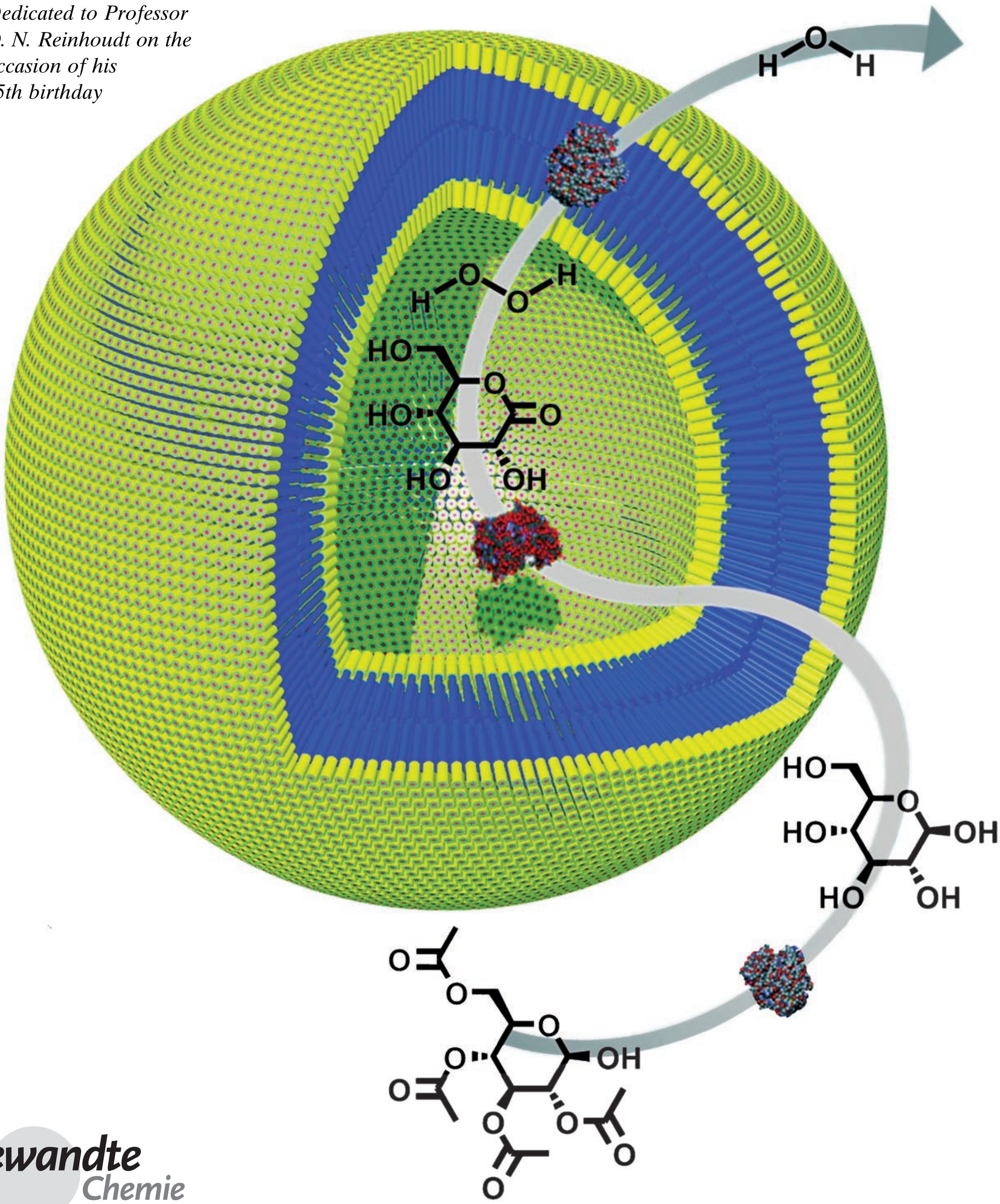
Nanoreactors

DOI: 10.1002/anie.200701125

Positional Assembly of Enzymes in Polymersome Nanoreactors for Cascade Reactions**

