Enhanced catalysis and enantioselective resolution of racemic naproxen methyl ester by lipase encapsulated within iron oxide nanoparticles coated with calix[8]arene valeric acid complexes

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In this study, two types of nanoparticles have been used as additives for the encapsulation of Candida rugosa lipase via the sol–gel method. In one case, the nanoparticles were covalently linked with a new synthesized calix[8]arene octa valeric acid derivative (C[8]-C4-COOH) to produce new calix[8]arene-adorned magnetite nanoparticles (NP-C[8]-C4-COOH), and then NP-C[8]-C4-COOH was used as an additive in the sol–gel encapsulation process. In the other case, iron oxide nanoparticles were directly added into the sol–gel encapsulation process in order to interact electrostatically with both C[8]-C4-COOH and Candida rugosa lipase. The catalytic activities and enantioselectivities of two novel encapsulated lipases (Enc-NP-C[8]-C4-COOH and Enc-C[8]-C4-COOH@Fe3O4) in the hydrolysis reaction of racemic naproxen methyl ester were evaluated. The results showed that the activity and enantioselectivity of the lipase were improved when the lipase was encapsulated in the presence of calixarene-based additives. Indeed, the encapsulated lipases have an excellent rate of enantioselectivity, with $E$ = 371 and 265, respectively, as compared to the free enzyme ($E$ = 137). The lipases encapsulated with C[8]-C4-COOH and iron oxide nanoparticles (Enc-C[8]-C4-COOH@Fe3O4) retained more than 86% of their initial activities after 5 repeated uses and 92% with NP-C[8]-C4-COOH.

Introduction

Researchers have developed various methods involving enzymatic and non-enzymatic catalysts to enrich a derivative for one of the enantiomers from the reaction product.1–9 Biocatalysis has been applied as a viable and preferred technique in organic synthesis for the production of enantiopure compounds, particularly for pharmaceutical compounds.10,11 Candida rugosa lipase has a wide range of natural substrates and is thus commonly chosen as a biocatalyst.12 Moreover, lipases are usually used as aqueous solutions, which makes their recovery and reuse problematic and can also result in contamination of the product.13 In an attempt to enhance the activity and enantioselectivity of C. rugosa, researchers have tried immobilizing C. rugosa using various types of carriers such as celite, kaolin, cyclodextrin, amberlite XAD 7, sporo-pollenin, chitosan, and calixarene.14–16

Recently, immobilizing the lipase using calixarenes has become a common way of increasing the lipase activity and enantioselectivity.14,17,18 Calixarenes are used to this end because they are strong building blocks and maintain functionalization at both lower-rim and upper-rim positions.19–24

Over the past few years, scientists have grafted certain calixarene derivatives onto the surface of silica-modified-iron oxide nanoparticles in order to provide easier separation and more reusable processes. Furthermore, in order to improve the activity and enantioselectivity of lipases in the hydrolysis reaction of racemic naproxen methyl ester, some calixarene-grafted magnetite nanoparticles have successfully immobilized the lipase via the sol–gel method, which opens up a wide range of opportunities for future research.14,25

In the present study, p-tert-butylcalix[8]arene was substituted with valeric acid, and selectively grafted onto aminoa silica-modified magnetite nanoparticles to afford the corresponding calix[8]arene-hepta acid-immobilized magnetite nanoparticles (NP-C[8]-C7-COOH). The C. rugosa lipase was encapsulated within a sol–gel system8,9,12,14,17,18 formed through polycondensation with tetraethoxysilane (TEOS) and octytriethoxysilane (OTES) in the presence or absence of octa valeric acid functionalized calix[8]arene (C[8]-C8-COOH) with magnetite nanoparticles to afford Enc-C[8]-C8-COOH@Fe3O4. Moreover, NP-C[8]-C7-COOH was also employed as an additive for the encapsulation of C. rugosa lipase to produce Enc-C[8]-
C₄-COOH. The activity and enantioselectivity of the encapsulated lipases were also evaluated under different conditions such as temperature and pH influences. The magnetite properties of these encapsulated lipases with nanoparticles provide easy separation with respect to reducing the labor force, as well.

