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Encapsulated Enzymes

Triggered Enzymatic Degradation of DNA within Selectively Permeable Polymer Capsule Microreactors**

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The compartmentalization of reactions, a strategy developed by living cells, enables multiple, simultaneous reactions to be carried out within spatially separated reaction vessels.[1] Various strategies for the compartmentalization of biochemical reactions in vitro have been applied, including their confinement in liposomes,[2] polymersomes,[3] water-in-oil emulsions,[4] and polyelectrolyte capsules.[5] A greater challenge for synthetic microreactors is the possibility of continuous reactions, for which the design of the vessel must accommodate for the constant intake of reagents and release of products. In the cell, these processes are often mediated by transmembrane proteins; however, examples of synthetic microreactors with continuous-reaction capabilities are scant.[6] The semipermeable nature of polymer capsules formed by layer-by-layer (LbL) assembly enables the selective confinement or exclusion of molecules,[7] primarily on the basis of their size: Small molecules, including ions and monomers, diffuse freely across the capsule walls, whereas larger macromolecules and crystals are diffusion-limited. This feature makes LbL capsules attractive candidates for use as synthetic microreactors. However, the field of encapsulated catalysis with these capsules is still in its infancy. The few successful studies reported have focused on the catalytic conversion of small molecules (e.g. carbonates, peroxides, phenols).[8] Herein, we report an essential step towards the mimicry of cellular processes[9] with synthetic LbL capsules: a triggered encapsulated enzymatic reaction with a nucleic acid substrate.

Within the living cell, the lysosomal degradation of proteins and nucleic acids is among the most prominent examples of compartmentalized enzymatic reactions. We have carried out this reaction in a synthetic microreactor by coencapsulating double-stranded DNA (dsDNA) and the enzyme DNase I in multilayered polymer capsules. We demonstrate herein that the semipermeable nature of the microreactor affords control over the reaction with external chemical stimuli, and that by conducting the enzymatic reaction within the polymer capsules with fluorescently labeled substrate DNA, the reaction can be monitored directly within each individual capsule by the high-throughput technique flow cytometry (FC). This reaction is the first example of a triggered, continuous chemical reaction for the modification of DNA within a polymer reaction vessel.

Bimodal mesoporous silica (BMS) particles were used for biomolecule immobilization and as templates for LbL assembly.[10] The surface modification of BMS particles[11] with a primary amine imparted a positive charge to the particles and thus created an electrostatic attraction between the particles and the introduced fluorescently labeled dsDNA. Confocal laser scanning microscopy (CLSM) images of the particles indicated the infiltration of DNA into the particles (see the Supporting Information). Subsaturation coverage of the particle surfaces by DNA, as confirmed by microelectrophoresis, was essential for the subsequent assembly of the polymer multilayers.[12] The infiltration of DNase I, a globular protein of approximate dimensions $4.5 \times 4.0 \times 3.5$ nm^3,[13] proceeded for 1 h following charge reversal by poly(methacrylic acid) (PMA; Figure 1). The amount of adsorbed enzyme was determined to be (0.23 ± 0.03) U of DNase I per milligram of silica by using a microbicinchoninic acid protein assay.

Subsequent polymer multilayers were assembled onto the particles through hydrogen bonding between a thiol-modified PMA (PMA_{SH}) and poly(vinyl pyrrolidone) (PVPON) by alternating the adsorption of the polymers at pH 4.0.[14] PMA_{SH} cross-linking and removal of the silica cores yielded intact polymer capsules, as confirmed by transmission electron microscopy (TEM) and differential interference contrast (DIC) microscopy (see the Supporting Information). Elevation of the pH value to 7 caused the removal of PVPON from the capsule walls by disrupting its hydrogen bonding to PMA_{SH} to produce single-component PMA capsules[14] with the nucleic acid chains confined within the interior of the capsules (Figure 2a). Although some enzyme leakage may occur from the capsules following core dissolution, the affinity of DNase I for dsDNA is likely to prevent any significant amount of the enzyme from escaping from the capsules.[13]

