Lipase Covalently Attached to Multiwalled Carbon Nanotubes as an Efficient Catalyst in Organic Solvent

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Lipase was covalently attached to multiwalled carbon nanotubes (MWNTs). Structural changes of the lipase upon attachment onto MWNTs were analyzed through circular dichroism and FTIR spectroscopy. The conjugate was utilized for the resolution of a model compound (R,S)-1-phenyl ethanol, and the reaction medium was n-heptane. The enzymatic resolutions were carried out at temperatures from 35 to 60°C. The results show that the lipase attached onto MWNTs has significantly affected the performance of the enzyme in terms of temperature dependence and resolution efficiency. The activity of MWNT–lipase was less temperature-dependent compared with that of the native lipase. The resolution efficiency was much improved with MWNT–lipase. MWNT–lipase retained the selectivity of the native lipase for (R)-1-phenyl ethanol. The consecutive use of MWNT–lipase showed that MWNT–lipase had a good stability in the resolution of (R,S)-1-phenyl ethanol. © 2010 American Institute of Chemical Engineers *AIChE J,* 56: 3005–3011, 2010
Keywords: multiwalled carbon nanotubes, lipase, resolution, organic solvent

Introduction

Lipases are widely used enzymes in biotechnology and extensively applied in pharmaceutical, food, or detergent industry.[1] Lipases exhibit an improved substrate specificity and operate in milder reaction conditions when compared with classical chemical catalysts. In the pharmaceutical industry, lipases can be used for racemic mixtures resolution or as biocatalysts for the synthesis of chiral synthesis intermediates.[1] Even though lipase can be used in organic solvents, the catalytic activity displayed by lipases in organic solvents is significantly lower than in aqueous media.[2] Immobilized lipase is an alternative to free lipase for the catalysis in organic media. Enzyme immobilization is well-known to improve enzyme thermal stability, activity, and recyclability, and it is critical for improving biocatalyst performance.

Nanometerials have been extensively studied for the immobilization of lipases.[3] In the case of surface attachment, nanoparticles can provide a larger surface area for the attachment of enzymes, leading to higher enzyme loading per unit mass of particles. Lipase was covalently attached on the magnetic nanoparticles via carbodiimide activation,[2,4,5] and a good enzyme stabilization was demonstrated. The final immobilization exhibited high stability, and it can be easily separated from the reaction medium by using a magnetic field. Polyacrylonitrile nanofibers with uniform fiber diameter were used for lipase immobilization.[6] Enzyme molecules

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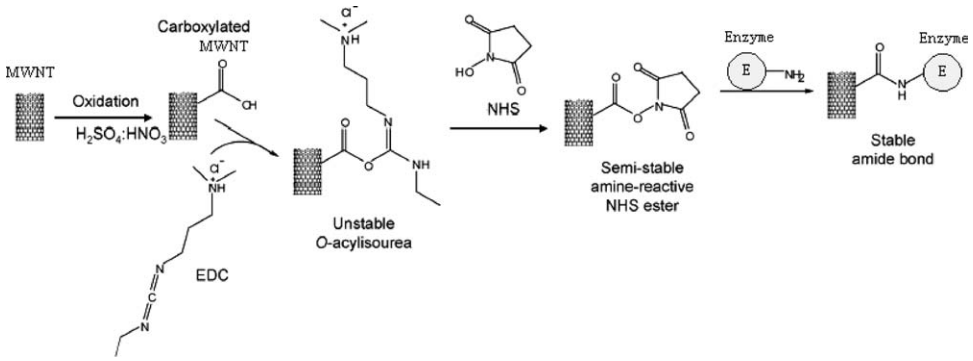


Scheme 1. Schematic representation of conjugation of enzymes to carboxylated MWNTs using EDC in the presence of NHS.

