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Immobilization of lipase from *Candida rugosa* on synthesized hydrogel for hydrolysis reaction

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ABSTRACT

Novel hydrogels based on *N*-acryloyl-tris(hydroxymethyl) aminomethane (NAT), 2-aminoethyl methacrylate (AEMA) and *N*,*N*′-methylenebisacrylamide (BIS) were prepared and applied for immobilization of lipase. Two procedures were previously performed on the hydrogels as follows: (a) modification with epichlorohydrin (ECH) or 1,4-butanediol diglycidyl ether (BDGE) or (b) grafting with AEMA, using ceric ammonium sulfate as an initiator, and subsequent reaction with ECH. In the hydrolysis reactions, high percentages of lipase activity were reached with products whose enzyme immobilization were performed on both BDGE-containing products and grafted matrices, since the enzyme was bound spaced from the matrices and more likely to interact with the substrate. The best derivatives preserved 86.7–89.5% of the activity corresponding to the soluble enzyme.

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1. Introduction

Enzymes are natural polymeric species that catalyze biochemical processes. They are characterized by high catalytic activity, specificity and selectivity and are able to catalyze many reactions under mild and environmentally friendly conditions. Currently, the use of enzymes applied to organic synthesis is growing. Compounds that are particularly difficult to prepare by purely chemical methods, are affordable and easily obtained with the help of enzymes. Enzymes have proved to be efficient catalysts especially for the resolution of chiral compounds providing a sustainable alternative to traditional catalysts. However, the extraction and purification of the enzyme catalyst is expensive and complex and, due to its instability in solution, it loses its activity after a few hours. Despite their advantages, biocatalysts are not usually used in industrial processes for their high cost, difficulty of the product purification and the instability of the enzymes which allows no reuse. Hence, different ways to improve the performance of the enzymes are being analyzed. Immobilization in a suitable solid support, by combining an enzyme with a polymeric support, would allow reusing the enzyme and facilitate the separation of the products, thus generating an economically viable bioconversion process technology [1–4]. Immobilized enzymes are protected by the solid matrix that limits their conformational changes diminishing variations of their properties. The immobilization of enzymes can be performed by different methods on a variety of supports. Therefore, the enzymes can be adsorbed on insoluble polymeric materials, encapsulated in gels, crosslinked with a bifunctional reagent, and covalently coupled or entrapped within an insoluble polymeric matrix as hydrogel [2,5–8].

Lipases are specially studied for their important applications in the oleo-chemical and renewable energy industries; particularly since they catalyze transesterification and/or hydrolysis of triglycerides and their derivatives can be an alternative to fossil fuel [4,9,10].

A typical hydrogel, as a special insoluble polymeric matrix, is a kind of three-dimensional network with the ability to absorb and swell in water, maintaining its form until a certain equilibrium balance is attained. It can create an adequate medium for the microenvironment and enzyme protection.

In the last years, several research groups, studied the immobilization of the *C. rugosa* lipase enzyme onto hydrogels [7–13].

The present study focuses on the synthesis, swelling behavior and rheological properties of new hydrogels obtained from two monomers by radical copolymerization: *N*-acryloyl-tris (hydroxymethyl) aminomethane (NAT) and 2-aminoethyl methacrylate (AEMA) and *N*,*N*′-methylenebisacrylamide (BIS) as crosslinking agent. The preparation of hydrogels was performed and then modified by different techniques to optimize the subsequent immobilization of the *C. rugosa* lipase enzyme (Fig. 1).

The matrix NAT-co-AEMA was modified in two different ways: on the one hand, epoxy groups were incorporated directly into

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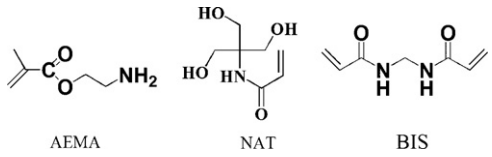

Fig. 1. Monomers and crosslinker used for the synthesis.

the matrix for further immobilization of the enzyme; on the other hand, grafting reactions were performed on the matrix incorporating AEMA, after which epoxy groups were also incorporated to immobilize the enzyme. These modifications were carried out since epoxy-activated supports seem to be almost ideal systems to develop very simple protocols for enzyme immobilization [14,15]. Epoxy groups can easily react with enzymes that contain amino groups giving stable linkage. The enzyme hydrolytic activity of the resulting matrices was determined from the hydrolysis of *p*-nitrophenyl palmitate (*p*-NPP).

2. Materials and methods

2.1. Materials

The following chemicals were purchased and used: *N*-acryloyltris(hydroxymethyl) aminomethane (NAT) (Aldrich); 2-aminoethyl methacrylate (AEMA) (Aldrich); *N*,*N*′-methylenebisacrylamide (BIS) (Mallinckrodt); ammonium persulfate (APS) (Anedra); tetramethylethylenediamine 99% (TEMED) (Anedra); epichlorohydrin (ECH) (Mallinckrodt); 1,4-butanediol diglycidyl ether (BDGE) (Sigma); Lipase from *C. rugosa* (Lip) (Biochemika); Ellman's reagent (Aldrich); Bradford reagent (Sigma–Aldrich); ceric ammonium sulfate [Ce(IV)](Merck) and *p*-nitrophenyl palmitate (*p*-NPP) (Sigma–Aldrich). Fourier Transform Infrared (FT-IR) spectra were obtained on a Nicolet 5-SXC FT-IR spectrometer on KBr discs. A rotational rheometer Anton Paar Physica MCR 301 was used to perform the rheological assays. The UV–visible spectra were recorded with a Shimadzu recording spectrophotometer UV-260.

