding $20 billion annually[2]. The autologous saphenous vein and internal mammary artery are the vascular grafts of choice for CABG. However, in at least 30% of patients these vessels cannot be used, due to disease or previous use[1].

Thus, large diameter synthetic vascular grafts (inner diameter larger than 8 mm) have been used successfully, where expanded polytetrafluorethylene (ePTFE) and polyethylene terephthalate (Dacron) are the most used material to fabricate these grafts[1,3]. However, small diameter constructs (smaller than 6 mm in the inner diameter) are

considered the most challenging ones with patency rates still low[4]. In fact, the patency rates of ePTFE and Dacron are significantly lower than those of the autologous saphenous vein and internal mammary artery. The most common problems involved with synthetic coronary bypass grafts include platelet adhesion/activation and a decreased compliance compared with the host coronary artery[5].

Cellulose and chitosan are the most abundant biopolymers found in nature. Both are biocompatible, non-toxic and very abundant, which make them good candidates for biomedical applications. Our group has successfully developed small diameter (less than 6 mm) hollow tubes made of cellulose and chitosan, where we found that a mixture of these polymers in equal proportions (CELL:CHIT 5:5) originated constructs with higher compliances when compared with those of Dacron, ePTFE and saphenous vein, but very close to that of human coronary artery[6]. However, although our results showed that the presence of chitosan had a favorable impact on the elasticity, hence compliance, of the hollow tubes, as well as on the myofibroblast cells attachment and proliferation, this polymer has been reported to be a thrombogenic material due to its polycationic nature[7]. Thus, the use of heparin, a highly sulfated glycosaminoglycan that has been extensively used as an antithrombogenic agent [8] would be an alternative to decrease the likelihood of thrombus formation. Since heparin can be covalently immobilized to amine groups [9,10] , which are present in the chitosan structure, we hypothesize that the immobilization of this antithrombogenic agent onto the surface of the CELL:CHIT grafts would prevent the thrombus formation.

In the present study, small diameter hollow tubes have been prepared by using cellulose and chitosan, where the ability of heparin to covalently bind to these tubes was investigated and the results were compared with that of the plain cellulose graft. In addition, a correlation between the heparin concentration and the immobilization efficiency was delineated.

METHODS

Preparation of the hollow tubes

A 2.2 to 2.6% w/w methylol cellulose solution was prepared following the method described by Schroeder et al.[11]. Briefly, a known amount of cotton linter was cut in small pieces and suspended in dimethyl sulfoxide (DMSO) for 2 hours in order to allow cellulose to swell. 7.5% (w/w with respect to the mass of cotton linter) of paraformaldehyde was slowly added to the suspension at 130°C. The reaction was performed until a clear and transparent solution was obtained, which indicates that cellulose was converted to methylol cellulose, allowing it to dissolve in DMSO. This solution was transferred to a bottle, capped and sealed with Parafilm® to avoid contact with moisture.

A 4% w/w chitosan (degree of deacetylation of 87%, viscosity-average molecular weight, M_v, 43,500, purchased from Polymar Ciência e Nutrição S/A, Fortaleza, Brazil)

solution was prepared by dissolving 2g of chitosan in 48g of HCl 2% v/v under constant stirring. The methylol cellulose and chitosan solutions were mixed at the same weight ratio (5:5) and the mixture was spread in a cylindrical mold with a centered rod (Figure 1). After 24 hours, the hollow tube was removed from the mold and soaked in a 1N NaOH solution for 4 hours, followed by washing with 3 x 500 mL of distilled water for 24 hours.

The hollow tubes were stored in a 70% ethanol solution until use.

