Material behaviour

Chitin/polyurethane networks and blends: Evaluation of biological application

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Abstract

Aiming at the combination of the biological properties of chitin and the mechanical and thermal characteristics of polyurethane, the two polymers were combined in two macromolecular configurations. In the first, the polymers were mixed forming blends and in the second tridimensional networks were built. The potential application of the two systems as biomedical materials was studied, showing that both blends and networks presented high stability with low mass loss in media simulating living tissue. No toxic products were released and the adhesion to Vero cells was low. These preliminary results in vitro indicated that the materials are potentially biocompatible, with potential biomedical applications.

1. Introduction

In previous contributions, we have described the synthesis and characterization of two bi-component polymeric systems containing chitin and polyurethane. In the first, the two polymers were blended in several component ratios [1] and in the other they were linked together in tridimensional networks of varying degrees of crosslinking. The thermal, thermomechanical and morphological properties of the two different macromolecular architectures were compared [2].

One of the main uses of biodegradable polymers is related to their clinical applications, and knowledge of the toxicity of the products released during their biodegradation is certainly of great concern. In vitro degradation tests in simulated physiological solutions are a means of forecasting the interactions between body fluids and the biopolymer, and also to study its stability and degradation rate [3]. Although perfect simulation of the chemical, mechanical and dynamic behavior of the human body fluids in vivo is almost unattainable, in vitro studies are an important tool to find potential materials to be used as implants [4]. To demonstrate that the degradation products are not harmful and can be eliminated through any organic pathway is also an important issue.

In this communication we present the first results concerning the behavior of bi-component systems of chitin/polyurethane in the form of blends and networks in biological tests. The chemical structure of the materials is illustrated in Fig. 1.

2. Experimental

2.1. Hydration degree and in vitro degradation in HBSS (Hanks’s balanced salt solution)

The degree of hydration was determined according to:

\[
\% \text{water} = \frac{m_2 - m_1}{m_1} \times 100
\]

where \(m_1\) and \(m_2\) stand for the dry and swollen samples before and after immersion in HBSS, the composition of which is shown in Table 1. The pH was set at 7.4, and the solution...
was sterilized by filtration through cellulose acetate membranes with a 0.22 μm pore diameter.

For the degradation tests, the samples had approximately 0.2 cm⁻¹ (surface/volume) with a volume of 30 cm³ and were sterilized in an autoclave at 121 °C for 15 min. They were immersed in the HBSS at 37 °C, in individual 50 cm³ flasks, and kept for 120 days with stirring (150 rpm) for 120 h. Tests were run in triplicate.

2.2. Cytotoxicity evaluation of the released products in degradation assay with HBSS

Vero cells (derived from *Cercopithecus aethiops* kidney) were provided by the Marcos Enrietti Diagnostic Center – SEAB, Brazil, and were cultured in 96-well microplates using minimum essential medium (MEM), supplemented with 10% fetal bovine serum (FBS), G-potassium penicillin
(100 IU/ml), streptomycin sulfate (100 μg/ml), and amphotericin B (1.25 μg/ml). The cell monolayers were incubated with maintenance MEM (2% FBS) containing 2-fold serial dilutions of the released material from the immersion of films of pure chitin, pure polyurethane and chitin/polyurethane networks in sterilized HBSS for 48 h in an atmosphere of 5% CO2 at 37°C. The compositions of the networks were 10/90, 20/80, 30/70, 40/60, 50/50, 60/40, 70/30 (w/w) chitin/polyurethane.

The viable cells were quantitatively evaluated according to Mosmann [5], using (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (MTT), which is converted into a purple formazan by mitochondrial dehydrogenases, and is detectable spectroscopically.

For those samples in which statistically significant variations were observed in the average absorbance, the percentage of viable Vero cells was determined in relation to the negative control (cells that were immersed in the culture medium only). It was assumed that the average absorbance corresponded to 100% viability in the negative control.

2.3. Biocompatibility evaluation of chitin/polyurethane networks

The evaluation of the in vitro biocompatibility was performed in Vero cells grown in 96-well microplates with an experimental protocol adapted from Sarasam and Sundararajan [6], using the MTT method previously mentioned [5].

Circular pieces (diameter ~ 6 mm in order to fit the 96-well microplates) were cut from the pure chitin film and chitin/polyurethane networks samples and sterilized by autoclaving (121°C, 15 min). Those samples were arranged in the wells (n = 8 wells/sample, run in triplicate). Additionally, the pure polyurethane film was put directly into the well through melting (60°C), and subsequently sterilized by ultraviolet radiation for 40 min. A suspension of Vero cells was added onto the polymer films (20,000 cells/well) and remained in contact with the samples for 48 h, at 37°C with 5% CO2 atmosphere.

After that, the culture medium was withdrawn and the MTT assay was carried out to quantify the viable cells that were adhered.

For the cases that showed significant differences between the average absorbance of the totally adhered cells (positive control) and that of the tests with polymer films, the percentage of the Vero cells adhered to the polymer films in relation to the positive control was determined, considering as 100% adhesion the average absorbance of the positive control (polystyrene surface of the plate containing the Vero cells only).

2.4. Statistical analysis

All experiments were statistically expressed as mean ± standard deviation, and analyzed by Student’s t-test with P = 0.01. Variables exceeding the upper quantification limit were considered statistically significant.

3. Results and discussion

3.1. Hydration degree and in vitro degradation

The degradation tests were run aiming to study the biodegradation behavior of the blends and networks when submitted to conditions that simulate as closely as possible the mechanisms that occur in living tissue. In order to identify possible toxic compounds released, the degradation
tests are also important to determine the lifetime of a biodegradable polymer when used as an implant [7].