Results and discussion

Synthesis of calixarene molecules

In our previous study,[26] we prepared an octa-acid derivative of p-tert-butylcalix[8]arene (6),[33] and examined its usability as an additive in the encapsulation of lipases via the sol–gel process.¹ To obtain 6, p-tert-butylcalix[8]arene was treated with methylbromoacetate to synthesize its octa ester derivative (5). Upon hydrolysis, 5 yielded an octa-acid derivative of p-tert-butylcalix[8]arene (6).[26] (Scheme 1). The focus of the current study was to prepare two novel encapsulated lipases (Enc-NP-C₄-C₄-COOH and Enc-C₄-C₄-COOH@Fe₃O₄) in order to evaluate their catalytic and enantioselective properties. To this end, p-tert-butylcalix[8]arene was initially treated with 5-bromovalerate to afford the octa-valerate derivative C₄-C₄-COOEt (2). The structure of C₄-C₄-COOEt was confirmed not only by the appearance of a new vibration band at 1732 cm⁻¹, which is the carbonyl group of the ester derivative (2) on the FTIR spectrum, but also by the appearance of the peak at 1.11 ppm (24H, –CH₃) and the peaks of –CH₂ which came from the valerate groups on the ¹H-NMR spectrum. The octa-valeric acid derivatives of p-tert-butylcalix[8]arene (C₄-C₄-COOH) (3) having a longer alkyl chain than 6 were synthesized by hydrolysis of 2 with an aqueous solution of NaOH for the first time (Scheme 1). The FTIR spectrum of C₄-C₄-COOH clearly shows that the ester carbonyl vibration shifted to an acid carbonyl

Scheme 1 Preparation of C₄-C₄-COOH and NP-C₄-C₄-COOH. Reaction conditions: (i) paraformaldehyde, NaOH; (ii) K₂CO₃, NaI, 5-bromovalerate; (iii) 15% NaOH solution; (iv) 1-hydroxybenzotriazole, DCC; (v) Fe₃O₄-APTES; (vi) methylbromoacetate, K₂CO₃; (vii) 15% NaOH solution.

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vibration band at 1704 cm$^{-1}$ (Fig. 1). The $^1$H-NMR spectrum of C[8]-C$_4$-COOH also proves the structure of 3 by disappearance of the –CH$_3$ peak of C[8]-C$_4$-COOEt.

One acid moiety of C[8]-C$_4$-COOH (3) was selectively treated with 1-hydroxybenzotriazol to afford the corresponding 5,11,17,23,29,35,41,47-octa-tert-butyl-49-mono-(benzotriazol-1)-oxycarbonyl/butoxy-50,51,52,53,54,55,56-hepta-hydroxycarbonyl butoxy-calix[8]arene (C[8]-C$_4$-COOBaz) (4) (Scheme 1). The $^1$H-NMR spectra of 4 confirmed exclusive functionalization by giving the expected splitting pattern (two doublets at 7.69 and 7.96 ppm and two triplets at 7.39 and 7.52 ppm for 1 integral ratio proton of ArH of the benzotriazole unit). Subsequently, C[8]-C$_4$-COOBaz was grafted onto the aminosilica-modified Fe$_3$O$_4$ nanoparticles (Fe$_3$O$_4$-APTES), which were prepared according to the literature, to produce NP-C[8]-C$_4$-COOH (Scheme 1). NP-C[8]-C$_4$-COOH was then used as an additive in the encapsulation of the lipase. FTIR spectra were used to elaborate the structure of NP-C[8]-C$_4$-COOH (Fig. 2). The characteristic peaks of NP-C[8]-C$_4$-COOH appeared at 1641 (COONa) and 1621 cm$^{-1}$ (COONH). Additional peaks centered at 1542, 1468, 1413 and 1363 cm$^{-1}$, which are stretching vibrations of the aromatic C=C bonds of the calix[8]arene derivative. Moreover, the FTIR spectrum also shows peaks of the magnetite nanoparticles at 1149, 1046, 957 and 790 cm$^{-1}$ for the Si–O group and at 578 cm$^{-1}$ for the Fe–O group (see Fig. 2).

Transmission electron microscopy (TEM) analysis was used to obtain more information about particle size and morphology (see Fig. 3) of Fe$_3$O$_4$-nanoparticles (Fig. 3a), and NP-C[8]-C$_4$-COOH (Fig. 3b). TEM images of Fe$_3$O$_4$-nanoparticles are observed as intensive aggregates due to the lack of any repulsive force between the magnetite nanoparticles, which is due to a single magnetic crystallite and the uniform nano-size of the Fe$_3$O$_4$, which has a typical size range of 8 ± 3 nm and is surrounded by a silica-based material and calixarene units that are about 19 ± 5 nm thick. Using calix[8]arene derivative immobilization, the dispersion of particles was improved greatly (Fig. 3b) due to the production of an electrostatic repulsion force and steric hindrance between the calix[8]arene and the surface of the Fe$_3$O$_4$ nanoparticles.