Dennis M. Vriezema, Paula M. L. Garcia, Núria Sancho Oltra, Nikos S. Hatzakis, Suzanne M. Kuiper, Roeland J. M. Nolte, Alan E. Rowan,* and Jan C. M. van Hest*

Dedicated to Professor D. N. Reinhoudt on the occasion of his 65th birthday



Angewandte Chemie

The cellular environment can be regarded as a highly complex synthetic medium, in which numerous multistep reactions take place simultaneously with unsurpassed efficiency and specificity. Nature employs several approaches to ensure the integrity of these, mostly enzyme-catalyzed, synthetic pathways, one of the most important ones being compartmentalization. This approach, which isolates the catalytic cycle, prevents interference by other compounds and enables regulation of the flux of molecules in and out of the microenvironment. Furthermore, to be efficient the biomolecular catalysts need not only to be separated but also positioned at specific sites within the cell.

Many studies have been reported in the literature with the objective of mimicking this natural concept of enzyme assembly and encapsulation,[1–4] with the earliest examples being based on phospholipid liposomes.[5–8] A major problem that accompanies the use of liposomes is their relative thermodynamic and mechanical instability. To overcome this limitation, other methods of enzyme compartmentalization based on sol–gel chemistry[1,2] and layer-by-layer deposition have been developed.[9]

Another method more closely related to the liposome approach is one that makes use of block-copolymer amphiphiles to construct a closed environment, a so-called polymersome.[10] These block copolymers have the same basic architecture as lipids, in that they possess a hydrophilic head group and a hydrophobic tail. The almost unlimited variety in monomers and polymerization methods makes it possible to precisely tune the properties of the polymersomes.[11–15] In general, polymersomes are less dynamic than liposomes, because of the larger dimensions of the amphiphilic block copolymers and their lower critical aggregation concentration.[10] The diffusion of water through their membranes is also slower as a result of the larger thickness of these membranes.[10] To resolve this problem, researchers have incorporated channel proteins[16] and proton pumps in polymersome membranes.[17]

In recent reports we have described the synthesis and properties of the diblock copolymer polystyrene$_{40}$-*b*-poly(L-isocyanoalanine(2-thiophen-3-yl-ethyl)amide)$_{50}$ (PS–PIAT), which consists of a rigid polyisocyanide block and a flexible polystyrene tail, thus making it a rod–coil type of diblock copolymer.[18,19] This well-defined block copolymer was shown to form stable polymersomes, which could be used to encapsulate a variety of guests.[18,19] A unique feature of these polymersomes is that they are sufficiently porous by themselves to allow diffusion of small molecules across their membranes, while large molecules, such as enzymes, remain trapped inside.[18] These polymersomes, therefore, are ideal to be used for compartmentalization, as they give protection to the enzymes inside, whereas low-molecular-weight substrates and products can diffuse in and out of the polymersomes.

Until now, in all cases enzyme encapsulation has been obtained without a high level of control over positional assembly. To mimic nature more closely it would be desirable not only to encapsulate enzymes, but also to position different types of enzymes in separate domains within the polymersome, for example, in the water pool and in the polymersome membrane.[20] Herein, we describe a procedure to achieve this aim. To demonstrate that our method is generic, three types of enzymes were selected as candidates for inclusion, that is, *Candida antarctica* lipase B (CALB), horseradish peroxidase (HRP), and glucose oxidase (GOX).

The previously reported procedure of encapsulating CALB in the inner aqueous compartments of the polymersomes was adopted for HRP and GOX.[18] Fluorescence microscopy was used to confirm that the incorporation of the enzymes labeled with Alexa Fluor in these compartments was successful. Electron microscopy clearly showed that encapsulation of these enzymes did not disrupt the structures of the polymersomes (Figure 1). The diameters of the enzyme-filled polymersomes ranged from 50 to 1100 nm with an average diameter of 517 nm, as determined by measuring the diameters of polymersomes from a representative number of TEM images (see Supporting Information). The possibility of using the enzyme-filled polymersomes as nanoreactors was investigated by enzyme-activity assays that could be monitored spectroscopically (Scheme 1).

