EFFECT OF IMMOBILIZED AND FREE *CANDIDA ANTARCTICA* B ON THE ENZYMATIC POLYMERIZATION OF MACROLACTONES

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ABSTRACT

Aliphatic polyesters derived from macrolactones are feasible alternatives in different branches of industry, from packing to biomedical applications. The possibility of employing bio-based monomers and green routes in their synthesis, such as enzyme catalysis, brings suitable polymer properties to these materials aligned with the green chemistry principles. Aiming to understand the role of different polymerization processes using different forms of the same enzyme in a ring-opening polymerization (e-ROP), the copolymer poly(globalide-co- ω-pentadecalactone) was synthesized via solution and miniemulsion polymerization using the enzyme Lipase B from *Candida antarctica* in its immobilized and free form, respectively. This study aimed to investigate the effect of the polymerization technique and the use of free or immobilized enzymes on the final copolymer properties. Lower molecular weights and narrow molecular weight distribution were obtained for the nanoparticles from miniemulsion copolymerization, which also presented a higher crystallinity. This work contributes to predicting the copolymer properties based on the enzyme form and type of polymerization, facts that dictate the final copolymer application, which should be chosen based on its properties.

1 INTRODUCTION

Aliphatic polyesters have been widely employed in different branches of industry, ranging from packing to biomedical applications, due to their excellent properties, such as mechanical resistance, crystallinity, and biocompatibility, allied with the synthetic versatility [1]. Besides, the possibility of polymerizing bio-based monomers through approaches with high control of molecular weight and polydispersity, such as ring-opening polymerization (ROP), allied with green catalysts such as enzymes, brings to polyester advantages in terms of non-toxicity and contributes to developing materials following the principles of green chemistry [2,3].

Using enzymes to obtain polyesters derived from macrolactones is particularly attractive when dealing with biomedical applications, once the resultant polymer does not require further purification to eliminate remaining toxicity, as can happen when using chemical catalysts [4]. Lipase B from *Candida antarctica* (CALB) is an enzyme widely reported in literature employed as a catalyst for the polymerization of several macrolactones via enzymatic ring-opening polymerization (e-ROP), resulting in faster reactions under milder temperatures. Novozym 435, an immobilized form of this enzyme, has been employed to polymerize macrolactones in solution polymerization [5–8] reaching polymers with higher molecular weights than those obtained with chemical catalysts. On the other hand, the free form of CALB has been reported so far by only one work, where it catalyzed miniemulsion homopolymerization reactions to produce nanoparticles [9]. In this sense, the effect of CALB in its free form in the final polymer properties is not well elucidated, nor is the effect of its high surface area in contact with a continuous phase in miniemulsion polymerization.

In ring-opening polymerization reactions, water plays a key role in determining the final polymer properties, such as molecular weight, as it acts as the initiator of the reaction. Novozym 435 is usually dried before use to reduce the amount of water in the support, which explains the high molecular weight polymers obtained when it is applied. In this context, this work aims to compare the use of CALB in its immobilized and free forms in the copolymerization of poly(globalide-co-ω-pentadecalactone) via solution polymerization and miniemulsion polymerization, respectively. Polymer intrinsic characteristics, such as thermal properties and molecular weight distributions, as well as monomer conversion and copolymer composition, will be thoroughly evaluated. This work could provide significant information on the final copolymer properties obtained via two different polymerization techniques using the same enzyme in immobilized and free forms, which is fundamental to dictate the final copolymer application based on these properties.

2 MATERIAL & METHODS

Dichloromethane P.A. 99.8% (DCM), propanone P.A., and ethanol P.A. were purchased from Vetec Química Fina Ltda. (Rio de Janeiro – Brazil). Toluene P.A was purchased from Dinâmica Química Contemporânea Ltda. (São Paulo – Brazil). All solvents were used without any purification. The immobilized enzyme Novozym 435 (lipase B from *Candida antarctica* immobilized on cross-linked polyacrylate beads) was donated by Novozymes A/S (Barigui – Brazil). The esterification activity of the enzyme was

28.5 U/g. [10] Enzymes were dried under vacuum before polymerization (65 °C, 24h), and stored at a desiccator over silica and 4Å molecular sieves. The free CALB was purchased from Sigma Aldrich (Germany) (Enzymatic activity of 7.2-10.8 U/mg). ω-pentadecalactone (ω-PDL) was purchased from Sigma Aldrich (São Paulo – Brazil), and globalide (Gl) was donated by Symrise (São Paulo – Brazil). The copolymerization using the immobilized CALB required drying of both monomers for 24 h at 80 °C under vacuum conditions and storage in a desiccator over silica and 4 Å molecular sieves. Hexadecane was purchased from TCI (Germany), Lutensol AT50 was purchased from BASF (Germany).