Thermogravimetric analysis (TGA) was used to determine the amount of C[8]-C$_4$-COOH (Fig. 4) on the aminosilica-modi-
fied Fe$_3$O$_4$ nanoparticles (Fe$_3$O$_4$-APTES). As depicted in Fig. 4, the TGA curve of NP-C$_{[8]}$-C$_4$-COOH reveals that the weight loss of 30% mass was due to the decomposition of C$_{[8]}$-C$_4$-COOH and the 3-aminopropyltrimethoxysilane groups in the range of 325–625 °C.

Sol–gel procedure for the encapsulation of C. rugosa lipase on additives

In our previous study, calix[8]arene octa acid (6) was used as an additive in the sol–gel encapsulation process of lipases in order to estimate its catalytic ability in the enantioselective hydrolysis of racemic naproxen methyl ester. It was found that the activity and enantioselectivity of the encapsulated lipase with 6 were extremely high when compared with the affinity of the encapsulated lipase without any additives. This increase in activity and enantioselectivity of the lipase was attributed to a complex between the −COOH groups of the calix[8]arene derivative and the lysine moieties of the enzymes as well as host–guest interactions and the cooperative affinities of calix[8]arene derivatives. The present study aimed to extend the number of calix[8]arene-based additives and compare their catalytic abilities. Furthermore, either a calix[8]-arene octa valeric acid derivative (C$_{[8]}$-C$_4$-COOH) and Fe$_3$O$_4$-nanoparticles or hepta valeric acid-substituted calix[8]-grafted iron oxide nanoparticles (NP-C$_{[8]}$-C$_4$-COOH) were employed as additives for the encapsulation of the lipase (see Fig. 5). A sol–gel procedure was used to encapsulate the lipase with or without additives, which provided mechanical entrapment of the enzyme according to the published procedure. The Bradford method regarding bovine serum albumin was used as a standard to determine the amount of protein in the solution and in the elution solute.
changes in activity of the encapsulated lipases were determined according to the literature. Changes in activity of the encapsulated lipases were determined according to the literature.  

Table 1 reveals the results of the initial attempt to associate the catalytic activity of the encapsulated lipases in the presence/absence of C₈-COOH, Fe₃O₄-nanoparticles, and Enc-C₈-C₄-COOH@Fe₃O₄. As seen in Table 1, in terms of catalytic activity and enantioselectivity, the encapsulated lipase in the presence of C₈-COOH (6) did not show higher affinity than the encapsulated lipase [Enc-(C₈-C₄-COOH@Fe₃O₄)]. However, the encapsulated lipase with C₈-COOH (6) was more efficient than the encapsulated lipase (Enc-Lipase) in the absence of additives (see Table 1). Moreover, in order to determine the role of the Fe₃O₄-nanoparticles in this reaction, the lipase was encapsulated in the presence of only Fe₃O₄-nanoparticles as an additive. However, the encapsulated lipase with iron oxide nanoparticles exhibited less activity (see Table 1). These findings clearly suggest that both octa COOH-substituted calix[8]-arene derivatives represent complexibility toward the lipase such as host–guest, hydrogen bonding, and ionic interactions that might increase the activity and stability of the lipase.

Table 1 also indicates the catalytic activity results of the encapsulated lipase in the presence of NP-C₈-C₄-COOH. The encapsulated lipase (Enc-NP-C₈-C₄-COOH) provided high catalytic activity and enantioselectivity as compared to the Enc-Lipase and Enc-NP.

Effect of pH and temperature on the lipase activity

The hydrolysis of p-NPP by the encapsulated lipases was examined to assess their catalytic activity at various pHs (4.0–9.0). Finding an optimum pH for the encapsulated lipases is the optimal point, since it is well known that the conformational change of the enzyme results in a different catalytic activity when the pH of the media is changed. With that in mind, the various pHs were altered so that the encapsulated lipases would show the most efficient hydrolysis behaviour towards p-NPP. It was found that both free and encapsulated (C₈-)
COOH, C[8]-C4-COOH) demonstrated high hydrolysis capability at pH 7.0, whereas the obtained efficient hydrolysis of p-NPP was observed to be at pH 6.0 for the encapsulated lipase in the presence of NP-C[8]-C4-COOH (see Fig. 6a).

