Enzyme Reactions in Microparticles

DOI: 10.1002/anie.201003244

Enzyme Reaction in the Pores of $CaCO_3$ Particles upon Ultrasound Disruption of Attached Substrate-Filled Liposomes**

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The intracellular delivery and controlled release of biomolecules[1] is significantly influencing the field of single-cell analysis. It is envisioned that this development will lead to a deeper understanding of fundamental biological processes such as cellular uptake and transport of biomolecules, and ultimately, to novel approaches for cancer therapy. The approach is also relevant for enzyme-catalyzed reactions and the production of microcapsules[2] through layer-by-layer (LbL)[3] assembly. Immune responses in vivo and in vitro have been investigated using microcapsules.[4–6] One example is the intracellular release[6] of small peptides forming complexes with MHC Class I molecules inside living cells. The surface presentation of peptides—an important process in immunology—was observed in real time.[6]

As a prelude to cellular applications, it is desirable to investigate biological reactions in model systems. In this connection, conducting biochemical reactions in protected compartments[7] provides the means for carrying out enzymatic reactions, thus mimicking activities in living cells. Multicompartmental concentric microcapsules[8] or capsule-based capsosomes[9] can be used for monitoring reactions under a microscope. However, microcapsules[10a] constituted of both non-biodegradable[10b] and biodegradable[10c] polymers are deformed upon phagocytic uptake by cells.[10a] For incorporation of microcapsules inside nonphagocytic living cells,[6] the requirements for mechanical stability are even more stringent. In this case the pressure is higher than that experienced after phagocytosis, such that conventional capsules rupture and release their contents under the mechanical stress[11a] exerted by the cells.[6] Owing to their enhanced mechanical properties,[11b] microcapsules that have been thermally shrunk to a small size have been shown to withstand the pressure experienced upon intracellular incorporation.[6] However, a general obstacle to the use of thermal shrinking is the degradation of most proteins at elevated temperatures. Moreover, simultaneous encapsulation and delivery of several molecules, for example differing in size, is hampered by the significantly different requirements for their encapsulation. Nonetheless, there remains great interest in mechanically robust systems capable of simultaneous delivery and controlled release of dissimilar types of molecules. The challenges in designing such carriers include the different requirements for the walls, and the need for selective permeability control for one type of molecule (generally the smaller), while retaining another type of molecule (generally the larger).

We introduce here an approach based on tailored multicompartmental, porous $CaCO_3$[12] particles as an alternative to microcapsules. The pores of the $CaCO_3$ microparticles can be filled with materials of choice, such as proteins. At the same time, the $CaCO_3$ inner core provides mechanical properties superior to those of microcapsules, the condition that must be met for successful intracellular incorporation including transfer into non-phagocytic cells.[10,11] An implementation of the concept of multicompartmentalization was tested by attaching smaller nanocontainers (NCs) to larger, micrometer-sized, microparticles. A number of different NCs at various concentrations were used for the construction of multicompartment delivery vehicles around porous $CaCO_3$ particles constituting the inner core (see the Supporting Information, Section 1). These studies were conducted to investigate the density of the coverage and the tendency to aggregate, issues of particular relevance for in vivo applications. The LbL self-assembly technique was applied for the surface functionalization of the $CaCO_3$ microparticles and NCs.

Figure 1a presents the general approach for constructing multicompartment microparticles. It shows the adsorption of a NC onto an inner secondary core or container. Confocal micrographs of an experimental system composed of a micrometer-sized $CaCO_3$ core surrounded by nanometer- or submicrometer-sized NCs are depicted in Figure 1b and c. The multicompartments were labeled with TRITC (tetramethylrhodamine isothiocyanate)-dextran (the inner core) and FITC (fluorescein isothiocyanate)-labeled PAH (polyallylamine hydrochloride) (outer NCs). In the case of structures

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[**] We thank A. Praast, H. Zastrow, and R. Pitschke for technical assistance. Support from the PICT-2006-01365 (MPI-SeCyt Argentina) is kindly acknowledged. A.M.Y. thanks the DAAD program (no. 325, A/09/03626) for support.

Supporting information for this article (Part I: complete experimental details, Part II: other information) is available on the WWW under http://dx.doi.org/10.1002/anie.201003244.