2.2. Synthesis of hydrogels

The hydrogels were prepared by free-radical solution crosslinking polymerization. A typical procedure for co-polymerization is described as follows: the monomers (AEMA and NAT) and the crosslinking agent (BIS) were dissolved in milliQ water in a glass tube (14 mm internal diameter and 15 cm long) used as a polymerization reactor and stirred for 15 min using an ultrasonic bath. The initiator (APS, 0.01 g) and the activator (TEMED, 0.1 mL) were then added to each solution and left to react for 24 h at room temperature. Table 1 summarizes the experimental conditions. After breaking the tubes, the long cylindrical-shaped matrices obtained were cut into pieces and immersed several times in excess of H_2O at room temperature for 24 h. Afterwards, they were exhaustively washed with water and dried under vacuum at 45 °C to constant weight for 2 days.

Table 1
Experimental reaction conditions to yield products T0–T5.

Product	NAT (mol × 10^{-3})	AEMA (mol × 10^{-3})	BIS (mol × 10^{-4})	H_20 (mL)
T0	3.00	–	1.69	4
T1	2.78	0.22	1.69	4
T2	2.60	0.40	1.69	4
T3	2.40	0.60	1.69	4
T4	2.06	0.94	1.69	4
T5	1.75	1.25	1.69	4

2.3. Network parameters

The swelling index in equilibrium state, q_w, was determined according to Eq. (1) where m_h and m_s are the mass of water-swollen hydrogel at equilibrium and the dry mass, respectively. To determine m_h, weighted dried samples were placed into distilled water and kept at 25 °C for swelling. The swollen samples were removed from the water bath at regular intervals and superficially dried with tissue paper, weighted by an electronic balance and placed in the same bath. The measurements were carried out until a constant weight was achieved.

$$q_w = \frac{m_h}{m_s} \tag{1}$$

The swelling index in relaxed state, q_r (after preparation) was determined according to Eq. (2), where m_r is the mass of the hydrogel after the synthesis.

$$q_r = \frac{m_r}{m_s} \tag{2}$$

The volume fraction of polymer network in the relaxed state (immediately after cross-linking of the polymer but prior to swelling), ν_{2r} [16] was obtained from Eq. (3), where ρ_1 is the density of the polymer, and ρ_2 is the density of water (1.0 g/mL). The determination of the density of the hydrogels was performed by measuring the weight in heptane using the Archimedes principle [17].

$$\nu_{2r} = \left|1 + \left[\frac{(qr-1)\rho_2}{\rho_1}\right]\right|^{-1} \tag{3}$$

The volume fraction of polymer network in the swollen hydrogel at equilibrium (ν_{2s}) was calculated according to Eq. (4)

$$\nu_{2s} = \left|1 + \left[\frac{(q_w-1)\rho_2}{\rho_1}\right]\right|^{-1} \tag{4}$$

The effective molecular weight of the polymer chain between cross-linking points, M_c, was calculated according to Eq. (5), based on the Phantom network model [18] where ϕ is the functionality of crosslinker ($\phi = 4$), V_1 is the molar volume of solvent (18 mL/mol) and χ is the polymer solvent interaction parameter calculated using Eq. (6) [19].

$$M_c = \frac{(1-2\Phi^{-1})V_1\rho_2{\nu_{2r}}^{2/3}{\nu_{2s}}^{1/3}}{\ln(1-\nu_{2s})+\nu_{2s}+\chi{\nu_{2s}}^2} \tag{5}$$

$$\chi = \frac{1}{2} + \frac{\nu_{2s}}{3} \tag{6}$$

In addition, the network pore size ξ was calculated as Eq. (7), in which C_n is the Flory characteristic ratio of the linear polymer (7.6 for AEMA) and l is the length of the bond along the polymer backbone (1.54 Å for vinyl polymers) [20]. This parameter was determined at 25 °C.

$$\xi = {\nu_{2s}}^{-1/3}\left[\frac{2C_nM_c}{V_r}\right]^{1/2} l \tag{7}$$

where V_r is the average molar volume of polymer repeat units. V_r can be calculated as,

$$V_r = \frac{M_1f_1 + M_2f_2}{\rho_1} \tag{8}$$

where M_1 is the molecular mass of AEMA, f_1 is the mole fraction of AEMA, M_2 is the molecular mass of NAT, f_2 is the mole fraction of NAT in the gel system.

2.4. Rheological studies

For the rheological studies, the hydrogels were swelled at equilibrium in water at 20 °C. Therefore, after the synthesis, the samples

were placed into the bath containing milliQ water for 24 h, after which they were removed from the bath and superficially dried with tissue paper. The elastic, G', and the viscous, G'', moduli of the matrices were measured in a rotational rheometer using parallel plates of 8 mm in diameter and gaps of approximately 2 mm. The strain sweeps at a constant frequency of $10\,s^{-1}$ were performed on each sample at 20 °C to determine G' values and the linear viscoelastic region. Then, frequency sweeps at a constant strain were applied to the samples over a wide range of frequencies ($0.1–100\,s^{-1}$) to study the viscoelastic performance of the hydrogels. No evidence of dehydration was found during the tests.

