Microcontainers

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Surface-Bound Microenclosures for Biomolecules**

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The fabrication of nano- and micrometer-sized containers has attracted significant recent interest. Such containers have wide applications, especially in biochemistry and biophysics, where they can mimic the confinements typical of a cell. Micrometer-sized enclosures have been made from phospholipids,[1] lipids,[2] polymers,[3] and emulsions.[4] Polyelectrolyte layer-by-layer (LbL) self-assembly is another commonly used method in the fabrication of small containers for encapsulating molecular and macromolecular components.[5] Typically, colloidal templates such as polymeric spheres, enzymes, organic crystals, or cells in the aqueous phase are encapsulated inside a layered polyelectrolyte assembly, thus limiting the process to water-insoluble substances.[6] Small molecular components, which trigger reactions within the enclosures, can then be added.[7] Recently developed reverse-phase LbL (RP-LbL) technology allows for easier encapsulation of water-soluble materials such as biomolecules.[8] The assembly of such nano- and micrometer-sized enclosures on flat substrates is important if the application requires observation with a microscope, single-molecule experiments, or multiplexing. Microparticles have been patterned onto solid substrates by using a variety of methods.[9] An example of microcapsule patterning was recently reported.[10]

Herein we demonstrate the RP-LBL-based fabrication of surface-bound microenclosures (SBMEs), with sizes in the range 1–20 μm and can trap water-soluble biomolecules. The fabrication of SBMEs from dried template material, and examples of the appearance of SBMEs in fluorescence and confocal microscopy are shown in Figure 1. The Brownian motion of fluorescently labeled DNA molecules from the bacteriophage lambda (λ-DNA) can be observed. The electrophoretic motion of DNA and proteins can be induced by applying an external electrical field, which leads to a steep concentration gradient, thus confirming efficient biomolecule trapping. The biofunctionality of encapsulated biomolecules was demonstrated in SBMEs. Small molecules can diffuse through the semipermeable membranes of the SMBEs, as demonstrated by diffusion of fluorescein isothiocyanate (FITC)-biotin to encapsulated NeutrAvidin, and by the control of enzymatic DNA digestion by DNase, mediated by Mg^{2+} and Ca^{2+} ions. SBMEs may find applications as a

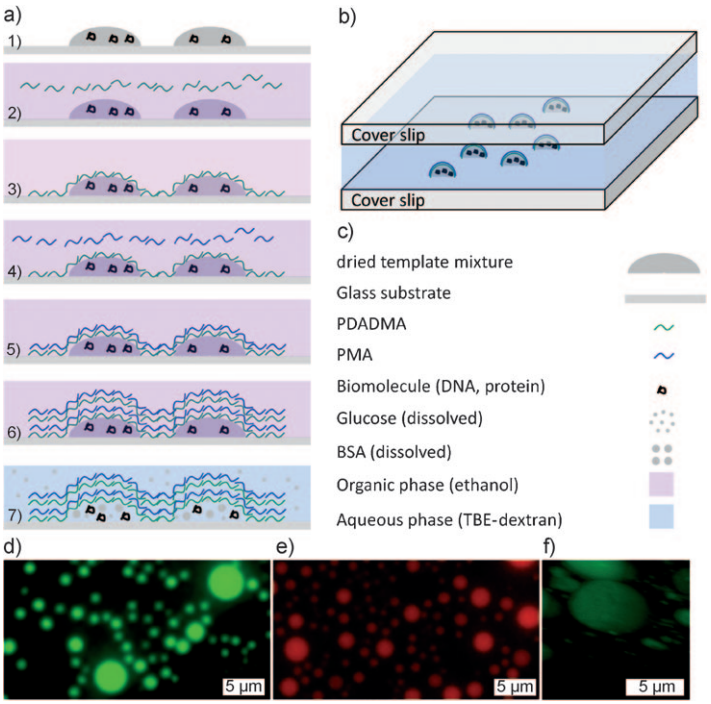

Figure 1. a) Fabrication of SBMEs on biological templates by using the RP-LbL method. 1) Dried template, which embeds biomolecules, is formed from a matrix (bovine serum albumin (BSA) and glucose); 2–6) stepwise assembly of polymer layers from organic phase. Washing steps between the successive additions of polyelectrolyte layers are not shown. 7) Exchange of the organic solvent with an aqueous buffer that dissolves biomolecules and glucose. Biomolecules and BSA remain encapsulated inside the SBMEs (but are largely mobile), glucose can diffuse out. b) Simple setup for buffer exchange used for experiments and microscopy. c) Legend (not to scale). d) SBMEs filled with Alexa Fluor 488 labeled DNA oligonucleotides. e) SBMEs filled with Texas Red labeled BSA. f) Confocal microscopy image of SBME, indicating a 3D dome shape.