The initial rate of substrate conversion by the enzyme-filled PS–PIAT polymersomes after filtration was compared with the initial rate of conversion of free enzymes in solution. The concentration of the latter enzymes was identical to that of the solution of enzyme used for the encapsulation, and therefore the concentration of enzyme in the inner aqueous compartments of the polymersomes was expected to be the same as that of the free enzymes in solution. The effective enzyme concentration in the total reaction volume (polymersomes and enzyme-free dispersion medium) was actually 1000

[*] Dr. P. M. L. Garcia,[+] N. Sancho Oltra,[#] Dr. N. S. Hatzakis,[$]
S. M. Kuiper, Prof. R. J. M. Nolte, Prof. A. E. Rowan,
Prof. J. C. M. van Hest
Institute for Molecules and Materials
Department of Organic Chemistry
Radboud University
Toernooiveld 1, 6525 ED Nijmegen (The Netherlands)
Fax: (+31) 24-365-2929
E-mail: a.rowan@science.ru.nl
j.vanhest@science.ru.nl
Homepage: http://www.science.ru.nl/orgchem
Dr. D. M. Vriezema
Encapson B.V.
Toernooiveld 100, 6525 EC Nijmegen (The Netherlands)
[+] Current address:
Department of Medicinal Chemistry
N.V. Organon
Molenstraat 110, 5342 CC Oss (The Netherlands)
[$] Current address:
Nano-Science Center
H.C. Ørsted Instituttet
Universitetsparken 5, Bygning D, 2100 Copenhagen (Denmark)
[#] Current address:
Department of Synthetic Organic Chemistry
University of Groningen
Nijenborgh 4, 9747 AG Groningen (The Netherlands)
[**] We thank Dr. E. Pierson for guidance with the fluorescence microscope and NWO-ACTS and Synthon B.V. for financial support.
Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

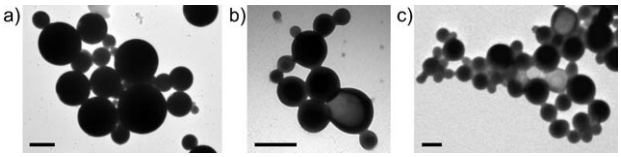


Figure 1. Transmission electron micrographs of PS–PIAT polymersomes containing a) CALB, b) HRP, and c) GOX. Scale bars: 500 nm.

times lower. The results of the three assays are summarized in Table 1.

Table 1: Difference in the rate of substrate conversion for encapsulated and free enzyme.

Enzyme	CALB	HRP	GOX
R_{out}/R_{in}[a]	2.8 ± 0.3	2.6 ± 0.3	6.8 ± 0.8

[a] R_{out}/R_{in} is the difference between the catalytic rate for free enzyme divided by the rate of catalysis for encapsulated enzyme. The rates of conversion in solution after 60 s were: CALB, $(4.2\pm0.4)\times10^{-2}\ h^{-1}$; HRP, $(8.1\pm0.7)\times10^{-4}\ h^{-1}$; GOX, $(3.2\pm0.3)\times10^{-4}\ h^{-1}$. See the Supporting Information for the specific conditions of each assay.

In all cases the apparent substrate conversion was found to be slower within the polymersome than for free enzyme. However, when corrected for the effective concentration, these results suggest that the encapsulated enzyme is actually 100-fold more active than free enzyme. Two explanations can be given for this phenomenon: 1) as a result of interactions between enzyme and polymer, more enzyme is encapsulated than statistically expected; and 2) as a result of interactions between the membrane and enzyme, the active site of the enzyme becomes more accessible for substrates.[21] Detailed studies are currently being performed to determine which of these explanations is the most plausible one. Investigation of the filtrate as a function of time showed that both substrate and product could readily diffuse through the polymersome membrane but that the enzymes could not leak out. The clear benefit of the encapsulation approach was observed in the lifetime of the enzymes, which showed only a marginal loss of activity after one month (87 ± 5 % activity), in contrast to the same enzymes in bulk solution, which lost their complete activity within a few days.