2.5. Modification

2.5.1. Incorporation of epoxide groups

After purification of the hydrogels, the amount of amino groups was determined colorimetrically by using Ellman's reagent [21]. Then, the hydrogels (100 mg) were modified with ECH in a ratio (in equivalents) of 16:1 of epoxide to amine group, respectively, in 4 mL NaOH 0.6 M during 24 h at room temperature or with BDGE in a ratio (in equivalents) of 16:1 of epoxide to amine group, respectively in 4 mL NaOH 0.6 M during 8 h at room temperature, to ensure the presence of oxirane groups for the subsequent immobilization of the enzyme [22]. The hydrogel T0 was also modified with both reagents. In this case, the hydrogel reacted with the same ratio of ECH and BDGE used for T1. Their epoxy equivalents were determined by using the pyridinium chloride method [23].

2.5.2. Grafting

The grafting reactions were carried out on T3 matrix, using AEMA as monomer. These were performed under N_2 in a 250 mL 3-necked flask equipped with a reflux condenser and a mechanical stirrer. The previously weighed dry matrix (200 mg) was placed to swell in 10 mL of water per g of matrix for 24 h. The flask was then placed in a bath at 30 °C and purged with N_2 for 1 h and, maintaining the closed system, 5 mL of 0.05 M Ce(IV) in 0.4 M HNO_3 was added through a syringe, after which, it was purged with N_2 again under stirring. The addition of the monomer AEMA dissolved in 5 mL milliQ water (30 min after the addition of the initiator) was performed at three different final concentrations [0.03, 0.06 and 0.18 M]. The reaction was carried out for 2.5 h. The purification of the grafted matrices was performed by repeated washing with milliQ water, maintaining the agitation. The grafting percentage (%G) was determined gravimetrically using Eq. (9), where m_{msu} is the mass of modified substrate and m_{su} is the mass of the substrate.

$$\%G = \left[\frac{m_{msu} - m_{su}}{m_{su}}\right] \times 100 \quad (9)$$

The grafted hydrogels were characterized by FT-IR. After grafting reactions on T3, the posterior modification was performed with ECH using 50 mg of hydrogel and a ratio (in equivalents) of 16:1 of epoxide with respect to amine group (T3 contained 1.10×10^{-4} mol amine/g), in NaOH 0.6 M for 24 h at room temperature, for subsequent immobilization of the enzyme. Their epoxy equivalents were determined by using the pyridinium chloride method [23].

2.6. Immobilization and quantification of the enzyme

The enzyme immobilization was carried out by placing each matrix (1 g) in contact with 3.9 mg of Lip from a solution of 1 mg/mL *rugosa* lipase in 0.05 M phosphate buffer (pH 7.25) for 24 h. The quantification of bound lipase onto the hydrogels was determined by subtracting unbound protein (in supernatant) from the total protein from the initial concentration. These concentrations were determined using the Bradford reagent. Then, the yielded products were extensively washed with miliQ water. Finally, an aliquot was taken and reacted with Bradford reagent to determine the absorbance at 465 nm confirming that there was no enzyme in the washing liquid because the absorbance was zero.

2.7. Determination of the activity

For the measurement of the enzyme activity, 0.2 g of the substrate *p*-nitrophenyl palmitate (*p*-NPP) was dissolved in 100 mL of ethanol at room temperature. Then, 0.1 mL of free lipase (0.1 mg/mL) or 300 mg of immobilized enzyme was incubated 1 h in 4 mL of 0.05 M phosphate buffer (pH 8.00) at 37 °C, followed by addition of 4 mL of the substrate (dissolved in ethanol). After 5 min, the reaction was finished by adding 2 mL of aqueous solution of Na_2CO_3 (0.25 M) followed by centrifugation for 20 min. The increase in the absorbance at 410 nm caused by the release of *p*-nitrophenol in the hydrolysis of *p*-NPP was measured spectrophotometrically from the supernatant extracted from the reaction media and the quantification was performed with an appropriate calibration curve.

2.8. Storage stability and reusability

The remaining activity of free and immobilized enzyme was measured at batch mode, using the experimental conditions given above. The remaining activity was assayed after storage of products T0-BDGE-Lip, T5-BDGE-Lip, T3-g-AEMA1-ECH-Lip, T3-g-AEMA2-ECH-Lip and T3-g-AEMA3-ECH-Lip for three weeks in phosphate buffer (0.05 M; pH 8.0) at 4 °C.

3. Results and discussion

3.1. Network characterization

Network structure was characterized as a function of its main parameters [24]: swelling index in equilibrium state (q_w); density (ρ); volume fraction of polymer network in the swollen state (v_{2s}); volume fraction of polymer network in the relaxed state (v_{2r}); polymer solvent interaction parameter (χ); effective molecular weight of the polymer chain between cross-linking points (M_c) and the network pore size (ξ) [24]. These are shown in Table 2.

It can be seen (Table 2) that as the proportion of NAT into the hydrogels increases, q_w decreases possibly because the presence of the NAT monomer could cause high intra molecular hydrogen bonding interaction by the presence of the three hydroxyl groups generating physical crosslinks within the network and diminutions of hydrogen bonding with water. Thus, as the AEMA monomer is incorporated into the networks, the intra molecular interactions are avoided, obtaining more expansible networks, consistent with the results obtained by other authors [25]. Thus as the proportion of AEMA increases, the v_{2s} values decrease and the parameters q_w, M_c and ξ increase. With the increase in the proportion of AEMA, v_{2r} (in the relaxed state) decreases although less significantly respect to swollen state.