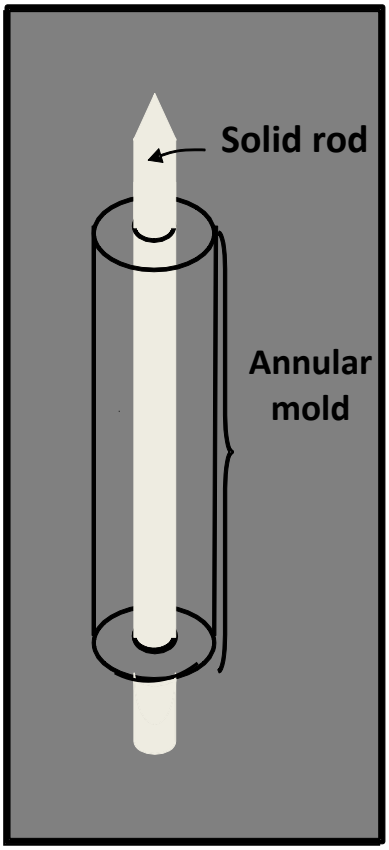

Figure 1: Tubular mold used for making the hollow tubes.

Heparin immobilization

In 2.5 mL of 0.05 M 2-morpholinoethanesulfonic acid (MES) buffer (pH 5.6), heparin sodium (150,000 units) was dissolved and its carboxylic acid groups were activated by adding 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) to the solution in a ratio of heparin:NHS:EDC of 1:1.2:2. The effect of the concentration of this activated heparin solution on the amount immobilized onto the hollow tubes was investigated. Thus, five different concentrations, named heparin 1X, 2X, 4X, 5X and 6X were used, as shown in Table 1.

Table 1: Amounts of heparin:NHS:EDC added to 2.5 mL of MES pH 5.60 buffer

Sample	Amount of heparin:NHS:EDC (mg)	Concentration of heparin (mg/mL)
1X heparin	2.5:3:5	1
2X heparin	5:6:10	2
4X heparin	10:12:20	4
5X heparin	12.5:15:25	5
6X heparin	15:18:30	6

The activation step occurred for 20 minutes at room temperature, under stirring. CELL:CHIT 5:5 hollow tubes were washed with distilled water in order to remove traces of ethanol and equilibrated with MES buffer (pH 5.6) for 30 minutes, prior to reacting with heparin. Next, about 3 cm of each hollow tube was cut and immersed in 1.25 mL of the activated heparin solution for 4 hours at room temperature. The samples were washed with 25 mL of distilled water, 5 times for 24 hours with the purpose of removing the physically bound heparin and the amount of covalently immobilized heparin was determined. In order to investigate the influence of chitosan on the heparin immobilization, the same procedure was done with the plain cellulose hollow tube.

Determination of immobilized heparin

The amount of heparin immobilized to the hollow tubes was determined using the toluidine blue method, where each hollow tube was incubated with 5 mL of a 0.04% toluidine blue solution (0.05M MES, 2% w/v NaCl, pH 5.38) for 4 hours at room temperature. Any heparin bound to the hollow tube would form a complex with the toluidine blue. Then, the toluidine blue solution was decanted and the samples were washed with 25 mL of distilled water, 5 times for 24 hours. The toluidine blue was extracted from the heparin-toluidine blue complex by immersing a piece (1 cm long) of each hollow tube in 5 mL of a 1:4 (v/v) mixture of 0.1N NaOH and ethanol under gentle shaking. The absorbance of the obtained solution was determined at 530 nm using a spectrophotometer. The total amount of immobilized heparin in the 1 cm hollow tube was calculated using a calibration curve. The immobilization density of heparin, defined as the amount of heparin per cm^2 of hollow tube, was determined as:

$$immobilization\ density\ (\mu g/cm^2) = \frac{amount\ of\ immobilized\ heparin}{total\ surface\ area\ of\ the\ hollow\ tube},$$

where the total surface area of the hollow tube was calculated as:

$$A = \pi L\ (D_{inner}\ +\ D_{outer}),$$

where L is the length of the hollow tube piece and D_{inner} and D_{outer} are its inner and outer diameters, respectively.