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platform for the parallel observation of large numbers of biochemical and biophysical processes in microenclosures of various sizes.

SBMEs were used to entrap λ-DNA, fluorescently labeled BSA, NeutrAvidin, fluorescently labeled oligonucleotides, and DNase. As shown in Figure 2, Brownian motion of the

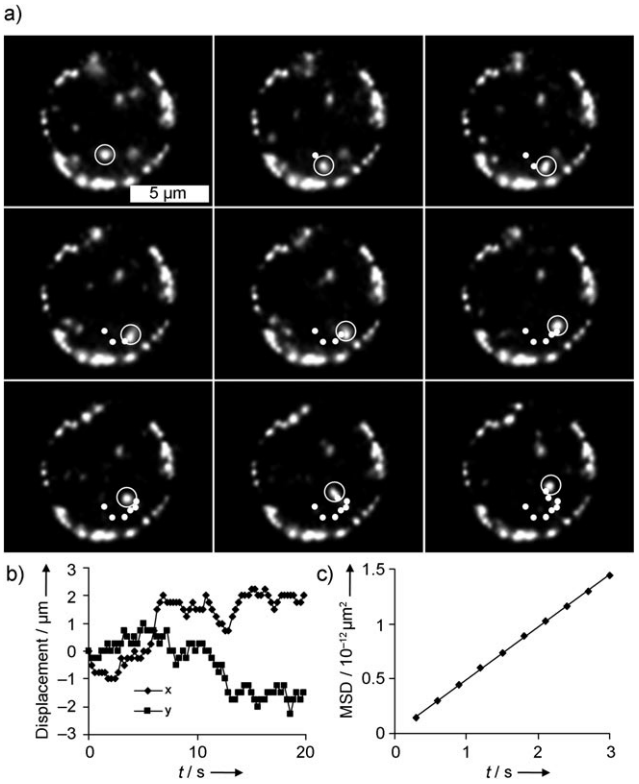

Figure 2. Brownian motion of λ-DNA inside SBMEs. a) Trace of encapsulated λ-DNA molecule over a time span of ca. 15 seconds. b) Spatial trace of λ-DNA molecule versus time. c) The linear fit of mean-square displacement (MSD) versus time indicates diffusive motion.

λ-DNA molecules encapsulated inside the SBMEs could be visualized by using fluorescence microscopy. The λ-DNA molecules were visible as bright fluorescent particles, which is consistent with their random coil conformation (for a video see the Supporting Information). The resulting SBMEs were stable, and, in most cases, encapsulation of the λ-DNA molecules was very efficient, as we could only rarely ($< 10\,\%$ of the SBMEs) observe that the molecules were able to escape; presumably the SBME was damaged in such cases. Particle tracking was used to estimate the diffusion coefficient D and viscosity η inside the SBMEs, assuming a molecular radius of 600 nm;[11] the analysis yields values of $D = (1.3 \pm 0.1) \times 10^{-13}\ m^2 s^{-1}$ and $\eta = (2.6 \pm 0.2)$ cP. This viscosity value lies between those of water and blood.

To further demonstrate the efficient trapping of the biomolecules inside the SBMEs, we subjected λ-DNA molecules and FITC-BSA to electrophoresis by applying an externally controlled electric field. Figure 3a shows a series of images that show the motion of encapsulated λ-DNA under

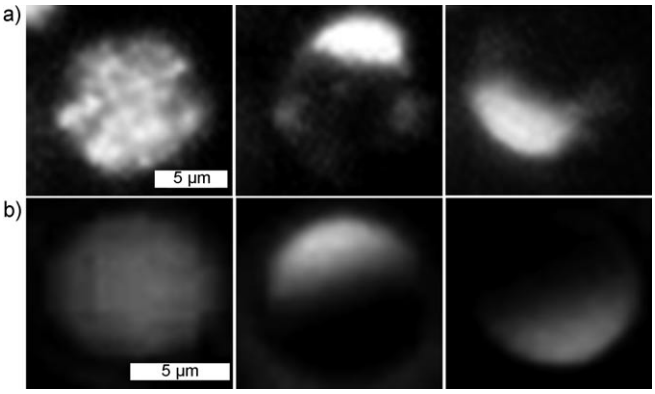

Figure 3. Fluorescence microscopy image sequence of λ-DNA and FITC-biotin inside SBMEs that are subjected to a slowly oscillating external electric field. a) λ-DNA SBME (left), electric field on (center), electric field reversed (right). b) FITC-BSA SBME (left), electric field on (middle), electric field reversed (right).