To calculate the affinity of the encapsulated lipase (Enc-Lipase) without calixarene derivatives or magnetite nanoparticles, as well as the affinity of lipase-encapsulated calixarene derivatives with temperature, the reaction was carried out under various temperatures (30–60 °C) at pH 7.0 (Fig. 6b). It was observed that encapsulated lipases with C[8]-C4-COOH or magnetite nanoparticles showed high activity at 45 °C, whereas the highest activity of encapsulated lipases without additives was at 35 °C. The highest percentage depending upon the activity of Enc-NP-C[8]-C4-COOH was at 40 ºC (see Fig. 6b). Immobilization shifted the optimum temperature from 35 °C for the free lipase to around 40–45 °C, due to either conformational limitations on enzyme movement as a result of multipoint interaction between the enzyme and the support or improved substrate diffusion at a high temperature. One of the main reasons for enzyme immobilization is the expected increase in stability toward various deactivating forces, due to the limited conformational mobility of the molecules after immobilization.9,13,32

Enantioselective hydrolysis of racemic naproxen methyl ester

Variations in pH and temperature can influence the conformation of the enzyme.31 In an effort to visualise the effects of these parameters on the activity of the encapsulated lipase, we carried out the hydrolysis reaction of (R,S)-naproxen methyl ester at a pH range of 5–7 (see Fig. 7a) and at temperatures of 35 and 40 °C (see Fig. 7b).

In an earlier study, the interaction of encapsulated lipases with octa acid derivatives of calix[8]arene (6) via the sol–gel encapsulation method was employed as a catalyst for the enantioselective hydrolysis of the racemic naproxen methyl ester.25 In order to understand and expand the range of encapsulated lipases available as enantioselective catalysts, in the present study, we describe two new encapsulated lipases (Enc-NP-C[8]-C4-COOH and Enc-C[8]-C4-COOH@Fe3O4). In describing these new encapsulated lipases, we also aim to evaluate their chiral catalytic affinities.

The hydrolysis reaction results of (R,S)-naproxen methyl esters by the encapsulated lipases are shown in Table 1. After 24 h treatment of the encapsulated lipases with racemic naproxen methyl ester in aqueous buffer solution and isooctane, the lipases produced R- naproxen methyl ester and the corresponding acid (eep) >98%, the percentage of conversion (x) 49.0 for Enc-C[8]-C4-COOH@Fe3O4 and 46.0 for Enc-NP-C[8]-C4-COOH. The treatment also resulted in enantioselectivities toward naproxen methyl ester (E value) of 371 for Enc-C[8]-C4-COOH@Fe3O4 and 265 for Enc-NP-C[8]-C4-COOH, as compared to an E value of 137 for the encapsulated lipase without additives (Enc-lipase). These results show strong evidence that the immobilization of lipases with calixarene derivatives led to high stereoselectivity, high conversion, and fast recovery of the catalyst owing to the magnetite properties of the encapsulated lipases (Enc-C[8]-C4-COOH@Fe3O4 and/or Enc-NP-C[8]-C4-COOH). This is not a surprising result, considering not only the highly effective complexing agent properties of the free COOH groups of C[8]-C4-COOH and NP-C[8]-C4-COOH by means of forming salt bridges with lysine groups of...
lipase, but also the host–guest interaction ability of calix[8]-
arene. A considerable increase in the activity and enantio-
selectivity of the encapsulated lipase in the presence of addi-
tives has also been observed in the literature.5,14,26,32

Increasing the recovery and reusability of the enzyme is 
essential for economical usage. In this sense, the encapsulated 
lipases with their unique magnetite properties should be paid 
much attention because of their easy separability with a 
simple magnet. Fig. 8 shows that, even after the 5th reuse 
cycle, the encapsulated lipases still retained 42% of their con-
version ratios for Enc-C[8]-C4-COOH@Fe3O4 and 42.4% for 
Enc-NP-C[8]-C4-COOH.

