Development, preparation and characterization of chitosan, gelatin and heparin membranes for biomedical applications

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Abstract

With the rising in life expectancy and the consequent population aging, degenerative chronic diseases have been figured as the major cause of morbidity and mortality in the world. It is believed that the production of a polymeric matrix composed of chitosan and gelatin may be an alternative to obtain a carrier base of heparin, with the prospective objective of applying these materials as an alternative to the conventional therapeutic resources biomedical. Based on the results, it can be concluded that the interaction between chitosan, gelatin, and heparin demonstrating a satisfactory result by the methodologies that were applied, making the study hypothesis concrete, certifying the use of a methodology for including heparin in a biodegradable polymeric base. Thus, expanding the alternatives for possible alternatives to the conventional therapeutic resources of vascular diseases with the application of the developed materials.

Keywords Heparin · Gelatine · Chitosan · Membranes · Biomaterials

1 Introduction

With the increase in life expectancy and the consequent population aging, degenerative chronic diseases have been figured as the major cause of morbidity and mortality in the world. They are called non-communicable diseases, which include cardiovascular disease, diabetes, obesity, cancer, and respiratory diseases. Approximately 50% of patients with degenerative chronic diseases do not obtain a clinical benefit due to their poor adherence to drug treatments and mainly because they do not assume the necessary lifestyle changes. At this point several pieces of research have been developed, in the field of the so-called biomaterials, to aid in the treatment of these diseases [1, 2].

It is considered biomaterial, any substance or combination of substances of natural or synthetic origin, designed to be implanted in the body for any period to replace living matter that is no longer in function and may or may not serve as a vehicle, matrix, support or enhancer for new tissue growth [3, 4].

Among the biopolymers stands out chitosan, which is biocompatible, biodegradable, and non-toxic. It is derived from chitin, a polysaccharide abundant in nature. Chitosan has been used for a variety of purposes, including metal ion complexing and controlled drug release matrices [5, 6].

Another biodegradable polymer widely used biomaterial is collagen that is capable of promoting healing. In the human body, collagen represents more than half of the total protein and 70% of the dry weight of the skin, besides

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being the most important constituent of the extracellular matrix [7–9].

The interaction between chitosan and collagen occurs most often by electrostatic forces, between the chitosan groups -NH_3^+ and the collagen groups -COO^- and in some situations, this interaction may occur through hydrogen bonds [10].

Thus, individuals with chronic diseases such as venous diseases and who need continuous treatment throughout their lives can benefit greatly from the use of biomaterials used as carriers of medications [11, 12].

Heparin can be used in high therapeutic regimens with low or doses. Its use, in low doses, is indicated when it is desired to prevent DVT (Deep Venous Thrombosis) in patients with a certain thrombotic risk (postoperative of abdominal and orthopedic surgeries, patients with neoplasms or sepsis, patients at prolonged rest, etc.) [12].

The high doses are used for therapeutic purposes, when it is intended to prevent the occurrence of a second thromboembolic episode in DVT already installed, in patients with DVT, PE (Pulmonary Embolism), thrombosis or arterial embolisms or that I am being related to procedures, which imply thrombotic risk, such as angioplasty, catheterization, hemodialysis and cardiovascular surgery with cardiopulmonary bypass and ECMO [12].

It is believed that the production of a polymeric matrix composed of chitosan and gelatin may be an alternative to obtain a carrier base of heparin, with the prospective objective of applying these materials as an alternative to the conventional therapeutic resource of biomedical application vascular disease through analyzes physical–chemical.

2 Methodology

The research was conducted at the Laboratory of Evaluation and Development of Biomaterials—CERTBIO, located at the Federal University of Campina Grande/UFCG.

2.1 Chitosan membrane preparation

The membranes were prepared by solvent evaporation method by dissolving the polymer in acetic acid (1% v/v) to a final concentration of the polymeric solution (1% m/v) under magnetic stirring for a period of 2 h at approximately 50 °C.

2.2 Incorporation of low molecular weight heparin in chitosan membranes

Heparin in the proportions of 20 mg and 40 mg in the 1% (w/v) gelatin solution and then mixed with the 1% (w/v)

chitosan solution, thus obtaining a 1 g solution: 1 g: 0.02 g and 1 g: 1 g: 0.04 g. The gelatin solution was obtained at a temperature of 50 °C. The membranes were obtained using the same methodology as the chitosan membranes described in the previous item.