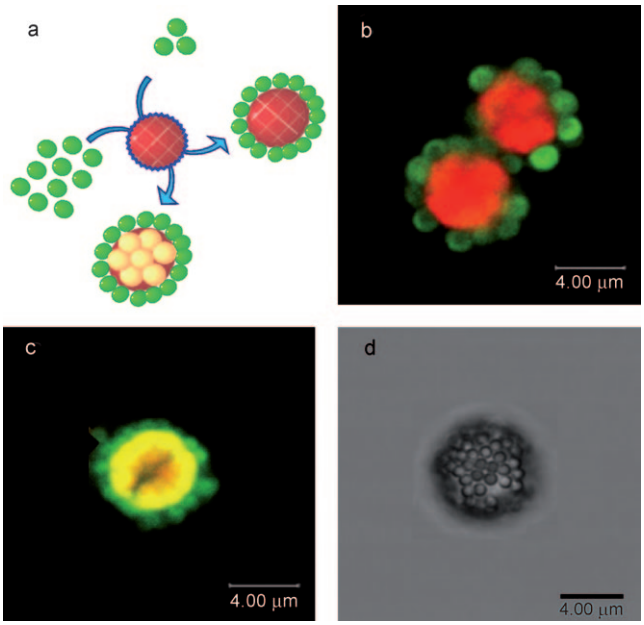

Figure 1. a) Schematic for the fabrication of multicompartment delivery vehicles. b,c) Fluorescent confocal images of multicompartment containers obtained at low concentration of polystyrene (8:1; b) and high concentration of silica NCs (1:58; c) (in both cases ratio refers to the weight of inner relative to that of the outer subcompartment particles; see the Supporting Information, Section 1). d) Transmission confocal microscope image of a multicompartment similar to that shown in (c). Colors are due to TRITC (red), FITC (green), and a combination of TRITC and FITC (yellow).

formed at low concentrations of NCs (Figure 1b), the latter (in green) were attached only at the periphery of the inner compartment, which thereby maintained their red color with a ball-bearing appearance. At higher concentrations (Figure 1c) the NCs integrated around the inner core, leading to a merged (yellow) color. The TEM image analysis confirmed the attachment of silica NCs to the calcium carbonate particle (see the Supporting Information). The overall size of the multicontainers corresponded to the initial diameter of the inner subcompartment with only a minimal influence of the NCs attached to its surface.

We note that the attachment of small colloids to the surface of large particles can be controlled by their initial concentration and also by the salt concentration and composition of the supporting polyelectrolyte. It was found that the number of NCs attached to the inner subcompartment assembled with polyelectrolyte polymers in 0.5 M NaCl was 30% higher than in 0.15 M NaCl (see the Supporting Information, Section 2). We attribute this difference to the formation of a thicker layer of polymers on the colloidal particles; that is, the polymers changed from an extended conformation at low ionic strength medium to a dense, collapsed conformation at high ionic strength.[13] Adsorption of NCs can be also performed using methods similar to those developed for nanoparticles.[14] In our studies we found that 1) decreasing the concentrations of inner and outer subcompartments reduces the aggregation of microparticles; 2) adjusting the relative concentrations of the outer and inner subcompartment can be used to control the density of coverage of the inner subcompartment; and 3) the porosity of microparticles is advantageous for the adsorption of materials, but it leads to greater aggregation of the microparticles (see the Supporting Information, Sections 1 and 2).

Multicompartmentalization demonstrated with an NC-based microparticulate system is of particular interest for enzymes and enzyme-catalyzed reactions.[15] Here, we encapsulated the substrate inside liposomes, which were subsequently attached as outer compartments to a $CaCO_3$ particle (the inner subcompartment) containing the enzyme peroxidase. It was possible to trigger the release of a small substrate molecule, while maintaining a larger molecule, the enzyme, within the confined and protected inner volume of the microparticles. The enzyme's catalytic activity, an important criterion of functionality, [16] was verified. The kinetics of the enzyme-catalyzed reaction inside the porous microparticles was compared with that carried out in polymeric microcapsules filled with enzymes.

