DOI 10.5012/bkcs.2010.31.03.650

Immobilization of Lipase on Single Walled Carbon Nanotubes in Ionic Liquid†

Han Ki Lee, Jae Kwan Lee, Mahn-Joo Kim,* and Cheol Jin Lee‡,*

Department of Chemistry, Pohang University of Science and Technology, Pohang 790-784, Korea
**E-mail: mjkim@postech.ac.kr*
‡Nanotube and Nanodevice Laboratory, School of Electrical Engineering and Department of Micro/Nano Systems, Korea University, Seoul 136-713, Korea
Received September 23, 2009, Accepted October 9, 2009

A lipase from *Pseudomonas cepacia* was immobilized onto single walled carbon nanotubes (SWNTs) in two different ways in each of two solvent systems (buffer and ionic liquid). The most efficient immobilization was achieved in ionic liquid (1-butyl-3-methylimidazolium tetrafluoroborate, BMIM-BF_4). In this procedure, carbon nanotubes were first functionalized noncovalently with 1-pyrenebutyric acid *N*-hydroxysuccinimide ester and then subject to the coupling reaction with the lipase in ionic liquid. The resulting immobilized enzyme displayed the highest activity in the transesterification of 1-phenylethyl alcohol in the presence of vinyl acetate in toluene.

Key Words: Lipase, Single walled carbon nanotube, Immobilization, Ionic liquid, Biocatalysis

Introduction

Transformations based on enzymatic catalysis provides a useful component of methodology in organic synthesis.[1,2] A wide range of enzymes have been utilized as the catalysts in organic synthesis. Among them, hydrolytic enzymes such as lipases and esterases have been most frequently employed as a result of their broad substrate specificity and good enantioselectivity. These enzymes have been particularly useful in the synthesis of optically active alcohols, acids, and esters.[3] They, however, have still a common limitation that they normally display low activity and stability in organic media.[4] In connection with our recent studies on the enzymo-metallic dynamic kinetic resolution of racemic substrates,[5-7] we became interested in developing enzymes with better activity or/and stability in organic solvent. As one of our efforts toward this end, we herein wish to report the immobilization of a lipase on single walled carbon nanotubes in ionic liquid leading to a nanobiocatalyst with significantly enhanced activity in organic solvent.

Single walled carbon nanotubes (SWNTs) are molecular wires that exhibit interesting structural, mechanical, electrical, and electrochemical properties.[8] They are increasingly attracting attention as the nano-scale materials for potential biological applications.[9-16] A useful type of applications is illustrated by their use as nano-scale supporters for immobilizing enzyme leading to biosensors and nanobiocatalysts. Carbon nanotubes, particularly single walled carbon nanotubes, have a large surface area, thus enabling the high loading of enzymes and lowering diffusional resistance. Recent studies have demonstrated that the enzymes onto carbon nanotubes could show the enhanced activity and stability relative to the solution counterparts.[17,18]

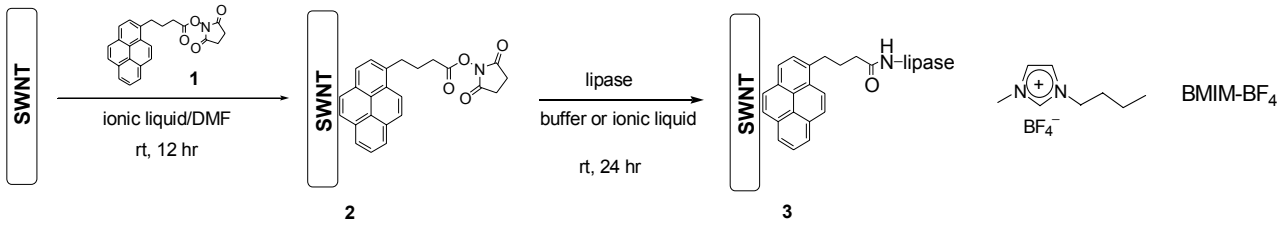
The immobilization of enzymes onto carbon nanotubes has been normally performed in aqueous media such as buffer. However, the inherent insolubility of carbon nanotubes in buffer, resulting from the intrinsic van der Waals forces of SWNTs,[19,20] often makes the immobilization inefficient. We envisaged that this limitation would be overcome by performing the immobilization in room-temperature ionic liquids (RTILs), in which carbon nanotubes becomes better soluble or dispersable[21,22] compared to aqueous media. In addition, RTILs are compatible with enzymes as demonstrated by many recent studies on enzymatic catalysis in ionic liquids.[23-25]

