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Hybrids of Polymer Multilayers, Lipids, and Nanoparticles: Mimicking the Cellular Microenvironment

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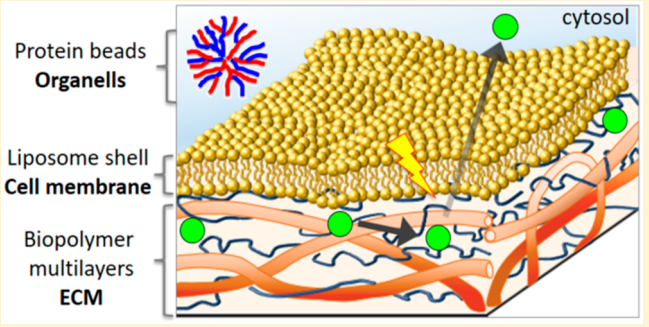
ABSTRACT: Here we address research directions and trends developed following novel concepts in 2D/3D self-assembled polymer structures established in the department led by Helmuth Möhwald. These functional structures made of hybrids of polymer multilayers, lipids, and nanoparticles stimulated research in the design of the cellular microenvironment. The composition of the extracellular matrix (ECM) and dynamics of biofactor presentation in the ECM can be recapitulated by the hybrids. Proteins serve as models for protein-based biofactors such as growth factors, cytokines, hormones, and so forth. A fundamental understanding of complex intermolecular interactions and approaches developed for the externally IR-light-triggered release offers a powerful tool for controlling the biofactor presentation. Pure protein beads made via a mild templating on vaterite $CaCO_3$ crystals can mimic cellular organelles in terms of the compartmentalization of active proteins. We believe that an integration of the approaches developed and described here offers a strong tool for engineering and mimicking both extra- and intracellular microenvironments.



Nowadays, self-assembled polymer-based structures represent a powerful and versatile tool that has attracted considerable attention from modern scientists and is widely employed for various biological and medical applications. Polymeric and hybrid self-assemblies can be composed from natural and synthetic biomolecules and recapitulate essential features of biological cells and/or the extracellular matrix (ECM). The concept of artificial cells and cellular microenvironments is not well established yet and is continuously developing. Although it is hard to predict further trends in the development of the "artificial cell" concept, one can already see the significant influence of original research developed in Helmuth Möhwald's group that to varying degrees brought the researchers closer to the recapitulation of major principle units of living cells and tissues.

Scheme 1 shows schematics highlighting these biological structures (ECM, the interface between the cell membrane and the ECM, and the cellular organelles) and their artificial analogues developed in Moehwald's group (polyelectrolyte multilayers, liposome−multilayer hybrids, and protein beads, respectively). The green spheres migrating within the multilayers represent soluble ECM biofactors (e.g., growth factors, cytokines, and hormones). Special attention has been paid to the external control over the presentation of biofactors to cell receptors and the biofactor diffusion through the cell membrane; both can remotely be achieved noninvasively via infrared (IR) light. This provides a unique opportunity to control the availability of biofactors for cell receptors up to the level of a single cell. This is a key to tackling fundamental questions in cell biology related to signal dynamics in the ECM and intracellular communication.

Below, the development of the individual directions in building the concept of an artificial cellular microenvironment are described together with expected future perspectives.

MULTILAYERS POSTLOADED WITH BIOFACTORS (ECM MIMICS)

Nowadays, the layer-by-layer (LbL) assembly of natural ECM components is well known and perhaps represents the most promising approach to mimicking the ECM to high accuracy and on the nanometer level.[1] Seeing the potential of the LbL technique, Helmuth Möhwald was one of the first scientists who took an interest in investigating the molecular dynamics of bioactive molecules (biofactors) in the LbL-assembled

