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Lipase-catalyzed synthesis and characterization of a novel linear polyamidoamine oligomer

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ABSTRACT

A linear polyamidoamine oligomer was obtained by polymerization of ethyl acrylate and *N*-methyl-1,3-diaminopropane, catalyzed by the *Candida antarctica* lipase. Depending on the reaction conditions such as substrates concentration, solvent and enzyme:substrate ratio, the enzyme catalyzes the polymerization reaction or Michael adducts formation. The polymeric material, characterized by FTIR, ^{1}H and ^{13}C NMR and UV-MALDI-TOF-MS, shows low molecular weight and high monodispersity. The activity showed by *C. antarctica* lipase in the polymerization reaction is highly selective and allows to obtain a product with potential biomedical applications in mild condition reactions and low environmental impact.

1. Introduction

Polyamidoamines (PAMAMs) are a family of polymers with interesting properties that can be exploited in biomedical and other applications [1]. These polymers are generally non toxic [2] and contain hydrolysable bonds in their main backbone that allow degradation in aqueous media within days or weeks depending on their structure [3]. Due to their versatile chemical structure and functionality, as well as to their variable aggregation behaviour in solution, such as the formation of micelles or vesicles, PAMAMs are applicable as carriers for the delivery of various products with biological activity [4–6]. They have already been used to deliver anticancer platinates and mitomycin C [7,8], and complex polyanions such as heparin [9] and their ability to promote pH-dependant membrane lysis has been used to enhance intracellular delivery of DNA and toxins [10]. Moreover, PAMAMs have a tremendous water solubilizing power, therefore, even complexes with extremely insoluble drugs are highly water soluble [11,12]. These polymers enter cells by pinocytosis and promote endosomal escape and intracellular trafficking of foreign substances [13]. Moreover, in cross-linked form, hydrophilic PAMAMs give rise to hydrogels, some of which exhibit excellent biocompatibility and are currently being studied as scaffolds for tissue engineering [14].

Regarding to the synthesis of linear polyamidoamines (PAAs), which can be considered as linear analogs of important family of PAMAM dendrimers [15], a large library of them has been designed and prepared for various biological applications, notably by Ferruti and co-workers [2–4,7,13,16–24,37]. A variety of linear polyamidoamines with various functional groups in the side chain could be synthesized by Michael addition of the appropriate primary or secondary amine to *N*,*N*′-bis(acrylamide) (Scheme 1). Their chemical structure generally alternates amide groups with tertiary amines, presenting an overall basic character due to the presence of free amines regularly arranged along the backbone.

The broad diversity of functionalities of PAAs allows the modulation of interactions with bioorganic macromolecules and the potential of tailor-made interactions makes this class of materials highly suitable for the reversible complexation and transport of biomacromolecules. However, the full potentials of these polymers cannot be fully exploited, since they are usually synthesized *via* polyaddition reactions. Due to the statistical step-growth mechanism, products with a polydispersity ~2 are obtained [24,25].

It has been reported that the synthesis of PAAs by the above mentioned traditional methods afford products having essentially a similar structure, but in which the amido- and amino groups are, in part, irregularly arranged along the macromolecular chain [25].

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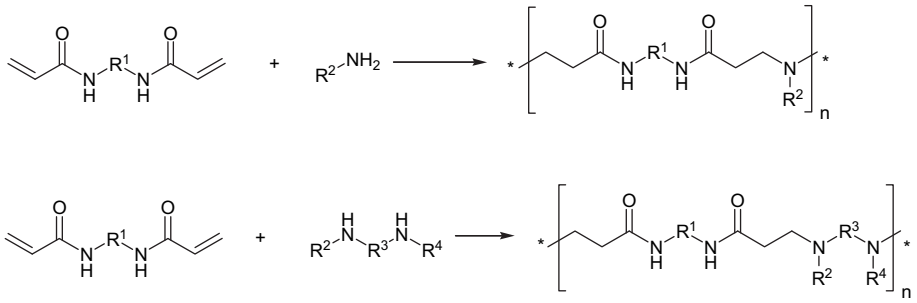


Scheme 1. Synthesis of polyamidoamines, containing two amide groups, from amines and bisacrylamides.

In the case of the piperazine-derived polyamidoamine, obtained by polyaddition of piperazine to 1,4 bis-acryloylpiperazine, contains two types of regularly alternating units, of bis-aminic (**a**) and bis-amidic structure (**b**) (Formula 1).

An alternative synthesis method which involved the reaction of acryloyl chloride and piperazine, contains also a third type of unit, having the structure of a mono-acylated piperazine (**c**) (Formula 1). However, this polymer still had an unexpectedly high degree of chain regularity, showing a ratio between the repeating units: a:b:c = 42:42:16, whereas, if the acylation and addition steps occurred randomly, the expected ratio should have been: 25:25:50 [24].

The rational design of multifunctional polymers is difficult, since the position of functionalities within the polymer chain cannot be controlled. Moreover, it is desirable to obtain more defined products for registration so that synthetic concepts resulting in well-defined structures are mandatory.

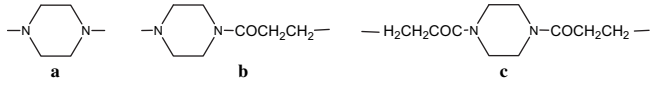
Here we present a straightforward strategy for the direct synthesis of well-defined PAAs by using enzymes as biocatalysts in a clean and easy procedure.

It is well known that biotransformations represent an effective and sometimes preferable alternative to conventional chemical synthesis for the production of fine chemicals and optically active compounds [26]. Moreover, enzymes have proven to be useful catalysts in polymer science, being these polymerizations examples of a new methodology of polymer syntheses and, in many cases, enabling the synthesis of polymers, which otherwise are difficult to prepare [27].