Calibration curve for heparin

The calibration curve for the heparin-toluidine blue complex was obtained using the method previously reported by Hinrichs et al.[12]. First, 2 mL of heparin solutions at different concentrations were prepared by dissolving appropriate amounts of heparin in aqueous 0.01N HCl/0.2% NaCl. Next, 2 mL of 0.04% w/w toluidine blue in aqueous 0.01N HCl/0.2% NaCl was added to each heparin solution. The mixture was gently shaken for 4 hours, where the excess of toluidine blue reacted with heparin forming and insoluble heparin/toluidine blue complex that readily precipitated. The mixture was centrifuged for 20 min and supernatant was carefully decanted. The amount of toluidine blue in the heparin/toluidine blue complex (precipitate) was determined after the precipitate was rinsed with aqueous 0.01N HCl/0.2% NaCl and then dissolved in 5 mL of a 1:4 (v/v) mixture of 0.1N NaOH and ethanol. This procedure was done for each heparin solution, separately. The absorbance of the obtained solution was measured spectroscopically at 530 nm.

RESULTS

Preparation of cellulose: chitosan hollow tubes

Mixing chitosan and methylol cellulose solutions allowed the displacement of the DMSO by the water present in the aqueous chitosan solution, inducing the coagulation and regeneration of cellulose and the eventual entrapment of chitosan between the cellulose chains. This process originated a solid and rigid structure (Figure 2).

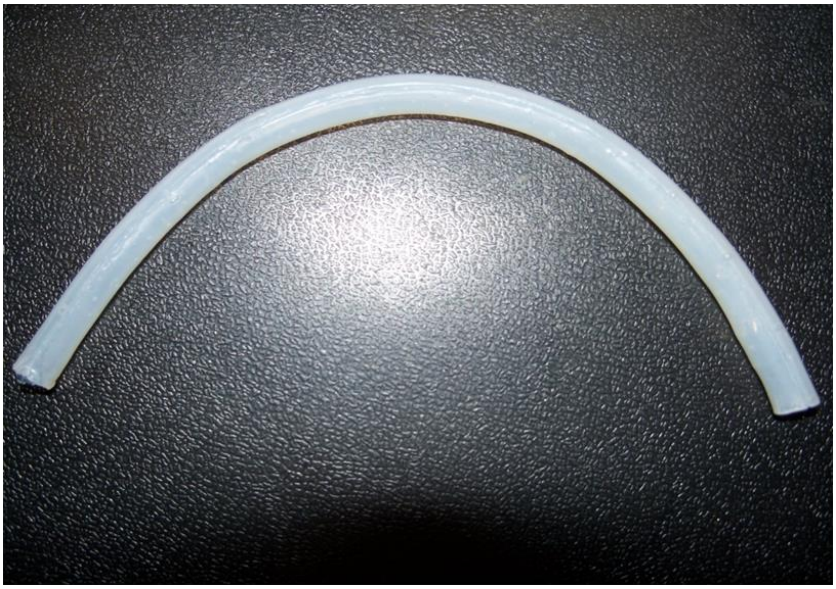

Figure 2: Picture of the CELL:CHIT 5:5 hollow tube (internal diameter: 4 mm; thickness: 1.2 mm; length: 100 mm).

Immobilization of heparin

Heparin has been covalently immobilized to collagen porous matrices[13], collagen films[14], albumin gels[15] and amine functionalized PLGA microspheres[8]. All these studies have in common the same method of immobilization, which consists in previously activating the carboxylic acid groups of heparin with EDC and NHS, followed by reacting these groups with the available free amine groups on the material's structure.

In this study, heparin was immobilized to CELL:CHIT 5:5 through the same method, where the CELL:CHIT 5:5 hollow tube was immersed in the activated heparin solution, allowing both the inner and outer surfaces of the sample to be exposed to the activated heparin. After the immobilization step, the hollow tube was immersed in a toluidine blue solution and all heparin that was immobilized on the hollow tube formed a complex with toluidine blue.

