CHEMICAL ENGINEERING TRANSACTIONS
VOL. 49, 2016
A publication of AIDIC
The Italian Association of Chemical Engineering
Online at www.aidic.it/cet
Guest Editors: Enrico Bardone, Marco Bravi, Tajalli Keshavarz

ISBN 978-88-95608-40-2; **ISSN** 2283-9216
DOI: 10.3303/CET1649059

Synthesis and Characterization of Bio-Based Polyurethane for Tissue Engineering Applications

Laís P. Gabriel*[a], Ana Carolina B. Benatti[b], André L. Jardini[a], Gilmara N. T. Bastos[c], Paulo Kharmandayan[b], Carmen G. B. T. Dias[d], Rubens Maciel Filho[a]

[a]School of Chemical Engineering, University of Campinas, Brazil
[b] School of Medical Sciences, University of Campinas, Brazil
[c]Laboratory of Neuroinflammation, Federal University of Pará, Brazil
[d] School of Mechanical Engineering, Federal University of Pará, Brazil
lagabriel@gmail.com

Tissue Engineering offers a new route for the reconstruction of new organs and tissues that have suffered trauma or injury. With this respect, the production of tridimensional scaffolds to allow cells to attach, migrate and proliferate is the base of Tissue Engineering. In this study, a polyurethane scaffold was synthesized in a batch reactor by the reaction of bio-based polyol extracted from *Euterpe oleracea* Mart. seeds (*açaí* berry), a renewable raw material present in Amazon Region of Brazil, and a polyisocyanate derived from hexamethylene diisocyanate. The chemical structure of the scaffold was characterized by using proton nuclear magnetic resonance spectroscopy, the distribution of average porosity and pore size was characterized by X-ray microtomography, the thermal properties were determined by thermogravimetric analysis and the biocompatibility was studied by *in vitro* assays. The results showed that the scaffold presents excellent morphological, chemical and thermal properties, with an appropriate porosity for cell attachment, cell growth proliferation, it also shows no inflammatory response, a good biocompatibility making it applicable as a new biomaterial for future applications in Tissue Engineering.

1. Introduction

Tissue engineering (TE) involves the study of the regeneration of organs or tissues that have suffered injury to restore, maintain or improve the tissue function (Langer and Vacanti, 1993), using a combination of cells, materials and growth factors. Researches directed to effective routes for the fabrication of biomedical devices through reactions of manufacturing processes are gaining attention nowadays.
The synthesis of scaffolds adequate to a tridimensional cell culture is a challenge to be overcome. They act as extracellular matrix that provides support to cells, stimulate rapid blood vessel ingrowth, and the reconstruction of the injured tissue. However, scaffolds should have a tridimensional porous structure that allows the vascularization process and tissue ingrowth, appropriate pore size, highly interconnected porosity, good mechanical properties and sufficient biocompatibility to make them implantable into the patient (Li et al., 2014). The development of different processing techniques such as solid free form fabrication, casting, coating, foaming, aims to develop scaffolds with these characteristics.
Polyurethanes (PUs) are known for their versatility. They are obtained by the reaction between molecules with two or more hydroxyl groups with molecules containing two or more isocyanate groups; additives, fillers, catalysts, chain extenders can also be added. In the last years, PUs have been considered excellent materials for biomedical applications (Blit et al., 2010), such as pacemakers, vascular grafts, stents, drug delivery devices, catheters, articular cartilage, because they exhibit various controllable properties, a good blood compatibility, some antibacterial properties (Atef El-Sayed, 2010).
Most of the works have employed conventional isocyanates, e.g. 2,4- toluene diisocyanate, 1,6-hexamethylene diisocyanate, isophorone diisocyanate and 4,4-diphenil methane diisocyanate. Another isocyanate is the Tolonate™ HDB 75 BX. This is an aliphatic polyisocyanate based on HDI biuret used to produce bicomponent polyurethanes. It is supplied at 75% solids in a mixture of butyl acetate/xylene (1:1).

