FULL PAPER

Hierachically Structured Hollow Silica Spheres for High Efficiency Immobilization of Enzymes

Shunsheng Cao, Long Fang, Zhiyuan Zhao, Yi Ge,* Sergey Piletsky, and Anthony P. F. Turner**

In this work, the first example of a hierarchically structured hollow silica system is reported without any chemical modification to the enzyme involved in the process. The leaching of the physically adsorbed enzyme is substantially restrained in comparison to pure hollow silica supports. The hierarchical architecture is composed of the ordered hollow silica spheres with a shell-in-shell structure. This rationally integrated architecture, which serves as the host for glucose oxidase immobilization, displays many significant advantages, including increases in mechanical stability, enzyme loading, and bioactivity, and a decrease in enzyme leaching compared to existing pure hollow silica matrices. This facilitates further multifarious applications for enhanced enzyme immobilization, biosensors, and biocatalysis.

1. Introduction

New advance in biocatalysis is determinant in lowering the environmental footprint of chemical processes by utilizing enzymes. However, the strategy of enzyme immobilization represents one of the main challenges that hamper the development of industrial-scale biocatalytic processes.[1] Essentially, the enzyme immobilization protocols can be achieved by physical or chemical attachment to a solid polymer or by entrapment or encapsulation within inorganic or organic host matrices.[2,3] It is reported that the adsorption of enzymes onto high-surface-area supports is the simplest and most inexpensive method.[2,4] Moreover, the bioactivities of the loaded enzyme can be retained better. However, the binding force (non-covalent bonding) based on this method, such as the van der waals force, electrostatic interaction, hydrophobic affinity and hydrogen bonding, is weak, resulting in the unwanted leakages of enzyme.[2,3,5] In situ sol–gel encapsulation can offer high retention of enzymatic activity and low enzyme leaching, however, the confining environment of the enzyme is very hard to control, which imposes large diffusion resistance on the transport of the substrate or product.[4–6] To conciliate the conflict, shrinking the pore openings to encapsulate enzymes is a good way to prevent enzymes from the direct contact with a harsh environment.[7] Previous investigations have shown that the ordered hollow mesoporous silica spheres are promising candidates for the encapsulation of enzymes, due to their inherent characteristics of large surface area and internal spaces inside the shell, as well as tunable pore size matching the dimensions of enzymes.[1,8] However, the major limitation of hollow silica matrices in a wide of applications is slowly kinetic response and low mechanical stability.[9,10]

Hierarchical fabrication at the nano- and microscale to integrate multi-functional composite materials has been of great challenge and increasing interest in chemistry, biology, and materials science.[5,11] The hierarchical composites have promising applications in the fields of adsorption, separation, sensing, drug release/delivery, and catalysis.[12] To meet a complex set of construction and performance requirements, improved properties and combined functions by fabricating a variety of multi-level architectures, such as core-in-shell, wire-in-tube, segmented tubes, and multi-channels, could be achieved.[12]

Recently, the rising of the multi-shelled structures becomes a powerful tool to achieve advanced performance of materials,[13] such as enhanced surface area, great thermal/mechanical stability, and optimized cargo loading. In this work, we report the first example of an ordered silica microspheres system, which has a shell-in-shell hollow structure, for high efficient immobilization of glucose oxidase. In comparison with pure hollow silica materials, hierarchical hollow silica spheres not only maintain a well-defined structure, high surface area, and pore volume, but also exhibit high hydrothermal and mechanical stability due to the hierarchical interaction caused by the two consecutive shell layers, resulting in high enzyme loadings and low leaching.[13,14] In order to show the potential of these novel materials as supports for bulky molecules, they were tested for enzyme immobilization by using glucose oxidase (GOx) as a model enzyme. GOx, with good characterizations in literature,[15] is one of the most extensively used enzymes in the development of bio-devices for

Dr. S. Cao. L. Fang, Z. Zhao
School of Materials Science and Engineering
Jiangsu University
Xuefu Road 301, Zhenjiang, 212013, P. R. China
E-mail: sscaochem@hotmail.com
Dr. S. Cao, Dr. Y. Ge, S. Piletsky, Prof. A. P. F. Turner
Cranfield Health
Cranfield University
Cranfield, Bedfordshire, MK43 0AL, UK
E-mail: y.ge@cranfield.ac.uk; anthony.turner@liu.se
Prof. A. P. F. Turner
Biosensors & Bioelectronics Centre, IFM
Linköping University
SE-58183, Linköping, Sweden