Conclusion
We have synthesized a new p-tert-butylcalix[8]arene derivative 
(C[8]-C4-COOH) and grafted it onto iron oxide nanoparticles 
to afford NP-C[8]-C4-COOH. Both C[8]-C4-COOH and Fe3O4-nano-
particles were used as additives for the encapsulation of 
lipase. Moreover, NP-C[8]-C4-COOH was also used as an additive 
for the lipase encapsulation in order to form a salt bridge 
between lysine moieties of the enzyme and free COOH groups 
of calix[8]arene derivatives, as well as to provide an easy way 
out for the separation processes. Two new encapsulated 
lipases (Enc-C[8]-C4-COOH@Fe3O4 and Enc-NP-C[8]-C4-COOH) 
were examined for the enantioselective hydrolysis of (R,S)-
apropanol methyl ester. In addition, the effects of some para-
eters such as pH and temperature were also investigated. It 
was found that the enantioselectivity of (R,S)-napropanol methyl 
ester improved more with Enc-C[8]-C4-COOH@Fe3O4 and 
Enc-NP-C[8]-C4-COOH than with the encapsulated lipase (Enc-
lipase), with E values of 371 and 265, respectively. These find-
ings demonstrate that the octa valeric acid-substituted calix[8]-
arene derivatives are useful supports for lipase encapsulation. 
Hence, an approach is opened for developing a new technique 
to regulate enantioselective studies. Moreover, due to the low 
price of sol–gel encapsulation, the excellent performance of 
the lipase-immobilization, and its ready recyclability, the 
method is industrially workable.

Experimental
Reagents
DC Aluofilen Kieselgel 60 F254 (Merck) was used for TLC analy-
sis. All chemical reagents and starting materials, and all sol-
vents were purchased from Aldrich, Fluka and Merck. HPLC 
grade organic solvents were used as the mobile phase without 
further purification or drying. A Millipore milli-Q Plus water 
purification system was used to receive deionized water. Candida rugosa, Bradford reagent, bovine serum albumin 
(99%), and p-nitrophenyl palmitate (p-NPP) were bought from 
Sigma Chemical Co. (St. Louis, MO).

Apparatus
A Varian 400 MHz spectrometer was used for NMR applica-
tions. FTIR spectra were evaluated on a Perkin Elmer spec-
trum 100 FTIR spectrometer. A Shimadzu 160A UV-visible 
recording spectrophotometer was used for UV-vis spectra. 
Thermogravimetric analysis (TGA) was carried out with a 
Seteram Evolution-1750 thermogravimetric analyzer. It was 
performed under an argon atmosphere. Transmission electron 
microscopy (TEM) analysis was carried out with FEI Tecnai G2 
Spirit. Melting points were determined on an E2-Melt appar-
atus in a sealed capillary. An Orion 410A+ pH meter was used 
for the pH measurements. High-performance liquid chromato-
graphy (HPLC) (Agilent 1200 Series) was carried out using a 
1200 model quaternary pump, a G1315B model diode array and 
multiple wavelength UV-vis detector, a 1200 model standard 
and preparative autosampler, a G1316A model thermostated 
column compartment, a 1200 model vacuum degasser, and 
an Agilent Chemstation B.02.01-SR2 Tatch data processor. The 
concentrations of S- and R-enantiomers of naproxen methyl 
ester were measured by HPLC (Agilent 1200 Series) using a 
Chiralecel OD-H column at a temperature of 25 °C. In the analy-

sis, n-hexane–2-propanol–trifluoro acetic acid (100/1/0.1, v/v/v) 
was used as the mobile phase at a flow rate of 1 mL min−1; and 
UV detection was done at 254 nm.

Synthesis

\[ \text{p-tert-Butylcalix[8]arene (1), Fe}_3\text{O}_4 \text{ nanoparticles (3) and} \]
aminosilica-modified Fe3O4 nanoparticles (Fe3O4-APTES) 
were synthesized according to the literature.14,27,33 The synthe-
theses of octa ester derivatives of p-tert-butylcalix[8]arene (2), 
5,11,17,23,29,35,41,47-octa-tert-butyl-49,50,51,52,53,54,55,56-
octahydroxycarbonylbutoxy-calix[8]arene (3), 
5,11,17,23,29,35,41,47-octa-tert-butyl-49-mono-(benzotriazol-1)-
(C[8]-C4-COOBaz) (4), and immobilization of C[8]-C4-COOBaz 
ono aminosilica-modified iron oxide nanoparticles (NP-C[8]-
C4-COOH) are reported for the first time.