Experimental

Immobilization of lipase on SWNTs. The immobilization of lipase on the functionalized SWNTs in ionic liquid is described as a representative procedure. To SWNTs (6 mg) suspended in BMIM-BF_4 (6 mL) were added 1-pyrenebutyric acid *N*-hydroxysuccinimide ester (**1**, 60 mg) in DMF (12 mL). The resulting solution was stirred at room temperature for 12 hours, followed by the addition of methanol (30 mL) to precipitate the functionalized SWNTs (**2**). The precipitate was washed three times with pure methanol (50 mL) to remove excess reagents. After dried under vacuum, the functionalized SWNTs (5 mg) were suspended in BMIM-BF_4 (5 mL), followed by the addition of *Pseudomonas cepacia* lipase[26] (54 mg of proteins in 10 mL of 20 mM phosphate buffer, pH 7.2). The heterogeneous mixture was stirred at room temperature for 24 hours and then filtered on a polycarbonate membrane (0.1 μm, Whatman). The solid was washed with phosphate buffer (25 mL × 2) and then dried under vacuum to give the final products (**3**, 5 mg).

Measuring the activities of native and immobilized lipases. The enzymatic activities were measured as the initial rates in the transesterication of 1-phenethyl alcohol. Assay-scale reactions were performed with 1-phenethyl alcohol (36 μL, 0.3 mmol), enzyme preparation (1 mg), and vinyl acetate (83 μL, 0.9 mmol) in toluene (3 mL). The heterogeneous mixture was shaken at 200 rpm and 25 °C. The reaction progress was followed by periodical HPLC analysis using chiral columns with the following conditions: Chiralcel OD, hexane/2-propanol 98/2, flow rate 1.0 mL/min, UV 217 nm for 1-phenylethyl al-

†This paper is dedicated to Professor Sunggak Kim on the occasion of his honorable retirement.



Scheme 1. Immobilization of PCL onto SWNTs *via* PBA ester

cohol; Whelk O1, hexane/2-propanol 98/2, flow rate 0.5 mL/min, UV 217 nm for 1-phenylethyl acetate. The conversions were calculated by the following equation, $C = ee_s/(ee_s + ee_p)$.

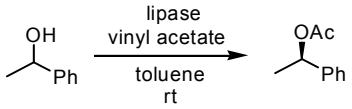
Preparative-scale transesterication of 1-phenethyl alcohol. A preparative scale reaction was performed with 1-phenethyl alcohol (72 μL, 0.6 mmol), SWNT-immolized lipase (2 mg), and vinyl acetate (166 μL, 1.8 mmol) in toluene (3 mL). The heterogeneous mixture was shaken at 200 rpm and 25 °C for 8 hours. The reaction mixture was then filtered to remove enzymes, concentrated, and finally subjected to silica gel chromatography to provide acetylated product (40 mg, 0.24 mmol, 40%, >99%ee) with recovered substrate (40.5 mg, 0.33 mmol, 55%, 76%ee).

Characterization of lipase immobilized on SWNTs. The synchrotron radiation source at the Pohang Accelerator Laboratory (photoemission electron microscopy beamLine, 4B1) was utilized for X-ray photoelectron spectroscopic (XPS) analysis. The photon energy was selected to give the optimized photoemission intensity and/or resolution. Binding energies were calibrated against the Au(4f) emission at $E_b = 86$ eV. The TGA data was collected using TG/DT analyzer (model : EXSTAR 6000, Seiko instruments, Japan) by heating 10 °C/min to 800 °C under nitrogen atmosphere. TEM examinations were performed by Hitachi-7600 (100 kV). For the TEM measurements, one drop of the sample suspended in toluene or methanol was placed on a carbon coating micro grid and examined after drying under vacuum.