DOI: 10.1002/adfm.201201793

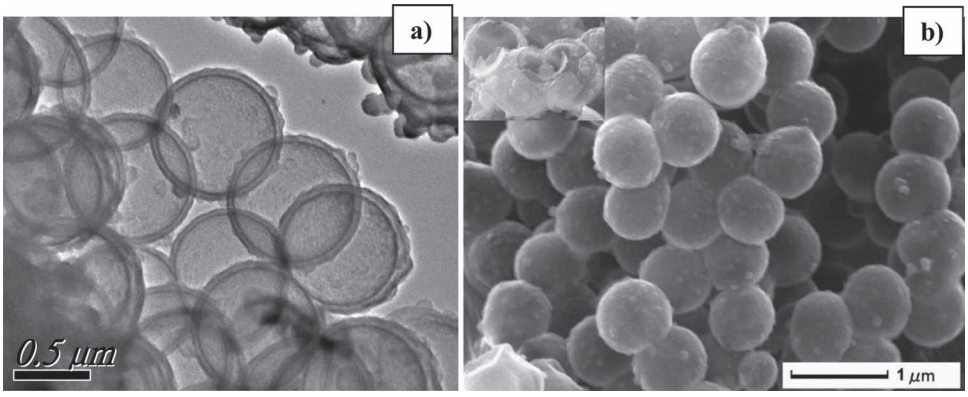

Figure 1. TEM and SEM images of hierarchically structured hollow silica supports.

the self-monitoring of diabetic patients,[16] the determination of glucose in food and beverages,[17] and in fermentation tanks.[18] the study has shown that the hierarchically structured hollow silica matrixes offer high GOx loading and bio-activity, improved stability, as well as low leaching, leading to faster kinetic response in comparison with pure hollow silica host.

2. Results and Discussion

2.1. The Fabrication of Hierarchically Structured Hollow Silica Supports

The hierachically architectured hollow silica spheres are synthesized by using a consecutive template-guided self-assembly as described in the Experimental Section. The high-magnification transmission electron microscopy (TEM) image in **Figure 1**a shows that all particles have two clear rings within one sphere and are composed of inner and outer small cavities with double walls, indicating the hierarchical hollow structures of the silica microspheres.[9,13] Interestingly, no free solid or pure hollow silica particles are observed in these samples, which suggests that the template-guided self-assembly technique can create a unique hierarchical structure, avoiding the formation of unwanted silica particles during the surrounding process of inner and outer silica walls. Another representative scanning electron microscopy (SEM) image presented in Figure 1b reveals that the hierarchical hollow silica spheres have a highly ordered and well-defined morphology, as fairly agrees with results determined in **Figure 2**a by dynamic light scattering (DLS). The hierarchical structure is further confirmed by the higher magnification SEM image of the several broken silica spheres (Figure 1b, inset), which allow one to see ordered double layer ring of the inner wall and outer wall. Unlike pure hollow silica,[19] the isotherm of the hierarchical architecture samples shown in Figure 2b exhibits a characteristic hysteresis loop observed over almost whole range (0.05–0.96 P/P_0),[13] and achieves higher BET (Brunauer-Emmett-Teller) specific area of ca. 365.482 m^2g^{-1} (pure hollow silica: 198.427 m^2 g^{-1}, Supporting information, Figure S1). Therefore, the above results confirm that the hierarchically structured hollow silica spheres have been successfully prepared in the present work.

2.2. Efficient Immobilization of GOx for Hierarchical Silica Supports

In order to highlight improved properties of hierarchical hollow silcia, the pure hollow silica spheres with well-defined size and

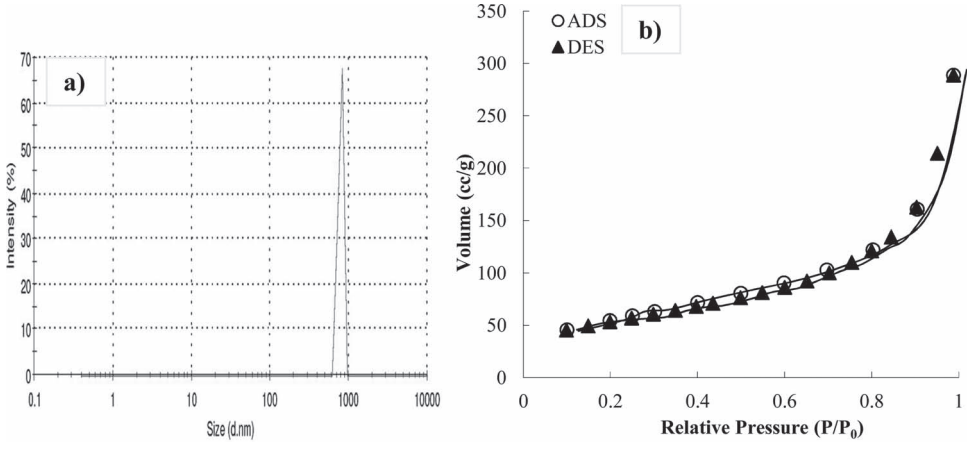

Figure 2. a) DLS curve and b) N2 adsorption/desorption isotherms of the prepared hierarchically structured hollow silica supports.