formed aggregates on the fiber surface. Large specific surface area was provided by the nanofiber, and protein loading could reach as high as 2.1% (w/w). Electrospun polymer nanofibers were used for enzyme immobilization by cross-linking additional enzyme molecules and aggregates onto the covalently attached seed enzyme molecules.[7] The apparent activity of enzyme coatings on nanofibers was nine times higher than that of nanofibers with just a layer of covalently attached enzyme molecules, and the operational enzyme stability of enzyme-aggregate-coated nanofibers was greatly improved. Carbon nanotubes (CNTs) represent an important group of nanomaterials and exhibit unusual electronic, mechanical, and adsorptive properties, as well as good chemical stability and efficient conductivity of heat.[8] Enzymes can be covalently attached or noncovalently absorbed onto CNTs.[9–18] The change of the secondary structure, stability, activity of the enzyme on attachment onto CNTs,[9,10] and the potential application of the conjugates have been investigated.[11–17] However, most of the catalysis of the CNTs-based enzymes are in aqueous medium. Few reports present the enzymatic catalysis of the conjugates in organic solvent.

Lipases exhibit high degree of selectivity over a broad range of substrates.[19–23] In this work, multiwalled carbon nanotubes (MWNTs) will be utilized to immobilize lipase. The conjugate of MWNT with lipase will be applied to the resolution of a model compound (*R*,*S*)-1-phenyl ethanol. *n*-Heptane will be used as the organic medium, as (*R*,*S*)-1-phenyl ethanol has a good solubility in it, and lipase remains active in *n*-heptane. Structural changes of the lipase upon attachment to MWNTs, and the activity of the conjugate will be studied.

Experimental

Materials

MWNTs were purchased from Nanotech Port Co., (Shenzhen, China). The purity was higher than 95%, and the catalyst residue was less than 0.2%. The lipase was isolated in Beijing Key Laboratory of Bioprocess.[24] (*R*,*S*)-1-phenyl ethanol, *N*-ethyl-*N*-(3-(dimethylamino)propyl) carbodiimide hydrochloride (EDC), 2-(*N*-morpholino) ethanesulfonic acid (MES), *N*-hydroxysuccinimide (NHS), hexane, heptane, and isopropyl alcohol were purchased from Sigma-Aldrich Chemical Co., China, and the chemicals were used without further purification. Deionized double-distilled water was used for making the solutions.

Purification and acid treatment of MWNTs

MWNTs were purified and oxidized as reported by Liu et al.[25] As-received MWNTs were purified by refluxing in an aqueous HNO_3 of 2.6 M at 70°C for 48 h. The nanotube suspension was diluted and washed with double-distilled water by filtering through a polycarbonate membrane (0.8 μm). The samples were dried at 80°C under vacuum. The purified MWNTs were further oxidized by sonication in 3:1 (v/v) concentrated H_2SO_4:HNO_3 mixture for 3 h. The nanotube suspension was diluted and washed with double-distilled water by filtering through a polycarbonate membrane (0.45 μm). The samples were dried at 80°C under vacuum.

Lipase covalently attached onto MWNTs

After acid treatment, carboxyl groups were produced at the defect sites of sidewall and tips of MWNTs. The covalent attachment of lipase onto MWNTs was based on the method described in literature,[9,18] as shown in Scheme 1. The oxidized MWNTs were dispersed in MES buffer (50 mM, pH 6.2), and then the mixture was added to the solution of NHS in MES buffer. The mixture was sonicated for 60 min followed by addition of EDC. The resulting mixture was shaken at 150 rpm for 40 min. The activated nanotubes were rinsed thoroughly with MES buffer to remove excess EDC and NHS by filtering through a polycarbonate membrane (0.45 μm). The nanotube film was transferred the aqueous solution of lipase and sonicated for 1 min to redisperse the nanotubes. The mixture was then shaken at room temperature during the conjugation of lipase to MWNTs. The nanotube-enzyme suspension was then washed six times with fresh buffer by filtering a polycarbonate membrane (0.2 μm) to remove unbound lipase. The amount of immobilized lipase was determined by elemental analysis of the acid-treated MWNTs and the MWNT–lipase conjugates.

