

A Microreactor with Thousands of Subcompartments: Enzyme-Loaded Liposomes within Polymer Capsules**

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Nanoengineered carriers that fulfill simple predefined cell activities have potential as a powerful therapeutic tool to replenish lost or missing cellular functions.^[1] The first key challenge in the creation of such systems lies in the design of a multifunctional carrier that accommodates controlled encapsulation and release of drugs and reagents ranging from small molecules to proteins and nucleic acids, and which simultaneously allows interaction with the surrounding environment. Two notable platforms that are currently considered as potential synthetic vessels, namely liposomes^[2,3] and multilayered polymer capsules,^[4,5] fulfill in part some of these requirements, however, both carrier systems have some inherent limitations. Polymer capsules possess the desired structural integrity and are well-suited for the encapsulation of macromolecular cargo. Furthermore, their semipermeable nature is an important feature that allows them to communicate with the external milieu. However, in their native form, their permeability makes them unsuitable in providing a protective barrier for small drugs and reagents, as they can freely diffuse across the capsule walls.^[4] On the other hand, small unilamellar liposomes provide effective encapsulation for small and medium-sized cargo, but can be susceptible to structural instability and are largely impermeable to their surroundings.^[3,6]

We report herein a method to create capsosomes, which are polymer capsules that contain liposomal subcompartments, to maximize the benefits offered by both polymer multilayer capsules and liposomes, and to deliver a new carrier platform (Figure 1). We show that the capsosomes inherit the structural stability of the polymer capsules, and have a semipermeable nature; the liposomes are capable of restricting the access of solutes to an encapsulated model enzyme, β -lactamase. To achieve these properties, we have

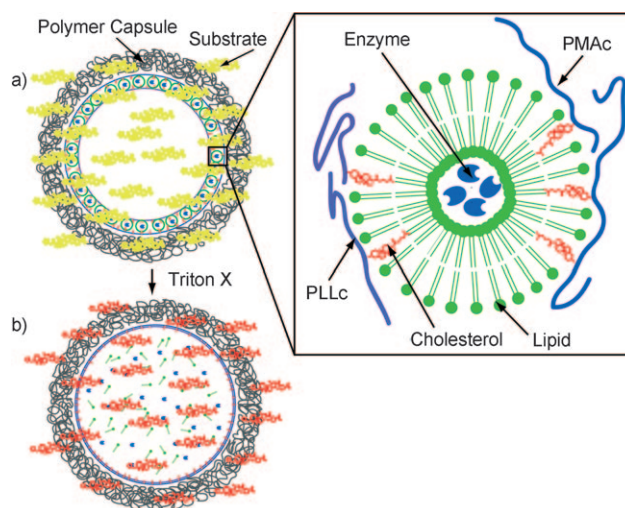


Figure 1. Representation of a capsosome: a) The enzyme β -lactamase is preloaded into liposomes and is sandwiched between two cholesterol-modified polymers, which are then embedded inside a polymer capsule. b) Upon addition of Triton X, the liposomes are destroyed and the enzyme is released, thus causing the hydrolysis of nitrocefin.

pioneered the use of cholesterol-modified polymers to maximize and control the loading of liposomes into polymer multilayer films, and have developed a benign technique to obtain disulfide-stabilized polymer capsules without the use of detrimental oxidizing reagents. We have independently verified the loading of the enzyme into the liposomes as well as the liposome loading in the polymer capsules. We have also performed a quantitative enzymatic assay to substantiate the encapsulation and functionality of the enzyme within the liposomal subcompartments and to estimate the number of subcompartments within the capsosomes. Each of these steps has fundamental merits, and, when taken together, this work represents significant progress toward the assembly of functional vehicles that can serve as artificial organelles.