Unlike the case of liposomes, incorporation of enzymes in the hydrophobic membrane of polymersomes has not been previously reported. We were able to accomplish this by the addition of a THF solution containing PS–PIAT to an aqueous solution of enzyme, after which the entire mixture was rapidly lyophilized. The resulting enzyme–polymer powder was then redissolved in THF and immediately injected into water. Initial experiments were performed using CALB labeled with Alexa Fluor 488 (CALB-AF488) and the resulting aggregates were studied by fluorescence microscopy. These studies showed that the CALB enzymes were localized in the polymersome membranes (Figure 2a). Subsequently, the lyophilized CALB-AF488–polymer sample was injected into an aqueous solution of a differently labeled

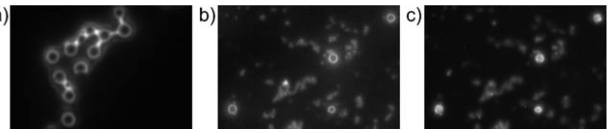
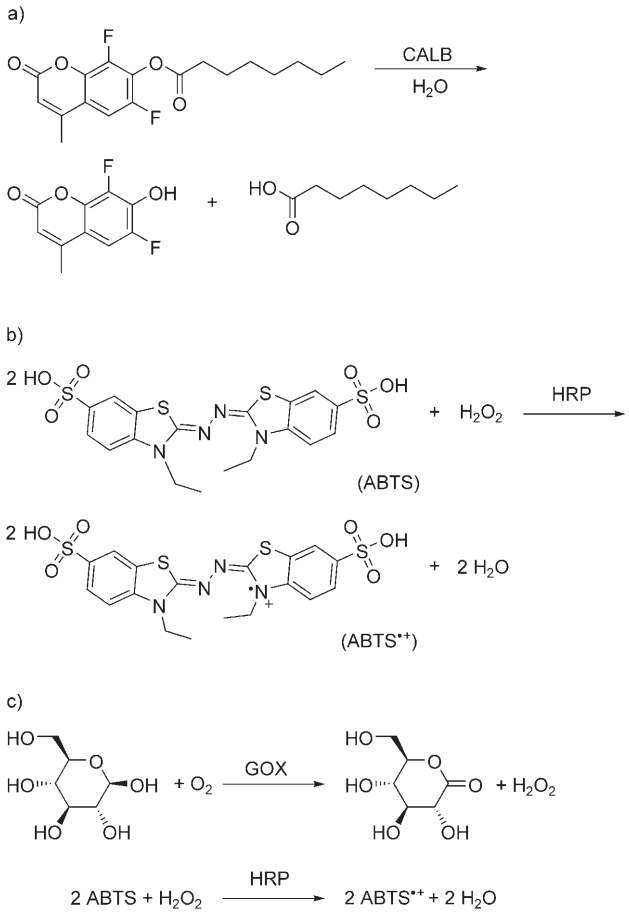


Figure 2. Fluorescence micrographs of Alexa Fluor labeled CALB inside polymersomes. a) CALB in the membrane of the polymersomes ($\lambda_{exc.}=488$ nm). b,c) Micrographs of the same area for polymersomes with CALB-AF488 in the membrane and CALB-AF633 in the water pool of the polymersomes ($\lambda_{exc.}=488$ and 633 nm, respectively). Samples were deposited on a glass surface. The width of the images corresponds to 6 μm.

CALB, that is, CALB labeled with Alexa Fluor 633 (CALB-AF633). The two enzymes could be independently visualized with fluorescence microscopy by switching the excitation wavelength from 488 to 633 nm (Figure 2b and c). CALB-AF488 was located exclusively in the membrane, whereas CALB-AF633 was only observed in the internal water pool of the polymersome. The enzyme-activity assay shown in Scheme 1a, performed on the filtrate of the polymersomes with membrane-bound CALB-AF488, revealed that no free enzyme was present, thus highlighting the encapsulation efficiency of this lyophilization approach.