DNase I is an endonuclease with a strong affinity for dsDNA. The catalytic nuclease activity of DNase I requires the presence of divalent cations.[13] Specifically, magnesium and calcium ions were shown to have a synergistic effect: A highly active enzyme–divalent cation complex was formed at pH 7.5.[15] In the absence of these ions, the enzyme remained inactive. Their introduction is an effective trigger for enzymatic activity. In the absence of Mg^{2+} and Ca^{2+}, CLSM images showed that the interior of the capsules was filled with DNA (Figure 2a). The capsules showed negligible leakage of DNA for at least 72 h. However, the addition of divalent cations and the elevation of the temperature to 35 °C led to activation of

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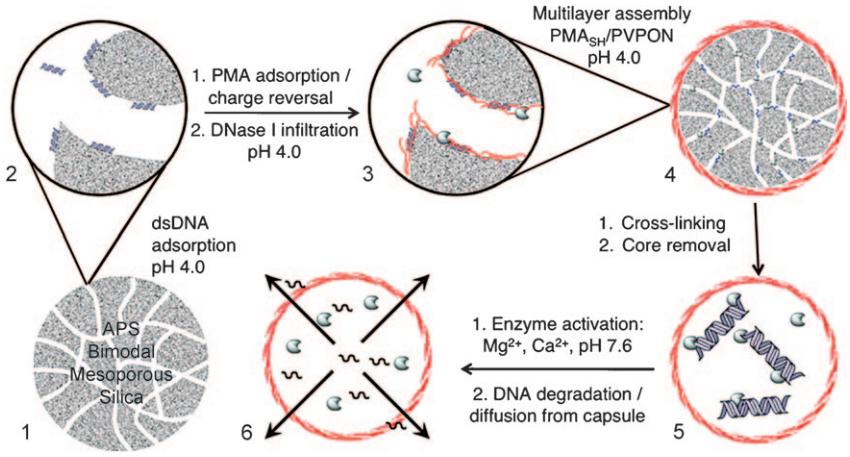

Figure 1. The assembly and function of the synthetic polymer microreactor: 1) Amine modification of BMS particles (APS = 3-aminopropylsilyl); 2) infiltration of dsDNA into the mesopores; 3) charge reversal through adsorption of poly(methacrylic acid) (PMA), then DNase I infiltration; 4) multilayer assembly of thiol-modified PMA (PMA$_{SH}$) and poly(vinyl pyrrolidone) (PVPON) through hydrogen bonding; 5) PMA$_{SH}$ cross-linking, BMS removal, and PVPON removal; 6) degradation of dsDNA by DNase I and diffusion of degraded DNA from the capsule.

that were heated to 35 °C exhibited a less than 5% decrease in fluorescence. This result indicates that heating alone does not cause significant DNA degradation or release from the capsules. In the absence of the enzyme, the capsules showed no loss of DNA in response to the introduction of magnesium and calcium cations. Thus, the divalent cations appear to have no adverse influence on the permeability of the capsules to the encapsulated nucleic acid. These results highlight the possibility of triggering an enzymatic reaction within a synthetic microreactor by an external stimulus. The addition of EDTA and cooling of the reaction mixture provides a means of halting the enzymatic reaction. The external control over the encapsulated enzymatic reaction is made possible by the semipermeable nature of the capsules.