Lipases are efficient catalysts in polyester synthesis through condensation polymerizations that do not require the careful and drastic reaction conditions employed in the reactions catalyzed by metal transition [28]. Biodegradable polyesters have been synthesized by incorporating lipase catalyst from various monomer combinations [29]. In our laboratory we have performed the polymerization of glycerol with ethyl adipate in dry dioxane using the lipase from *Candida antarctica* B as catalyst [30].

We have also described the synthesis of a family of acrylic copolymers, containing at random sequences of ethyl polyacrylate and poly(*N*-(2-hydroxyethyl)acrylamide). In this case, ethyl acrylate was the only monomer starting material in a chain polymerization process, catalyzed by the *C. antarctica* lipase B [31].

Following the application of lipases in polymer synthesis, in this paper we describe the polymerization of ethyl acrylate (**1**) with *N*-methyl-1,3-diaminopropane (**2**), catalyzed by immobilized *C. antarctica* lipase B. We obtained a low molecular weight polyamidoamine with a high monodispersity and a well-defined arrangement along the macromolecular chain.



Formula 1.

2. Experimental

2.1. Materials

Ethyl acrylate, *N*-methyl-1,3-diaminopropane, (Sigma–Aldrich), diisopropy ether, methanol, toluene, acetone, dioxane, hexane, acetonitrile chloroform, ethanol and trifluoroacetic acid (Merck) were used as purchased without further purification. Water of very low conductivity (Milli Q grade; 56–59 nS/cm with PURIC-S, ORUGANO Co., Ltd., Tokyo, Japan) was used. Lipase from *Candida rugosa* (CRL) (905 U/mg solid) was purchased from Sigma Chemical Co.; *C. antarctica* lipase B is abbreviated as CAL B. CAL B immobilized on a macroporous acrylic resin of poly(methyl methacrylate-co-butyl methacrylate): Novozym® 435 (7400 PLU/g) (CAL B-1) was purchased from Codexis Inc., lipase from *Rhizomucor miehei* in the immobilized form on a microporous anion exchange resin: Lipozyme RM 1M (7800 U/g) (LIP) was a generous gifts of Novozymes A/S. Lipase from *C. antarctica* lipase B immobilized by adsorption onto porous polypropylene Chirazyme® L-2, c-f, C3 lyo (3000 U/g) (CAL B-2) was purchased from Roche Diagnostics GmbH. *Pseudomonas cepacia* lipase, PS-C Amano II (804 U/g) (PSL) was purchased from Amano Pharmaceutical Co. All enzymes were used as supplied. Calibrating chemicals for UV-MALDI-TOF-MS analysis [proteins (aprotinin, bovine insulin, angiotensin 1 and angiotensin 2); carbohydrates (α-cyclodextrin, β-cyclodextrin and γ-cyclodextrin)] and matrices [β-carboline (9H-pyrido[3,4-*b*]indole) *nor*harmane (*nor*-Ho), 2,5-dihydroxybenzoic acid (DHB), gentisic acid (GA) and *trans*-3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid (SA))] were obtained from Sigma–Aldrich.

Precautions for handling ethyl acrylate: Avoid contact with eyes, skin, and clothing. Use only with adequate ventilation. Keep away from heat, sparks and flame. Avoid breathing vapour or mist [32].

2.2. Analysis of products

FT-IR measurements were performed on a Shimadzu FTIR-8300 spectrophotometer in film and in solution using a cell for liquids with KBr windows. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AC-200 NMR instrument operating at 200.1 and 50.2 MHz for ^{1}H and ^{13}C. Bidimensional ^{1}H–^{13}C heteronuclear chemical shift correlation spectra (HSQC), ^{1}H–^{13}C heteronuclear multiple bond correlation spectra (HMBC) and ^{1}H–^{1}H homonuclear shift correlated spectra (COSY) were recorded in Bruker AM-500 NMR instrument operating at 500.14 and 125.76 MHz for ^{1}H and ^{13}C, using standard pulse sequences. Solvents are indicated. Elemental analysis was performed in an elemental analyzer CE-440 (Exeter Analytical, Inc). Aqueous solutions of the polymer were titrated with 0.1 N HCl, measuring the pH variation with a Hanna pH 209 pH-meter (Hanna Instruments) equipped with a combined glass/AgCl electrode. The electrode was calibrated with buffer pH 10.00 and pH 7.00 standard solutions (Anedra) and anhydrous sodium

carbonate (CarloErba) was used as primary standard. UV-MALDI-TOF Mass Spectrometry. Measurements were performed using a Shimadzu Kratos, Kompact MALDI 4 laser-desorption/ionization time-of-flight mass spectrometer (Shimadzu, Kyoto, Japan) equipped with a pulsed nitrogen laser (λ_{em} = 337 nm; pulse width = 3 ns), tunable PDE and a secondary electron multiplier (SEM) detector. The samples were irradiated just above the threshold laser power; the irradiation used for producing a mass spectrum was analyte-dependent with an acceleration voltage of 20 kV. Usually 50–100 spectra were accumulated. All samples were measured in the linear and the reflectron modes, in both positive- and negative-ion mode. The stainless steel polished surface twenty-sample-slides were purchased from Shimadzu Co., Japan (P/N 670-19109-01). Spectra were calibrated using as external calibration reagents: (i) proteins (10 μM in MeCN:H_2O–TFA 0.1% (2:3, v:v)) solution with SA as matrix (saturated solution in MeCN:H_2O–TFA 0.1% (2:3, v:v)) in positive ion mode and (ii) the carbohydrates (10 μM in MeOH:H_2O (1:1, v:v)) with *nor*-Ho (saturated solution in MeOH:H_2O (1:1, v:v)) as matrix in positive- and in negative-ion mode. The Kratos Kompact calibration program was used.

