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Simultaneous single-step immobilization of *Candida antarctica* lipase B and incorporation of magnetic nanoparticles on poly(urea-urethane) nanoparticles by interfacial miniemulsion polymerization

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ABSTRACT

In this work *Candida antarctica* lipase B (E.C: 3.1.1.3), was immobilized on magnetic poly(urea-urethane) nanoparticles (MNPs-PUU) in a single-step during the interfacial miniemulsion polymerization. Transmission electron microscopy images showed the morphology of synthesized magnetic nanoparticles encapsulated in poly(urea-urethane) nanoparticles and fluorescence microscopy images confirmed the enzyme immobilization onto MNPs-PUU. After the immobilization process, the immobilized enzyme on support (MNPs-PUU) was attracted by an external magnetic field and used as biocatalyst for the synthesis of esters ethyl oleate, geranyl propionate and geranyl oleate. Ester conversions above 85% were obtained for all systems based on the free fatty acids contents measured by titration until pH 11.

1. Introduction

Among various types of magnetic nanoparticles (MNPs), the most used are iron oxide. MNPs exhibit superparamagnetic properties in diameters under 20 nm, therefore MNPs in this diameter range have a single magnetic domain when an external magnetic field is applied. This ability to maintain the magnetic poles in a particular position is denominated coercivity and in MNPs larger than 20 nm the magnetic domains are aligned in several directions.

Another interesting property of MNPs is related to low toxicity, which make them suitable for several applications such as: drug delivery systems [1], magnetic resonance [2,3], hyperthermia and contrast agents for biomedical applications [4].

Furthermore, research in biotechnology showed high magnetic response of MNPs containing enzymes and proteins when an external magnetic field is applied. Thus, MNPs can be used for enzyme immobilization [5–10], for further application on biocatalysis, being able to mediate synthesis of aromas and biodiesel [6,11,12]. Enzymes are limiting factors in some processes due to their high cost, and then the use of immobilized enzymes on MNPs encapsulated in a polymer matrix is an attractive process, once after one reaction cycle they can be recovered by application of a magnetic field and applied to a new reaction cycle.

The coating of MNPs with materials such as surfactants and polymers prevents agglomeration of nanoparticles and these stabilized MNPs can be dispersed in different solutions [1]. MNPs encapsulated in a polymeric shell as support for enzyme immobilization has an advantage preserving the MNPs in a size range with superparamagnetic behavior [13]. Before encapsulation process, MNPs are usually functionalized with a surfactant which allows dispersion in monomers [14]. In MNPs synthesis by precipitation of Fe^{3+} and Fe^{2+} in aqueous solution with a base, is common the coating of obtained MNPs with oleic acid (OA), adsorbed on the surface of the MNPs [14–16], this coating allows the dispersion of MNPs in hydrophobic monomers. After functionalization, MNPs can be encapsulated *in situ* during the synthesis of polymers by different polymerization techniques, including miniemulsion polymerization [15–18].

Miniemulsion polymerization is the mixture of two immiscible liquids by homogenization with high-shear force. Typically miniemulsion formulations consist in water, monomer (or monomer mixture), costabilizer, surfactant and initiator (when polymerization occurs via free radicals) [19]. Polyurethane (PU) and poly(urea-urethane) (PUU) submicrometric particles can be prepared directly via step polymerization in miniemulsion. When water soluble polyols and hydrophobic diisocyanates are used as monomers the reaction occurs at the interface between the

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hydrophobic submicrometric droplets and the continuous aqueous phase [20,21]. The main reaction, between the isocyanate and hydroxyl (from polyol) groups leads to the formation of urethane. However, secondary reactions can occur simultaneously. The reaction between diisocyanate and water produces amino groups that can react with diisocyanate groups leading to urea formation as byproduct. Then, diisocyanates with low reactivity in water, as isophorone diisocyanate (IPDI),can be used, reducing urea formation [13].

Most works reported in the literature regarding lipase immobilization on magnetic materials are performed in two-steps: first, the synthesis of the support and after the immobilization of enzyme on the synthesized support [5,12,22–24]. One of the few works describing the immobilization of lipase on polymer by miniemulsion polymerization in one-step was reported by Cipolatti et al. [25]. The authors immobilized *Candida antarctica* lipase B (CALB) on PEGylated poly(urea-urethane) nanoparticles by step miniemulsion polymerization using isophorone diisocyanate (IPDI) and polycaprolactone (PCL) as monomers. Another work was reported by Valério et al. [26], the authors reported the immobilization of CALB on poly(methyl methacrylate), (PMMA), nanoparticles by miniemulsion polymerization. Both studies showed satisfactory results in relation to lipase immobilization in one-step.

Results regarding two-steps immobilization of CALB on magnetic poly(urea-urethane) were already reported by our research group [27] and the immobilized CALB showed high stability at different pH and temperature values. In face of this scenario, this work shows the simultaneous incorporation of MNPs and immobilization of CALB in a single-step during the synthesis of poly(urea-urethane) nanoparticles with superparamagnetic properties by interfacial miniemulsion polymerization, and after synthesis, the immobilized CALB was used as catalyst in esterification reactions.