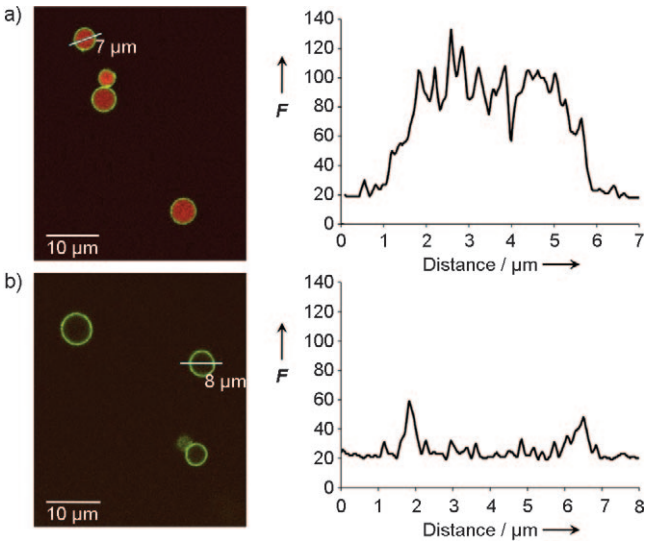

Figure 2. CLSM images of capsules a) before and b) after activation of encapsulated DNase I. The encapsulated DNA was labeled with Alexa Fluor 546 (red emission), and the capsule wall was labeled with Alexa Fluor 488 (green emission). The adjacent graphs show the intensity of red fluorescence, *F*, along the blue lines in the images. The peaks in the bottom graph are due to emission into the red spectrum from the Alexa Fluor 488 fluorophores on the capsule walls.

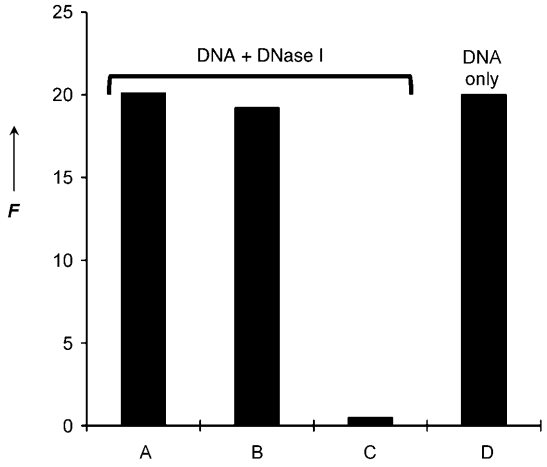

Figure 3. FC fluorescence, *F*, for PMA$_{SH}$ capsules containing 600 bp DNA labeled with Alexa Fluor 546. Measurements A–C were for capsules containing DNA and DNase I. Measurement D was for capsules containing DNA only. A) Measurement made prior to DNase I activation. B) The capsules were incubated at 35 °C for 45 min in the absence of divalent cations. C,D) The capsules were incubated at 35 °C for 45 min in a buffer containing 1.25 mM $MgCl_2$ and 0.25 mM $CaCl_2$.

the enzyme: Within 45 min, the interior of the capsules became free of DNA (Figure 2b), which indicated that the products of degradation had been released.

To obtain quantitative data on DNA degradation, we analyzed the capsules by FC, which enables fluorescence measurements of individual capsules. In agreement with the observations by CLSM, the activation of DNase I within the capsules led to an almost complete loss of fluorescence within 45 min (Figure 3). In the absence of divalent cations, capsules

To determine the kinetics of the process, the encapsulated enzymatic reaction was monitored by quenching the reactions at specified time points by the addition of EDTA to a concentration of 10 mM to sequester the divalent cations and cooling of the capsules on ice. The fluorescence of the capsules was measured after 70 min for all samples to allow a similar time period for the diffusion of digested DNA from the capsules. In agreement with previous in vitro studies of the DNase I reaction,[16] the profile for the degradation of the fluorescently labeled DNA and its release from the capsules was found to be linear (Figure 4). However, the timescale of