the influence of an externally applied low-frequency ac electric field (0.1 Hz, 15 $V\,cm^{-1}$, step function). Negatively charged λ-DNA molecules accumulate at either the upper or the lower side of the enclosure, depending on the field orientation (λ-DNA molecules always accumulated towards the positive electrode). Upon switching the field direction, the distribution of the λ-DNA molecules inside the SBMEs can be reversed. Figure 3b shows the electrophoretic movement and containment of FITC-BSA (pI at pH 4.8) in the SBMEs. In contrast to experiments with the large (ca. 32 MDa) and hence relatively slow-moving λ-DNA, the smaller (68 kDa) FITC-BSA recovers its random distribution much faster (less than 1 s for FITC-BSA compared with approximately 5 s for λ-DNA) after the field has been switched off (videos of these electrophoresis experiments can be found in the Supporting Information).

In the next step, unlabeled NeutrAvidin, a tetramer that is able to bind up to four biotin molecules, was encapsulated in the SBMEs together with Texas Red labeled BSA. As shown in Figure 4a, the SBMEs could be observed in the red channel, but not in the green channel of the fluorescence microscope. A small quantity of FITC-biotin (1:40 FITC-biotin/NeutrAvidin) was then added to the aqueous buffer solution. Because FITC-biotin has an approximate molecular weight of only 650 Da, it is able to diffuse through the membranic polymer layers of the SBMEs and bind to the encapsulated NeutrAvidin molecules, thereby making the SBMEs fluorescent (Figure 4a). The control experiment without NeutrAvidin (Figure 4b), shows no increase in the green fluorescence. Electrophoresis experiments led to accumulation of the enclosed NeutrAvidin (pI at pH 6.3) towards the anode, thus further confirming the specific binding of FITC-biotin to NeutrAvidin (see video in the Supporting Information).

The occurrence of a reaction in SBMEs was demonstrated by trapping an Alexa Fluor 488 labeled DNA oligonucleotide with a molecular weight of 10 kDa and the DNA-digesting enzyme DNase I. The digestion of encapsulated DNA by DNase can be achieved by addition of Mg^{2+} and Ca^{2+} ions,

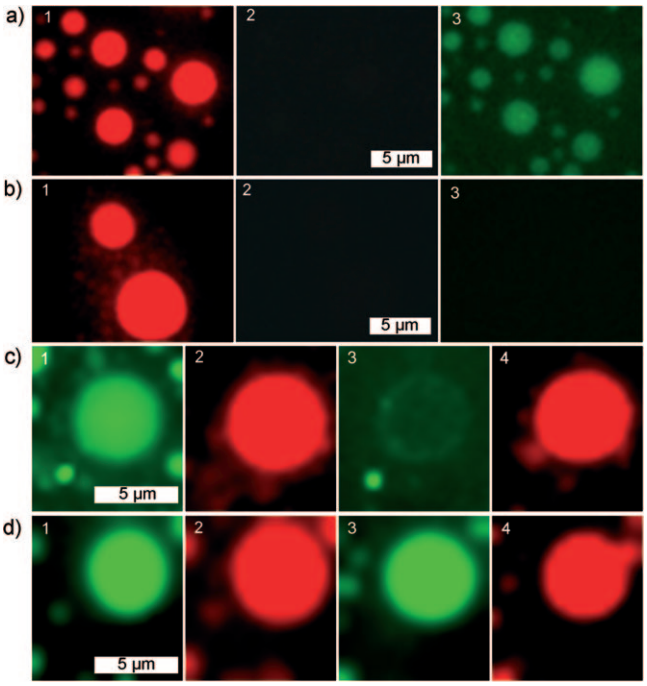

Figure 4. Applications of SBMEs observed with fluorescence microscopy. a) Labeling of enclosed NeutrAvidin molecules with FITC-biotin from outside the SBMEs. 1) SBMEs filled with Texas Red labeled BSA and NeutrAvidin (red channel); 2) Image of the same area before adding FITC-biotin (green channel); 3) same area 3 min after adding FITC-biotin (green channel). b) Control experiment without NeutrAvidin. Same sequence of events as in (a). c) Enzymatic DNA digestion. 1) SBME filled with Alexa Fluor 488 labeled oligonucleotide, Dnase I, and Texas Red/BSA (green channel); 2) same area as in (1) (red channel); 3) same area 10 min after adding buffer containing Mg^{2+} and Ca^{2+} ions to start the enzymatic reaction (green channel) 4) same area as in (3) (red channel). d) Control experiment of enzymatic DNA digestion with SBMEs containing no DNase. Same sequence as in (c).

which are required cofactors, to the external solution. Figure 4c shows the appearance of the SBMEs before and after addition of TBE buffer (TBE: tris(hydroxymethyl)aminomethane boric acid ethylenediaminetetraacetat) containing 5 mM $MgCl_2$ and 1 mM $CaCl_2$ and placing it on a temperature control stage set to 37 °C (gel electrophoresis confirmed DNA digestion under these conditions, data not shown). The green fluorescence from the SBMEs is much lower after addition of the metal ions as small digested fragments escape from the SBMEs. A control experiment, in which the buffer containing magnesium and calcium salts was added to oligonucleotide-filled SBMEs without the DNase, shows no reduction of green fluorescence (Figure 4d).