Matrix stock solutions were made by dissolving 1 mg of the selected compound, GA and norharmane, in 0.5 ml of MeOH:H_2O (1:1, v:v). Analyte solutions were freshly prepared by dissolving the sample 1–4 (1 mg) in water (1 ml). To prepare the analyte-matrix deposits two methods were used [33]. Method A: (sandwich method), typically 0.5 μl of the matrix solution (M) was placed on the sample probe tip, and the solvent was removed by blowing air at room temperature. Subsequently, 0.5 μl of the analyte solution (**A**) was placed on the same probe tip covering the matrix and partially dissolving it, and the solvent was removed and the tip was dried with a stream of forced room temperature air. Then, two additional portions (0.5 × 2 μl) of the matrix solution (M) were deposited on the same sample probe tip. Method B (mixture method), the analyte stock solution was mixed with the matrix solution in 1:1 to 1:12 v/v ratio. A 0.5 μl aliquot of this analyte-matrix solution was deposited onto the stainless steel probe tip and dried with a stream of forced room temperature air. Then, an additional portion of 0.5 μl was applied to the dried solid layer on the probe and the solvent was removed by blowing air. Among the different UV-MALDI matrices tested (GA and *nor*-Ho) and the different experimental conditions used, best results were obtained with *nor*-Ho and GA as matrix in positive ion mode and sample preparation method A.

2.3. Synthesis of polyamidoamine

In a typical polymerization, to 7.5 ml a 0.12 M solution of ethyl acrylate in diisopropyl ether, 0.1 ml of *N*-methyl-1,3-diaminopropane and 100 mg of lipase were added. The suspension was stirred (200 rpm) at 30 °C. After 72 h, a viscous oil separated from the solution. The solvent was discarded and the reaction flask was washed with chloroform:ethanol 2:1 (3 × 2 ml). The enzyme was filtered off and the washing solvent evaporated. The polymeric material was obtained as a viscous liquid. IR (film, cm^{-1}): 3278, 3084, 2949, 2850, 2804, 1647, 1560, 1466, 1389, 1311, 1261, 1240, 1132, 1049, 754. ^{1}H NMR (200.13 MHz; D_2O): δ 1.2 (t, J = 6.9 Hz), 1.6 (m), 2.2 (s), 2.3 (m), 2.4 (m), 2.6 (m), 3.2 (m), 3.6 (q, J = 6.9 Hz). ^{13}C NMR (50.2 MHz; D_2O): δ 26.8, 33.5, 37.2, 41.4, 53.7, 54.7, 172.5.

2.4. Synthesis of N-(4-azapentyl)-acrylamide (**5**)

To 75 ml a 0.012 M solution of ethyl acrylate in diisopropyl ether, 0.1 ml of *N*-methyl-1,3-diaminopropane and 100 mg of CAL B-1 were added. The suspension was stirred (200 rpm) at 30 °C. After 24 h, the enzyme was filtered off and washed with diisopropyl ether (2 × 5 ml). The supernatant and the washing solvent were combined and evaporated. The product was purified by flash chromatography. Viscous liquid (68 mg, 53% yield). IR (film, cm^{-1}): 3446, 3278, 3005, 2951, 2858, 2804, 1664, 1522, 1465, 1435, 1365, 1232, 1157, 1065, 978, 962, 607. ^{1}H NMR (200.13 MHz; $CDCl_3$): δ 1.71 (2H, m, J = 6.2 Hz), 2.42 (3H, s), 2.70 (2H, t, J = 6.2 Hz), 3.43 (2H, m, J = 6.2 Hz), 5.60 (1H, dd, J = 9.9, 1.8 Hz), 6.06 (1H, dd, J = 16.8, 9.9 Hz), 6.24 (1H, dd, J = 16.8, 1.8 Hz). ^{13}C NMR (50.2 MHz; $CDCl_3$): δ 28.3, 35.4, 37.3, 48.7, 126.0, 130.4, 172.3. Elemental analysis calcd. for $C_7H_{14}N_2O$: C 59.12%, H 9.92%, N 19.70%. Found: C 58.56%, H 9.89%, N 19.02%.

3. Results and discussion

3.1. Enzymatic reactions

Firstly, we tried several commercial lipases in the reaction of ethyl acrylate (**1**) with *N*-methyl-1,3-diaminopropane (**2**): *C. rugosa* lipase (CRL), two commercial forms of lipase B from *C. antarctica*: (CAL B-1 and CAL B-2); Lipozyme (LIP) from the fungus *R. miehei* and *P. cepacia* lipase (PSL). Reactions were carried out at 30 °C using an enzyme:substrate ratio and a diamine (**2**):ethyl acrylate (**1**) ratio of 1 and ethyl acrylate concentration of 3 M.

After 72 h under these reaction conditions, instead of a polymeric material, a mixture of Michael adducts (**4a**–**d**) was obtained through CAL B-1, CAL B-2, Lip and PSL catalysis (Scheme 3). ^{1}H NMR spectra of crude mixtures did not show signals above 4.5 ppm, demonstrating the absence of vinyl protons in the products. A series of unresolved signals at 2.3, 2.4 and 2.6 ppm were assigned to the two new methylene groups obtained by Michael addition to ethyl acrylate. Signals belonging to ethyl ester moieties were also identified (a triplet at 1.25 ppm and a quartet at 4.11 ppm). Aminolysis reaction also occurred in some extent, as demonstrated by a signal at 3.25 ppm corresponding to the methylene attached to the amide nitrogen. As a proof that the whole process was enzyme-catalyzed, no reaction products were obtained in absence of lipase.