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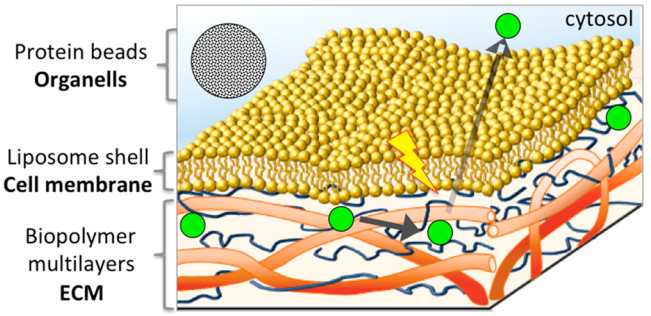
Scheme 1. Schematics Showing Polymer-Based Structures Used for Mimicking Components of the Intra- and Extracellular Microenvironment[a]



[a]Biopolymer multilayers mimic the ECM, the liposome shell (lipid bilayer) mimics the cell membrane, and protein beads assembled on vaterite $CaCO_3$ crystals mimic cellular organelles. The liposome–multilayer hybrids described below mimic the interface between the cell membrane and the ECM. Biofactors (in green) diffuse into multilayers and through the liposome shell, also under control of externally applied IR-light stimulation (yellow lighting).

structures. These studies are tightly associated with the approach of the postloading of biomacromolecules into the preformed multilayers. In this case, the multilayers serve as an insoluble matrix that can host soluble biomacromolecules that migrate between binding cites of the matrix. This recapitulates very well the real ECM where an insoluble scaffold of glucosaminoglucans and other still polymers serves as a matrix for the diffusion of soluble signals/biofactors such as growth factors, cytokines, hormones, and so forth.

The assembly of matrix-type capsules, as 3D analogues of the planar multilayers, was first shown in 2004.[2] It was demonstrated that the LbL polymer coating of porous vaterite $CaCO_3$ crystals results in filling the pores with multilayers, and after core elimination, it yields the nonhollow structures made of polymer network–matrix-type capsules (Figure 1A).[2] These capsules have been employed to host such biomacromolecules as various proteins and dextranes.[3] The loading mechanism is based on complex electrostatic and nonelectrostatic interactions of the loaded molecules with the polymer network possessing free charges due to the extrinsic (driven mostly by counterions but not permanent polymer charges) charge compensation into multilayers. This compensation results in a number of defects in the multilayers or simply pores for the loading of externally added molecules. Such a simple concept of postloading proteins has further been translated from 3D capsules to planar multilayers, with an increasing number of publications showing loading of growth factors into the multilayers and a biofunction of the multilayers (cellular response in cellular differentiation, proliferation, adhesion, so forth.) i. For an overview, see ref 4.

Furthermore, the multilayers have been successfully utilized as reservoirs for not only biomacromolecules but also rather small bioactives such as drugs. The first demonstration of the loading and release of the indicator dye methylene blue was shown in 2002,[5] but extensive use of the multilayers as reservoirs for small drugs has attracted attention together with the employment of the multilayers for the loading of bioactive macromolecules such as proteins. Later on, a number of bioactives and model dyes were postloaded into the multilayers, including antibiotics and hydrophobic compounds.[6−11] This makes the postloading principle extremely powerful for the assembly of the artificial ECM with biomolecules of various natures, thus bringing about a high level of complexity.

It is of note that the soft and highly hydrated hyaluronic acid/polylysine or HA/PLL multilayers are probably the most studied artificial ECM system with regard to the fundamental understanding of the multilayer structure, internal dynamics of the film components including postloaded ones, and cellular adhesion to this soft multilayer film.[12−17] HA is a real and very important ECM component, and the HA/PLL multilayers are one of first studied multilayers made of biopolymers.[13] PLL is a mobile polymer, and HA is immobile, which makes this system similar to ECM where a network of immobile polymers serves as a scaffold for soluble biofactors.