Please cite this article as: Gabriel L., Benatti A., Jardini A., Bastos G., Kharmandayan P., Dias C., Maciel R., 2016, Synthesis and characterization of bio-based polyurethane for tissue engineering applications, Chemical Engineering Transactions, 49, 349-354
DOI: 10.3303/CET1649059

Figure 1 presents different isocyanates that have been applied in TE. Aromatic isocyanates are more reactive than aliphatic ones, and this choice will change the final properties of the PUs (Cherng et al., 2013). However, PUs based on aromatic isocyanates are considered less biocompatible than PUs based on aliphatic isocyanates because the products of degradation of aromatic isocyanates are toxic, such as aromatic amines from the rigid segments of PUs (Alishiri et al., 2014).

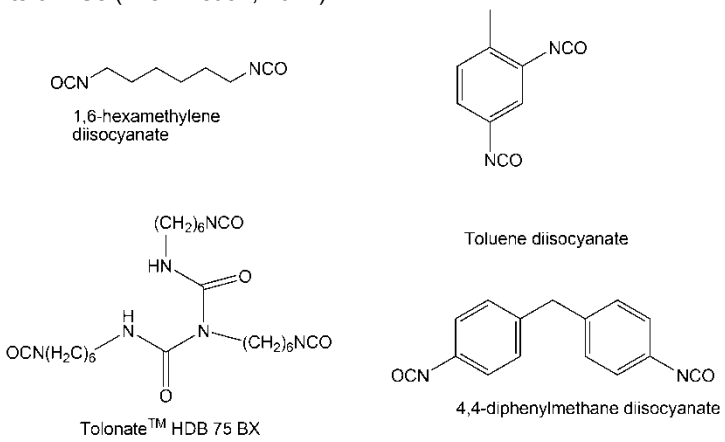

Figure 1: Chemical structures of isocyanates applied in TE.

The most common polyols applied in PU synthesis are hydroxylated polyethers obtained by anionic polymerization of a propylene oxide or by the block copolymerization of propylene and ethylene oxides, such as 1,4-butanediol, poly(ethylene glycol), polycaprolactone. However, the major disadvantage in the synthesis of PU nowadays is the dependence on petroleum-based products; therefore, in order to contribute to global sustainability, the use of renewable biomass as raw materials was recognized as viable (Dubé and Salehpour, 2014). In this way, different types of vegetable oils have been applied, such as sunflower oil (Kalita and Karak, 2014), soybean oil (Heinen et al., 2014), peanut and linseed oil (Garrison et al., 2014).

Euterpe oleracea Mart., popularly known as *açaí*, is a palm tree widely distributed in the northern part of South America, especially in the Amazon Region of Brazil. The *açaí* fruits are spherical dark purple berries with a high content of flavonoids. Each berry is covered by a thin pulp layer, covered by a shell and also has one large seed covered with a layer of fibers. Biochemical studies have shown that the *açaí* seeds contain cellulose and hemicellulose (Wycoff et al., 2015), present antioxidant and anti-inflammatory characteristics due to compounds such as flavonoids and polyphenolic molecules, such as catechin and epicatechin (Bonomo et al., 2014; Hu et al., 2014) (see Figure 2). It was also reported by Barros et al. (2015) that the *açaí* seeds present cytotoxic activity.

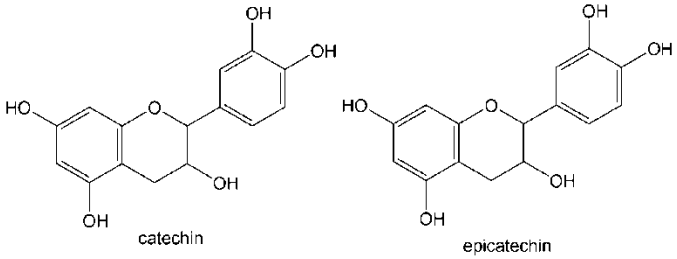

Figure 2: Polyphenolic compounds found in açaí seed.