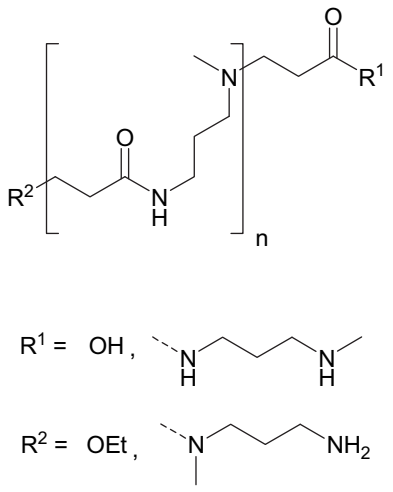
Under similar conditions, in a previous work on the CAL B-1 lipase-catalyzed polymerization of ethyl acrylate in the presence of ethanolamine, we had obtained copolymers of poly(ethyl acrylate) and poly(*N*-hydroxyethyl acrylamide). The substitution of ethanolamine by diamine **2** drove the reaction to afford a mixture of Michael adducts.

Taking into account the work by Gotor et al. [34] and our results on enzymatic synthesis of acrylamides [35], in which the ethyl acrylate concentration was lower, we decided to study the effect of concentration in the reaction between ethyl acrylate (**1**) and *N*-methyl-1,3-diaminopropane (**2**). We observed that at a concentration of ethyl acrylate 0.12 M, in the reaction catalyzed by CAL B-1 and CAL B-2 and using diisopropyl ether as solvent, spectroscopic data were consistent with a polymeric material. *C. antarctica* lipase B was the only lipase giving satisfactory results in terms of polymer obtention. At lower concentration of ethyl acrylate (0.012 M) it was possible to obtain *N*-(4-azapentyl)-acrylamide (**5**) (Scheme 3).

The solvent effect on the polymerization was evaluated in a comparative study between hexane, diisopropyl ether, acetonitrile, acetone and dioxane, working with a diamine:ethyl acrylate ratio of 1.

Attempts to carry out the reaction in polar solvents such as acetone, acetonitrile or dioxane were unsuccessful, recovering the starting materials.

On the other hand, it was observed that non polar and medium polar solvents such as hexane and diisopropyl ether favoured the



Scheme 2. Repetitive unit and ending groups for compound **3**.

polymerization reaction. Therefore diisopropyl ether turned out to be the best choice.

The optimal enzyme:substrate ratio was studied for *C. antarctica* lipase B in hexane using a diamine **2**: ethyl acrylate ratio of 1, varying the enzyme:substrate ratio between 0.1 and 5. The best results in terms of polymerization were achieved with E/S = 1. Control experiments with *N*-methyl-1,3-diaminopropane and ethyl acrylate in standard conditions in the absence of enzyme did not afford polymeric products.

It was observed that enzyme support also played an important role on this reaction. Commercial CAL B-1 immobilized on a macroporous acrylic resin (Novozym® 435) allowed us to recover the reaction product in 60% yield at the most. On the other hand, when the same enzyme was employed immobilized on polypropylene (Chirazyme® L-2, c-f, C3, lyo), polyamidoamine was obtained quantitatively.

Considering the results of enzymatic screening and the study of different parameters of the reaction, CAL B-1 immobilized on polypropylene (CAL B-2) was the catalyst of choice for the polymerization reaction, using hexane as solvent, temperature of 30 °C, ethyl acrylate concentration of 0.12 M and E/S ratio of 1.

3.2. Product characterization

3.2.1. NMR spectroscopy

Regarding its structure, the spectroscopic analysis of the polymeric material showed the presence of a repetitive unit of polyamidoamine as it can be seen in Scheme 2. This proposal is consistent with ^{1}H and ^{13}C NMR spectra. The ^{1}H NMR in Fig. 1 (top) shows a series of signals between 1.2 and 3.6 ppm. The presence of a singlet methylene signal at 3.2 ppm (f) demonstrates that only primary amine was acylated. This fact is also supported by previous results on the lipase-catalyzed acylation of the same diamine [36]. The region between 2.2 and 2.6 ppm shows three partially overlapped methylene signals (one comes from the diamine (d), the other two were formed by the Michael addition (c and e)) and a singlet from the methyl attached to nitrogen (b). The methylene at a two-bond distance from both nitrogen atoms generates the signal at 1.6 ppm (a). We assume that the triplet at 1.2 ppm (R^2) and the quartet at 3.6 ppm (R^2) reveal the presence of ethoxyl residues as ending Michael adducts (R^2, Scheme 2). ^{13}C NMR spectrum contains only seven signals (assigned in Fig. 1 (bottom)), which match the proposed repetitive unit in number of carbons and chemical shift. Moreover, a DEPT 135 sequence showed that all signals, with the exception of those corresponding to methyl (41.4 ppm) and carbonyl (172.5 ppm) carbons, belong to methylene carbons (Supplementary Material). This piece of information favors the proposed structure over a possible polyacrylamide-type constitution. Complete assignation of proposed structure could be achieved by means of bidimensional NMR techniques (Supplementary Material).

3.2.2. FT-IR

This analysis confirms the presence of a polyamide motif with CONHR units. Strong absorption bands from N–H stretching (3278 cm^{-1}) and bending (1560 cm^{-1}), and amide C=O stretching band (1647 cm^{-1}) were observed (Fig. 2).

3.2.3. Physical and acid–base properties

The product was obtained as yellowish viscous oil. It is sparingly soluble in water and chloroform, and very soluble in chloroform-methanol mixtures. These properties are according to those reported for other linear polyamidoamines [37].