The stability of the materials in solution, the evaluation of the toxicity of the released compounds and the amount of absorbed water (hydration degree) were determined. Some materials undergo dramatic changes in mechanical properties such as tensile strength, fatigue resistance, creep, elastic modulus, torsion and flexural moduli when slightly humid [8]. Degradation is also affected by water absorption: hydrophilic materials tend to degrade from the inside to the surface, whereas hydrophobic ones tend to begin to degrade from the outside [9]. The results of the absorption tests presented in Fig. 2, show that for both the networks and the blends the degree of hydration decreases with increasing PU content, as compared to the pure chitin. Apparently, the networks present better water absorption capacity in comparison to the blends, which could be due to the chain separation induced by the tridimensional array that would permit the uptake of the water molecules. In spite of their lower degree of hydration, the networks and blends presented a hydration capacity greater than 50%.

The results of the degradation tests with the HBSS are shown in Fig. 3. A low degradation rate was observed: 1 - 11% for the blends and 1-7% for the networks. Release of the PU in the blends to the solution is not expected due to its low solubility and molecular weight. Only small chains could be released by a diffusion process, as well as reaction by-products such as the LiCl salt used to improve the chitin solubility and traces of solvent. The networks were more resistant to degradation than the blends due to the improved stability provided by the chemical crosslinks [10].

In biological terms, it is important to give assurance that a new material to be employed in a biomedical application will not release toxic compounds nor bring about adverse reactions. This can be verified in principle by means of in vitro cytotoxicity tests. We have used a culture of Vero cells, which according to the literature is one of the cell lines recommended to study the interaction of biomaterials with living tissue [11].

The results of the direct analysis of the solutions submitted to the degradation process, done in order to evaluate the cytotoxicity of the released products, are shown in Fig. 4. The tests were run with the seven compositions of PU/Chitin films, along with those of the pure components. The concentration of the reaction product of MTT, the purple compound formazan, can be directly related to the number of viable cells, since the transformation of the MTT into formazan crystals occurs through the action of the enzymes of the mitochondria present in the viable and active cells [5].
The calculated values demonstrated 100% viability for the pure components (PU and Chitin), while for the networks with a PU/Chitin ratio of 10/90, 20/80 30/70 40/60 50/50 and 60/40 no significant decrease in relation to the control was found in the absorbance, indicating that no toxic products were released. An exception was found for sample 70/30 which showed 81% viability, indicating release of a slight amount of toxic product. This could be due to the presence of solvent traces that were not completely removed in the washing process of the film with distilled water.

3.2. Biocompatibility tests

The biocompatibility tests were run using the MTT reactant as used previously. The results presented in Fig. 5 illustrate the behavior of the Vero cells after 48 h in contact with the networks. All the compositions showed lower absorbance values as compared to the positive control (polystyrene surface of the 96-well microplates used in the cell culture), and only the 70/30 networks showed no significant difference in relation to the positive polystyrene control, with \( P = 0.01 \). The pure polyurethane showed 34% more viable cells than the positive control. Hsu et al. [12] showed no difference in the attachment and proliferation of rat skin fibroblasts on polyurethane and polystyrene. The higher percentage of cellular viability observed here for polyurethane could be related to differences in the thickness of this sample. It was expected that the 70/30 network would allow better adhesion than the other PU:Chitin ratios, as a macroscopic separation was previously observed for this network ratio [1, 2], was closer to the pure PU structure. These results lead to the conclusion that the Vero cells show poor adhesion to the networks, as compared to the positive control. However, this does not imply that the materials are not biocompatible, since they have so far shown no toxic effect.

Reported data have also shown that, for chitosan with degree of deacetylation up to 46% and cells of the fibroblasts and keratinocytes, the higher the deacylation degree the lower is the adhesion capacity of the cells onto the material’s surface and, consequently, the lower the cell multiplication [13]. Other data related to the biological behavior of fibroblasts [14], (L929 and BHK21) revealed that when in contact with chitosan with degrees of deacetylation in the 76 - 90% range, cellular adhesion was favored by increases in the degree of deacetylation. The discrepancy in reported data is in part due to the use of chitin from different sources, the differences in the conditions used for obtaining the biomaterials and also to the different methods of characterization employed.

The photomicrograph of Fig. 6a shows the positive polystyrene control with plenty of cells with normal morphology, whereas in those of (b), (c) and (d) only cell fragments were detected. It is noteworthy that the poor adhesion of the Vero cells to the substrates could be associated with the film’s morphology. It has been reported [15] that the anchoring of a biomaterial to the adjacent tissue is influenced by the presence of pores in the biomaterial, their size and morphology. Materials with pores smaller than \( 1.5 \pm 0.5 \mu m \) have shown lower adhesion and stimulated inflammatory processes [16]. On the other hand, materials with pores of larger dimensions, in spite of the favoring of anchoring and growth, have brought about severe

![Fig. 6. Vero cells seen in an inverted phase contrast microscope, after 48 h at 37 °C and 5% CO₂ in contact with positive polystyrene control (a), pure PU (b), Chitin (c) and network with 10/90 PU/Chitin ratio. Total magnification of 100×.](image-url)
inflammatory reactions [17]. In spite of the promising features of chitin and its combination with different materials for biomedical applications and the large number of papers exploring the subject, information is still lacking for a definitive description of the mechanisms involved and the relationships between morphology and performance.

4. Conclusions

Chitin/Polyurethane blends and networks were prepared and tested aiming at their application as biomedical materials. Both blends and networks presented high stability with a low mass loss in media simulating living tissue. No toxic products were released and the adhesion to Vero cells was low. These preliminary results in vitro indicated that the materials are potentially biocompatible, with promising applications in biomedical applications.

References