Results and Discussion

The immobilization was explored with a lipase from *Pseudomonas cepacia* (PCL)[26] and SWNTs produced by the chemical vapor deposition (CVD) method.[27,28] The enzyme immobilizations were carried out in two ways in each of two solvent systems (buffer and ionic liquid): (a) by simple adsorption on neat SWNTs and (b) by chemical bonding with functionalized SWNTs. The former was done simply by mixing an enzyme solution with neat SWNTs in buffer (20 mM phosphate, pH 7.2) or ionic liquid (1-butyl-3-methylimidazolium tetrafluoroborate, BMIM-BF_4). The latter was done in two steps. SWNTs were first functionalized by the reaction with 1-pyrenebutyric acid (PBA) *N*-hydroxysuccinimide ester **1**[9] in an ionic liquid-DMF mixture at room temperature. The functionalized SWNTs **2** were then coupled with the lipase in buffer (20 mM phosphate, pH 7.2) or ionic liquid (BMIM-BF_4) to yield the enzyme-nanotube conjugates **3** (Scheme 1).

The activities of native and immobilized lipases were examined with the transesterification of 1-phenylethyl alcohol in the presence of vinyl acetate in tolune (Scheme 2). Each reaction



Scheme 2. Lipase-catalyzed transesterification of 1-phenylethyl alcohol.

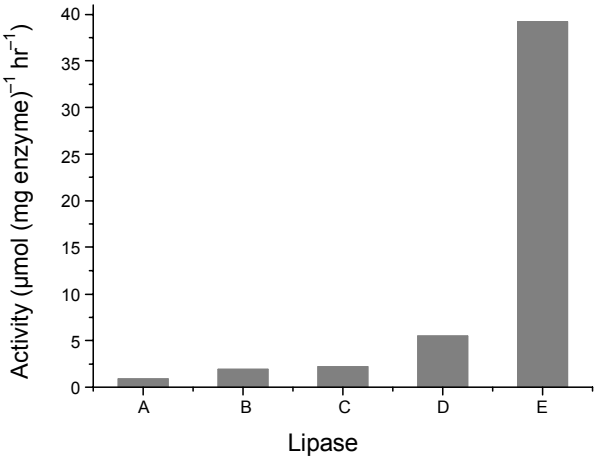


Figure 1. Activities of lipases: (**A**) native, (**B**) immobilized by simple adsorption in buffer, (**C**) immobilized by chemical reaction in buffer, (**D**) immobilized by simple adsorption in ionic liquid, and (**E**) immobilized by chemical reaction in ionic liquid.

was performed at 200 rpm at 25 °C with a solution containing 1-phenylethyl alcohol (0.3 mmol), enzyme preparation (1 mg), and vinyl acetate (3 equiv) in toluene (3 mL), followed by periodical HPLC analysis using a chiral column. The enzymatic activity (μmoles per hour per mg of enzyme preparation) in each reaction was determined as an initial rate based on conversion %.

The results from the activity measurements are presented in Figure 1 for comparison between five different types of lipases: **A** (native), **B** (immobilized by physical adsorption in buffer), **C** (immobilized by chemical reaction in buffer), **D** (immobilized by physical adsorption in ionic liquid), and **E** (immobilized by chemical reaction in ionic liquid). The enzymatic activities are 0.9 U, 1.9 U, 2.2 U, 5.5 U and 39 U, respectively, for **A**-**E**. These data clearly indicate that the immobilizations in the presence of ionic liquid were more efficient than those in buffer and the former took place more efficiently by noncovalent bonding *via* an anchoring reagent **1** than by simple physical adsorption.