3.2. Rheological characterization

Fig. 2 shows the amplitude sweep for each sample. The range of deformation, where the deformation imposed on the structure of hydrogels in the rheological experiments is completely reversible, was determined. After measuring the linear viscoelastic range, an appropriate value range of approximately 1% was chosen; this remained constant when the frequency sweep shown in Fig. 3

Table 2
Main network parameters.

Product	q_w	ρ (g/mL)	v_{2s}	v_{2r}	χ	M_c (g × 10^4/mol)	ξ (nm)
T1	10 ± 1	1.55	0.09158	0.093	0.5305	6.39	0.2
T2	12 ± 1	1.55	0.08439	0.090	0.5281	8.52	0.4
T3	15 ± 1	1.55	0.05737	0.088	0.5191	36.3	0.9
T4	19 ± 1	1.55	0.03388	0.086	0.5113	103	1.6
T5	22 ± 1	1.55	0.02487	0.082	0.5083	793	5.6

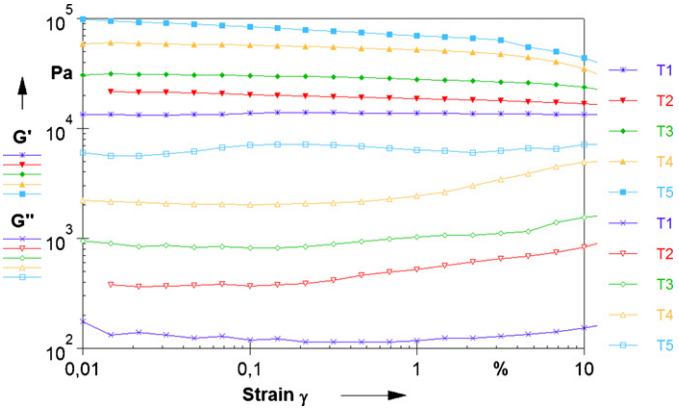

Fig. 2. Strain sweep.

was performed. In the frequency sweep, the variation of G' and G'' was determined as a function of frequency at room temperature for the matrices. The storage modulus G' increased slightly, being greater than G'' for all samples in the frequency range. This behavior indicates that the elastic behavior of the material was stronger than the viscous response, showing a predominantly solid performance.

By comparing hydrogels with the same composition and different degree of crosslinking, those hydrogels that present more crosslinking and lower degree of swelling generally have better mechanical properties. However, in this case the situation is different because the products were prepared with the same proportion of crosslinking agent (see Table 1) but with different monomers (NAT and AEMA) composition. The degrees of swelling are influenced to a greater extent but it must be reminded that they differ in their composition (T1 contains the lower proportion of AEMA while T5 contains the largest). In this particular case, it was observed that G' increased as the incorporation of AEMA was major in the products, indicating a rise in the rigidity of the materials.

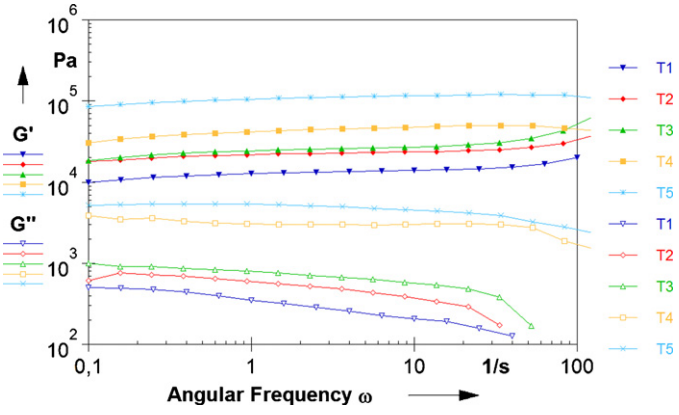

Fig. 3. Frequency sweep.

3.3. Modification

3.3.1. Incorporation of epoxide groups

Direct modification of hydrogels was carried out with epichlorohydrin (ECH) or 1,4-butanediol diglycidyl ether (BDGE) with the objective of incorporating epoxide groups into the structures to immobilize the enzyme (turn acting as spacer arms), and to evaluate the influence of the length of the chain on its immobilization. So, two types of materials were modified: matrix T0, containing only hydroxyl groups (modified with ECH and BDGE to yield T0-ECH and T0-BDGE, respectively) and matrices T2–T5, containing amino and hydroxyl groups (modified with ECH and BDGE to yield (T2–T5)-ECH and (T2–T5)-BDGE, respectively).

The reactions of hydroxyl groups with ECH or BDGE, under basic medium are presented in Fig. 4. As an example, Hermanson et al. [26] presented the activation reaction of the hydroxyl containing-Sepharose 4B with ECH to yield epichlorohydrin-activated Sepharose 4B. Bisoxiranes like BDGE reacts with hydroxylic polymers to yield derivatives containing a long-chain hydrophilic spacer molecule with a reactive oxirane on the end. The bond between the biosoxirane and the matrix becomes a stable ether bond, while the other end provides the ligand coupling potential. This terminal epoxy can then react with ligands containing hydroxyl, amine or thiol groups [26].

The reactions performed to incorporate oxirane groups onto the matrices were carried out through different assays until to reach the better results. However they were realized taking into account experimental conditions studied on other matrices from previous works, which yielded good results [27–29].

On the other hand, the oxirane groups react with nucleophiles as amine-containing compounds through ring opening in basic medium to give amino alcohols. Different authors reported assays to carry out the reaction between an amine containing compound on epoxy-containing matrices under different experimental conditions [30–32].