The solution copolymerization of globalide and ω-pentadecalactone using the immobilized CALB was carried out in a 25 mL vial in a proportion of 2:1 of the total monomers to the solvent (toluene). Monomers feed mass ratio (Gl: PDL) was fixed at 75:25. Monomers, solvent, and enzyme were weighted on a precision balance (ATX224 Shimadzu, Japan). Novozym 435 was employed at 5 wt%, relative to the total amount of the monomers. The reaction was carried out at 65°C, during 2h, under magnetic stirring. After the polymerization the copolymer was submitted to a purification, that comprises (1) solubilization of the copolymer in DCM, (2) enzyme removal by filtration, and (3) precipitation in a proportion of 1:6 (v/v) to the copolymer, in a cold solution of ethanol: propanone (3:1), where the copolymer was separated from residual monomers and oligomers. In the end, the copolymer was dried in an oven at 60 °C overnight. Typical yields of the copolymerization reactions were 70%.

The miniemulsion copolymerization using the free CALB consisted of the preparation of the organic and aqueous phases before polymerization. The organic phase was prepared with 2.4 g of monomers at the proportion of 75:25 of Gl:ω-PDL mass ratio and hexadecane as costabilizer (4.16 wt% to the monomers). 15 mL of aqueous phase were prepared using distilled water with 1wt% of Lutensol AT50 (related to water). This aqueous phase was portioned into 10 mL and 5 mL: in the first one the organic phase was emulsified by stirring for 1h at 45°C and the formed coarse emulsion was then sonicated using an ultrasonic probe (Branson Digital Sonifier SFX 550) for 3 min in a pulsed mode (10s on, 10s off, amplitude of 90%) to prepare the miniemulsion. The latter portion was employed to solubilize the CALB enzyme (1 wt% to the monomers) and added to the miniemulsion. The reaction was carried out under magnetic stirring, at 45°C. After 5 h of reaction, 0.1 mL of NaOH 1 M solution (pH 14) was added to the reaction to end the polymerization by enzyme denaturation. The nanoparticles (NPs) were purified by 3 centrifugation cycles (1h each) in Amicon filtration tubes (MWCO: 100KDa, 4°C, rotor 12154). Purified dispersions were stored in a refrigerator (4°C).

Monomer conversion and copolymer composition for both strategies were determined using Nuclear Magnetic Resonance (‘H NMR, Bruker Company, USA) operating at 200 MHz. Samples weighing 10 mg were solubilized in 0.55 mL of CDCl3 (δ=7.26 for 1H NMR). Copolymer composition was determined based on the area of the peak related to the double bond of the Gl monomer (around 5.5 ppm) to the methylene peak (4.01 - 4.15 ppm). Copolymer properties were evaluated in terms of Molecular Weight Distribution using Gel Permeation Chromatography (HPLC, LC 20-A, Shimadzu– Brazil); and thermal properties using Differential Scanning Calorimetry (DSC, Jade DSC Perkin Elmer®, USA), at a heating and cooling rate of 10 °C min^{-1}, in an inert atmosphere of nitrogen at 50 mL min^{-1}.

For the nanoparticles resulting from miniemulsion polymerization, the morphology, size, and charge were evaluated. Dynamic Light Scattering (DLS – Malvern Instruments, Zetasizer Nano S) was employed to determine the intensity average particle diameter (D_p) and the polydispersity index (PDI). Zeta Potential (ζ) was measured on a Zeta Sizer Nano Series (Malvern Instruments) equipment in a 1mM potassium chloride solution (1mL) to determine the surface charge of the NPs. Transmission Electron Microscopy (TEM) was carried out with a FEI Tecnai F20 transmission electron microscope operated at an acceleration voltage of 20 kV.

3 RESULTS & DISCUSSION

Aiming to understand the effect of the enzyme form (free or immobilized) on the final copolymer properties, two different polymerization techniques were compared: the first one in solution in toluene, using the immobilized form; and the second, using free CALB in a miniemulsion polymerization. It is worth mentioning that in the last it was not possible to control the amount of water in the enzyme, once the miniemulsion was formed of submicrometric monomer droplets dispersed in a continuous aqueous phase with a very high interfacial area between both phases. Regarding monomer conversion and copolymer composition, information calculated by NMR analysis, the solution polymerization reached 100% monomer conversion after 2h of reaction, while the miniemulsion polymerization reached 95.5% after 5h. The different temperatures employed for polymerization may explain this difference. Both reactions presented the feed copolymer composition similar to the composition calculated using NMR (Table 1), evidencing that there was no preference in interacting with the enzyme active site between both monomers.