Circular dichroic spectroscopy

Circular dichroism (CD) spectroscopy was used to monitor the secondary structure of the native lipase and the lipase attached to MWNTs. The CD spectra (200–260 nm) were recorded on a JASCO J-810 CD instrument (JASCO) with a bandwidth of 0.5 nm and a scan speed of 50 nm/min. Cell length was 10 mm. In measurements, the lipase concentration was kept at 50 μg/ml. Each scan was repeated five times, the spectra were then averaged.

FTIR spectroscopy

FTIR spectrum of sample was recorded on a Fourier transform infrared spectrometer 3100 (Varian) at room temperature. A sample, prepared for KBr determinations, consisted of 1 mg of lipase powder mixed with 100 mg of spectroscopic grade KBr. A total of 512 scans at 2 cm^{-1} resolution were averaged to obtain each spectrum. Spectra were analyzed by using the Varian Resolutions Pro 4.0.

Hydrolytic activity measurements

The enzyme concentrations used were 0.13 mg/ml for the native lipase and 0.20 mg/ml for MWNT–lipase. The enzymes were dissolved in 50 mM phosphate buffer (pH 7) to make the enzyme solutions. Olive oil (4 ml) was added to 8 ml of the enzyme solutions, and the hydrolysis reaction was carried out in an incubator at 200 rpm and various temperatures. The reaction proceeded for 15 min. And then, 8 ml of toluene was used to stop the reaction and extract the fatty acid. After centrifugation at 4000 rpm for 10 min, the free fatty acid was determined in the upper organic phase. The activities of the native lipase and MWNT–lipase for olive oil hydrolysis were measured by a Shimadzu spectrophotometer (model UV 2550) at an absorbance of 715 nm.[26]

Enantioselective resolution of (R,S)-1-phenyl ethanol

Racemic (*R*,*S*)-1-phenyl ethanol (0.5 mmol) and a certain amount of acyl donor were mixed with 10 ml heptane in a reaction vessel of 50 ml. The native lipase (12.3 mg) and 20 mg of MWNT–lipase were separately used as catalysts. The solution was continuously stirred at 100 rpm at a certain temperature. The enantiomeric excess and conversion were analyzed by high-performance liquid chromatography (HPLC). The HPLC analysis was performed using Shimadzu 10AVP instrument equipped with a UV detector on a Chiracel OJ-H column (0.46 mm diameter, 250 mm long, 5 μm, Chiracel). The mobile phase consisted of hexane–isopropyl alcohol at 99:01 (v/v) with a flow rate of 1.0 ml/min. At 220 nm, the substrate and product were detected. The retention time of (*R*)-1-phenylethyl acetate was 9.5 min and that of (*S*)- and (*R*)-alcohol enantiomers were 27.4 and 29.1 min, respectively. The enantiomeric excess of (*S*)-1-phenylethnaol (ees) is defined as ees $= ([S] - [R])/([S] + [R]) \times 100\%$, where [*R*] and [*S*] are the concentrations of *R*- and *S*-alcohol, respectively. The enantiomeric excess of (*R*)-1-phenylethyl acetate (eep) is defined as eep $= ([P] - [Q])/([P] + [Q])$, where [*P*] and [*Q*] are the concentrations of *R*- and *S*-phenylethyl acetate, respectively.[27] The conversion is calculated by Conversion = ees/(ees + eep).

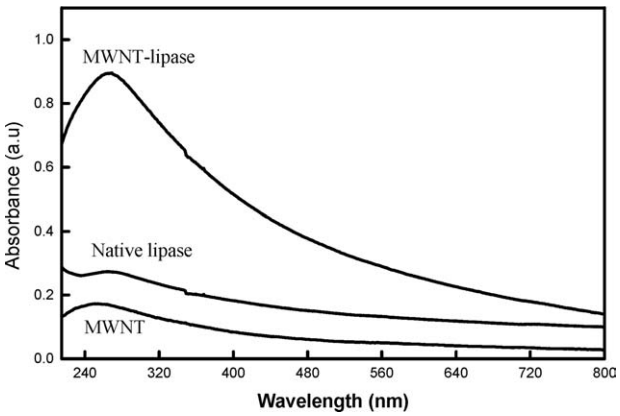


Figure 1. UV–vis absorption spectra of MWNT–lipase, native lipase, and MWNTs.