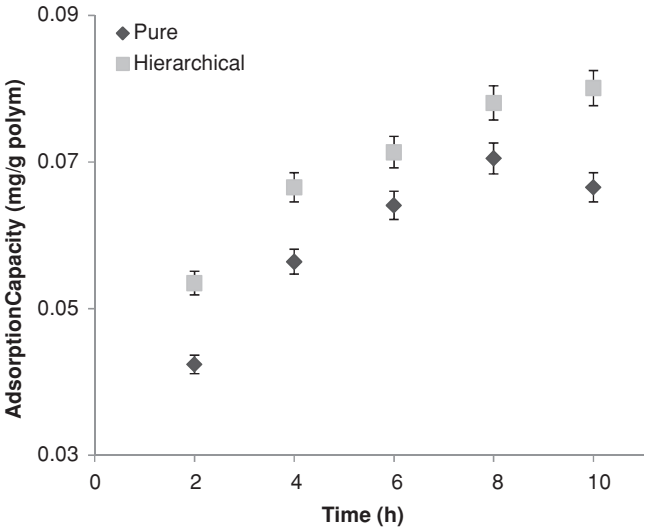

Figure 3. Time profile for GOx immobilization.

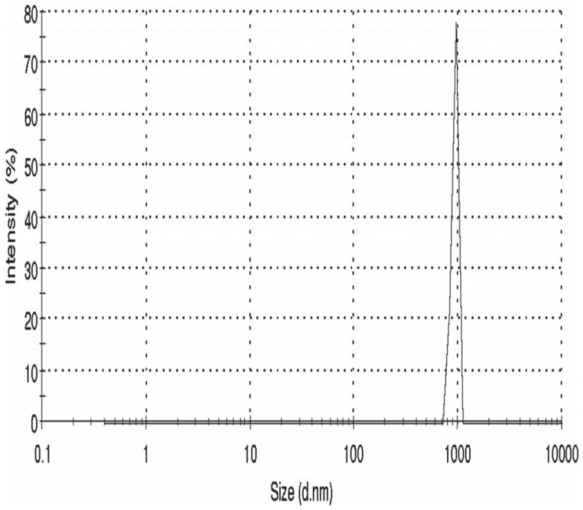

Figure 5. The DLS curve of GOx-loaded hierarchical hollow spheres.

morphology (Supporting Information, Figure S2) were synthesized according to the methods described in our previous works,[19] and used for enzyme immobilization of glucose oxidase.

Figure 3 (initial GOx concentration: 4.56×10^{-4} mg mL^{-1}) shows that there is an initially fast period of increase and then a slow increase in the GOx loading. Moreover, the GOx loading at about 8 h shows no clear difference from that at 10 h, indicating the GOx immobilization approaches equilibrium within 8 h. In practice, the encapsulation process is very complicated, comprising adsorption of GOx onto the external surface of hollow silica followed by further encapsulation of GOx inside the inner channel.[20] Therefore, it can be expected that well-defined morphology and higher surface area of the hierarchical hollow silica supports would enhance GOx loading, as shown in Figure 3. Surprisingly, the GOx adsorption-amount of pure hollow silica at 10 h has a markedly decrease than that at 8 h, which is probably ascribed to its weakly mechanical stability because it has only a wall. This can be further confirmed by TEM and SEM images and the DLS curve of GOx loaded. After the GOx immobilization, most originally intact (Supporting information, Figure S2) hollow silica spheres have been deformed and broken as shown in **Figure 4**a,c. In contrast, the morphology and dispersity of the hierarchical hollow silica have no obvious damages (Figure 4b,d and **Figure 5**) before and after the GOx immobilization, denoting that the hierarchically structured supports present higher mechanical stability, which is vital for high efficient immobilization of enzymes.[2,4,14]