To link studies on NCs-based multicompartment carriers with those containing liposomes, we adsorbed silica particles covered with lipids onto polyelectrolyte-covered $CaCO_3$ cores. The adsorption of palmitoyl oleoyl phosphatidyl choline (POPC) and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) lipids onto silica particles was previously analyzed using NMR spectroscopy.[17a] Our study revealed a decrease in the Z-potential of the silica particles from −50 mV to −8 mV. Adsorption of liposomes onto polyelectrolyte-coated particles is consistent with results reported by other groups.[9,17b] Although a mixture of acidic and zwitterionic lipids resulted in the formation of a better bilayer than that formed from individual lipids, a weak interaction between liposomes comprising zwitterionic lipids has been observed.[17c,d] The presence of charged lipid groups leads to stronger interaction with charged surfaces.[17d–f] By adjusting the charge and composition of liposomes it is possible to control the adsorption of the liposomes as well as the wrapping of polyelectrolyte multilayers by lipids.[17d]

Liposomes are attractive carriers because of their small size and capability to enclose small molecules.[18] The adsorption of liposomes onto $CaCO_3$ particles was monitored by fluorescence confocal microscopy (see the Supporting Information). In the case of polyelectrolyte-coated particles, the results were consistent with those obtained using capsules.[9] We selected ultrasound[19–21] from a number of stimuli[22] that can be used to trigger release and thus initiate the enzymatic reaction. Upon disruption of liposomes, the substrate was released and diffused into the inner subcompartment containing the enzyme. The course of the reaction was monitored with a microscope (Figure 2). The substrate, Amplex Red, in the presence of peroxidase, reacts with H_2O_2 to produce the red-fluorescent product, resorufin [Eq. (1)]. The insets to Figure 2b–d show that the fluorescence intensities of the capsules (the red channel) increased with time. At 15 minutes (white trace in Figure 2d) the signal reached its highest value.

$$H_2O_2 + \text{Amplex Red} + H^+ \rightarrow H_2O + \text{resorufin} + CH_3COOH \quad (1)$$

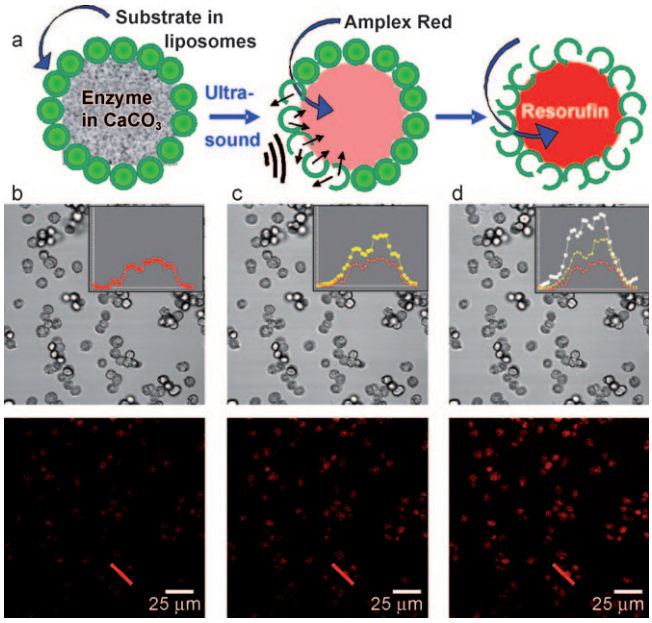

Figure 2. a) Evolution of the enzymatic reaction inside porous microparticles. b–d) Confocal transmission (middle row) and fluorescence (bottom row) images of microparticles after 1 min (b), 10 min (c), and 15 min (d) of ultrasonication and addition of H_2O_2. The insets show fluorescence profiles drawn across the same microcontainer (the red lines in the bottom row). The red profiles in the insets corresponds to emission 1 min after sonication, the yellow and white profiles to emission 10 and 15 min after sonication, respectively (see the Supporting Information, Section 3).

An important aspect of porous microparticles is that the time course of the reaction can be controlled by the composition of the inner core and the concentration of electron donors. In contrast to multicompartment microcapsules,[8,9] the particle-like inner core of such delivery vehicles enhances the mechanical stability of the containers. We note that the enzyme-catalyzed reaction in particles is significantly slower than that in microcapsules of polyelectrolytes[8] (Figure 3). This difference can be rationalized by assuming that in $CaCO_3$ particles only the fraction of surface-exposed enzyme molecules is active.