Consecutive use of MWNT–lipase in the resolution of (R,S)-1-phenyl ethanol

A batchwise fashion was adopted to investigate the operational stability of the immobilized lipase. MWNT–lipase was recovered by filtration and washed by 3 × 5 ml of heptane and dried under vacuum. Before each reuse, the dried MWNT–lipase was conditioned for 2 days in a constant humidity chamber containing a saturated solution of potassium acetate in order to readjust the water activity. The conversion of (*R*,*S*)-1-phenylethnaol and the enantiomeric excess were analyzed after each cycle.

Results and Discussion

UV–vis spectroscopy study

The lipase was attached onto MWNTs using carbodiimide activation of the nanotube-bound carboxylic acid groups. The solution of MWNT–lipase conjugates was monitored by UV–vis spectroscopy using a Shimadzu spectrophotometer (model UV 2550). Figure 1 shows UV–vis spectra of the native lipase, MWNT–lipase conjugates, and oxidized MWNTs. The peak of UV–vis spectrum of oxidized MWNTs is at 254 nm. After the lipase is covalently attached to MWNTs, the peak of UV–vis spectrum of MWNT–lipase is shifted to 269 nm. By elemental analysis, the lipase loading was 1.6 mg of lipase/mg of MWNTs. The high lipase loading facilitates the performance of spectroscopic analysis of lipase structure change upon the attachment onto MWNTs.

CD spectroscopy study

CD spectroscopy was used to analyze the influence of covalent attachment on the secondary structure of lipase. Figure 2 shows CD spectra of the native lipase, lipase attached to MWNTs, and oxidized MWNTs. The CD spectra were measured at 25°C. As can be seen, oxidized MWNTs had no contribution to the spectrum of the MWNT–lipase conjugates. In the measurement of the CD spectrum of the conjugates, the CD spectrum of oxidized MWNTs was recorded as a control.[9] CDPro software package (CONTIN)[28]

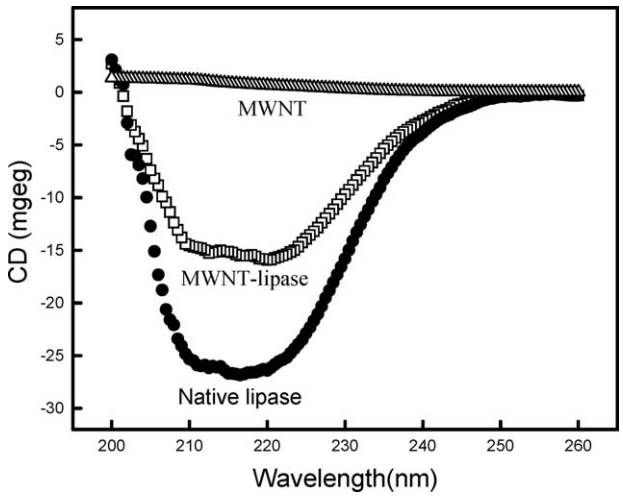


Figure 2. CD spectra of native lipase (solid circle), lipase attached to MWNTs (open square), and bare oxidized MWNTs (open up triangle).

was used to analyze the protein CD spectra for determining the secondary structure fractions. From the mean residue ellipticity at 222 nm, we know that the lipase attached to MWNTs retains about 62% of its native α-helix content, this value is similar to that for horseradish peroxidase, which retains 68% of its native α-helix content when covalently attached to single walled nanotubes.[9]

FTIR spectroscopy study

The amide I (1600–1700 cm^{-1}) region of the FTIR spectrum of a protein has been widely used to quantify the individual elements of the secondary structure.[29,30] In the present study, the percentage of α-helices determined from the amide I spectral region was employed to look at the structural perturbations of the lipase when attached onto MWNTs. In the measurement of the FTIR spectrum of the lipase attached to MWNTs, FTIR spectrum of activated MWNTs was recorded as a control. Figure 3 shows FTIR spectra of the native lipase and MWNT–lipase in the amide I region. Using the second-derivative method, the secondary structural features were calculated from the amide I spectra for both the native lipase and MWNT–lipase. α-Helical and β-sheet structures were assigned following the reports in literature,[24] and their relative amounts were determined by computing the areas under the assigned bands. Gaussian deconvolution of the spectrum revealed an α-helix content of 24% for MWNT–lipase compared to an α-helix content of 38% for the native lipase. The lipase endures a loss of 37% in the helical content of its secondary structure. This result is comparable with the secondary structural change obtained by CD spectroscopy.