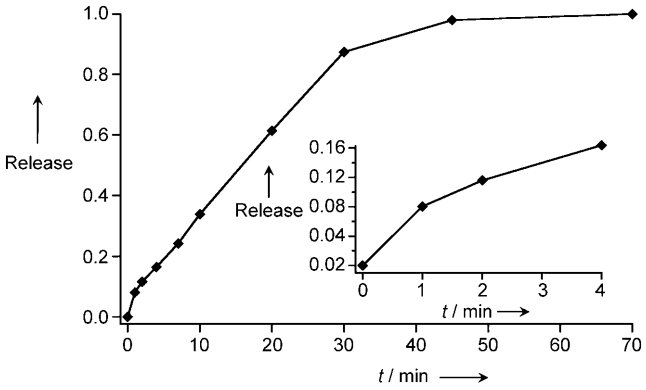

Figure 4. Normalized release of DNA following activation of coencapsulated DNase I, as measured by FC. The inset shows the initial release. Reaction temperature: 35 °C.

the reaction was significantly longer than expected; our own measurements showed that DNase I (0.1 U) was able to digest 1000 ng of dsDNA in 20 μL samples in 2 min (data not shown). The rate of DNA degradation depends on a number of factors, including the concentration of the enzyme and substrate, the reaction conditions, DNA conformation, and substrate inhibition.[17] The slower reaction kinetics may be due to the accumulation of DNA degradation products, which are known to inhibit DNase I,[17] whereby the inhibitory effect is more pronounced in the small volumes of capsules. This effect may also explain the measured drop in the reaction rate beyond the first 60 s.

In conclusion, we have demonstrated the first successful enzyme-catalyzed reaction of a nucleic acid substrate within the confines of nanoengineered polymer capsules assembled by an LbL process. Coencapsulation of DNase I and dsDNA enabled the degradation of the DNA within multilayered LbL-assembled polymer capsules under the control of an external stimulus. High-throughput monitoring of the encapsulated, fluorescently labeled DNA provided a novel means of measuring the kinetics of the reaction. In contrast to other encapsulation techniques, polymer capsules enable the simple diffusion or exclusion of reaction components primarily on the basis of size, as well as simple control of the reaction through the use of chemical stimuli.

Experimental Section

A suspension of APS–BMS (1 mg) in 25 mM sodium acetate buffer (pH 4) was prepared with 0.5 wt%. This suspension was incubated with 500 ng of a labeled dsDNA PCR amplicon for 15 min, and the resulting particles were washed three times. The DNA-loaded particles were suspended in a solution of PMA (M_w 7700, 0.1 g L^{-1}) for 15 min and then washed three times (pH 4). The particles were then suspended in a buffer (150 μL, pH 4) containing DNase I (10 U) and incubated for 1 h at 4 °C. Enzyme immobilization was monitored by using a microbicinchoninic acid protein assay. Following the infiltration of DNase I, PVPON (M_w 360 000) was added to a polymer concentration of 1.0 g L^{-1}, and the mixture was mixed at 4 °C for 15 min. The particles were washed three times, incubated in a solution of PMA_{SH} (1.0 g L^{-1})[14] for 15 min at 4 °C, washed three times in chilled buffer (pH 4), and then suspended in a solution of PVPON (M_w 55 000, 1.0 g L^{-1}) for 15 min at 4 °C. PMA_{SH} and PVPON were added sequentially until 10 layers had been deposited. The particles were then treated with 2.5 mM chloramine T in 50 mM 2-morpholinoethanesulfonic acid (MES) buffer (pH 6) for 1 min, washed with fresh buffer, and then dispersed in buffer (50 μL, pH 4). A mixture of 2 M HF and 8 M NH_4F (100 μL, pH ≈ 5) was added to this suspension, and the resulting capsules were washed by centrifugation (4500 g for 5 min) and dispersed in 10 mM Tris-HCl buffer containing 50 μM EDTA (pH 7.6; Tris = 2-amino-2-hydroxymethylpropane-1,3-diol, EDTA = ethylenediaminetetraacetic acid). This washing process was repeated until the pH of the supernatant was equal to the pH of the fresh buffer solution. The encapsulated enzyme was activated by dispersing the capsule microreactors in a buffer containing 1.25 mM $MgCl_2$ and 0.5 mM $CaCl_2$. See the Supporting Information for details.

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