In order to provide further insight into the mechanism of liposome disruption we performed the following experiment. Liposomes containing the substrate not associated with

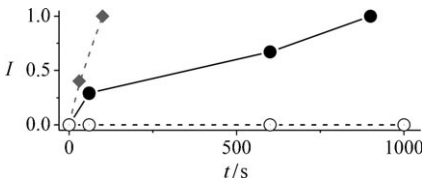

Figure 3. Kinetics of the enzyme-catalyzed reaction inside the microcapsules (gray squares) and porous microparticles (black circles) following ultrasound-triggered disruption of the liposomes attached to the polyelectrolyte shell. The kinetics of the reaction without ultrasound (empty circles) is also shown. The enzyme content in particles and capsules was assumed to be similar since protein loss could not be detected upon microcapsule formation.

microparticles were subjected to ultrasound under conditions identical to those employed for the observed enzyme-catalyzed reaction in individual microparticles. Subsequently, the solution was mixed with microparticles containing the enzyme. Under these conditions no reaction was observed, revealing that the ultrasound intensity used for disruption of liposomes not connected to particles was below the threshold of release (roughly 2 $W cm^{-2}$).[20a] Yet, when microparticles with enzyme in their pores and substrate-carrying liposomes attached to their surface were subjected to identical ultrasound conditions, the enzyme-catalyzed reaction again took place. This experiment confirmed that disruption of the liposomes occurred at the sites of attachment to the microparticles, a result that was expected since ultrasound acts on the density gradient at such junction points of the liposomes with the polymeric shells of the microparticles. In contrast, the lipid shells of liposomes freely suspended in solution do not provide a pronounced density gradient and liposomes are not disrupted. Absorption of ultrasound by particles is minimal (for silica particles, < 0.1 $dB cm^{-1} MHz^{-1}$)[20b] at the operating low-frequency range, but the higher particle density in the vicinity of the liposomes suffices for disrupting the lipid membrane. The mechanism of transport through the lipid membrane has been proposed to involve the formation of transient hydrophilic or hydrophobic pores.[20c] Further improvement should be possible by varying the type of lipids, for example, those utilized close to their phase transitions.

A distinctive and unique feature of our approach is the delivery of enzymes and substrates simultaneously in one and the same container with enhanced mechanical stability. In previously reported methods, primarily developed for studying reactions in situ, the delivery vehicles only carried proteins. For example, in one approach, the substrate was added to a solution containing microcapsules,[8,9] while in a second method the enzyme was attached to the surface of microcapsules[23a] or core–shell nanoparticles[23b] or used to monitor coupled enzyme reactions.[23c] Another interesting attribute of the system described in this work is the ability to trigger enzyme-catalyzed reactions by ultrasound; the power at which the liposomes were disrupted (50 $mW cm^{-2}$) was significantly lower than that previously reported for disruption of the liposomes.[20a] In fact, the intensity of ultrasound that we applied was in the range of that used in vivo in medical ultrasound conditions (0.08 to 8 $W cm^{-2}$).[20d] Furthermore, the methods described in this work are of significant importance for the simultaneous delivery of several molecules inside cells and may potentially be used in vivo as well.

In conclusion, we have demonstrated a controllable enzyme-catalyzed reaction triggered inside a multicompartmentalized, porous $CaCO_3$ particle, and have compared the dynamics of the reaction with that carried out in polyelectrolyte multilayer microcapsules. Subcompartmentalization was achieved by decorating a larger subcontainer with smaller subcompartments (liposomes). It was found that porosity, ionic strength, and particle concentration are critical factors controlling the adsorption of NCs and liposomes onto the larger inner core of containers. The strategy proposed here

allows the simultaneous incorporation of small and large molecules, for example, an enzyme and its substrate, in the same particle or capsule so as to induce a specific biochemical reaction in a well-defined three-dimensional architecture. Enhanced mechanical stability of the particles is a characteristic feature of this system. Furthermore, it was shown that an enzyme–substrate reaction can occur in the same porous $CaCO_3$ particle upon disruption of the outer subcompartments; thus the substrate is released while the enzyme is maintained in the confined and protected volumes of the microparticles or microcapsules. The disruption of liposomes attached to microparticles was achieved by ultrasound under conditions similar to those used in medical ultrasound treatment; the key parameter was the density gradient around the lipid membrane. The time course of the reaction in a microparticle is more than an order of magnitude slower than that in a hollow polymeric microcapsule filled with the enzyme. We anticipate that such entities used together with various types[24] of capsules will serve as very useful tools for the simultaneous delivery and activation of molecules of various sizes in such vastly different disciplines as biomedicine[25a] sensors,[25b] and corrosion protection.[25c]

Received: May 28, 2010
Published online: September 21, 2010

Keywords: drug delivery · enzyme reactions · microparticles · nanocontainers · ultrasound

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