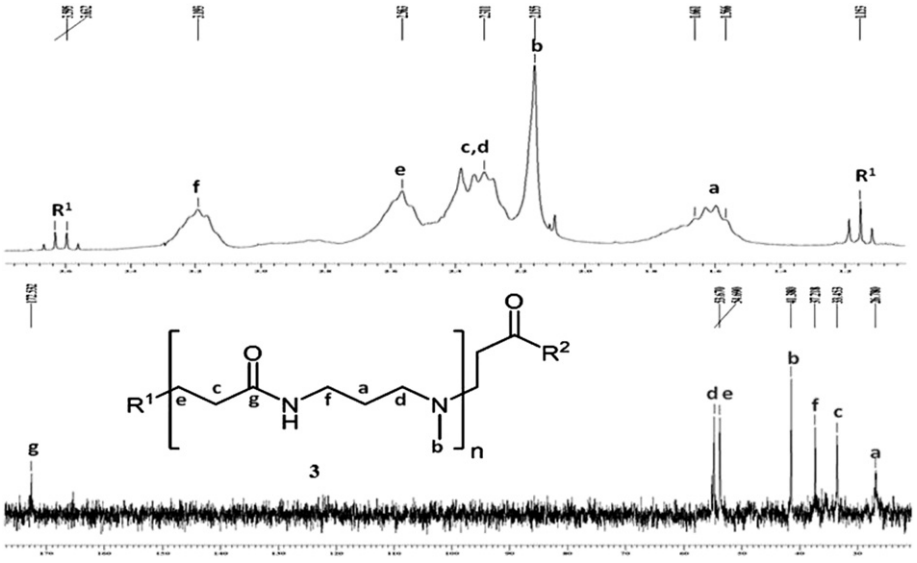


Fig. 1. ^{1}H and ^{13}C NMR spectra of **3**.

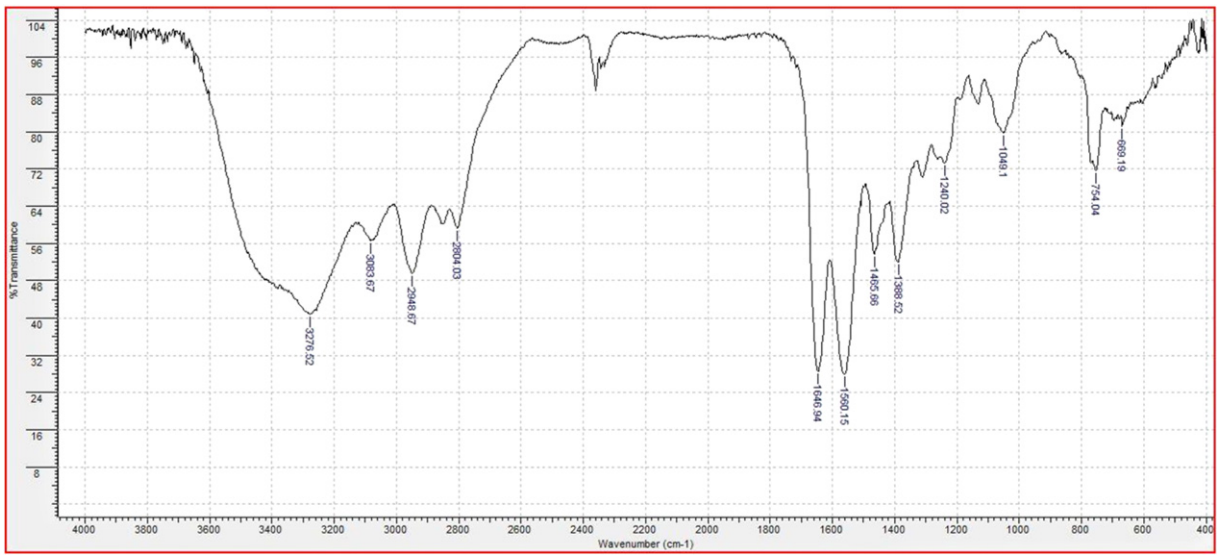


Fig. 2. IR spectrum of polyamidoamine **3**.

The pH of a 1% aqueous solution of the product is 9.5. The amino groups could be titrated with 0.1 N HCl (Supplementary Material). The buffer capacity of the solution was roughly the same between pH 9.5 and 6.5.

3.2.4. UV-MALDI-TOF-MS

In order to determine molecular weight of the product, several mass spectra of the product were collected. All experiments were conducted in positive- and negative-ion mode and two different