According to the nature of the reactants and reactivity of isocyanate, PUs present a thermodynamically incompatible structure influenced by the polymerization method. In general, PUs may be synthesized by one-step and more commonly by a two-step method. Usually two-step method is more precise, reliable, with a highly controllable reaction, and a final product is more reproducible. During the two-step method which is also known as prepolymer method, the excess of diisocyanate reacts with the polyol to form the NCO-terminated prepolymer. In the second step, the chain extension occurs, where the prepolymer reacts with a short organic diol to obtain the high molecular weight PU. Generally, the microphase separated structure consisted of a hard segment domain composed of the diisocyanate and the diol, and a soft segment domain composed of the polyol (Mishra et al., 2014). The two-step polymerization method for the formation of PU is presented in Figure 3.

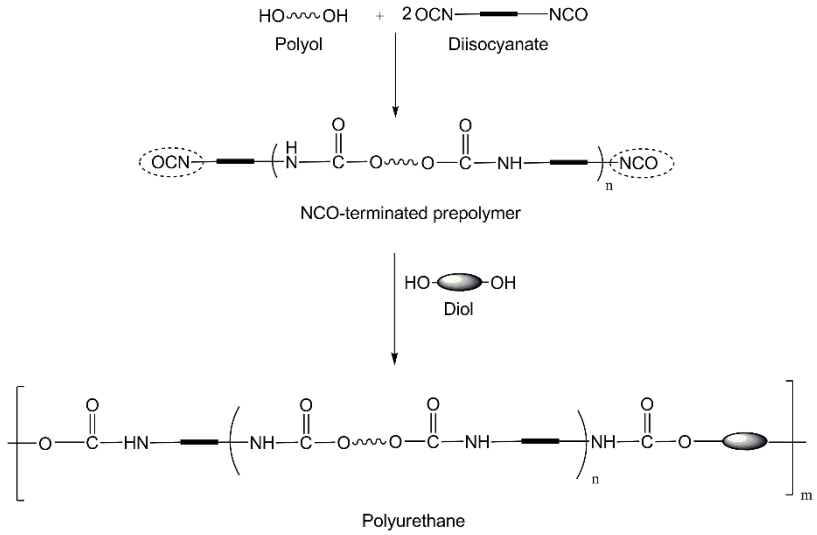

Figure 3: Two-step polymerization method for the formation of polyurethane.

The present work reports the synthesis and characterization of PU synthesized from *açaí*'s polyol and Tolonate™ HDB 75 BX via prepolymer method. The obtained PU was studied through chemical, thermal and in vitro characterizations in order to evaluate the structure, morphology and final properties of the polyurethane.

2. Experimental Section

2.1 Materials

Tolonate™ HDB 75 BX, a polyisocyanate derived from hexamethylene diisocyanate (HDI) was provided by Perstorp (Sweden). *Açaí* berry polyol was kindly provided by the Laboratory of Eco-composites from the Federal University of Pará, Brazil. The extraction method was previously described by Barreira (2009).

2.2 Polyurethane synthesis

PU was synthesized by two-step reactions under dry nitrogen. In the first step, the synthesis was carried out in a batch reactor by starting with a mixture of one mole of polyol with two moles of HDB with a heating rate of 10 ^{o}C/min up to a temperature of 76 ^{o}C by stirring at 100 rpm under 4 kgf/cm^2 of pressure in order to form an NCO-terminated prepolymer. At 76 ^{o}C, a sample of prepolymer was taken out of the reactor and placed in a high density polyethylene bottle for cooling. In the second step, the resulting mixture in the reactor was heated from 76^{o}C to 120 ^{o}C, in the same conditions, to obtain the expansion and crosslinking and also the complete polymerization of PU.

2.3 Characterization

2.3.1 Characterization of the chemical structure

Proton nuclear magnetic resonance spectroscopy (^{1}H NMR) was used to characterize the structure of the sample. The chemical structure of the prepolymer of PU was determined by ^{1}H NMR spectra (Bruker AV 250 NMR Switzerland), using deuterated tetrahydrofuran as a solvent.