The assembly of polymer capsules by the sequential deposition of interacting macromolecules onto sacrificial template particles is a versatile technique that provides the means to incorporate other nanosized materials as integral components of the film.^[7–9] However, electrostatic interaction alone has been shown to provide insufficient affinity between liposomes and the underlying polymer surface on both planar^[9] and colloidal^[7] substrates, and the subsequently introduced polymer displaces the liposomes from the surface. To facilitate liposome incorporation into polymer multilayers, we have introduced a novel concept based on cholesterol-modified polymers as noncovalent anchors for the attachment

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of liposomes to the underlying surfaces. The incorporation of cholesterol, a natural lipid membrane constituent, into the lipid bilayers leads to two distinguishing features in this strategy: 1) it is a fast and spontaneous process,^[10] regardless of the lipid composition, thus enabling the incorporation of synthetic and native (cell-derived) liposomes;^[11] and 2) it is insensitive to the solution properties (pH, presence of buffer salts and reactive groups), thus making it a facile alternative to covalent attachment strategies.

The incorporation of saturated phospholipid liposomes (DMPC/DMPC 80:20 wt %) into a polymer film was accomplished by sandwiching the liposomes between a cholesterol-modified poly(L-lysine) (PLL_c) precursor layer and a poly(methacrylic acid)-*co*-(cholesteryl methacrylate) (PMA_c) capping layer. The use of PLL_c ensured stable and reproducible adsorption of the liposomes onto the underlying surfaces. Quartz crystal microbalance with dissipation monitoring (QCM-D) experiments on the film buildup on planar silica substrates revealed a 30 % increase in the amount of adsorbed liposomes for a PLL_c layer compared to unmodified PLL layer (data not shown). In turn, PMA_c, unlike unmodified PMA, was found to be a suitable capping layer, which caused negligible liposome desorption while priming the assembly for the subsequent deposition of poly(*N*-vinyl pyrrolidone) (PVP) and thiol-modified poly(methacrylic acid) (PMA_{SH}) by hydrogen bonding (see Figure S1 in the Supporting Information). The choice of this polymer pair for the creation of capsosomes stems from their ability to create disulfide-stabilized single-component PMA capsules as carriers for gene^[12] and peptide^[13] vaccines, as well as drug-loaded oil droplets.^[14] We have shown that these capsules are (bio)degradable^[15] and nontoxic,^[13] therefore they are promising for the creation of resorbable synthetic organelles. Having determined that the film buildup proceeds on planar substrates, the multilayer assembly buildup on colloidal particles was followed by using flow cytometry to monitor both the fluorescently labeled liposomes and their enzyme cargo. This technique allows measurement of the fluorescence of each individual particle (Figure 2a). The fluorescence intensity of the particles associated with the lipids was constant (Figure 2a, ■), thus substantiating their stable incorporation into the multilayered thin film on the particles. On the other hand, the constant level of the fluorescence intensity of the enzymes (Figure 2a, □) suggests that the liposomes did not rupture or release their cargo.

Controlled conversion of the PMA_{SH} thiol groups into bridging disulfide linkages is an essential step in creating capsosomes that are stable under physiological conditions. Upon increasing the pH value from 4 (multilayer assembly conditions) to 7.4, the hydrogen bonding between PVP and PMA_{SH} becomes ineffective, which results in the release of PVP and thus creates single-component PMA capsules held together by disulfide linkages.^[15] The current cross-linking strategy relies on the use of an oxidizing agent, chloramine T.^[15] Although effective, this method is potentially harmful to therapeutics and biomolecules that are susceptible to oxidation, and, in the particular case of β -lactamase, the use of this reagent led to a loss of function of the enzyme (data not shown). We therefore sought an alternative method to