For the neutralization of the membranes two techniques were used, one placing 20 mL of 1 mol/L sodium hydroxide solution over the dry membranes and remaining 1 h and another was subjecting the membranes to a 2% ammonium hydroxide atmosphere (v/v).

The solution was placed under a glass dome along with the membranes for 2 h. The neutralized sodium hydroxide membranes were washed with distilled water to remove excess NaOH and those neutralized with ammonium hydroxide were left open until the ammonia odor on the membranes disappeared.

3 Characterization

3.1 X-ray diffraction

The X-ray diffraction analyzes were conducted at room temperature on an XRD- 6000 Shimadzu apparatus using copper K α radiation (1.5418 Å), 40 kV voltage, and 30 mA current. Samples were examined at a range of 2θ between 5 and 30.0 degrees at a rate of 2°/min.

3.2 Fourier transform infrared spectroscopy

The analyzes using the Fourier Transform Infrared Spectroscopy (FTIR) technique of the samples were performed at room temperature and the equipment used was a Perkin Elmer Spectrum 400, using the scan range from 4000 to 600 cm^{-1} .

3.3 Scanning electron microscopy

For scanning electron microscopy analyzes, samples of approximately 0.5 cm were used. Countertop scanning electron microscope, model Hitachi TM-1000, 1000 $\times$ magnification, without metal coating, even on non-conductive samples were used.

3.4 Energy dispersive X-ray spectroscopy

The chemical identification of the material a scanning electron microscope, model TM-1000, Hitachi brand coupled with a system for chemical analysis by Energy Dispersive X-Ray Spectroscopy (EDS) was used.

3.5 Surface tension by contact angle measurements

Before weighing the membranes, they were placed in a 37 °C oven for 4 h. They were then weighed and submerged in distilled water for 24 h. After this period, the membranes were quickly placed on paper towels to remove excess water and weighed. The degree of swelling was calculated by Eq. 1.

The Equation for the degree of swelling

$$\%I = \left[\frac{m_f - m_i}{m_i} \right] \times 100 \quad (1)$$

m_i =initial mass, m_f =swollen mass.

3.6 Biodegradation assay

For this test, we used ASTM F1635-04 Standard Test Method for in vitro Degradation Testing for Hydrolytically Degradable Polymer Resins and Fabricated Forms for Surgical Implants and ASTM F2103-01 Standard Guide for Characterization and Testing of Chitosan Salts as Starting Materials Intended for Use in Biomedical and Tissue- Engineered Medical Products Applications.

4 Results and discussion

All chitosan membranes with and without gelatin and heparin were characterized by X-ray diffraction to observe the influence of gelatin and heparin on crystallinity and consequently on membrane properties as observed in Fig. 1.

The chitosan membrane diffractogram showed typical peaks of semicrystalline material, with a broad base around $2\theta = 10^\circ$ and $2\theta = 20^\circ$ and corroborates with Barbosa [13] e Dantas [14]. In gelatin, typical characteristic peaks of partially crystalline material were also observed, with a predominant peak at $2\theta = 22^\circ$ and a small peak at $2\theta = 8^\circ$.

No significant changes in membrane crystallinity were observed with gelatin and the drug. However, in ammonium hydroxide neutralized diffractograms, a peak at $2\theta = 18^\circ$ and two small peaks at $2\theta = 8^\circ$ and $2\theta = 12^\circ$ were observed, which made the material a little more crystalline when compared to the others.

The FTIR technique was used to demonstrate the spectra (characteristic bands of the functional groups) obtained from chitosan membranes with and without gelatin and also with different heparin concentrations. According to Fig. 2, it can be stated that the addition of heparin to the chitosan membranes with gelatin caused

Fig. 1 X-ray diffraction of gelatin powder and chitosan membranes with gelatin and heparin

changes in the membrane profile when compared to pure chitosan, indicating the presence of gelatin and heparin in them.

The characteristic spectrum of chitosan is in agreement with some studies, since they present all the chitosan absorption and, according to the spectrum of the chitosan membrane, it can be stated that it is not 100% deacetylated, as it is in the region of 1658 cm^{-1} , a characteristic band of amide I ($\text{O}=\text{C}-\text{NHR}$) [12, 14].