One has to provide additional support for the use of postloading to assemble the artificial ECM. Postloading is often called postdiffusion or postinfiltration. The obvious way that comes to mind in order to load a biomacromolecule inside the multilayers is to add it during the deposition steps as one of the constituents. This approach was employed in the 1990s in the assembly of multilayers made of proteins.[18] Postloading has significant benefits because of no need to add an excess of typically expensive biomacromolecule (otherwise, at a lack

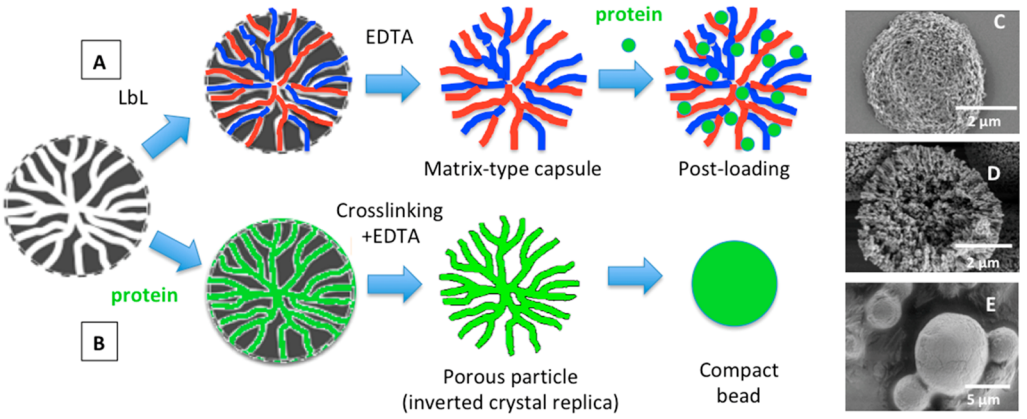


Figure 1. Schematics of fabrication of (A) matrix-type multilayer capsules with postloaded protein and (B) porous and spontaneously formed compact protein beads. Protein molecules are in green. (C–E) Scanning electron microscopy (SEM) images of the matrix-type (polystyrene sulfanate/poly(allylamine hydrochloride))$_3$ or (PSS/PAH)$_3$ capsules, $CaCO_3$ crystals, and insulin beads, respectively. Adopted from refs 2 and 27 with permission from the American Chemical Society and Elsevier.

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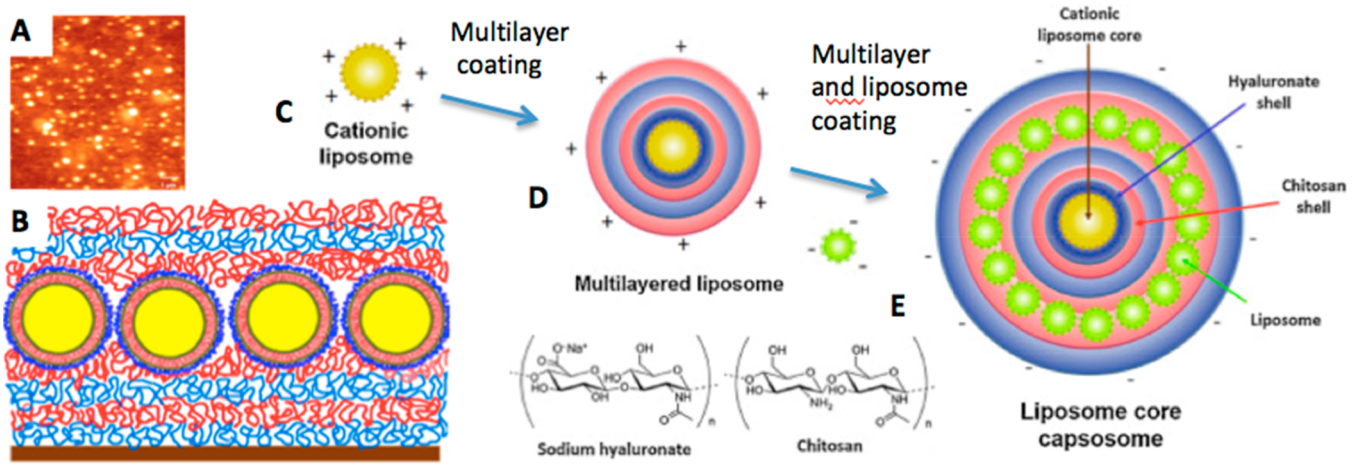