2.3.2 Characterization of the porosity, average pore size and pores distribution

Porosity, average pore size and pore size distribution were evaluated by X-ray microtomography using a 1074 microCT imaging system (Skyscan 1074, Aartselaar, Belgium) at 1000 mA, and 40 KV. The specimens were rotated through 360^{o} around the long axis of the sample, with rotation steps of 0.9^{o}.

2.3.3 Characterization of the thermal properties

The thermal behaviour of the PU was studied by thermogravimetric analysis (TGA) with a TGA-50 (Shimadzu Instruments Kyoto, Japan). A sample mass of about 8 mg was heated under a nitrogen atmosphere (50 mL/min) from 30 to 600 ^{o}C, at a heating rate of 10 ^{o}C/min.

2.3.4 In vitro characterization

2.3.4.1 Cell culture

Human normal lung fibroblasts (MRC-5) were obtained from ATCC (Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS, Gibco). Growth media

contained 100 units/mL penicillin and 50 µg/mL streptomycin in a 37 ^{o}C, humidified, 5% CO_2/95% air environment.

2.3.4.2 In vitro cell viability assays

To determine the toxicity of the polyurethane, cells were seeded onto 96-well culture plates at $0{,}5\times10^5$ cells/mL and were incubated for seeding 24 hours. After that, cells were incubated with 100 µL of medium for 24 hours. The amount of formazan crystals formed was measured after 2 hours of exposure to the MTT solution in PBS and absorbance values were measured at 540 nm by a scanning multi well spectrophotometer plate reader (ELISA reader). Cytotoxicity experiments were performed in triplicate and results were presented as mean ± standard deviation.

3. Results and discussion

3.1 Synthesis of porous polyurethane

In this work, PU has been synthesized in a batch reactor, using a polyol derived from renewable biomass and an aliphatic polyisocyanate. In the first step, a NCO-terminated prepolymer was obtained. In the second step, the expansion and crosslinking of the polymer was performed , producing a tridimensional molecular network of urethane groups, which form the high molecular weight thermoset PU scaffold.

3.2 Characterization of the structure

The chemical reaction of PU formation (Fig. 4a) and ^{1}H NMR spectrum of prepolymer (Fig. 4b) are presented.

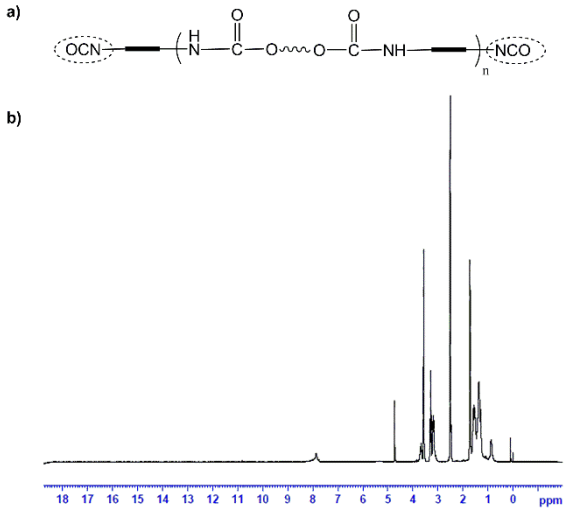

Figure 4: Schematic of the chemical reaction (a) and ^{1}H NMR spectrum of the PU prepolymer (b).

As can be seen in Fig. 4, the signal at around δ 7.8 ppm can be ascribed to amino protons. The methylene groups appeared as peaks at δ 1.3-3.7 ppm. The proton signal of imino group -OOCNH- appeared at δ 4.7 ppm. ^{1}H NMR spectra of prepolymer sample was in accordance with proposed structure.

3.3 Porosity, average pore size and pore size distribution characterization

The 3D model generated by X-ray microtomography of the polymer is shown in Figure 5.

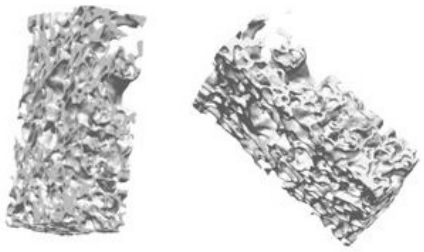

Figure 5: X-ray microtomography of the PU.