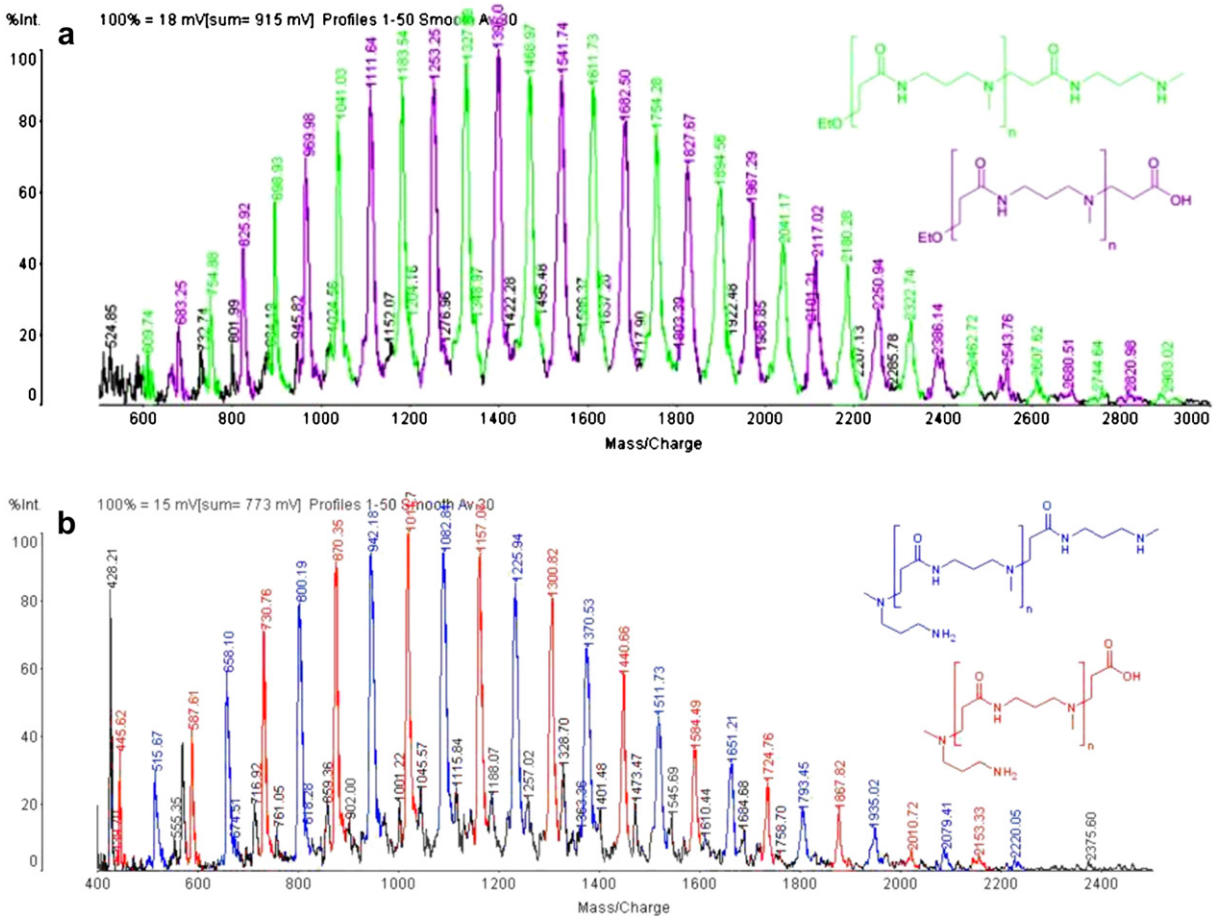
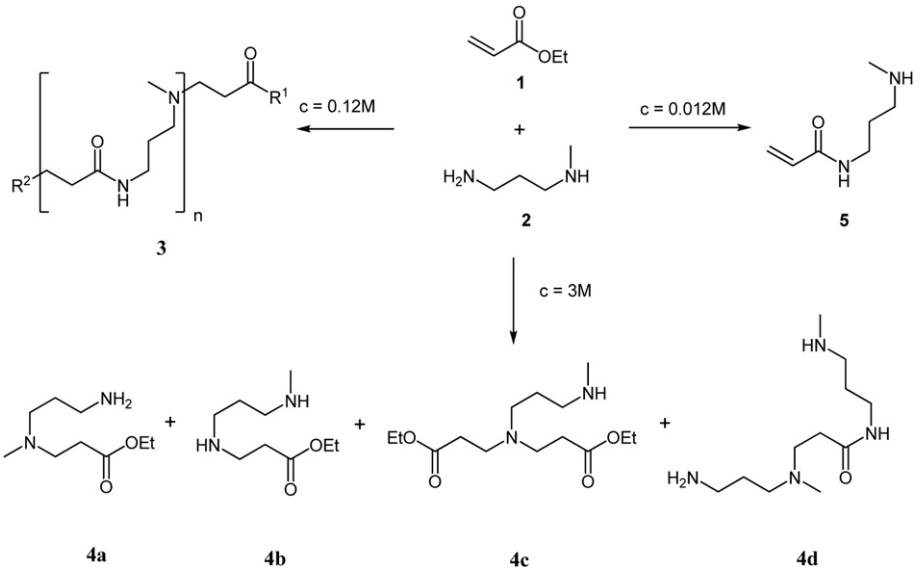


Fig. 3. a. UV-MALDI-TOF spectrum of **3** (matrix: GA, positive ion mode). Green: R^1 = EtO–, R^2 = $-NH(CH_2)_3NHCH_3$. Violet: R^1 = EtO–, R^2 = –OH. b. UV-MALDI-TOF spectrum of **3** (matrix: *nor*-Ho, positive ion mode). Blue: R^1 = $-N(CH_3)(CH_2)_3NH_2$, R^2 = $-NH(CH_2)_3NHCH_3$. Red: R^1 = $-N(CH_3)(CH_2)_3NH_2$, R^2 = –OH.



Scheme 3. Products obtained by lipase-catalyzed reaction between **1** and **2** at various ethyl acrylate concentrations.

sample preparation methods were tested: sandwich (or successive dry layers) and mixture. Moreover, several MALDI matrices with different chemical structure were tested in order to select those that showed better performance (highest sensitivity, signal-to-noise ratio, signal reproducibility) and various matrices: poly-amidoamine concentration ratios were tested.

From all these experiments, best experimental results were obtained with gentisic acid (GA) and with norharmane (*nor*-Ho) as matrices using sandwich preparation method in positive ion mode.

Surprisingly, two different patterns were obtained when using GA (Fig. 3a) or *nor*-Ho (Fig. 3b) as matrices in positive ion mode. As conclusion from the *m/z* values of peaks on both spectra, GA seemed to promote selective desorption of molecules having ethoxyl as Michael ending group (R^2: −OEt, Scheme 3), whereas molecules bearing a diamine as ending group (R^2: $-N(CH_3)(CH_2)_3NH_2$, Scheme 3) could be seen when *nor*-Ho was employed. Two acyl ending groups were also identified: amide (R^1: $-NH(CH_2)_3NH(CH_3)$) and carboxylic acid (R^1: −OH)(Scheme 3).

It is very important to underline that the results obtained with GA and norharmane were complementary. GA as matrix induces desorption/ionization of oligomers with R^2 = ethoxyl as end-group at m/z range 600–3000 in a specific manner.