To show the synthetic application of the most active immobilized lipase **E** as the practical catalyst, we performed a preparative scale transesterification of 1-phenylethyl alcohol (0.6

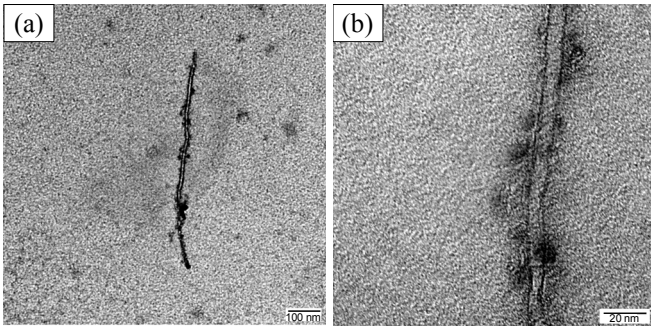


Figure 2. TEM images of lipase immobilized on SWNTs. Scale bar = 100 nm (a) and 20 nm (b).

mmol) with **E** (2 mg) according to Scheme 2. The reaction proceeded smoothly to afford the acetylated product of high optical purity (> 99%ee, 40% isolated yield) with the recovery of unreacted substrate (76%ee, 55% isolated yield). The results thus indicate that the immobilized lipase **E** is catalytically intact with high enatioselectivity (E = > 200).

The immobilized lipase **E** was characterized with several analytical techniques. X-ray photoelectron spectroscopy (XPS) analysis disclosed a peak at 400.6 eV corresponding to nitrogens in the amide bonds of proteins, indicating the existence of enzymes on carbon nanotubes. Thermal gravimetric analysis revealed that the lipase-nanotube conjugate **E** lost 42% of total weight but its precusor **2** lost 15%. These data suggest that the amount of enzyme on carbon nanotubes could reach approximately 30% of the total weight. Finally, the transmission electron microscopy (TEM) analysis gave clear images of single enzymes of approximately 5 nm present on carbon nanotubes (Fig. 2).

The enzymes normally exist as aggregates in organic solvents such as toluene and *t*-butyl methyl ether because they are insoluble in organic media. The aggregated enzymes thus become catalytically less efficient in organic solvents. The enzymes immobilized on SWNTs however exist as separate single molecules (Figure 2) and display high activities relative to their native counterpart (Figure 1). Accordingly, it is thought that the deaggregation of enzymes by their immobilization on SWNTs with a large surface area is a useful approach for enhancing their performance in organic media. We believe that the further improvements of this approach could lead to the discovery of biocatalysts highly active in organic media.

Conclusion

We explored the immobilizations of a lipase from *Pseudomonas cepacia* on SWNTs under several different conditions. The kinetic, synthetic, and characterization studies have revealed that the enzyme immobilization took place more efficiently in case using a room temperature ionic liquid as the medium for the better dispersion of carbon nanotubes, and the resultant immobilized enzyme displayed a good performance in a synthetic reaction. The immobilization procedure is simple and straightforward. It thus provides a practical route to nanobiocatalysts for synthetic applications. We believe that this procedure should be applicable to other enzymes.

Acknowledgments. This work was supported by the Korean Ministry of Education, Science and Technology (KMEST) *via* National Research Foundation (formerly Korea Research Foundation, KRF-2004-005-C00007 and KRF-2006-005-J01201). We also thank KMEST for the support of our graduate program (the BK 21 program), and Mr. T.-H. Kang and Mr. K.-W. Ihm at PLS for their help with the XPS experiments.