In order to quantify the amount of immobilized heparin, the heparin-toluidine blue complex was broken and all toluidine blue was dissolved, where the absorbance values of the obtained colored solutions were determined. Therefore, this procedure represents an indirect method of determining the amount of the immobilized heparin by quantifying the amount of toluidine blue that formed a complex with heparin. Figure 3 shows the calibration curve for heparin, where the obtained equation was used to convert the absorbance into concentration (μg/mL).

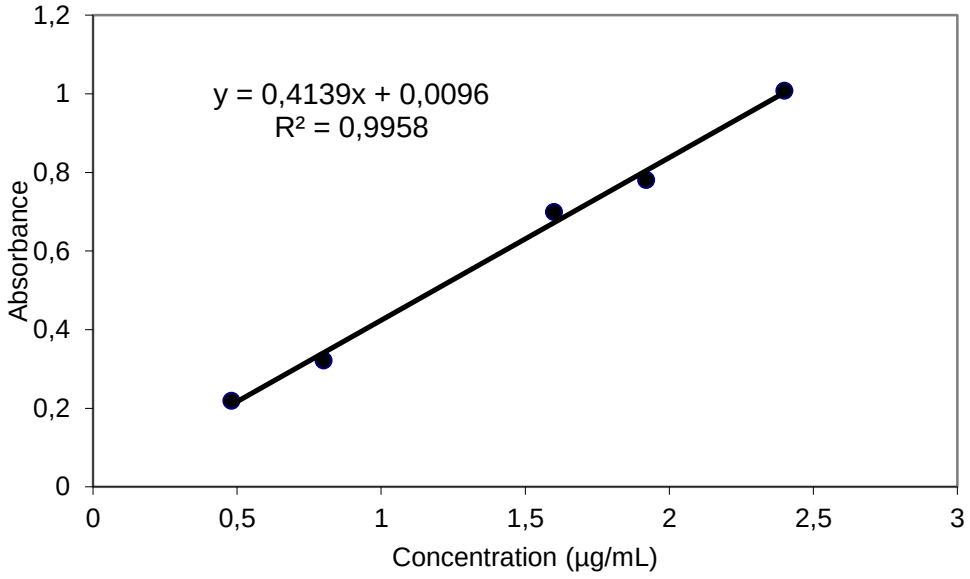

Figure 3: Calibration curve for heparin.

The concentration of the activated heparin solution affected the immobilization density. As shown in Figure 4, concentrations of heparin from 1 to 4 mg/mL showed a linear relationship (R^2=0.9914) with the immobilization density. A plateau was reached at

heparin concentrations above 4 mg/mL, which indicates that this is the concentration where the maximum heparin immobilization occurs in the CELL:CHIT 5:5 hollow tube.