Scheme 1. Assays used to establish enzyme activity inside polymersomes for a) CALB, b) HRP, and c) GOX. ABTS = 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid).

The concept of positional assembly was further extended by using two different enzymes that can operate in a tandem fashion, namely, GOX and HRP. Through the above-mentioned lyophilization approach, HRP was associated with the membrane and GOX was entrapped within the water pool of the PS–PIAT polymersomes (nonencapsulated enzyme molecules were removed by filtration). As HRP is a hydrophilic enzyme, it is expected that it will be positioned in the hydrophilic domains of the polymersome membrane. The enzyme-activity assay in Scheme 1c was used to measure the activity of the entrapped HRP–GOX couple (Figure 3). It can be concluded that the reaction proceeds smoothly and that glucose added to the dispersion of polymersomes can readily permeate the membrane and be converted by GOX to its lactone, thus resulting in the release of H_2O_2, which is subsequently utilized by HRP to convert ABTS to $ABTS^{\bullet+}$. The highest activity was observed at pH 5.5, near the optimum working pH for GOX; glucose was converted 3.1 times faster at pH 5.5 than at pH 7.5 after 1 h. Control measurements, carried out by repeating this experiment in the absence of glucose at pH 5.5 and 7.2, or without ABTS at pH 5.5, showed no absorption change.

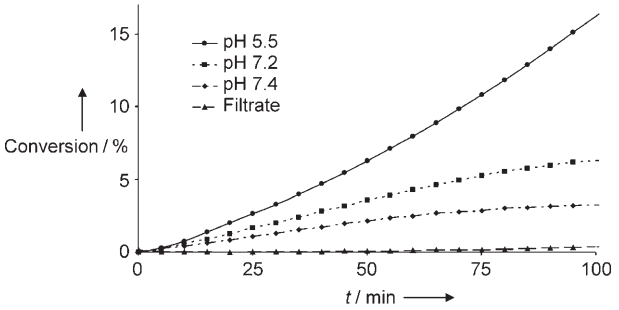
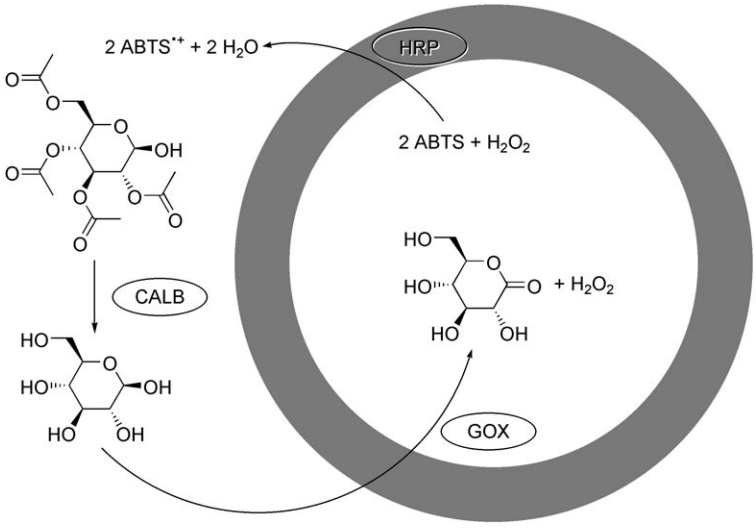


Figure 3. Plots of UV/Vis absorption versus time for the conversion of glucose at different pH values in the presence of ABTS by HRP–GOX–polymersomes. The increase in conversion in the filtrate is a result of autooxidation of ABTS.

The measured enzyme activity cannot be directly correlated with the number of enzyme molecules obtained from UV absorption, as the enzyme is in a different environment. The average amount of encapsulated enzyme was therefore determined by calculating the volume of the PS–PIAT polymersome water pool and the total amount of polymersomes present in the dispersion,[22] assuming that the enzyme concentration inside the polymersomes was equal to that initially used outside. The calculated average amounts of HRP in the membrane and GOX in the water pool were estimated to be 4.1 and 14 enzymes, respectively.