References

1. Wong, C.-H.; Whitesides, G. M. *Enzymes in Synthetic Organic Chemistry*; Pergamon: Oxford, UK, 1994.
2. Drauz, K.; Waldmann, H. *Enzyme Catalysis in Organic Synthesis*; VCH: Weinheim, Germany, 1995; Vols. I and II.
3. Bornscheuer, U. T.; Kazlauskas, R. J. *Hydrolases in Organic Synthesis*; Wiley-VCH: Weinheim, Germany, 1999.
4. Koskinen, A. M. P.; Klivanov, A. M. *Enzymatic Reactions in Organic Media*; Blackie Academic & Professional: Glasgow, Scotland, 1996.
5. Kim, M.-J.; Ahn, Y.; Park, J. *Curr. Opin. Biotechnol.* **2002**, *13*, 578.
6. Kim, M.-J.; Ahn, Y.; Park, J. *Bull. Korean Chem. Soc.* **2005**, *26*, 515.
7. Ahn, Y.; Ko, S.-B.; Kim, M.-J.; Park, J. *Coord. Chem. Rev.* **2008**, *252*, 647.
8. Dresselhaus, M. S.; Dresselhaus G.; Eklund, P. C. *Science of Fullerenes and Carbon Nanotubes*; Academic Press: San Diego, 1996; pp 1-985.
9. Chen, R. J.; Zhang, Y.; Wang, D.; Dai, H. *J. Am. Chem. Soc.* **2001**, *123*, 3838.
10. Huang, W.; Taylor, S.; Fu, K.; Lin, Y.; Zhang, D.; Hanks, T. W.; Rao, A. M.; Sun, Y.-P. *Nano Lett.* **2002**, *2*, 311.
11. Azamian, B. R.; Davis, J. J.; Coleman, K. S.; Bagshaw, C. B.; Green, M. L. H. *J. Am. Chem. Soc.* **2002**, *124*, 12664.
12. Rege, K.; Raravikar, N. R.; Kim, D.-Y.; Schadler, L. S.; Ajayan, P. M.; Dordick, J. S. *Nano Lett.* **2003**, *3*, 829.
13. Jiang, K.; Schadler, L. S.; Siegel, R. W.; Zhang, X.; Zhang, H.; Terrones, M. *Mater. Chem.* **2004**, *14*, 37.
14. Yim, T.-J.; Liu, J.; Lu, Y.; Kane, R. S.; Dordick, J. S. *J. Am. Chem. Soc.* **2005**, *127*, 12200.
15. Gómez, J. M.; Romero, M. D.; Fernàndez, T. M. *Catal. Lett.* **2005**, *101*, 275.
16. Prakash, R.; Superfine, R.; Falvo, M. R. *Appl. Phys. Lett.* **2006**, *88*, 063102.
17. Heering, H. A.; Williams, K. A.; de Vries, S.; Dekker, C. *Chem. Phys. Chem.* **2006**, *7*, 1705.
18. Reich, S.; Thomsen, C.; Ordejón, P. *Phys.Rev. B* **2002**, *65*, 155411.
19. Girifalco, L.; Hodak, M.; Lee, R. S. *Phys. Rev. B* **2000**, *62*, 13104.
20. Fukushima, T.; Kosaka, A.; Ishimura, Y.; Yamamoto, T.; Takigawa, T.; Ishii, N.; Aida, T. *Science* **2003**, *300*, 2072.
21. Price, B. K.; Hudson, J. L.; Tour, J. M. *J. Am. Chem. Soc.* **2005**, *127*, 14867.
22. Kragl, U.; Eckstein, M.; Kaftzik, N. *Curr. Opin. Biotechnol.* **2002**, *13*, 565.
23. Park, S.; Kazlauskas, R. J. *Curr. Opin. Biotechnol.* **2003**, *14*, 432.
24. van Rantwijk, F.; Sheldon, K. R. A. *Chem. Rev.* **2007**, *107*, 2757.
25. Cantone, S.; Hanefeld, U.; Basso, A. *Green Chem.* **2007**, *9*, 954.
26. This enzyme is available from some commercial suppliers such as Fluka, Roche, and Amano. We used the one provided by Amano.
27. Lyu, S. C.; Liu, B. C.; Lee, T. J.; Liu, Z. Y.; Yang, C. W.; Park, C. Y.; Lee, C. J. *Chem. Commun.* **2003**, 734.
28. Liu, B. C.; Lyu, S. C.; Jung, S. I.; Kang, H. K.; Yang, C.-W.; Park, J. W.; Park, C. Y.; Lee, C. J. *Chem. Phys. Lett.* **2004**, *383*, 104.