Figure 2. (A) AFM image of the film with embedded vesicles. (B) Schematics of HA/PLL films with embedded liposomes assembled by the LbL method. PLL, in blue; HA, in red. The film composition is $(PLL/HA)_{12}$/PLL-liposome/HA/PLL/HA. (C–E) Schematics of the fabrication of capsosomes using HA and chitosan as polymers. Adopted from refs 31 and 39 with permission from The Royal Society of Chemistry and Elsevier.

of adsorbing moelcules the LbL assembly is not promoted). The multilayer component is typically "contaminated" by another component during the assembly because the disassembly process also takes place. This would require a step of purification of the biomacromolecule to be used after the assembly procedure. For postloading, the yield of the loading is typically very high, reaching the ratio between the protein/charged substance in the bulk and in the multilayers of more than 2 orders of magnitude.[4,15,19,20] Thus, almost complete loading can be achieved within a single step if the multilayers are incubated in the protein solution.

Nowadays, the mimicking of the ECM using the polymer multilayers has been significantly improved, and the linkage between the mobility of the loaded biofactors and their presentation to cell receptors is the main challenge and the main fundamental question. Different approaches, including fluorescence recovery after photobleaching, have been employed to probe the internal structure and mobility of the multilayer-loaded substances on various time scales, including the assessment of lateral diffusion and multifractional molecular diffusion.[14,21−24] Moreover, approaches for the reproducible and fast (deposition time down to seconds) fabrication of multilayers (also patterned) at highly controlled polymer mass transport have been reported using microfluidics.[25,26]

In our opinion, the issue of the bioactive presentation/mobility of biofactors in the multilayers will define the success of the employment of the multilayers as artificial ECMs to develop a tissue in a controlled manner and to serve as effective biocoatings of implants guiding tissue regeneration. However, the stimuli to tune the presentation/mobility of the loaded biofactors have to be noninvasive and ideally will have a remote effect that does not have any influence on functions of cells in contact with the multilayers. Light is an ideal stimulus because it can be well focused to affect a single cell, and light irradiation modalities can be well adjusted and controlled. The next section is devoted to the employment of light for the light-triggered release of biofactors for external control over the presentation of the biofactors in the multilayers.

MULTILAYER-LIPOSOME HYBRIDS (CELL–ECM INTERFACE MIMICS)

After the recognition of the power of the LbL deposition approach to precisely assemble complex multicomponent structures, the necessity to mimic the interface between the ECM and the cell has arisen. The cell membrane can be well reconstituted as the intact lipid bilayer. However, the integration of the LbL approach with lipid bilayer reconstruction faced a serious obstruction of low stability and spontaneous disruption of the bilayer in contact with the multilayers that is governed by the electrostatic interactions of lipids with the polymer cushion of polyelectrolyte multilayers.

In 2008, Volodkin et al. successfully constructed and investigated quasi-2D liposome–multilayer hybrids (Figure 2A,B) compartmentalized with the strata of intact unilamellar liposomes that were anticipatorily stabilized[28,29] with polycation poly-L-lysine.[30,31] These papers demonstrate the potential of such assembles for the inclusion of biofactors into the liposome lumen as well as controlled-release opportunities.

These works have driven the development of a new research direction for the design of functional compartmentalized polyelectrolyte multilayers that provides the multilayer films with a higher level of hierarchy and an option to include small and large molecules into liposomes and multilayers, respectively.[32,33] The loading of both hydrophobic and hydrophilic molecules can be achieved via the liposome–multilayer hybrids.[34] After the works of Volodkin et al., the subsequent studies introduced alternative assembly concepts for the entrapment of the liposomes without the need for their stabilization prior to immobilization, for instance, using cholesterol- or oleic acid-modified polymers.[35] In recent years, hybrid liposome-containing multilayers have been extensively used for surface-mediated drug delivery.[32] Externally triggered release from surface-immobilized liposome–multilayer hybrids has been reported.[36,37]

On the other hand, aiming at the mimicry of the cellular environment, the initial approach to liposome integration into planar multilayers has been modified toward the 3D spatial organization. Thus, capsosomes, which are polymer-based multilayer capsules that contain liposomal subcompartments, have been constructed. Encapsulated enzymatic reactions within capsosomes were successfully carried out with the aim