The deacetylated chitosan units have three characteristic functional groups, namely a C-2 primary amine, a C-3 primary hydroxyl and a C-6 secondary hydroxyl. In the chitosan spectrum, the band around 3332 cm^{-1} refers to the axial OH stretch, superimposed on the N-H stretch band. The band at 2885 cm^{-1} is attributed to the asymmetric stretch of the C-H group. The 1646 cm^{-1} band is associated with the C=O axial deformation of the primary amide. The band at 1581 cm^{-1} refers to the vibrational deformation of the protonated amine group (NH_3^+). The band at 1379 cm^{-1} can be attributed to the axial -CN deformation of amino groups. The intense band at 1016 cm^{-1} is associated with the C-O-C stretching of the pyramided rings.

Fig. 2 Fourier Transform Infrared Spectroscopy of gelatin powder and chitosan membranes with and without gelatin and heparin

The gelatin spectrum showed characteristic bands at 1656 cm^{-1} , typical of amide I, due to carbonyl stretching; at 1556 cm^{-1} related to amide II due to vibrations in the N–H bond plane and the C–N stretch; at 1229 cm^{-1} , corresponding to the vibrations in the plane of amide III due to the C–N stretch and N–H strain; 1454 cm^{-1} , corresponding to the stereochemistry of the pyrimidine rings and close to 3268 cm^{-1} , due to the O–H stretch and corroborates with Sionkowska et al. [15].

In the heparin spectrum we can see characteristic bands in 3692 and 2880 cm^{-1} regarding the asymmetric stretching of OH and NH groups, in 1635 cm^{-1} referring to angular deformation NH, in 1240 cm^{-1} referring to stretching of CN, CO and S groups, C=O, at 1067 cm^{-1} for the HSO₃– group stretch and 648 cm^{-1} for the S=O [16].

For chitosan with gelatin and the drug, peaks were observed for all membrane components, indicating their presence in the membranes.

However, it was observed in the spectrogram of the samples that were neutralized with ammonium hydroxide

a slight decrease of the peak intensity in 1628 cm^{-1} for group C=O and an increase in the peak in 1541 cm^{-1} for group C=C, besides the appearance of an intense peak at 1408 cm^{-1} that may be related to the CO and CC groups.

These changes may be related to the presence of acetate, derived from acetic acid, because possibly the neutralization of the membranes was not effective and this result corroborates the results obtained in the wettability test, swelling and especially in biodegradation, where membranes dissolved over the 15 days.

The Scanning Electron Microscopy is intended to observe the morphology of all membranes obtained in this research and to compare them. The Fig. 3 shows the photomicrographs of the chitosan membranes with gelatin and different heparin proportions.

In images, **a** and **c** of Fig. 3 are observed microspheres that are the result of electrostatic bonding of chitosan and heparin which are polycationic and polyanionic respectively. It is also observed that membrane **c** has a higher amount of microspheres because it has a higher amount of heparin. In membranes **b** and **d** these microspheres are not observed as clearly as in images **a** and **c**. It is believed that this fact occurred because of the distilled water washing, which was used to remove excess sodium hydroxide. This result is confirmed by the FTIR assays, where a 648 cm^{-1} decrease in the peak for S=O and in the EDS was observed, where a decrease in the sulfur percentage was observed.

The X-ray dispersive energy spectroscopy (EDS) was used to identify the constituents of each of the samples. Heparin was identified by the presence of sulfur as observed in Fig. 4.

Through the results observed in the EDS assay, it was possible to confirm different amounts of the drug in the membranes and that the sodium hydroxide neutralized membranes had a significant decrease in sulfur percentage, which is a substance that characterizes the presence of the drug in the membrane. This was possibly due to washing with distilled water to remove excess sodium hydroxide, which dragged the water-soluble drug. This fact was confirmed by the SEM and the FTIR.

With the development of new surface modification techniques, the use of materials has been expanded for the most diverse industrial sectors, especially in the biomedical sector, because of the contribution that these techniques make in the modification of surface properties such as wettability, biocompatibility, adhesion is great cell differentiation, cell differentiation, etc. All of these physicochemical properties are always related to contact angle measurements.