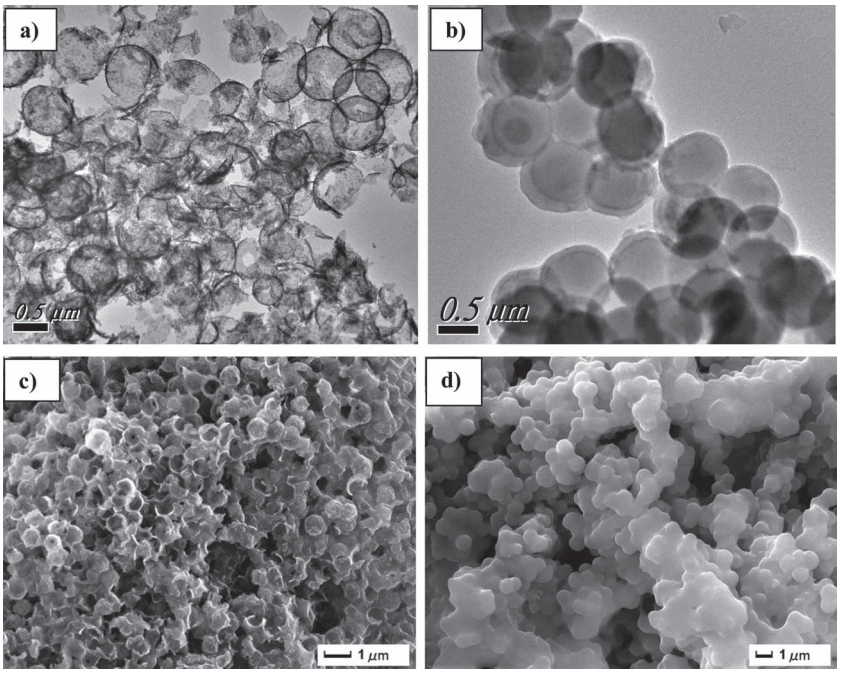

Figure 4. TEM and SEM images of GOx loaded on the pure and hierarchical hollow silica supports.

2.3. Analysis of Isotherms

The adsorption isotherms of GOx loaded onto hollow supports are shown in **Figure 6**. The general trend is increase in the adsorption capacity with the increase in initial GOx concentration. Compared with pure hollow silica, the novel host not only offers the higher GOx loading due to its improved BET surface area, but also retains lower equilibrium concentration and improves the immobilization rate in the relative initial concentration range of 0.456–1.368 (10^{-3} mg mL^{-1}), indicating that the hierarchically structured matrix achieves high efficient immobilization of glucose oxidase.

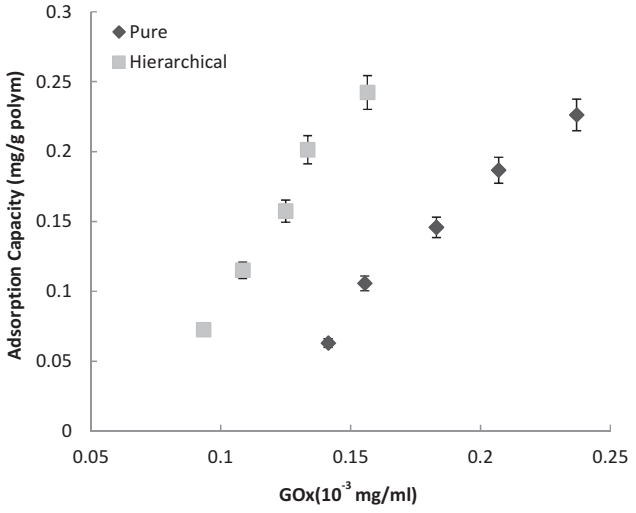

Figure 6. GOx adsorption isotherms after 8 h.

In this work, the kinetics of GOx immobilization onto two matrixes are evaluated based on two classical models, the Langmuir and Freundlich models, which have been widely used to fit the experimental results.[21]

Langmuir model:

$$\frac{C}{Q} = \frac{1}{bQ_m} + \frac{1}{Q_m}C$$

Freundlich model:

$$\frac{Q}{Q_m} = kC^{1/n}$$

or

$$\ln Q = \frac{1}{n}\ln C + \ln kQ_m$$

where C, Q, and Q_m are the equilibrium concentration of GOx, the adsorption capacity at a certain time, and the maximal loading of GOx, respectively, and b, n, and k are constant under specific conditions. As shown in **Figure 7**, the correlation coefficients of the curve from Langmuir isotherm fitting are very poor, indicating the immobilization of GOx does not follow the first-order kinetic model. On the contrary, Freundlich isotherm fitting gives good correlation coefficients, suggesting that the adsorption of GOx obeys the pseudo-second-order kinetic model.[21] In other words, the encapsulation process comprises an adsorption of GOx onto the external surface of hollow silica hosts followed by a further encapsulation of GOx inside the inner channel/cavity along with the increasing loading of GOx. Therefore, the better correlative coefficients from Freundlich fitting than Langmuir fitting appear to be logical and reasonable.