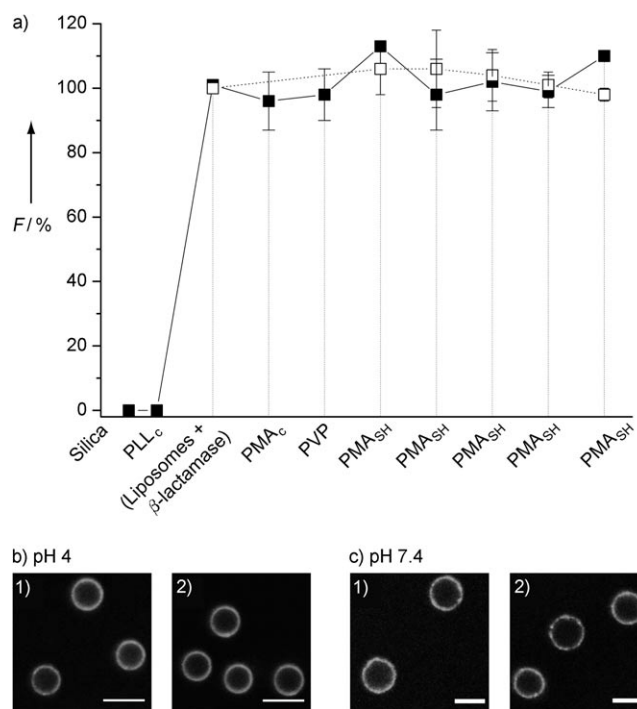


Figure 2. a) Development of the normalized fluorescence intensity (F) of NBD-labeled liposomes (■) and AF-488 β -lactamase-loaded liposomes (□) during the film assembly, as measured by flow cytometry. After deposition of the first PVP layer, the intensity was measured only after deposition of the PMA_{SH} layers. CLSM images of 1) AF-488 β -lactamase-loaded liposomes and 2) NBD-labeled liposomes in b) NaOAc buffer (pH 4) or c) HEPES buffer (pH 7.4). Scale bars are 5 μ m.

crosslink the PMA_{SH} chains within the polymer film, and established a novel technique to obtain disulfide-stabilized hydrogels based on a thiol–disulfide exchange reaction, which is benign, specific to thiols, and does not affect the fragile cargo. We used a polymeric crosslinker PMA_{SH} with pyridyl disulfide activated thiol groups, which was infiltrated into the polymer thin films of PMA_{SH}/PVP assembled on particles to obtain stable capsosomes upon template core removal. Their structural characteristics (e.g., swelling) and integrity was found to be similar to their oxidized counterparts, and the functionality of the encapsulated cargo, β -lactamase, was not affected. Fluorescent labeling of the liposomes and encapsulated cargo allowed visualization of the capsosomes at both pH 4 (Figure 2b) and pH 7.4 (Figure 2c). The images reveal stable, intact capsosomes with homogeneously distributed liposomes (Figure 2b2,c2) and their β -lactamase cargo (Figure 2b1,c1).

The choice of β -lactamase as the model enzyme made it possible to visualize and quantify its activity by monitoring the conversion of nitrocefin (yellow), the enzymatic substrate, into its hydrolyzed product (red) in a colorimetric assay (Figure 3). To confirm the presence and activity of the β -lactamase confined within the liposomal compartments of the capsosomes, Triton X (TX), a surfactant, was used to release the enzyme molecules, which made them accessible to their nitrocefin substrate (Figure 1b). Equal numbers of core-shell particles (Figure 3, ●) and capsosomes (Figure 3, ■) exposed

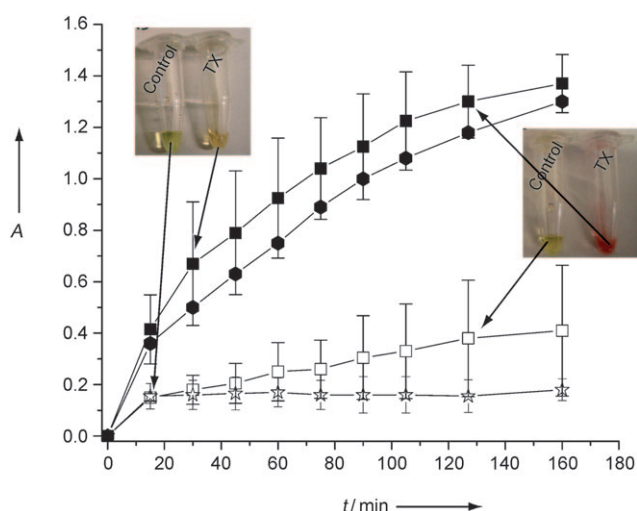


Figure 3. Absorbance measurements of the quantitative β -lactamase assay using core-shell particles (●) and capsosomes (■ in the presence TX, □ in the absence of TX). A negative control (substrate only) is also shown (☆).