(C[8]-C4-COOEt) (2). A mixture of p-tert-butylcalix[8]arene (1 g, 
0.771 mmol), K$_2$CO$_3$ (3.41 g, 24.672 mmol), NaI (1.85 g, 
12.336 mmol) and 5-bromovalerate (24.672 mmol) in 90 mL of 
acetone was heated at 80 °C. The reaction was monitored
using TLC. After 5 days, the reaction was cooled to room temperature, filtered off, and the filtrate was evaporated to dryness. The remaining solid was dissolved in 100 mL EtO and washed with water to adjust to pH 7.0. The organic phase was dried over MgSO₄ and then filtered. The filtrate was evaporated under reduced pressure. The crude residue was recrystallized from MeOH. Yield 78.2%, M.p. 165–166 °C. FTIR (ATR): 1732 cm⁻¹ (C=O). ¹H NMR (CDCl₃): δ 1.05 (s, 72H, Bu'), 1.11 (t, 24H, J = 6.8 Hz, −CH₃), 1.72–1.76 (m, 32H, −CH₂−), 2.27 (t, 16H, J = 6.8 Hz, −CH₂−CO), 3.63 (brs, 16H, ArCH₂−Ar), 3.99–4.04 (m, 32H, ArO−CH₂− and O−CH₂−), 6.92 (s, 16H, ArH).

Sol-gel encapsulation of lipase with/without C[8]-C₄-COOH and magnetite nanoparticles or NP-C[8]-C₄-COOH

The method of Reetz was modified for sol-gel encapsulation of the lipases. Typically, a mixture of lipase powder (lyophilized) such as CRL-type VII (245 mg) in phosphate buffer (1.56 mL, 50 mM) adjusted at pH 7.0 was vigorously shaken. Either NP-C[8]-C₄-COOH (0.2 g) or C[8]-C₄-COOH (3) (0.1 g) and Fe₂O₃ (0.1 g) was added to the mixture, together with 400 µL of polyvinyl alcohol (4% w/v), 200 µL of NaF solution (0.1 M) and 400 µL of isopropyl alcohol. After obtaining homogeneity, tetramethoxysilane (460 µL, 0.55 mmol) and octyltrimethoxysilane (3.2 mL, 2.5 mmol) were added and left to be shaken for 10–15 s. Then, 15 mL of isopropyl alcohol was poured onto the dried white solid. The gel was washed with water (10 mL) and isopropyl alcohol (10 mL), and then it was lyophilized to produce the encapsulated lipases.

Lipase activity and protein assay determination

p-NPP in an aqueous phosphate buffer (100 mM sodium phosphate, pH 7.0) was used to determine the hydrolytic activities of the encapsulated lipases. A UV-visible spectrophotometer was scanned at 410 nm in order to measure the concentration of the corresponding p-nitrophenol.

Protein content was defined by the method of Bradford using a Bio-Rad protein dye reagent concentrate. Bovine serum albumin was used as the standard.

pHs and temperatures on activity

Activity was determined between pH 4 and 9 in 50 mM phosphate buffer to see the changes in activity of free and immobilized lipases. Moreover, the thermal inactivation of the free and immobilized lipases was investigated at 30–60 °C. Both forms of enzymes were incubated in PBS (50 mM, pH 7.0) for 20 min at various temperatures and, after lowering the temperature, the remaining activity was assayed under the standard conditions and analyzed.

Thermal stability

Each encapsulated lipase (with or without additives) was stored in the buffer (50 mM, pH 7.0) at 60 °C for 2 h, in order to estimate their activity as outlined above.

Enantioselective hydrolization of racemic naproxen methyl ester by encapsulated lipases

A reaction system in an aqueous phase/organic solvent was used for the hydrolization reactions according to the literature procedure. The conversion and enantioselectivity of naproxen methyl ester by the encapsulated lipases in the presence or absence of additives such as either C[8]-C₄-COOH (3) and Fe₂O₃, or NP-C[8]-C₄-COOH were expressed as the enantiomeric ratio (E), which was calculated from the conversion (x)
and the enantiomeric excess of the substrate (ee_{s}) and the product (ee_{p}) using HPLC:\textsuperscript{34}

\[ E = \frac{\ln[(1-x)(1-ee_{s})]}{\ln[(1-x)(1+ee_{s})]} \]

\[ x = \frac{ee_{s}}{ee_{s} + ee_{p}} \quad \frac{ee_{p}}{C_{R} - C_{S}} \quad \frac{C_{R} - C_{S}}{C_{R} + C_{S}} \]

\[ ee_{s} = C_{R} - C_{S} \quad ee_{p} = C_{S} - C_{R} \]

\[ C_{P} = C_{R} + C_{S} \]

\[ E, ee_{s}, ee_{p}, x, C_{R} \text{ and } C_{S} \text{ stand for enantiomeric ratio for irreversible reactions, enantiomeric excess of substrate, enantiomeric excess of the product, racemate conversion, concentration of R-enantiomer and concentration of S-enantiomer, respectively.} \]

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Notes and references