Hydrolytic activity retained by MWNT–lipase

Figure 4 shows the effect of temperature on the hydrolytic activity retained by MWNT–lipase. In the temperature range of 40–55°C, MWNT–lipase retained 76–82% of the hydrolytic activity of the native lipase. The high percentage of the

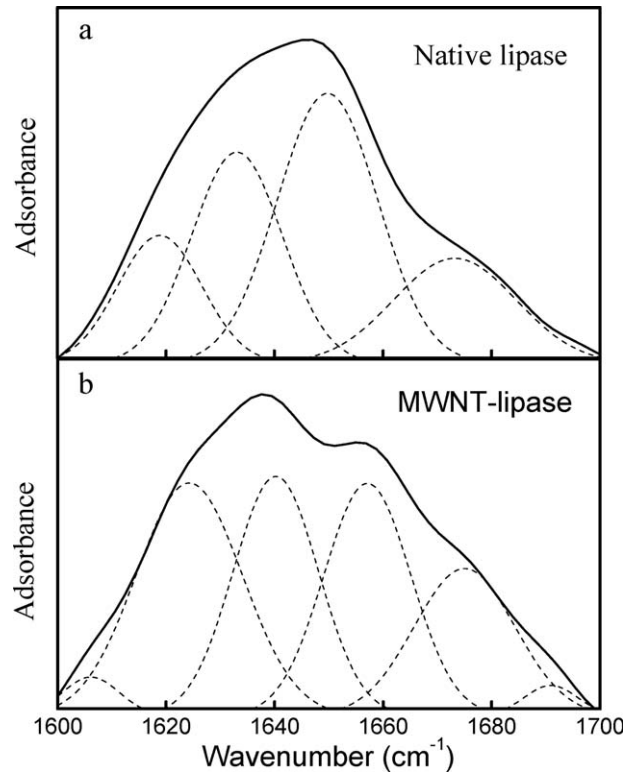


Figure 3. Fourier self-deconvoluted FTIR spectra and Gaussian curve-fitting: (a) native lipase; (b) MWNT–lipase.

activity retained by MWNT–lipase may be due to the efficient conductivity of heat by CNTs. This property of CNTs can promote the heat transfer between substances. The hydrolytic activity by MWNT–lipase is higher than that by the enzymes immobilized on single-walled CNTs.[9]

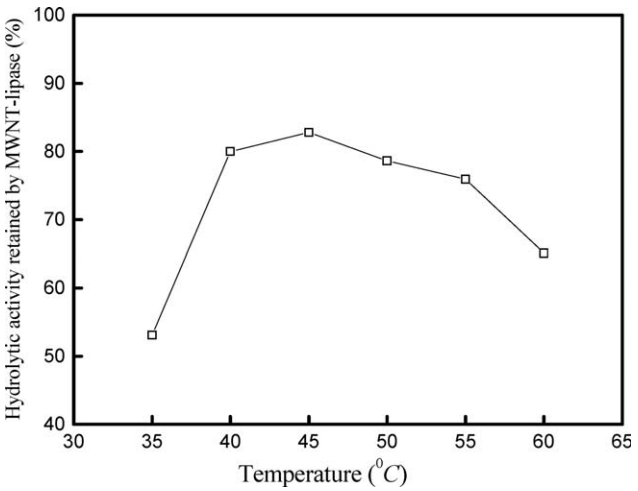
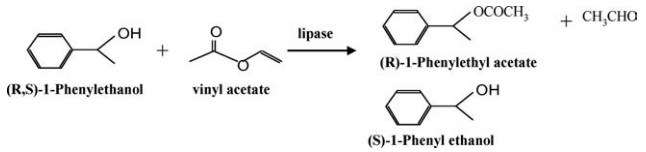


Figure 4. The hydrolytic activity retained by MWNT–lipase at various temperatures.