Scaffolds exhibit homogeneous morphology and high porosity, due to the reaction of the NCO groups with air humidity. An average pore size of 250 micrometers and porosity of 63,6 % ± 1,4 were found. The obtained

results indicated that PU scaffold exhibit size, morphology, and interconnectivity of pores allowing the vascularization process and tissue ingrowth, so this PU scaffold satisfies the conditions to be applied in TE.

3.4 Thermal characterization

The thermal degradation of polyol (Figure 6a) and PU (Figure 6b) are presented in Figure 6.

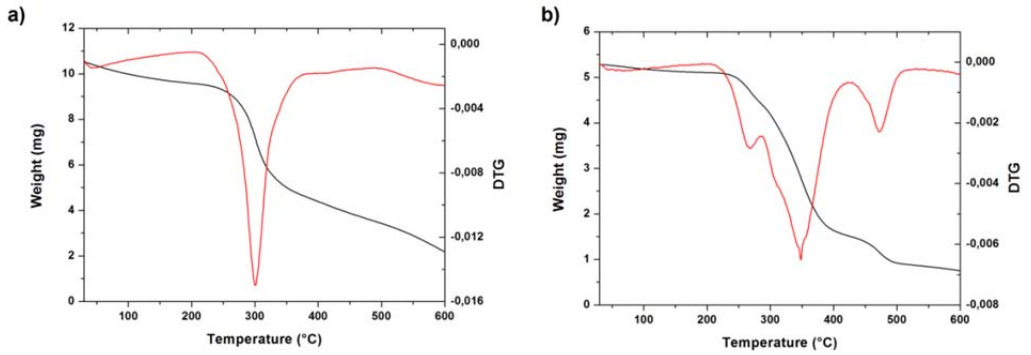

Figure 6: TGA/DTG curve of polyol (a) and polyurethane (b).

For the polyol sample, TGA/DTG curves show 65% mass loss between 279 and 312°C, which can be attributed to the decomposition of oligo-polyssacarides. In PU analysis, the TGA/DTG curves show three steps of mass loss. The first step was attributed to the decomposition of the oligo-polyssacarides, between 233 and 270°C with 16% mass loss. In the second step, 54% of mass loss between 322 and 376°C is attributable to the decomposition of rigid segments of PU, and the third step of 13% mass loss occurs between 427 and 590°C, due to the decomposition of flexible segments of PU. The PU developed in this work is a thermoset polymer and the covalent bonds are responsible for crosslinking between chains formed in the polymerization process. This polymer can be normally sterilized by using different process, including autoclave.

3.5 Biological studies

3.5.1 *In vitro* study

In vitro study is necessary to evaluate the cytotoxicity of the polyurethane. For this purpose, a control and the PU were tested for 24 hours (Figure 7).

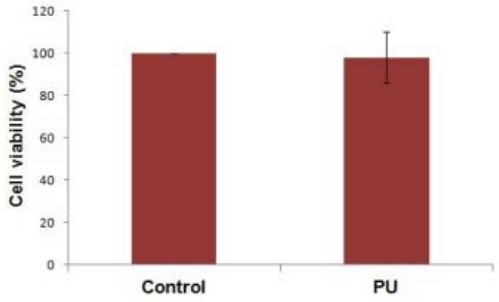

Figure 7: Cell viability of PU.

After 24 hours, PU had 98% ± 12 MRC-5 viability. The result indicated that the PU did not induce any toxicity to the MRC-5 cell line, and it showed potential to be applied in TE.

4. Conclusion

A new bio-based polyurethane was successfully developed by using a natural and renewable seed from Brazil and a polyisocyanate derived from hexamethylene diisocyanate. In summary, the experimental results showed that the material is appropriate for use as TE scaffold because it exhibits high porosity, open, interconnected and well-distributed pores structure, with an average size of porous of 250 micrometers; *in vitro* studies showed appropriate characteristics for cell growth and cell proliferation, promising for future tissue engineering applications.

Acknowledgments

This work was financed by Capes and INCT-Biofabris.

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