2. Material and methods

2.1. Materials

The following reactants were used for the synthesis of magnetite (Fe_3O_4) nanoparticles: distilled water (H_2O), ferric chloride ($FeCl_3{\cdot}6H_2O$), ferrous sulfate ($FeSO_4{\cdot}7H_2O$), ammonia hydroxide (NH_4OH), and oleic acid, (OA), ($C_{18}H_{34}O_2$) all purchased from Vetec. In the interfacial miniemulsion polymerizations isophorone diisocyanate (IPDI, 98%,) and 1,6-hexanediol ($C_6H_{14}O_2$), both from Sigma-Aldrich, were used as monomers, Crodamol GTCC (Alfa Aesar) was used as costabilizer, sodium dodecyl sulfate (SDS, Vetec) was used as surfactant and cyclohexane (Sigma-Aldrich) was used as solvent for the IPDI monomer.

Sorbitol solution of free *Candida antarctica* B (Novozymes NZL-102, CALB) was donated by Novozymes Latin América Ltda (Araucária, PR, Brazil). Ethanol (Dinâmica, 99.5% purity), geraniol (Vetec, 97% purity), oleic acid and propionic acid, from Vetec (97% purity) were used as substrates for esterification reactions. Sodium hydroxide (Quimex, 97% purity) was used for free fatty acids contents determination. Molecular sieves of 4 Å were purchased from Sigma-Aldrich (USA). Sodium phosphate monobasic anhydrous and sodium phosphate dibasic anhydrous, from Vetec (99% purity), were used to prepare sodium phosphate buffer.

2.2. Methods

2.2.1. Candida antarctica *lipase B: purification*

The purification of free *Candida antarctica* B in sorbitol solution was conducted by dialysis of the liquid enzyme using cellulose membrane in sodium phosphate buffer 0.05 M (pH 7.0) during 24 h.

Table 1
Recipes of miniemulsion polymerizations for the in situ immobilization of CALB on magnetic PUU NPs.

Reactants and conditions	M0	M4	I1	I2
IPDI (g)	4.9	4.9	2.46	2.46
1,6-hexanediol (g)	1.07	1.07	0.53	0.53
Cyclohexane (mL)	5.0	5.0	2.5	2.5
SDS (g)	0.6	0.6	0.3	0.3
Crodamol (g)	–	1.08	0.54	0.54
MNPs-OA (g)	–	0.49[a]	0.25[a]	0.25[a]
CALB enzyme (g)	–	–	0.07[b]	0.35[c]
H_2O (mL)	20.0	20.0	10.0	10.0

[a] 10 wt% in relation to IPDI.
[b] 2.
[c] 10 wt% in relation to monomers (IPDI,1,6-hexanediol) and crodamol.

After purification, the enzyme was lyophilized for 24 h and stored under refrigeration at 4 °C for further analysis.

2.2.2. Synthesis of magnetite nanoparticles

Magnetite nanoparticles (MNPs) coated with oleic acid (OA) were prepared by co-precipitation method as described by Feuser et al. [16]. $FeCl_3{\cdot}6H_2O$ (5 g) and $FeSO_4{\cdot}7H_2O$ (6 g) were dissolved in 140 mL of distilled water using a mechanical stirrer (800 rpm) and NH_4OH (11 mL) was subsequently quickly added. One hour after the synthesis of MNPs, 20 mL of OA was added to protect them against aggregation and to modify their surface for subsequent encapsulation. The MNPs coated with oleic acid (MNPs-OA) were separated by applying an external magnetic field and repeatedly washed with ethanol to remove the excess of OA.

2.2.3. Immobilization of CALB lipase on magnetic poly(urea-urethane) by interfacial miniemulsion polimerization

PUU nanoparticles with encapsulated MNPs was based on the step miniemulsion polymerizations as described by Chiaradia et al. [17]. The miniemulsion formulations of the present work are shown in Table 1. The dispersed phase (organic) was prepared using monomer IPDI as diisocyanate, cyclohexane, magnetic nanoparticles coated with oleic acid (MNPs-OA) and Crodamol GTCC. Continuous phase (aqueous) was prepared using 1,6-hexanediol as polyol, water, SDS and free enzyme in different concentrations. Diisocyanate:polyol (NCO:OH) molar ratio was 2.5:1.

MNPs-OA were added to the organic phase using an ultrasound bath during 20 min. After, organic and aqueous phases were mixed and coarse emulsions were prepared by mechanical stirring for 20 min. In the sequence, the coarse emulsions were sonified using an ultrasonic probe (Fisher-Scientific-Ultrasonic Dismembrator 500, 400 W) for 180 s at 70% power intensity to prepare the miniemulsions. Polymerizations were performed at constant temperature (70 °C) for 3 h. The schematic representation of the immobilization process is shown in Fig. 1.

2.2.4. Enzymatic synthesis of esters by immobilized CALB on magnetic PUU nanoparticles

The magnetic PUU nanoparticles with immobilized CALB were attracted by an external magnetic field and used as catalyst for ethyl oleate, geranyl oleate and geranyl propionate production in a solvent free-system, performed according to Bernardes et al. and Paroul et al. [28–30]. These reactions were performed with magnetic PUU NPs with 2 wt% (Reaction I1) and with 10 wt% (Reaction I2) of CALB.

For ethyl oleate production, oleic acid and ethanol were used as substrates in a molar ratio 3:1, 7 wt% of magnetic PUU NPs with immobilized CALB (fraction attracted by an external magnetic field) was used as biocatalyst and molecular sieves (20 mg/mL of substrates) were also used. The experiments were performed in an orbital shaker at 150 rpm at 50 °C during 4 h. Two different method-