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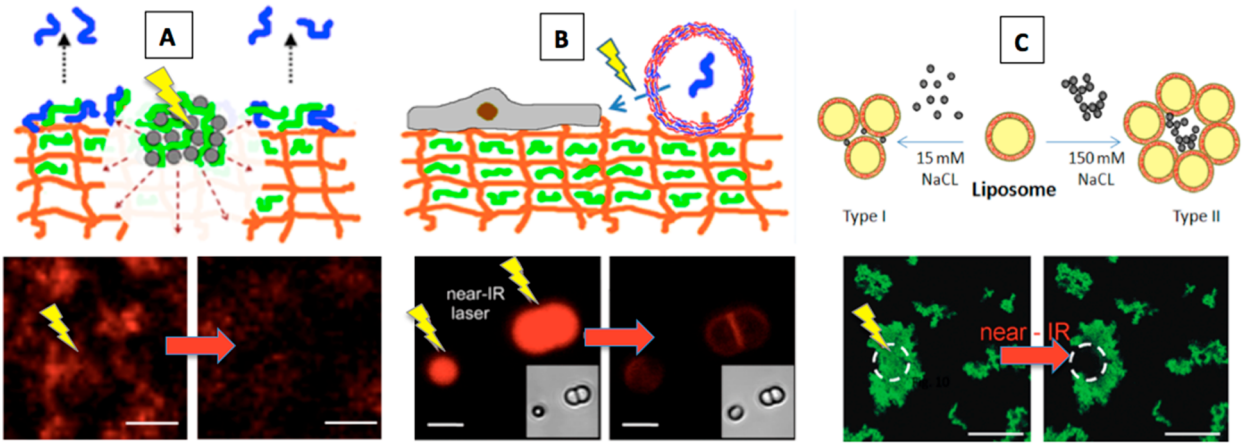

Figure 3. Schematics of IR-light-triggered release from gold nanoparticle (AuNP, gray spheres) containing planar multilayers, multilayer capsules, and liposomes supported by the following confocal laser scanning microscopy (CLSM) images underneath the schematics before and after IR-light irradiation (at a wavelength of 830 nm; for more light modalities, see the references below). (A) $(PLL/HA)_{24}/PLL$ film with embedded DNA and gold AuNPs. DNA is labeled with ethidium bromide. The blue molecule is dextran released from the capsule into a cell. (B) AuNP-coated $(poly(diallyldimethylammonium\ chloride)/PSS)_4$ microcapsules loaded into the $(PLL/HA)_{24}/PLL$ multilayers. (C) Scheme of formation of liposome–AuNP assemblies by the variation of the AuNP aggregation state induced by salt. Remote release of carboxyfluorescein from the assemblies of type II. Adopted with permission from (A) ref 41, (B) ref 41, and (C) ref 53. The scale bars are (A) 1, (B) 5, and (C) 10 μm.

of mimicking metabolic activities.[38] Recently, the multicompartment 3D architecture that has been assembled via additional liposome adsorption onto the core capsosome has generated almost infinite possibilities for the construction of compartmentalized microreactor and artificial cells (Figure 2C–E).[39]

Nowadays, the enormous potential for the translation of multilayer–liposome hybrids for the concept of artificial cell is evident. Further improvement of the initial concept of multilayer-coated liposomes is expected. This might be focused on the coupling of the LbL technique with giant liposome formulation as one of the central modern approaches for the concept of artificial cell. Herein, recent advancements in the powerful microfluidic approach might play a crucial role. At the same time, the step from introducing different assembly concepts toward implementing biofunctions into the engineered assemblies is expected. This includes not only the development of multilayer–liposome hybrids as microreactors that have a high potential for biomedical application but also the generation of novel cell-mimicking platforms that will allow the integration of the features of different components of the cell and the cellular microenvironment in order to decipher the fundamental mechanisms of essential cellular processes.

LIGHT-TRIGGERED PRESENTATION OF BIOFACTORS (ECM SIGNALING MIMICS)

The control over the presentation of biofactors in multilayers as artificial ECM is indispensable. This gives an option to mimic the dynamics of the biofactors as in the real ECM with a high precision in space and time. The localization of biofactors and their controlled release has been proposed as an effective way to control the biofactor presentation down to the level of a single cell. The IR light serves a noninvasive external stimulus that is able to permeate the highly hydrated multilayers and biological cells without affecting cellular functions.