2.4. Enzymatic Activity Tests

The slowly kinetic response of hollow silica supports is very easy to confirm by adding a minute amount of GOx (i.e., 0.000456 mg mL^{-1}) for enzyme immobilization. The residual activity of GOx loaded into the pure and hierarchical hollow silica supports approach to about 88% and 62% even after 24 h (Supporting Information, Figure S3), respectively, indicating that the kinetic response is closely related to the structures of hollow silica supports. Evidently, GOx being strongly encapsulated inside the inner cavity is key factor to lower the activity of GOx loaded. In this work, the drawback can be effectively overcome by slightly increasing the amount of GOx added. In fact, from GOx adsorption isotherms (Figure 6), the increase in GOx loading is very evident with the increase in the concentration of GOx added. Understandably, the higher GOx loading certainly results in much better GOx activity, as shown in **Figure 8**. There exists a good linear relation between the amount of GOx added and the activity of GOx loaded for pure and hierarchically structured hollow silica matrixes, in which their correlation coefficients are as high as 0.976 and 0.993. This indicates that higher GOx loading are favorable to create higher GOx activities. More interestingly, there is a crossing point between two curves: below the critical content (≈0.0031 mg) of GOx added, the synthesized hollow silica obtains lower GOx activity; above the critical content, it offers higher GOx activities, indicating that the hierarchical host can achieve faster kinetic response through its higher GOx loading. It should be pointed out that such a GOx loading is far from fully occupied the large area

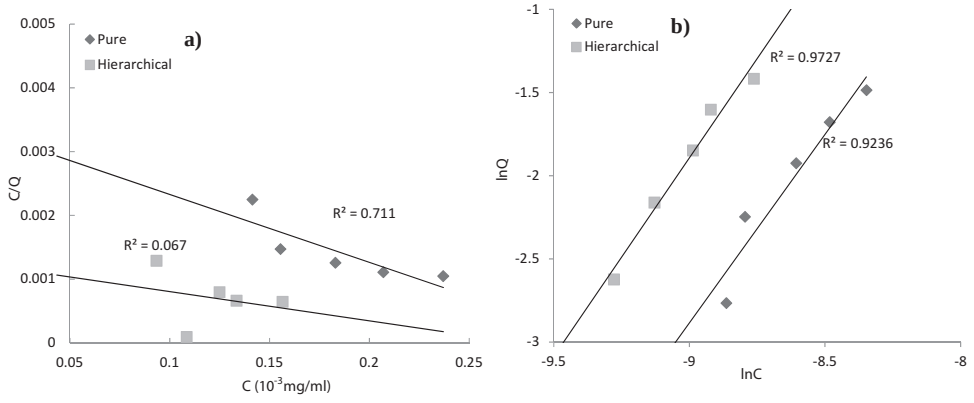

Figure 7. a) Langmuir and b) Freundlich fitting curves.

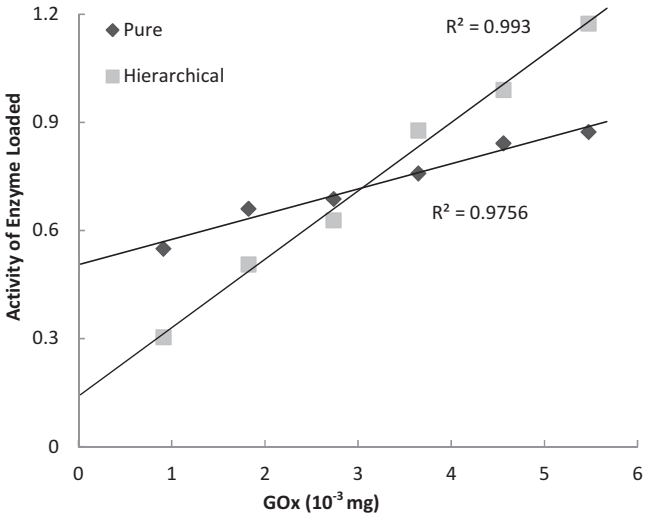

Figure 8. Activity of GOx loaded on pure and hierarchical hollow silica matrixes.

of the synthesized hollow support, and thus, it can be expected that some advanced systems such as biosensors and biocatalysis could be fabricated by employing the novel materials, offering a fast kinetic response within a short time.