Thus, contact angle measurements have been widely used to monitor surface properties such as critical surface tension, free surface energy polar and dispersive

Fig. 3 Electron Microscopy of chitosan membranes with gelatin and heparin: **a** Chitosan with gelatin with 20 mg heparin (Q/G/2H); **b** Chitosan with gelatin with 20 mg heparin (Q/G/2H/NaOH); **c** Chi-

tosan with gelatin with 40 mg heparin (Q/G/4H); and **d** Chitosan with gelatin with 40 mg heparin (Q/G/4H/NaOH)

components, surface acid–base interactions, surface crystallinity, the surface orientation of functional groups, roughness surface contamination and wettability [17, 18].

From the contact angle (θ) measurements between the substrate and water, the hydrophobicity of the membranes can be evaluated.

The contact angle analyzes of this work were performed with distilled water dripping on the membrane surface, with subsequent analysis of the measurements of the angles formed by the membrane water bubble, using the Biomaterials Group's software.

It is observed in Fig. 5 that the presence of gelatin in the membranes increased the average contact angle values (52° – 62°), that is, the membranes became less

hydrophilic. However, the decrease in hydrophobicity cannot be considered significant when the standard deviation is observed.

It can also be seen in Fig. 5 that the decrease in hydrophobicity occurred by the addition of the drug since all samples with heparin had an increase in contact angle, and there was a small and non-significant decrease in membrane angle with a greater amount of drug neutralized with NaOH. This may be related to the amount of the drug removed in the membrane wash or by the action of the sodium hydroxide itself. However, these changes were not significant in the hydrophilic profile of the membranes.

According to Thein-Han and Kitiyanant [19], the ability to absorb and retain water is an important factor for

Fig. 4 Dispersive Energy Spectroscopy of chitosan membranes with gelatin and heparin: **a** chitosan with gelatin with 20 mg heparin (Q/G/2H); **b** chitosan with gelatin with 20 mg heparin (Q/G/2H/

NaOH); **c** chitosan with gelatin with 40 mg heparin (Q/G/4H); and **d** gelatin chitosan with 40 mg heparin (Q/G/4H/NaOH)

Fig. 5 Surface tension by contact angle measurements of chitosan membranes with and without gelatin and heparin

implantable materials, as it allows the absorption of body fluids and the transfer of nutrients and metabolites, being important for controlled drug release. However, this high absorption rate can have undesirable consequences such as reduced structural stability of the polymer, that is, the constant presence of moisture in the polysaccharide structure causes the matrix to swell with consequent membrane degradation.

THEIN-HAN [20], observed that the pure chitosan membrane presented a high degree of swelling for the first hour of immersion, reaching over 850% for one of the samples. This degree of swelling was reduced for the 3 h and 24 h

times. This may be because the water absorption process has reached equilibrium.

During the test, the samples were weighed before being placed in distilled water. This assay was performed with triplicate of each sample type analyzed. After 24 h, all membranes were weighed on the same scale used for the previous weighing. Then calculations were performed to find out how much the membranes swelled.

The presence of gelatin in the membranes increased the swelling rate of the samples. However, this rate had a more significant increase in membranes that were neutralized with ammonium hydroxide, possibly because neutralization was not effective, as already observed in FTIR and biodegradation assays. The increase in swelling rate may also have been influenced by the addition of the drug, since, according to Fig. 6, heparin samples had a swelling rate of around 700%.

The hydrophilicity of chitosan is due to its deacetylated groups, which, naturally associated with hydroxyl, and amino groups characterize this strong affinity for polar molecules. These groups have a great influence on the amount of water retained, considering that, the increased chitosan concentration in the membrane interferes with the absorbed water [5]. The membranes that were neutralized with NaOH presented a lower percentage of swelling, this may be related to their effective neutralization and consequently fewer free reactive points to bind with the

Fig. 6 Gelatin and heparin chitosan membrane swelling assay

Table 1 Percentage mass loss membranes subjected to biodegradation tests and their standard deviations

Test	Membrane mass loss 15 days trial			
	PBS (pH 6.78)		Lysozyme/PBS (pH 6.80)	
	Mean (%)	SD	Mean (%)	SD
Chitosan	34	4	38	0.6
Chitosan neutralized with sodium hydroxide	26	5	22	1
Chitosan with Gelatin	*	*	*	*
Q:G NaOH	**	**	**	**
Q:G:2F	*	*	*	*
Q:G:2F NaOH	**	**	**	**
Q:G:4F	*	*	*	*
Q:G:4F NaOH	**	**	**	**

*The samples dissolved or crumbled at the time of collection for weighing and were unable to be weighed. **The samples did not dissolve but degraded or fragmented and were unable to be weighed

water molecule, since chitosan at pH above 6.4 is present, neutral, that is, lose the polycationic profile.