As seen in Table 3, a major proportion of AEMA into the structures (T2–T5) increases the amount of amino groups in each matrix, their swelling indexes (q_w) and the number of epoxide groups yielded by modification with ECH or BDGE (T2-ECH–T5-ECH and T2-BDGE–T5-BDGE). The matrix containing only hydroxyl groups (T0) was also modified with ECH and BDGE to yield T0-ECH and T0-BDGE, respectively.

Product T0 (of composition 100% of NAT, crosslinked with BIS) reached low q_w probably due to the intra molecular hydrogen bonding interaction by their three hydroxyl groups, as previously described. When these groups reacted with ECH or BDGE, oxirane groups were formed and the possibility of intra molecular physical interactions was diminished, favoring the expansion of the network. On the other hand, the decrease of q_w in T4-ECH and T5-ECH from T4 and T5, respectively, could arise from covalent crosslinks although a high amount of epoxy groups remained intact. The modification with BDGE causes increase in q_w in most cases due to, as described above, intra molecular physical interactions are less likely to occur and because the increase in chain length in all cases inhibits the formation of crosslinks.

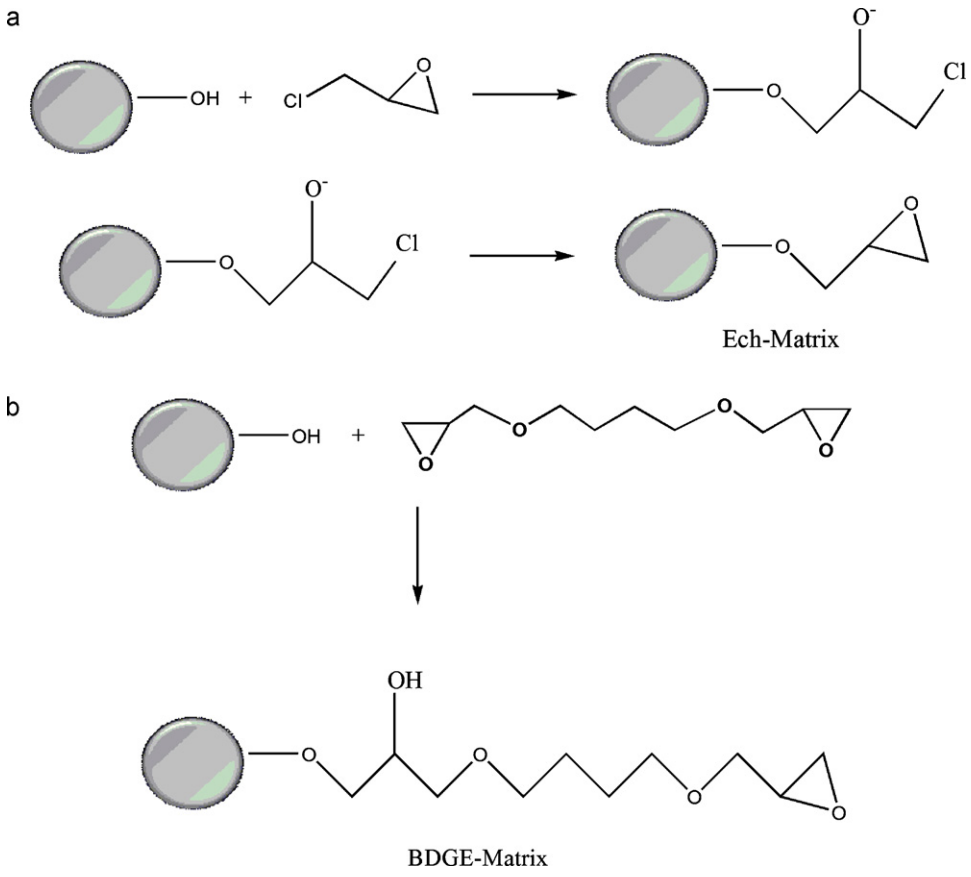

Fig. 4. Mechanism of epoxy incorporation. (a) Reaction of hydroxyl groups with ECH, (b) reaction of a hydroxyl containing-matrix with BDGE

3.3.2. *Grafting*

Redox systems, such as ceric ammonium nitrate and potassium persulfate, have been usually used to produce free radical sites on many kinds of polymer [33]. The use of ceric ions to initiate graft copolymerization of vinyl monomers on synthetic and natural polymers has been reported by many workers [34–36]. Grafting reaction proceeds through redox mechanism in which Ce(IV) ions are reduced to Ce(III) ions, Fig. 5, by the transfer of electron from hydroxyl containing-molecule and hence free radical sites are generated on the surface at which monomer radicals attack to form graft copolymers as tentacles [37–39].

Table 3
Quantification of functional groups and variation of swelling ratios.

Product	Amine groups (mol × 10^{-4}/g)	Oxirane groups (mol × 10^{-4}/g)	q_w
T0	–	–	8 ± 1
T0-ECH	–	3.1	34 ± 1
T0-BDGE	–	1.9	22 ± 1
T2	0.71		12 ± 1
T3	1.10		15 ± 1
T4	1.81		19 ± 1
T5	2.05		22 ± 1
T2-ECH		1.8	28 ± 1
T3-ECH		2.6	22 ± 1
T4-ECH		3.6	16 ± 1
T5-ECH		3.9	12 ± 1
T2-BDGE		1.8	27 ± 1
T3-BDGE		2.5	26 ± 1
T4-BDGE		3.5	24 ± 1
T5-BDGE		4.3	23 ± 1

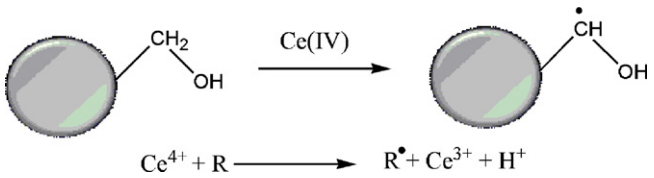

Fig. 5. Mechanism of grafting reaction using Ce(IV).