The SBMEs are much smaller than the initially deposited drops of template material; this observation may be explained by drying effects and by the partial solubility of glucose in ethanol. The concentration of biomolecules inside the SBMEs was found to be proportional to their concentration in the template solution. Also, by using an adjusted experimental protocol that employs emulsifiers, it is possible to exert more control over the size of the SBMEs. The controlled release of the contents of the SBMEs[6e,12] can also be achieved (see the Supporting Information for further details).

In summary, we have enclosed highly water soluble biomolecules in SBMEs with a size range of 1–20 μm by using a simple RP-LBL protocol. Low-molecular-weight species such as FITC-biotin can diffuse in and out through the membrane walls of the SBMEs. The biofunctionality of the encapsulated biomolecules in the SMBEs was demonstrated. Enzymatic reactions, such as DNA digestion by DNase mediated by Mg^{2+} and Ca^{2+} ions, can be controlled. Electrophoresis can be used to create sharp concentration gradients inside the SBMEs; the time required for recovery of the equilibrium distribution after switching off the field depends on the size of the encapsulated molecules. This method may hence be used to observe binding events inside the SBMEs, and could be the scope of future work. This cost-effective system may find applications in biochemistry and biophysics[13] as a platform to study and control biomolecules, and their reactions with each other or with other molecules, within enclosures of various sizes in parallel and in real time. This is important as most biological reactions occur in closed confinements.

Experimental Section

Glass treatment: Glass surfaces were cleaned in a solution of 30% NH_4OH, 30% H_2O_2 and deionized H_2O (1:1:5). Template solution: 60 μL of template solution contain: λ-DNA (NEB, MA, USA), labeled with YOYO-1 (Invitrogen, Singapore): 0.5 μg mL^{-1}, 1 μL; Alexa Fluor 488 labeled oligonucleotide (IDT, IA, USA): 1 mg mL^{-1}, 5 μL; FITC-BSA (Sigma, Singapore): 10 mg mL^{-1}, 10 μL; Texas Red labeled BSA (Invitrogen): 1 mg mL^{-1}, 8.5 μL; NeutrAvidin (Thermo Scientific, MA, USA): 1 mg mL^{-1}, 10 μL; DNase I (NEB): 2000 units mL^{-1}, 3.5 μL; BSA (Sigma): 10 mg mL^{-1}, 10 μL for protein experiments and 19 μL for λ-DNA experiments; Glucose: 200 mg mL^{-1}, 40 μL, dissolved in 1 × TBE. Preparation of poly-(diallyldimethyl ammonium chloride) (PDADMA): 20% PDADMA (Sigma) in H_2O was dried at 150 °C for 4 h followed by dissolution in hot ethanol. Fabrication of SBMEs: The glass surface and SBMEs were never allowed to dry during all experiments. Template solution was deposited in small volumes (ca. 1 μL) on the treated glass surfaces and dried in an oven at 45 °C for around 1 h (a vacuum oven at lower temperature can be used for very heat sensitive proteins). RP-LbL encapsulation was initiated by submerging the glass slip in an ethanolic solution of PDADMA (5 mg mL^{-1}) for 20 min followed by 5 successive submersions (5 s each, with gentle shaking) in pure ethanol in order to wash off unbound PDADMA. The procedure was repeated with a solution of poly(methylacrylic acid) (PMA, 5 mg mL−1; Polysciences, Germany) to deposit the second polymer layer. To deposit more polymer layers, the procedure can be repeated (typically, 6 layers were used for DNA digestion experiments, 2 layers for all other experiments). Buffer exchange: The glass slip with SBMEs was immediately covered with a small cover slip followed by displacement of ethanol by addition of aqueous buffer (1 × TBE with 150 mg mL^{-1} dextran). Imaging: The covered glass slip was placed on an inverted fluorescence microscope (Olympus, oil immersion, 100 × objective) or a confocal microscope (Olympus, water immersion, 60 × objective). Further experimental details can be found in the Supporting Information.

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