2.5. Leaching Investigation

For enzyme immobilization via the adsorption or encapsulation approach, the leakage of enzyme is usually a big accompanied problem and disadvantage.[4] **Figure 9** shows that the leaching of GOx increases along with time. A relatively fast desorption is observed within the first 8 h. After then, since some of the desorbed GOx can be re-absorbed onto the matrixes, the absorption rate will drop slightly. More importantly, the unique hierarchically structured remarkably decreases the GOx leaching and

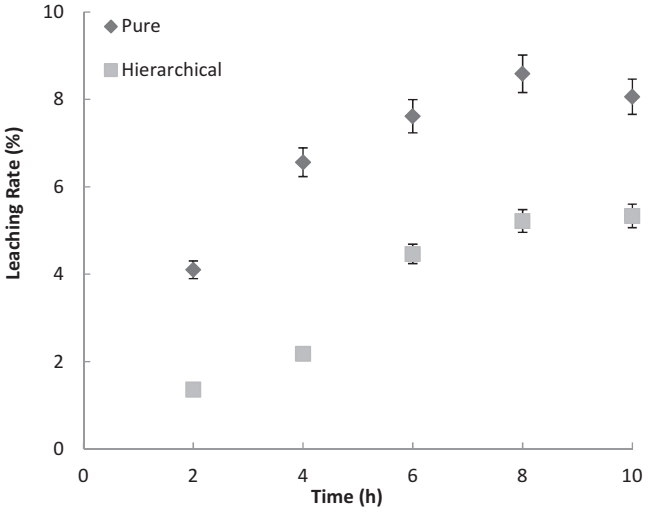

Figure 9. Leaching profiles of GOx loaded on pure and hierarchical hollow silica spheres.

achieves a lower desorption rate (≈4.8%) of GOx after 8 h, which can be mainly ascribed to their hierarchical protection because a higher activation energy is needed for desorbing enzyme. In contrast, a higher desorption-rate (≈8.8%) of GOx is observed after 8 h by using the pure hollow silica matrix. Therefore, the higher surface area and intact hierarchical structure of the synthesized hollow silica is more efficient to suppress the enzyme leakage, resulting in a more stable system.

3. Conclusions

In summary, we reported the first effort of utilizing ordered hierarchically structured hollow silica supports as a host for high efficiency enzyme immobilization. The larger surface area and higher mechanical stability of the novel supports could benefit the achievement of a high enzyme loading, bioactivities, sensitivity, and lower enzyme leaching, leading to faster kinetic response, which thus makes it highly versatile for a broad range of applications such as biosensor, biocatalysis, separation, encapsulation, drug-delivery, and controlled release.

4. Experimental Section

Hierarchically Structured Hollow Silica: The ordered hierarchical hollow silica microspheres were synthesized by using a consecutive template-guided self-assembly according to our previous work.[9] In the first step, the cationic polystyrene particles (CPS), which were prepared via emulsifier-free polymerization (Supporting Information), were dispersed into ethanol medium, and tetraethyl orthosilicate (TEOS) and ammonia were then added quickly and the mixture was reacted at 50 °C for around 6 h with constant stirring in the presence of methacryloxy-propyltrimethoxysilane (MPS), generating the core-shell structure of CPS/SiO_2 particles. In the second step, the synthesized CPS/SiO_2 particles were further used as the template and were *in situ* polymerized with styrene and co-monomer DMC (2-(methacryloyloxy) ethyltrimethylammonium chloride), resulting in the sandwich-like CPS/SiO_2/CPS particles. In the third step, the sandwiches-like particles were further treated with TEOS in ammonia to create the exterior silica layer. Finally, the two CPS layers were removed by calcination at 450 °C, forming a hierarchical hollow silica.

Enzyme Assay: Free and immobilized GOx activities were determined spectrophotometrically at 420 nm[22] by UV-vis spectroscopy (F-4500, Hitachi, Japan) by monitoring H_2O_2 using ABTS substrate (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) in a secondary reaction with horseradish peroxidase (HRP). A calibration curve of glucose oxidase in phosphate buffer solution (4 mL; pH = 7.0) was constructed (Supporting Information, Figure S4).