Table 1. PGlPDL Copolymer properties from miniemulsion and solution polymerization

Polymerization type (enzyme)	M_n (g mol^{-1})	M_w (g mol^{-1})	Đ	T_m (°C)	ΔH_m (J g^{-1})	X_c (%)[b]	Gl:PDL (mol:mol)[a]	Polymerization degree (N)
Solution (NVZ 435)	9220	42800	4.6	63	80	34	70.4:29.6	39
Minemulsion (free CALB)	1660	4270	2.57	66.4	112.7	48.3	74.1:25.9	6.94

[a] based on NMR data
[b] based on a 100% crystalline PPDL sample [11].

Miniemulsion polymerization reached lower molecular weights, but with a narrower chain size distribution when compared to solution polymerization. It is known that the molecular weight in e-ROP of macrolactones is strongly related to the amount of water

at the beginning of the reaction, once water acts as the initiator of the polymerization [12]. Water dictates the polymer's molecular weight during the reaction once it governs the initiation of the reaction. The higher the amount of water, the more polymer chains will be initiated, resulting in a lower M_n. For this reason, excess water favors the degradation of polymeric chains, resulting in polymers with low molecular weights. [13]. Miniemulsion polymerization employing free CALB exhibits a remarkably high interfacial area associated with the high number of nanoparticles. It facilitates extensive contact between the enzyme's active sites and water molecules. Consequently, it enhances the initiation of numerous polymer chains, resulting in a reduction in molecular weight. Conversely, solution polymerization employs the CALB enzyme in an immobilized form and diminished water content in the reaction medium, yielding fewer initiation events compared to miniemulsion polymerization. Morphology and NPs properties are shown in Figure 1.

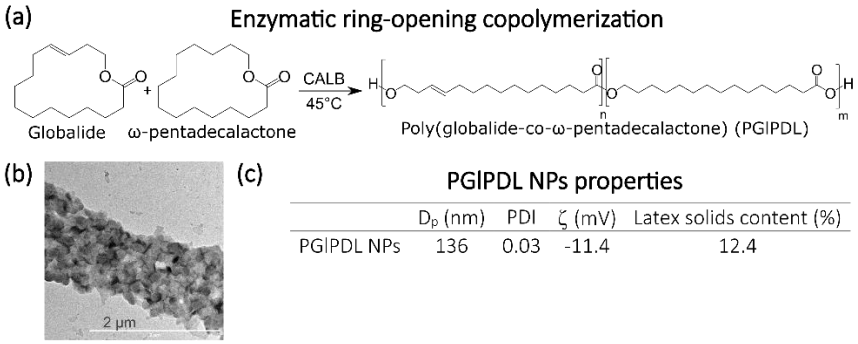

Figure 1 (a) Scheme of the e-ROP of ω-PDL and Gl, (b) TEM image of PGlPDL NPs, (b) NPs properties

Regarding the thermal properties, copolymer crystallinity was estimated using the melting enthalpy of copolymers and the melting enthalpy of a standard 100% crystalline poly(pentadecalactone) [11]. The higher crystallinity obtained for the nanoparticles can be explained by a more compact and organized structure induced by the nanoparticle's formation. The presence of a single intermediate melting temperature in the copolymer serves as an indication of isomorphic crystallization, suggesting that they are indeed random copolymers. Several studies, including those on enzyme-catalyzed copolymerization of lactones and macrolactones, yielded copolymers with random microstructures [14,15]. This observed microstructure is attributed to the rapid transesterification induced by the enzyme during the copolymerization.

2 CONCLUSION

In the present work, the polyester PGlPDL was obtained via solution and miniemulsion polymerization to understand the effect of the polymerization technique and the use of immobilized or free enzyme on the final copolymer properties. Lower molecular weights and narrow molecular weight distribution were obtained for the nanoparticles resulting from miniemulsion copolymerization, which also presented a higher crystallinity. The possibility of controlling the water amount depending on the form of the enzyme proved to be a determinant factor affecting the molecular weight of the final copolymer. This work pointed out the versatility of e-ROP in reaching different polymer properties depending on the form of the enzyme. Thus, it could provide significant information in predicting the final copolymer properties based on the polymerization route and the enzyme form.

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