In 2009, in the department of Helmuth Möhwald, the IR-light triggered release of biofactors (e.g., DNA, dextranes) in multilayers has been developed, demonstrating both film- and capsule-assisted release at a low light irradiation power (Figure 3A,B).[40,41] The mechanism is both cases is based on the local heating of multilayers with integrated metal nanoparticles. The nanoparticles can convert light energy into heat, which results in either the redistribution of polymers in multilayers (film-assisted release) or the local destruction of the capsule shell by overheating (capsule-assisted release). In these cases, the heating is slightly above room temperature to enhance polymer diffusion into multilayers or very strong (hundreds of °C) but highly localized, respectively. The temperature variation in multilayers may significantly affect the multilayer growth that is caused by both a change of the multilayer structre and enhancement of molecular transport within the multilayers.[42,43] Microcapsules capable of targeted drug delivery have been reported to open new biofunctional opportunities.[44]

It is of note that the localization of biofactors in capsule planar multilayers allows us to protect the biofactors from biodegradation (degradation by enzymes) and complexation with other undesired molecules that can inactivate the biofactors. Light-responsive capsules based on a localized temperature rise, measured using fluorescent probes, were designed earlier[45] and have been used for intracellular delivery as covered in another another article in this special issue. The light-responsive capsules have also been integrated into biopolymer-based HA/PLL multilayers in a manner similar to that for smaller carriers (i.e., liposomes).[31,46] This integration allows us to construct surface-based carriers made of only multilayers that are able to host biofactors in the empty cavities of the embedded capsules. Such systems have been employed for extracellular delivery triggered by light.

The multilayer capsules are themselves mimics of ECM, being made of a multilayer film. Light-triggered delivery from the nanoparticle-modified multilayer capsules can mimic biofactor presentation in the presence of multilayers as the artificial ECM. The capsules, along with those immobilized onto a surface, can be used for the controlled delivery of biofactors into cells noninvasively at a low dosage of IR light. A number of light-triggered multilayer-based structures have also been developed for light-triggered delivery to a cell. For a review, see refs 47–49. For instance, multilayer disruption by light and ultrasound has been demonstrated to be an effective

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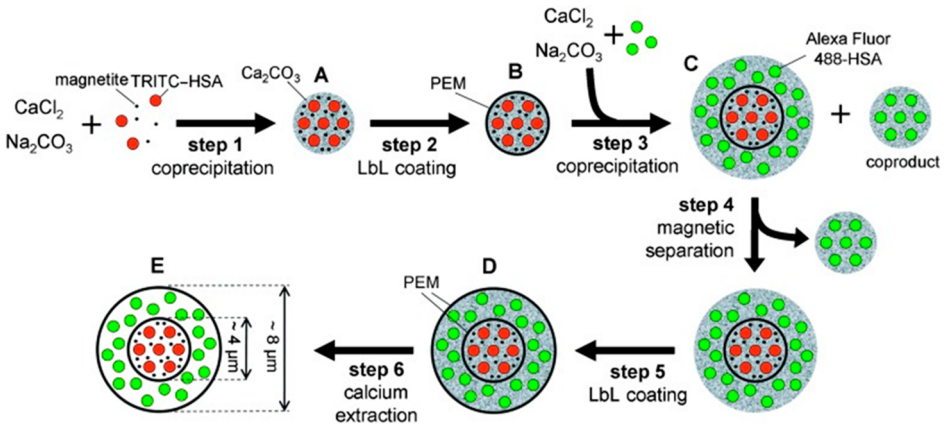

Figure 4. General route for the synthesis of shell-in-shell microcapsules. A = initial core; B = core–shell particle; C = ball-in-ball particle (type I); D = ball-in-ball particle (type II); and E = shell-in-shell microcapsule. Adopted from ref 61 with permission.

externally triggered release system.[50] Lipid-coated polymer multilayer capsules have been used for in vivo anticancer drug delivery induced by IR light.[51] Chlorhexidine composite capsules can effectively release antimicrobial components in a stepwise manner.[52]

In the same year, 2009, light-triggered release from liposome–polymer complexes was demonstrated in Möhwald's department.[53] Figure 3C shows the structure of the complexes of liposomes and nanoparticles (with sizes that can be adjusted by the ionic strength) formed by mixing the components in the presence of salt. Such structures can be selectively activated by light, resulting in local heating over the phase-transition temperature followed by enhancing the permeability of the liposome bilayer and finally the release of the liposome cargo (Figure 3C).