to TX exhibited similar activities. This result indicated that neither the amount of the β -lactamase within the capsosomes nor its activity were affected by removal of the silica core using buffered hydrofluoric acid. In the absence of TX, intact liposomes provided an effective barrier for the diffusion of nitrocefin, and the enzymatic activity was measured at a low level. Furthermore, this quantitative assay allowed the estimation of the number of incorporated liposomes, that is, the number of subcompartments within the capsosomes. From the experimental value of the enzymatic activity and taking into account the content of enzyme per liposome defined in the synthesis, we found that each of the capsosomes contains approximately 8×10^3 subcompartments. This value comprises approximately 70% of the theoretical maximum (i.e., the number of 50 nm spheres packed in a square lattice on the surface of a 3 μ m colloidal particle), which confirms the success of the developed strategy to load the liposomes into polymer capsules by anchoring them to cholesterol-modified polymers.

In summary, we have developed a multifunctional carrier that successfully encapsulates biological components within the liposome subcompartments of a polymer carrier capsule. A quantitative enzymatic reaction confirmed the presence of active enzyme within the capsosomes and the number of liposomal subcompartments within the capsosomes was determined to be approximately 8×10^3 . This novel and versatile platform (capsosomes) can be optimized to accommodate diverse biomedical applications. We are currently extending this system to coencapsulate different enzymes, while equipping the liposomes with specific triggers toward encapsulated enzymatic cascade reactions without destruction of the subcompartments. Furthermore, our preliminary data show that capsosomes have no adverse effect on the viability of model cell lines, which make them an attractive platform not only for microencapsulated catalysis but also for drug delivery. We are currently investigating the potential of

capsosomes for coencapsulation and the combined delivery of hydrophobic and/or hydrophilic drugs and nanoparticles. In addition, the liposomal subcompartments of the capsosomes can be prepared from synthetic liposomes, which are well-suited to carry both hydrophobic and hydrophilic cargo, or can also be made from native liposomes that are equipped with intact membrane proteins. As the number of subcompartments and the active cargo (drugs and/or reagents) within each liposome are effectively controlled by the assembly protocol, capsosomes are particularly attractive as novel systems for a range of biomedical applications, including drug and gene delivery, and as microreactors.

Experimental Section

Polymer capsules with embedded liposomes were assembled using the layer-by-layer (LbL) technique. Briefly, silica particles (5 wt %) were washed (1060 g for 30 s) three times in *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (10 mM HEPES, 150 mM NaCl, pH 7.4). The particles were suspended in a solution of PLL_c (1 mg mL⁻¹, 15 min), washed three times, suspended in the liposome solution (0.8 mg mL⁻¹, 60 min), washed three times, and a capping layer of PMA_c (1 mg mL⁻¹, 15 min) was adsorbed. The buffer was changed to NaOAc (20 mM, pH 4) and five bilayers of PVP (1 mg mL⁻¹, 10 min) and PMA_{SH} (12–14 mol % thiolation, 1 mg mL⁻¹, 10 min) were sequentially adsorbed. The core-shell particles were then cross-linked using PMA_{SH} with pyridyl disulfide activated thiol groups (6 mol % thiolation, 1 mg mL⁻¹, 15 h) and the template core was removed using a solution HF/NH₄F (2 M:8 M) for 2 min, followed by multiple centrifugation (4500 g for 3 min)/NaOAc buffer washing cycles. (Caution! hydrofluoric acid is highly toxic and great care must be taken when handling it.) Flow cytometry (Becton Dickinson FACS Calibur flow) was performed using an excitation wavelength of 488 nm for all experiments. At least 20 000 particles were analyzed for each fluorescence intensity measurement. The capsosomes loaded with NBD-labeled liposomes or loaded with unlabeled liposomes but with Alexa fluor 488 (AF-488) labeled β -lactamase were imaged using a Leica TCS SP2 AOBs confocal microscope. The progress of the nitrocefin hydrolysis was measured by taking absorbance readings at 492 nm using a NanoDrop 1000. Prior to the measurements, the core-shell particles and the capsosomes were counted and a suspension of 3×10^7 particles or capsosomes mL⁻¹ in 50 μ g mL⁻¹ nitrocefin was used.

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