Furthermore, grafting reactions using Ce(IV) redox initiation method were carried out on T3 varying the concentration of monomer AEMA from 0.03 to 0.18 M. The matrix T3 was selected because it presented intermediate values of: swelling index in equilibrium state, molecular weight of the polymer chain between cross-linking points, network pore size, amount of amine groups and good mechanical properties.

The results of this modification are summarized in Table 4. The evident gain in the weight of the final products gives strong evidence for grafting of AEMA onto T3 base-matrix. As seen in Table 4, the increase in the concentration of monomer added in the reaction medium raised the percentage of grafting yielded (%*G*).

The swelling indexes in equilibrium state, q_w were determined again (after the grafting reactions on T3) and no significant changes were observed respect to the initial matrix, so presum-

Table 4
Percentage of grafting.

Product	AEMA concentration (M)	*G* (%)
T3-g-AEMA 1	0.03	7.2
T3-g-AEMA 2	0.06	17.5
T3-g-AEMA 3	0.18	92.4

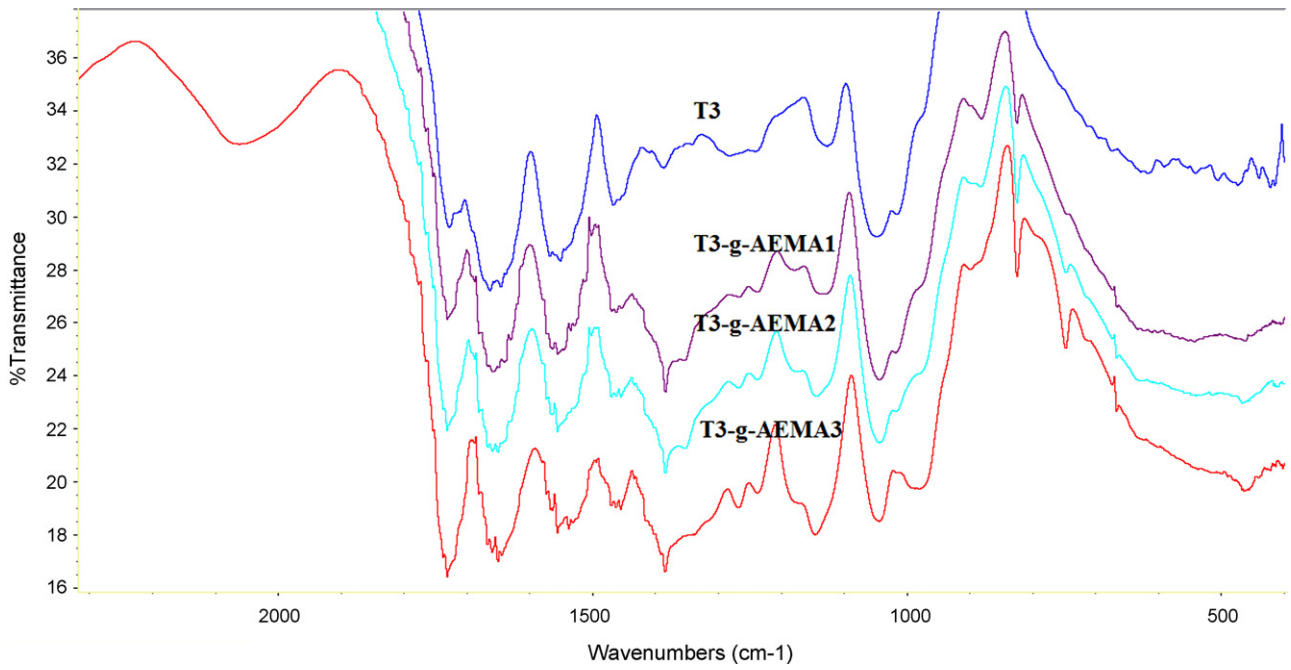

Fig. 6. IR spectra of grafted hydrogels.