In a final increase in complexity, a three-enzyme couple was designed containing the enzymes CALB, GOX, and HRP. For this experiment, 1,2,3,4-tetra-*O*-acetyl-β-glucopyranose (GAc_4) was chosen as substrate, of which the acetate groups have to be hydrolyzed first by CALB in bulk before the above-described tandem reaction can occur (Scheme 2). The same HRP–GOX–polymersome system was used as described above, and CALB was added to this system together



Scheme 2. Schematic representation of the multistep reaction taking place in the three-enzyme–polymersome system.

with GAc_4 and ABTS. The plots of the formation of $ABTS^{\bullet+}$ as a function of time are given in Figure 4. Compared to the experiment in which glucose is converted by the HRP–GOX–polymersomes, the increase in absorption after 3 h was 14

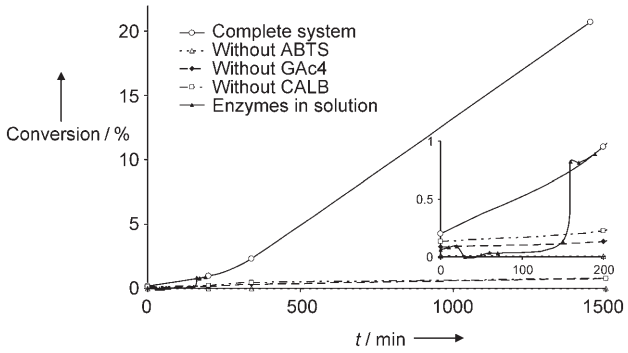


Figure 4. Formation of $ABTS^{\bullet+}$ in the three-enzyme system at pH 7.2, as followed by UV/Vis spectroscopy.

times smaller and the initial increase in absorption was slower. As all substrate and enzyme concentrations were unchanged with respect to the previous experiments, it is likely that in the three-enzyme system the hydrolysis of GAc_4 is the rate-limiting step of the process.

From the plot in Figure 4 it is evident that the three different enzymatic reactions proceed in one pot; $ABTS^{\bullet+}$ can only be formed when the entire cascade (Scheme 2) is operational. Control experiments confirmed that if one of the components was absent no $ABTS^{\bullet+}$ was formed (Figure 4). Moreover, when the cascade reaction with the three enzymes was performed in solution, after 170 min the conversion stopped at only 0.8%, thereby showing the beneficial effect of encapsulation.

In summary, we have shown that nanoreactors constructed from porous enzyme-containing polymersomes can be used to perform one-pot multistep reactions. Three

different enzymes were selectively encapsulated inside PS–PIAT polymersomes. Spectroscopic studies confirmed that the enzymes retained their activity. The use of lyophilization allowed us for the first time to position enzymes exclusively in the polymersome membrane and to combine this with other enzymes in the water pool of these species. Addition of glucose to a dispersion of such polymersomes resulted in a tandem system in which the activities of the GOX and HRP enzymes were coupled. In a more complex three-enzyme system, external CALB was used to first convert the substrate GAc_4 into glucose, which was then used by GOX and HRP to generate $ABTS^{\bullet+}$.

Experimental Section

General procedure for the encapsulation of two different enzymes inside PS–PIAT polymersomes: A PS–PIAT solution in THF (1.0 mL, 1.0 g L^{-1}) was injected into a HRP solution (200 μL, 100 mg L^{-1}) in phosphate buffer (20 mM, pH 7.2). Ultrapure water (12 mL) was added to this mixture, and after homogenization the sample was lyophilized. The off-white powder obtained was redissolved in THF (1.0 mL) and this solution (500 μL) was injected into a GOX solution (2.5 mL, 100 mg L^{-1}) in phosphate buffer (20 mM, pH 7.2). After equilibration for 30 min the mixture was transferred to an Eppendorf tube equipped with a 100-kDa cutoff filter and centrifuged to dryness. The polymersomes were redispersed in phosphate buffer (500 μL, 20 mM, pH 7.2) and centrifuged again. This step was repeated until no enzyme activity was observed in the filtrate. The resulting biohybrid was redispersed in phosphate buffer (500 μL, 20 mM, pH 7.2).