GOx Immobilization: The immobilization capacity of GOx was determined according to our previous work.[8d] 0.2 mL GOx (0.00912 mg mL^{-1}) and 0.02 g matrix were mixed in a disposable plastic tube. A typical batch-type experiment was conducted in phosphate buffer solution (pH = 7.0) at 25 °C. The sample solutions (4 mL) were stirred for 8 h to allow the equilibrium of immobilization to be reached for each incubator, and they were then separated from solution by centrifugation, decantation, and washing with 2 mL fresh buffer solution for three times in order to remove the free enzymes completely. The amount of loaded GOx was calculated by measuring the concentration of enzymes in the supernatant and then subtracting from the free-enzyme amount added in the experiment. The GOx concentration in the supernatant was determined based on the GOx assay.[22]

Stability/Leaching Studies: The samples of immobilized GOx on the pure and hierarchically structured hollow silica spheres were examined by desorption experiments in aqueous solution under the magnetic

agitation. In the case of a normal desorption, the GOx immobilized particles (0.02 g) were redispersed in 4 mL of fresh phosphate buffer solution (pH = 7.0), and then equilibrated for a desirable time (about 12 h) to ensure a steady state, prior to centrifugation. After that, the supports were rinsed three times with 4 mL of phosphate buffer solution. The concentration of the desorbed GOx in the supernatant was determined according to the GOx assay. The rate of leaching was then obtained through the formulation of the amount of desorption divided by the total amount of GOx loaded.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

The authors would like to thank the Research Directorate-General of European Commission for financial support to carry out this work under the framework of Marie Curie Action FP7-People-IIF-2010 (No. 275336)

Received: July 1, 2012
Revised: September 13, 2012
Published online: November 22, 2012