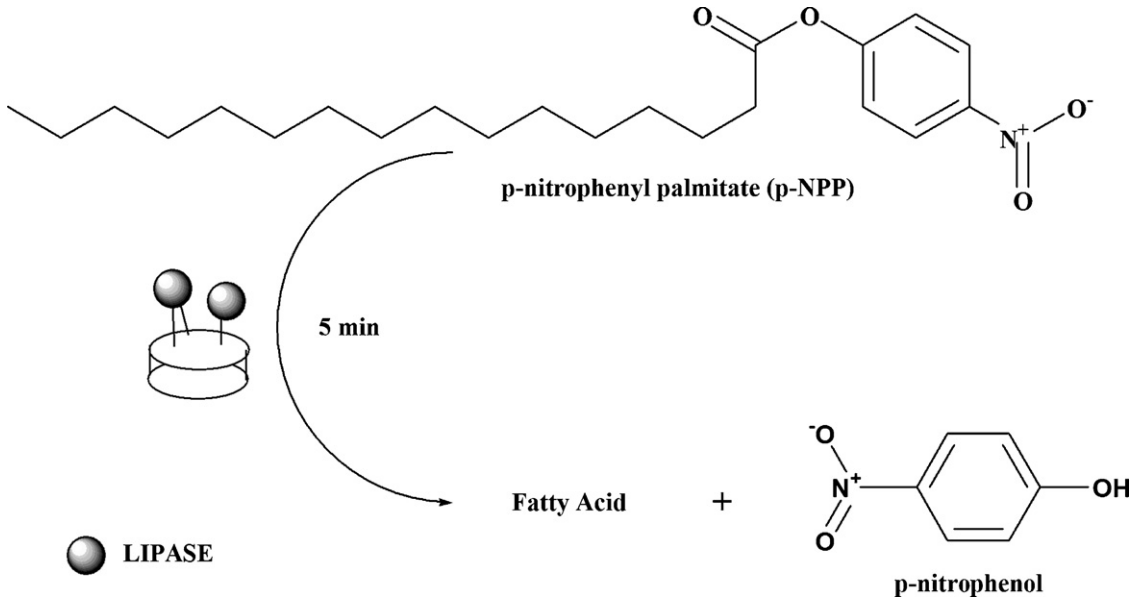

Fig. 7. Scheme of determination of lipase activity from the hydrolysis of *p*-nitrophenyl palmitate.

Table 5
Results of binding of the enzyme and enzymatic activity percentages.

Product	Lip[a] (mg)	Immobilized protein (mg/g hydrogel)	Activity of immobilized Lip ($U \times 10^{-2}$/g hydrogel)	Activity[b] (%)
T0-ECH-Lip	3.9	1.30	42.5	40.8
T2-ECH-Lip	3.9	1.54	14.0	11.4
T3-ECH-Lip	3.9	1.93	16.0	8.3
T4-ECH-Lip	3.9	1.80	12.0	8.3
T5-ECH-Lip	3.9	1.67	16.0	12.0
T0-BDGE-Lip	3.9	0.74	53.0	89.5
T2-BDGE-Lip	3.9	1.28	47.0	45.9
T3-BDGE-Lip	3.9	1.71	63.0	46.0
T4-BDGE-Lip	3.9	1.30	80.0	57.5
T5-BDGE-Lip	3.9	1.33	94.0	88.3
T3-g-AEMA 1-ECH-Lip	3.9	1.59	16.0	12.6
T3-g-AEMA 2-ECH-Lip	3.9	1.20	27.5	28.6
T3-g-AEMA 3-ECH-Lip	3.9	0.75	52.0	86.7

[a] Amount of lipase in contact with 1 g of polymer.
[b] Activity of 1 mg of immobilized enzyme compared to 1 mg of free enzyme, taking into account that 1 mL of free Lip, containing 0.4 mg of enzyme, has an activity of $32\,U \times 10^{-2}$.

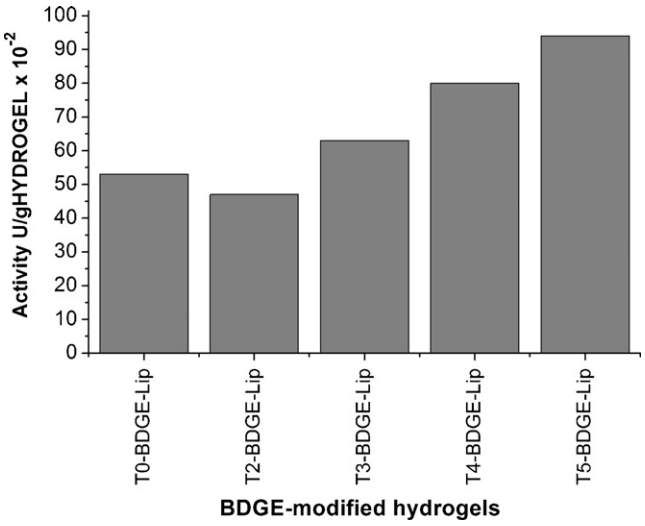

Fig. 8. Activity of immobilized lipase using BDG-modified hydrogels.

ably grafting reactions should not change the properties within the matrix.

The grafted matrices were analyzed by FT-IR (Fig. 6). The spectra show a relative increase of the bands at 1735 cm^{-1} from the stretching vibrations of carbonyl (C═O, from the ester group of AEMA) and at 1385 cm^{-1} from the deformation vibrations of C–H (from C–CH_3 of AEMA methyl group) with respect to the band of stretching of the carbonyl (C═O, from the amide group) at 1650 cm^{-1} while the percentage of grafting increases.

3.4. Quantification of bound enzyme and enzyme activity

Table 5 summarizes the amount of immobilized enzyme onto epoxide-modified hydrogels. The activities of the enzyme immobilized onto matrices were determined from the hydrolysis of *p*-nitrophenyl palmitate (Fig. 7). The results showed increased activity of the products in which the enzyme was immobilized through BDGE (Fig. 8) compared to those performed through ECH (Fig. 9), possibly due to the chain length. The enzyme coupled onto BDGE-modified hydrogels whose spacer arm is longer would be