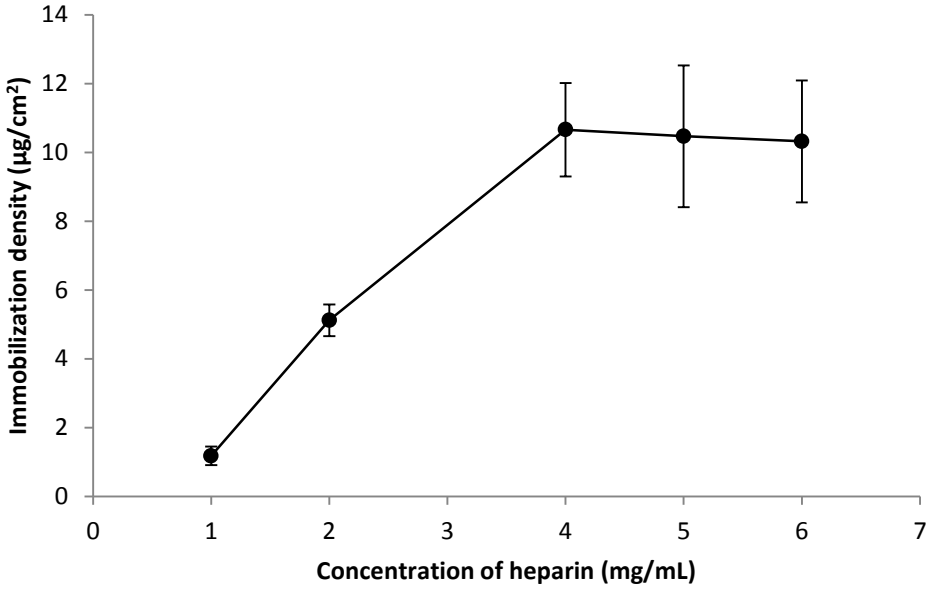

Figure 4: Immobilization density of heparin into the CELL:CHIT 5:5 hollow tubes according to the concentration of the activated heparin solution. n=3; Error bar: standard deviation.

In order to verify the importance of chitosan for heparin immobilization, the same experiment was performed with a hollow tube prepared with only cellulose, without chitosan, and using a 2 mg/mL heparin solution. No toluidine blue was bound to the plain cellulose hollow tube, suggesting that the immobilization of heparin did not occur. Therefore, we can assume that the immobilization of heparin onto the CELL:CHIT 5:5 hollow tube occurred through a reaction between the activated carboxylic acid groups of heparin and the amino groups of chitosan.

DISCUSSION

The poor patency rate of the currently used synthetic arterial bypass grafts has been attributed mainly to a difference in the elasticity (compliance) between the graft and the host artery[16]. Cellulose and chitosan, mixed at the same weight ratio (CELL:CHIT 5:5), have been used by our group to produce hollow tubes with great potential for use as coronary bypass grafts[6]. Besides being more compliant than ePTFE, Dacron and sapheneous vein, but with a compliance very close to that of a human coronary artery, the CELL:CHIT 5:5 hollow tube (Figure 2) did not form any kink, even when it was extremely

bended and twisted. This is an important feature when designing any coronary artery bypass grafts as the absence of kink formation with bending is desirable in order to avoid any disturbance of flow or stenosis[17].

However, besides being mechanically strong and reasonably compliant, the CELL:CHIT 5:5 hollow tube must be antithrombogenic in order to be used as a vascular bypass graft. As a rule of thumb, any foreign material that is exposed to blood will induce the adsorption of plasma proteins, followed by the adhesion and activation of platelets, which eventually leads to the formation of thrombus [9] . In fact, stenosis and thrombus formation, in addition to compliance mismatch, are the leading factors responsible for the poor patency rates of small-diameter vascular grafts.

We have previously shown that the presence of chitosan not only increased the compliance of the hollow tubes, but also enhanced the cell adhesion, as shown by a better cell (myofibroblasts) attachment into CELL:CHIT 5:5 hollow tubes in comparison with the plain cellulose sample[6]. Although presenting these benefits, the presence of chitosan on the CELL:CHIT hollow tube may lead to thrombous formation as this polymer has been reported as being thrombogenic[7]. One approach that can be used to decrease the thrombus formation on the lumen of any graft is through the immobilization of heparin onto the graft's surface[18].

In this work, heparin was successfully immobilized through covalent bond onto the CELL:CHIT 5:5 hollow tubes. The amount of heparin bound to the hollow tube's surface (represented by the immobilization density) was linearly proportional to the concentration of the activated heparin solution. However, a plateau was reached at the concentration of 4 mg/mL, which seems to indicate that the saturation of the available amine groups of chitosan might have contributed to the observed plateau. In addition, the absence of any immobilized heparin onto the plain cellulose hollow tubes seems to indicate that the immobilization takes place through the interaction between the activated heparin and the chitosan's amine groups.

CONCLUSIONS

In conclusion, this work demonstrated that the presence of chitosan in the hollow tube allowed heparin to be covalently immobilized onto their surfaces, where the immobilization density depended on the concentration of the activated heparin solutions. Although we could prove that heparin can be immobilized onto the CELL: CHIT hollow tubes, the platelet adhesion test needs to be performed in order to show that this hollow tube is in fact antithrombogenic.

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