[1] a) R. A. Sheldon, *Adv. Synth. Catal.* **2007**, *349*, 1289; b) S. Hudson, J. Cooney, E. Magner, *Angew. Chem. Int. Ed.* **2008**, *47*, 8582.
[2] A. Popat, S. B. Hartono, F. Stahr, J. Liu, S. Qiao, G. Lu, *Nanoscale* **2011**, *3*, 2801.
[3] a) A. Macario, M. Moliner, A. Corma, G. Giordano, *Microporous Mesoporous Mater.* **2009**, *118*, 334; b) Y. Deng, C. Deng, D. Qi, C. Liu, J. Liu, X. Zhang, D. Zhao, *Adv. Mater.* **2009**, *21*, 1377–1382.
[4] a) U. Hanefeld, L. Gardossi, E. Magner, *Chem. Soc. Rev.* **2009**, *38*, 453; b) M. Tsai, Y. Tsai, *Sens. Actuators B* **2009**, *141*, 592.
[5] a) S. Jun, J. Lee, B. C. Kim, J. E. Lee, J. Joo, H. Park, J. H. Lee, S. Lee, D. Lee, S. Kim, Y. Koo, C. H. Shin, S. W. Kim, T. Hyeon, J. Kim, *Chem. Mater.* **2012**, *24*, 924; b) S. Lu, Z. An, J. He, B. Li, *J. Mater. Chem.* **2012**, *22*, 3882.
[6] X. S. Zhao, X. Y. Bao, W. Guo, F. Y. Lee, *Mater. Today* **2006**, *9*, 32.
[7] a) F. Fernandez-trillo, J. M. van Hest, J. C. Thies, T. Michon, R. Weberskirch, N. R. Cameron, *Adv. Mater* **2009**, *21*, 55; b) P. Tartaj, J. M. Amarilla, *Adv. Mater* **2011**, *23*, 4904; c) C. Baldacchini, M. A. H. Chamorro, M. Prato, S. Cannistraro, *Adv. Funct. Mater* **2011**, *21*, 153; d) F. Hoffmann, M. Froeba, *Chem. Soc. Rev* **2011**, *40*, 608.
[8] a) H. R. Luckarift, J. C. Spain, R. R. Naik, M. O. Stone, *Nat. Biotechnol.* **2004**, *22*, 211; b) W. Fu, A. Yamaguchi, H. Kaneda, N. Teramae, *Chem. Commun.* **2008**, 853; c) Y. Bai, H. Yang, W. Yang, Y. Li, C. Sun, *Sens. Actuators B* **2007**, *124*, 179; d) S. Cao, B. Liu, *Macromol. Biosci.* **2009**, *9*, 361; e) J. Fan, C. Z. Yu, T. Gao, J. Lei, B. Z. Tian, L. M. Wang, Q. Luo, B. Tu, W. Z. Zhou, D. Y. Zhao, *Angew. Chem. Int. Ed.* **2003**, *42*, 3146; f) Y. J. Wang, F. Caruso, *Chem. Mater.* **2005**, *17*, 953.
[9] S. Cao, Z. Zhao, X. Jin, W. Sheng, S. Li, Y. Ge, M. Dong, W. Wu, L. Fang. *J. Mater. Chem* **2011**, *21*, 19124.
[10] a) V. Vamvakaki, N. A. Chaniotakis, *Biosens. Bioelectron* **2007**, *22*, 2650; b) V. Bulmus, H. Ayhan, E. Piskin, *Chem. Eng. J.* **1997**, *65*, 71.
[11] a) W. Wei, Z. Yang, *Adv. Mater.* **2008**, *20*, 2965; b) C. Sanchez, C. Boissière, D. Grosso, C. Laberty, L. Nicole, *Chem. Mater.* **2008**, *20*, 682.
[12] a) J. Perez-Ramırez, C. H. Christensen, K. Egeblad, C. H. Christensen, J. C. Groen, *Chem. Soc. Rev.* **2008**, *37*, 2530; b) X. Zhang, T. Zhang, J. Ng, D. D. Sun, *Adv. Funct. Mater.* **2009**, *19*, 3731; c) Y. Zhao, L. Jiang, *Adv. Mater.* **2009**, *21*, 3621; d) C. Chen, S. Yang, W. Ahn, R. Ryoo, *Chem. Commun.* **2009**, 3627.
[13] a) X. W. Lou, C. Yuan, L. A. Archer, *Small* **2007**, *3*, 261; b) O. Kreft, M. Prevot, H. Mohwald, G. B. Sukhorukov, *Angew. Chem. Int. Ed.* **2007**, *46*, 5605; c) Q. Zhang, W. Wang, J. Goebl, Y. Yin, *Nano Today* **2009**, *4*, 494; d) M. Y. Bai, Y. Xia, *Macromol. Rapid Commun.* **2010**, *31*, 1863.
[14] a) R. Winkler, M. E. A. Richter, U. Knupfer, D. Merten, C. Hertweck, *Angew. Chem. Int. Ed.* **2006**, *45*, 8016; b) Z. Zhou, R. N. K. Taylor, S. Kullmann, H. Bao, M. Hartmann, *Adv. Mater.* **2011**, *23*, 2627; c) A. Schmid, J. S. Dordick, B. Hauer, A. Kiener, M. Wubbolts, B. Witholts, *Nature* **2001**, *409*, 258.
[15] a) R. Wilson, A. P. F. Turner, *Biosens. Bioelectron.* **1992**, *7*, 165; b) S. Cao, B. Liu, *Macromol. Biosci.* **2009**, *9*, 361.
[16] a) M. Shichiri, N. Asakawa, Y. Yamasaki, R. Kawamori, H. Abe, *Diabetes Care* **1986**, *9*, 298; b) S. A. Jaffari, A. P. Turner, *Physiol. Meas.* **1995**, *16*, 1.
[17] a) I. Tothill, J. Newman, S. F. White, A. Turner, *Enzyme Microb. Technol.* **1997**, *20*, 590; b) M. Yang, Y. Yang, B. Liu, G. Shen, R. Yu, *Sens. Actuators B* **2004**, *101*, 269.
[18] S. Brooks, R. Ashby, A P. F. Turner, M. R. Calder, D. J. Clarke, *Biosensors* **1987**–**1988**, *3*, 45.
[19] a) S. Cao, X. Jin, X. Yuan, W. Wu, J. Hu, W. Sheng, *J. Polym. Sci., Part A: Polym. Chem.* **2010**, *48*, 1332; b) W. Wu, X. Yuan, S. Cao, Y. Ge, S. Li, Z. Zhao, L. Fang, *Aust. J. Chem.* **2011**, *64*, 1541.
[20] a) J. Yu, L. Ge, P. Dai, S. Ge, S. Liu, *Biosens. Bioelectron.* **2010**, *25*, 2065; b) Q. Xiao, X. Tao, H. J. Zou, *Chem. Eng. J* **2008**, *137*, 38.
[21] a) F. B. O. Daoud, S. Kaddour, T. Sadoun, *Colloid Surf. B* **2010**, *75*, 93; b) M. Ghiaci, H. Aghaei, S. Soleimanian, M. E. Sedaghat, *Appl. Clay Sci* **2009**, *43*, 289; c) B. Liu, S. Cao, X. Deng, S. Li, R. Luo, *Appl. Surf. Sci* **2006**, *252*, 7830.
[22] S. B. Bankar, M. V. Bule, R. S. Singhal, L. Ananthanarayan, *Biotechnol. Adv.* **2009**, *27*, 489.