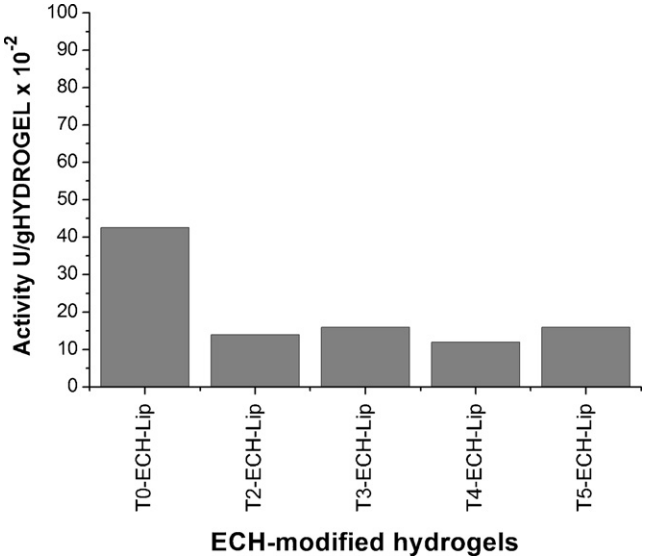

Fig. 9. Activity of immobilized lipase using ECH-modified hydrogels.

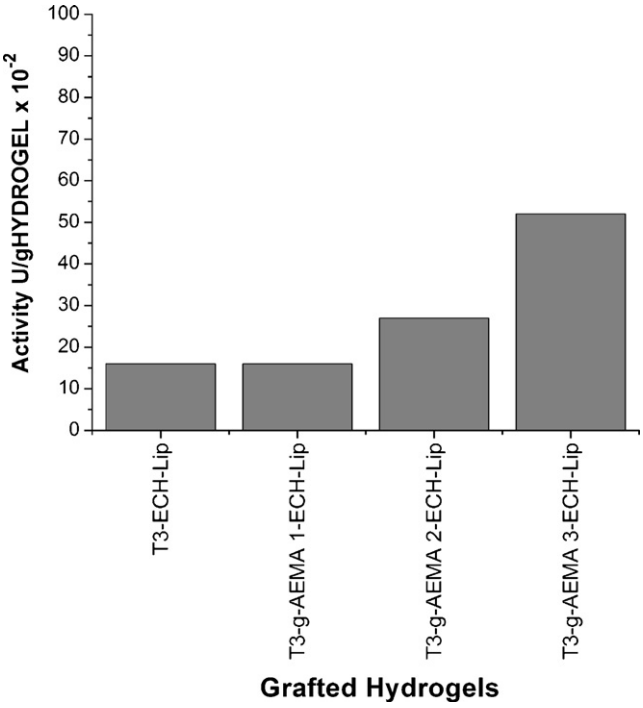

Fig. 10. Activity of immobilized lipase using grafted hydrogels.

distanced from the matrices with increased availability of motion, which would allow greater interaction with the substrate. In the case of grafted matrices modified with ECH (T3-g-AEMA 1-ECH-Lip, T3-g-AEMA 2-ECH-Lip and T3-g-AEMA 3-ECH-Lip) (Fig. 10), it was observed that the activity of the enzyme enhances as the grafting percentage of the matrices increases, although those matrices reached a lower amount of immobilized enzyme. In the grafting reactions, the monomer is usually incorporated into the active sites generated by the initiator; the polymer is formed grafted onto the surface, allowing the separation of enzyme from the matrix and enabling a better interaction with the substrate.

On the other hand, Table 5 summarizes the percentages of activity for each matrix with respect to the activity of the free enzyme. The best derivatives are the following: T0-BDGE-Lip, T5-BDGE-Lip and T3-g-AEMA 3-ECH-Lip preserved 89.5, 88.3 and 86.7% of activity corresponding to the soluble and free enzyme.

It is important to note that in all cases, epoxy groups were yielded for the covalent coupling of the protein. However, it is known that this covalent immobilization is promoted via physical adsorption of the protein by other functional groups and remains in the matrices (like amine or hydroxyl) before their covalent linkage. Other authors reported [14,40] the use of multifunctional epoxy supports.

3.5. Storage stability and reusability

One of the more important parameter to be considered when an enzyme is immobilized on a support is its storage stability and posterior reusability. The stabilities of the free and the immobilized lipase preparations were measured then of storage of T0-BDGE-Lip, T5-BDGE-Lip, T3-g-AEMA1-ECH-Lip, T3-g-AEMA2-ECH-Lip and T3-g-AEMA3-ECH-Lip in phosphate buffer at 4 °C for three weeks. The products T0-BDGE-Lip, T5-BDGE-Lip, T3-g-AEMA1-ECH-Lip, T3-g-AEMA2-ECH-Lip and T3-g-AEMA3-ECH-Lip preserved the activity (respect to the activity of immobilized Lip by gram of hydrogel), about 70, 60, 98, 97 and 88%, respectively while the free enzyme preserved about 40% of the initial activity. It can be concluded that

the covalent immobilization definitively holds the enzyme in a stable position in comparison to the free counterpart.

4. Conclusions

New rod-shaped hydrogels were prepared by co-polymerization of NAT, AEMA and BIS. All products were homogeneous and water-swellable. In all cases, chemical modifications performed onto the yielded hydrogels allowed the immobilization of lipase. High percentages of activity were reached in products in which the enzyme was immobilized on BDGE-containing matrices and grafted matrices. In both cases, although the enzyme was bound to a lesser proportion with respect to the others, it was further spaced from the matrix and therefore more likely to interact with the substrate. The best products kept 86.7–89.5% of the activity corresponding to the soluble and free enzyme. The assays performed to analyze the storage stability and reusability allowed to conclude that various Lip-containing matrices preserved the activity at higher percentages values compared with the free enzyme.

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