On the other hand, norharmane as matrix, also specifically, induces desorption/ionization of oligomers with R^1 = amine as end-group at m/z range 400–2400. This so detailed information about the structure of oligomers between m/z 400 and 3000 could only be obtained by UV-MALDI analysis after screening different MALDI matrices.

The obtained results show the presence of four different ending group combinations. M_w, M_n and PD could be determined for each combination separately (Table 1). This analysis confirms that the degree of polymerization is slightly bigger when R^2 is an ethoxyl group (DP: 9–10) than when is diamino (DP: 6–7). It could be also concluded that all combinations are nearly monodisperse.

Table 1
Molecular weight, degree of polymerization and polydispersity of product **3**.

R^1	R^2	DP	M_n	M_w	PD
−OEt	$-NH(CH_2)_3NH(CH_3)$	9–10	1499.7	1620.6	1.08
−OEt	−OH	9–10	1472.6	1589.0	1.08
$-N(CH_3)(CH_2)_3NH_2$	$-NH(CH_2)_3NH(CH_3)$	6–7	1130.4	1249.3	1.11
$-N(CH_3)(CH_2)_3NH_2$	−OH	6–7	1104.7	1230.5	1.08

To estimate the relative abundance of each combination, we considered the results obtained from NMR spectroscopy, mass spectrometry and volumetric analysis. The ratio between *N*-methyl-3-aminopropyl and ethoxyl units could be determined through the quotient among the areas of the signals at 1.6 and 3.6 ppm in the ^{1}H NMR. Equivalent weight and *N*-methyl-3-aminopropyl:ethoxyl ratio for every combination of ending groups can be predicted as a function of the degree of polymerization. In optimal conditions, the amount of ethoxyl groups was negligible, the equivalent weight was 135 g and the degree of polymerization for ethoxyl-free molecules is about 6. Under these conditions, the proportion of acyl terminal groups (R^2) could be estimated as 60% amide and 40% carboxylic acid.

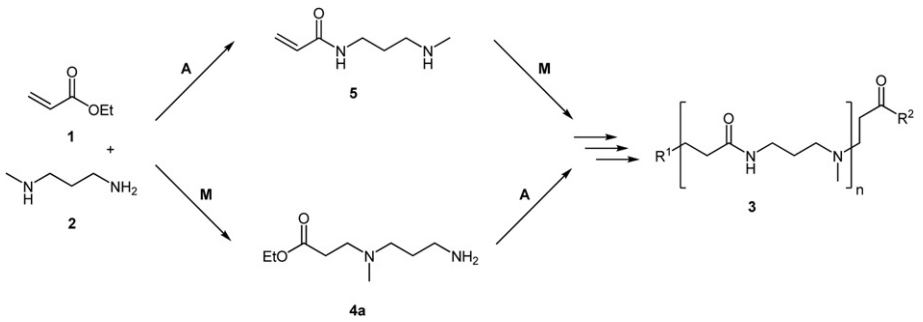
3.3. Mechanism of polymerization

The participation of CAL B in the polymerization process gave the polyamidoamine **3** distinctive characteristics related to this type of polymers that previously were synthesized using traditional chemical methods:

1. Narrow molecular weight distribution, as shown by their low PD values (1.08–1.11).
2. A completely regular structure with a novel repetitive unit. This repetitive unit contains only one amide function instead of the previously reported polyamidoamines which show two amide groups in the repetitive unit.

The second characteristic shows that CAL B could catalyze both aminolysis and Michael addition in a regioselective fashion, as the primary amine group in **2** leads to the amide formation, for example in product **5**, whereas the secondary one acted as nucleophile in Michael addition reactions as in the case of product **4a** (Scheme 4).

Therefore, we decided to investigate the sequence of reactions that takes place in product formation. Two possible mechanisms are feasible and both are depicted in Scheme 4: the formation of the acrylamide monomer **5** prior to the aminoester monomer **4a** or *viceversa*.



Scheme 4. Possible routes for obtaining the product **3**. Two intermediates, acrylamide **5** and aminoester **4a**, are indicated. **M**: Michael addition, **A**: aminolysis.

The formation of both products (**4a** and **5**) to afford the polymer **3**, only can be explained through the regioselective behavior showed by the enzyme.

With the aim to know the sequence of reactions and to obtain a reasonable explanation for the experimental results we follow its progress by FT-IR.

The mixture of reactants dissolved in DIPE shows three IR bands that are diagnostic for the functional groups that are present: (a) a strong band at 1732 cm^{-1} that indicates the presence of an ester, (b) two weak bands at 3327 and 3392 cm^{-1} due to N–H stretching of amines. As the reaction takes place the polymeric material separates from solution. Consequently, once the reaction is complete none of these bands are observed. The reaction progress followed by FT-IR showed three important events: (a) a fast decrease of the band at 1732 cm^{-1}, (b) a change in the profile of the N–H stretching bands. The above mentioned bands were replaced by two new bands at 3336 and 3493 cm^{-1}, (c) a new band appeared at 1684 cm^{-1} during the first 20 h of reaction. After this period this band started to decrease and disappeared completely after 72 h (Fig. 4a and b).