Received: March 14, 2007
Revised: April 18, 2007
Published online: August 17, 2007

Keywords: cascade reactions · catalysis · enzymes · polymersomes · supramolecular chemistry

[1] D. Avnir, T. Coradin, O. Lev, J. Livage, *J. Mater. Chem.* **2006**, *16*, 1013.
[2] V. B. Kandimalla, V. S. Tripathi, H. X. Ju, *Crit. Rev. Anal. Chem.* **2006**, *36*, 73.
[3] D. M. Vriezema, M. Comellas Aragones, J. A. A. W. Elemans, J. J. L. M. Cornelissen, A. E. Rowan, R. J. M. Nolte, *Chem. Rev.* **2005**, *105*, 1445.
[4] P. Walde, S. Ichikawa, *Biomol. Eng.* **2001**, *18*, 143.
[5] G. Weissman, G. Sessa, M. Standish, A. D. Bangham, *J. Clin. Invest.* **1965**, *44*, 1109.
[6] T. Oberholzer, K. H. Nierhaus, P. L. Luisi, *Biochem. Biophys. Res. Commun.* **1999**, *261*, 238.
[7] A. Fischer, A. Franco, T. Oberholzer, *ChemBioChem* **2002**, *3*, 409.
[8] S. M. Nomura, K. Tsumoto, T. Hamada, K. Akiyoshi, Y. Nakatani, K. Yoshikawa, *ChemBioChem* **2003**, *4*, 1172.
[9] A. P. R. Johnston, C. Cortez, A. S. Angelatos, F. Caruso, *Curr. Opin. Colloid Interface Sci.* **2006**, *11*, 203.
[10] D. E. Discher, A. Eisenberg, *Science* **2002**, *297*, 967.
[11] L. Luo, A. Eisenberg, *Langmuir* **2001**, *17*, 6804.
[12] J. Wu, A. Eisenberg, *J. Am. Chem. Soc.* **2006**, *128*, 2880.
[13] B. M. Discher, H. Bermudez, D. A. Hammer, D. E. Discher, Y.-Y. Won, F. S. Bates, *J. Phys. Chem. B* **2002**, *106*, 2848.
[14] P. P. Ghoroghchian, G. Z. Li, D. H. Levine, K. P. Davis, F. S. Bates, D. A. Hammer, M. J. Therien, *Macromolecules* **2006**, *39*, 1673.
[15] F. H. Meng, C. Hiemstra, G. H. M. Engbers, J. Feijen, *Macromolecules* **2003**, *36*, 3004.
[16] M. Nallani, S. Benito, O. Onaca, A. Graff, M. Lindemann, M. Winterhalter, W. Meier, U. Schwaneberg, *J. Biotechnol.* **2006**, *123*, 50.
[17] H.-J. Choi, C. D. Montemagno, *Nano Lett.* **2005**, *5*, 2538.
[18] D. M. Vriezema, J. Hoogboom, K. Velonia, K. Takazawa, P. C. M. Christianen, J. C. Maan, A. E. Rowan, R. J. M. Nolte, *Angew. Chem.* **2003**, *115*, 796; *Angew. Chem. Int. Ed.* **2003**, *42*, 772.
[19] D. M. Vriezema, A. Kros, R. de Gelder, J. J. L. M. Cornelissen, A. E. Rowan, R. J. M. Nolte, *Macromolecules* **2004**, *37*, 4736.
[20] P. P. Ghoroghchian, J. J. Lin, A. K. Brannen, P. R. Frail, F. S. Bates, M. J. Therien, D. A. Hammer, *Soft Matter* **2006**, *2*, 973.
[21] K. Faber, *Biotransformations in Organic Chemistry*, Springer, Berlin, **1992**.
[22] The details of this calculation can be found in the Supporting Information.