The symbols are the ratios of the activity of MWNT–lipase to that of the native lipase.



Scheme 2. Resolution of (*R,S*)-1-phenyl ethanol catalyzed by lipase.

Resolution of (R,S)-1-phenyl ethanol

Lipase catalysis for the resolution of (*R*,*S*)-1-Phenyl ethanol is shown in Scheme 2. In the reaction, (*R*)-1-phenyl ethanol is converted to (*R*)-1-phenylethyl acetate, while (*S*)-1-phenyl ethanol is unconverted. Therefore, for this reaction, the maximum conversion that can be achieved is 50%. Figure 5 shows the results with the native lipase and MWNT–lipase at different temperatures, the reactions were carried out for 40 h. In each experiment, the amount of the native lipase was equivalent to that of the lipase immobilized on MWNTs. As can be seen, the conversion with MWNT–lipase is less temperature-dependent compared to that with the native lipase. For example, in the temperature range from 40 to 55°C, the conversions with MWNT–lipase are not changed so much. While in this temperature range, for the reactions with the native lipase, the conversions are increased from lower to higher values. On the other hand, at the same temperature, the conversion achieved by MWNT–lipase is much higher than that by the native lipase. The results are based on a mass/mass comparison. If the loss of effective lipase due to denaturation is corrected, the ratio of the conversion with MWNT–lipase to that with the native lipase can be better. For example at 55°C, the conversion ratio was increased from 1.4 (without correction) to 1.7 (with correction). The results demonstrate that the lipase attached onto MWNT has significantly affected the performance of the enzyme. The improvement of catalysis efficiency may be attributed to the physical properties of CNTs. CNTs have an efficient conductivity of heat.[8] This property can improve the heat transfer efficiency between *n*-heptane and lipase, and hence accelerate the lipase catalysis. Another reason may be due to the mass transfer promoted by MWNTs. The covalent attachment of lipase occurred at the defect sites of sidewall and the tips of MWNTS.[18] The uncovered surfaces of MWNTs were exposed to the solvent *n*-heptane and

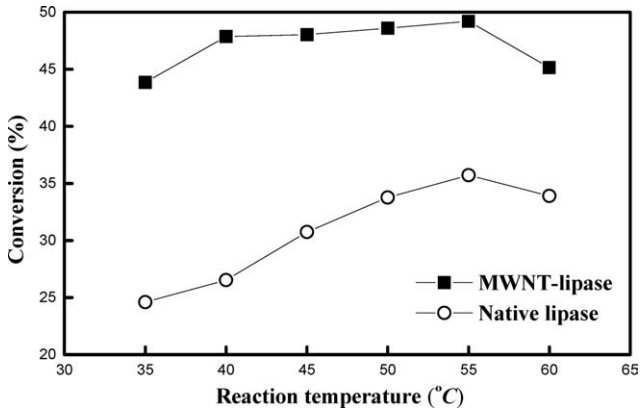


Figure 5. Effect of reaction temperature on the conversions by the native lipase and MWNT–lipase.

The molar ratio of vinyl acetate to (*R*,*S*)-1-phenyl ethanol was 4:1.

the substrate. Hexagon rings along the tubular surface of MWNTs are naturally easy to interact with the benzyl rings of (*R*,*S*)-1-phenyl ethanol and *n*-heptane molecules. This facilitates the interaction of the substrate (*R*,*S*)-phenyl ethanol with the lipase, and hence promotes the catalysis. Figure 6 shows the change of conversion, ees, and eep over the course of the enantioselective resolution experiments with MWNT–lipase and the native lipase. As can be seen, along the course of resolution reaction, the conversion and ees with MWNT–lipase are higher than that with the native lipase. The result means that MWNT–lipase exhibited a better resolution efficiency than the native lipase. The selectivity of the lipase is measured as ''eep.'' It does not change significantly with reaction time. The values of eep are indicated by solid up triangles. It was demonstrated that MWNT–lipase retained the excellent selectivity of the native lipase for (*R*)-1-phenyl ethanol.