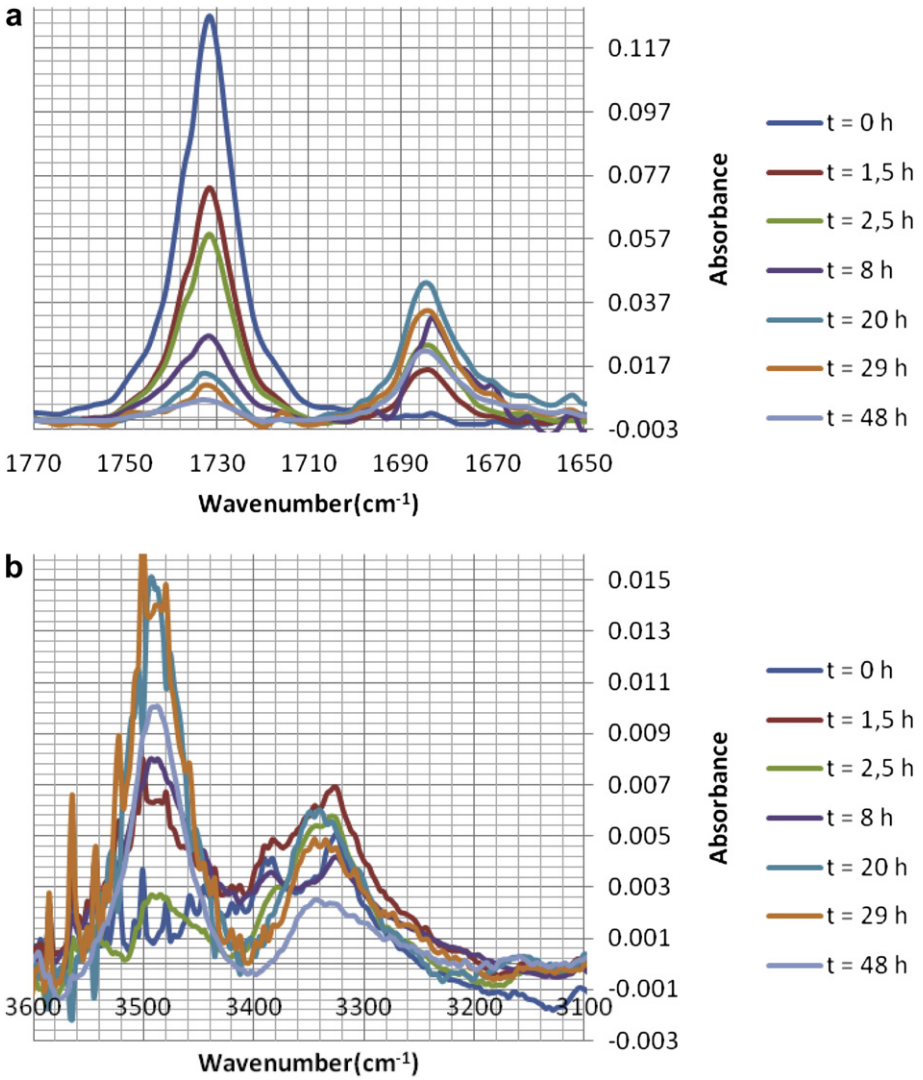


Fig. 4. a. Temporal evolution of FTIR C=O stretching bands of species in solution. b. Temporal evolution of FTIR N–H stretching bands of species in solution.

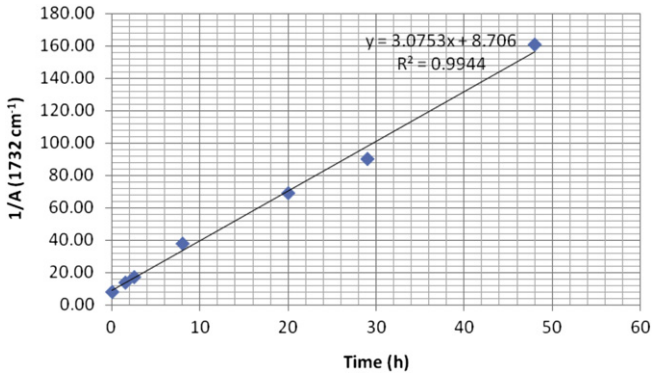


Fig. 5. 1/A (1732 cm^{-1}) vs. time plot. A linear trendline fits reasonably well to experimental data points.

These new bands could be attributed to the presence of the acrylamide intermediate **5**, indicating that amide formation is much faster than Michael addition reaction.

Moreover, a plot of 1/A (1732 cm^{-1}) vs. *t* shows a linear behaviour (Fig. 5), suggesting that ethyl acrylate reacts through a bimolecular mechanism.

These observations illustrate some aspects of the selectivity of this reaction. First, aminolysis occurs always prior to Michael addition and the acylation of primary amine groups is highly regioselective. This leads to the formation of only acrylamide monomer **5** in the first step of the process. Then, this monomer polymerizes *via* a lipase-catalyzed Michael addition. It is interesting to notice that despite both ethyl acrylate and the acrylamide **5** can act as Michael acceptors; the former reacts selectively in the first step yielding **5**.

4. Conclusion

This paper reports the synthesis and characterization of a novel polyamidoamine oligomer (**3**) through the polymerization of ethyl acrylate (**1**) with *N*-methyl-1,3-diaminopropane (**2**), catalyzed by immobilized *C. antarctica* lipase B. The polymeric material, characterized by FTIR, ^{1}H and ^{13}C NMR and UV-MALDI-TOF-MS, shows a low molecular weight and a high monodispersity. Moreover, it is interesting to remark its well-defined arrangement along the macromolecular chain with a novel repetitive unit containing only one amide function. The enzymatic catalysis is highly selective: first, CAL B catalyzes the synthesis of the acrylamide monomer **5**, which in a second step is polymerized through an enzyme-catalyzed Michael addition. This sequence of reactions is reported for the first time and opens a novel pathway for the synthesis of polyamidoamines.

Among the advantages of the present method, we can mention mild reaction conditions, easy handling of the biocatalyst and low environmental impact. The work-up is simple, only filtration of the enzyme and evaporation of the solvent is needed. All these characteristics make the biocatalysis an appropriate means for preparing products with biomedical applications such as polyamidoamines.

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Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.polymer.2010.04.071.

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