The Biodegradation Assay aims to observe the action of phosphate-buffered solution (PBS) without and with lysozyme on the process of membrane degradation, as well as to verify the influence of gelatin and drug in this process. For the evaluation of this assay, the weight loss of the degraded samples was considered as observed in Table 1.

Lysozyme is the major enzyme responsible for the in vivo degradation of chitosan through the hydrolysis of acetylated residues, although other proteolytic enzymes have shown a low level of degradation activity on the molecule. The degradation rate of chitosan is inversely proportional to the degree of crystallinity and the degree of deacetylation [20, 21]. This enzyme is present in mammalian tissues, organs, and body fluids, including in lacrimal fluid with contents above 1% [22].

In this work, membrane degradation was basically due to the presence of PBS buffer and not lysozyme, as all membranes lost mass.

For Dallan [23], the values of membrane degradation when exposed to lysozyme enzyme for a period of one month and when exposed only to PBS buffer, it was noted that, in most compositions, membrane degradation also occurred, due to the presence of PBS buffer and not to the action of lysozyme.

However, after two months of exposure to lysozyme, there was an increase in membrane degradation, and unlike contact for one month, in most assays, a statistically significant difference was observed between degradation when the enzyme was a present and absent solution.

According to Tomihata and Ikada [24], chitosans with a deacetylation degree of up to 69% present considerable enzymatic degradation even in short periods of lysozyme exposure, while those with a deacetylation degree above 69% are hardly degraded. In addition, the morphological characteristics of the materials also interfere with their enzymatic degradation.

The presence of gelatin in the chitosan membranes was a factor that accelerated their degradation process; this may be related to the amount of gelatin used in this work. Another important factor in membrane degradation was the type of neutralization, as those that were neutralized with ammonium hydroxide possibly dissolved because neutralization was not effective as observed in the FTIR assay. Chitosan membranes with gelatin and the drug that were neutralized with sodium hydroxide also dissolved or degraded, but membrane fragments were presented at the end of the assay, making it impossible to weigh them.

Chitosan is considered a polymer degradable by lysozyme, but such degradability is dependent, among other factors, on the degree of deacetylation of chitosan, since lysozyme binds to the *N*-Acetylglucosamine groups present in the structure of this polysaccharide (SUH) [25, 26].

5 Conclusion

The wettability test demonstrated a decrease in membrane hydrophilicity with gelatin and heparin. However, this decrease was not considerable and did not affect the absorption and retention capacity of the liquid found in the swelling test where the gelatin and heparin membranes presented a higher degree of swelling, and this swelling was very significant in the ammonium hydroxide neutralized membranes.

However, the NaOH neutralization is no longer indicated for membrane neutralization due to drug drag during washing and the time of 2 h for membrane

neutralization in the 2% NH₄OH atmosphere was not effective, as observed in characteristic peak analyzes of the FTIR.

The biodegradation test showed significant membrane mass loss, mainly those neutralized with ammonium hydroxide. This can be explained by the high degree of swelling, because the higher the degree of swelling, the greater the polysaccharide degradability.

Also, conclude that the degradation was due to the action of PBS and not lysozyme, so in future studies, we can test another system.

Based on the results, it can be concluded that the interaction between chitosan, gelatin, and heparin demonstrating a satisfactory result by the methodologies that were applied, making the study hypothesis concrete, certifying the use of a methodology for including heparin in a biodegradable polymeric base. In future studies, biological tests will be carried out to research the action of this compost in experimental models of vascular diseases.

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Declarations

Conflict of interest We have no conflict of interest to declare.

Ethics approval No ethical approval is required according to research practices.

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