In the control experiments, pristine, purified, and oxidized MWNTs were used for the resolution of (*R*,*S*)-1-phenyl ethanol (without using lipase). HPLC analysis showed that (*R*,*S*)-1-Phenyl ethanol was not converted at the conditions only with MWNTs. This result demonstrated that MWNTs did not absorb (*R*,*S*)-1-phenyl ethanol in the presence of heptane.

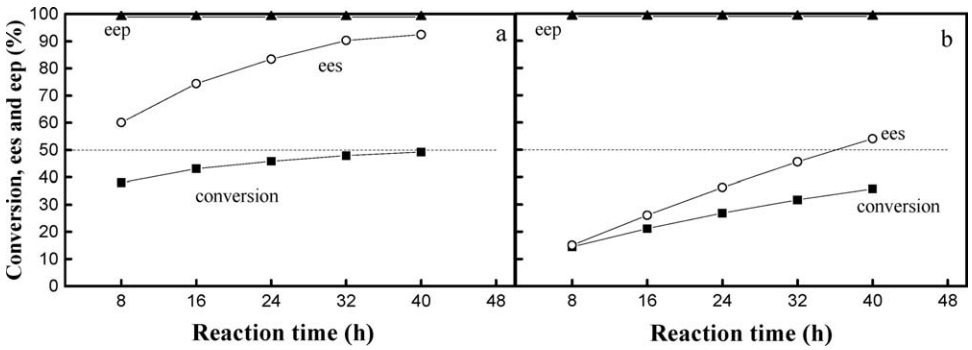


Figure 6. Change of conversion, ees, and eep with reaction time (a) MWNT–lipase (b) native lipase.

The reaction temperature was 55°C.

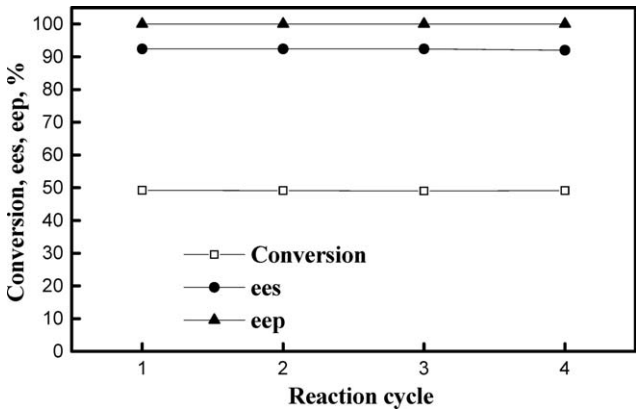


Figure 7. Activity of MWNT–lipase after use in several consecutive cycles of the resolution of (*R*,*S*)-1-phenyl ethanol.

The molar ratio of vinyl acetate to (*R*,*S*)-1-phenyl ethanol was 4:1. The reaction temperature was 55°C. The reaction time of each cycle was 40 h.

Consecutive use of MWNT–lipase in the resolution of (R,S)-1-phenyl ethanol

The stability of MWNT–lipase was investigated. For each batch of MWNT–lipase, the resolution reaction was carried out at a constant temperature of 55°C. The reaction time employed for each cycle was 40 h. As can been in Figure 7, the conversion, ees and eep have little change during the four reaction/cleaning cycles.

Conclusions

Lipase was covalently attached to MWNTs. CD spectra and FTIR spectroscopy showed that the lipase attached to MWNTs retained 62% of the α-helix content of the native lipase. For the resolution of (*R*,*S*)-1-phenyl ethanol in the organic solvent of heptane, the activity of MWNT–lipase was less temperature-dependent compared with that of the native lipase, and the resolution efficiency was much improved with MWNT–lipase. The lipase attached to MWNTs retained the selectivity of the native lipase. The consecutive use of MWNT–lipase showed that MWNT–lipase had a good stability in the resolution of (*R*,*S*)-1-phenyl ethanol.

Acknowledgments

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