The integration of such liposome-based light-sensitive structures into multilayers can obviously be done as described in the Multilayer-Liposome Hybrids section in order to endow the multilayers with externally activatable features. Such systems can definitely serve as strong mimics of real ECM where the temporally and spatially controlled presentation of biofactors in 2D and 3D can be achieved. For instance, electrochemically induced release from liposomes embedded into planar multilayers (and also into cells) has been demonstrated.[36,37] The light-induced release of doxorubicin from liposome–nanorod assemblies in vitro and in vivo has been shown.[54]

The review of the light-triggered release from multilayers highlighting aspects of light as a trigger and a presentation of biofactors to a single cell is given elsewhere.[55,56] The formation of nanoparticle–polymer complexes for light-triggered release has been discussed.[57]

PROTEIN BEADS VIA HARD TEMPLATING ON VATERITE $CaCO_3$ (CELL ORGANELLES MIMICS)

Cellular metabolism is a cascade of chemical reactions (mostly enzymatic catalytic reactions) within a living cell. To mimic the metabolism, one needs to design artificial organelles where the reactions take place in highly compact compartments mainly composed of proteins with a concentration of up to tens of percent by mass. Moreover, the organelles should be divided into separate compartments with simultaneously occurring reactions, similar to natural processes taking place in confined volumes of organelles.[58] Thus, there was a strong need to assemble protein structures with well-defined architecture and separate compartments.

In 2010, we developed the approach to assemble pure protein particles using hard templating on vaterite $CaCO_3$ crystals (Figure 2B).[27] The approach is similar to the idea behind that proposed for the assembly of multilayer matrix-type capsules described in the Multilayers Postloaded with Biofactors section. However, the infiltration of the crystal pores (by cosynthesis or solvent exchange) is done using only one component (i.e., protein molecules) instead of at least two polyelectrolytes. Protein molecules infiltrating the pores should be cross-linked (physically or chemically) to retain the integrity of the formed porous protein particles after the core elimination by EDTA (Figure 2B). In the case of physical cross-linking such as for insulin particles,[27] the porous particles are spontaneously shrunk to compact particle as a result of the hydrophobic interactions between protein molecules. Later, the approach of formulating protein particles was extended to be applied to virtually any protein.[59,60]

Below, we show how the approach proposed above has been utilized for the assembly of multicompartment structures in order to mimic cellular organelles. Coupled enzyme reactions inside microparticles separated by the multilayer membrane to make compartments have been reported. The first example of a capsule in a capsule nanoreactor involves the glucose oxidase (GOX) and horseradish peroxidase (HRP) bienzymatic system assembled by means of the double cosynthesis of vaterite $CaCO_3$ crystals.[61] The polyelectrolyte shell retained the enzymes in separate compartments, while small substrate and product molecules diffused in and out (Figure 4). Moreover, the mixing of the contents of microcapsules upon laser illumination demonstrates the applicability of remote control over bioreactions in multicompartment capsules.[62]

Later, coupled reactions inside five concentrically built $CaCO_3$ compartmentalized particles with three separated cross-linked enzymes (i.e., HRP, GOX, and β-glucosidase) have been shown,[63] demonstrating an influence of the spacing on the enzymatic reaction rate, which can be delayed from seconds to minutes.

The assembly of non-protein-based structures using the hard templating on vaterite $CaCO_3$ has also been shown using functional biologically relevant molecules such as poly(ethylene glycol)[64] and poly(*N*-isopropylacrylamide).[65] Such structures can endow the protein-based particles with a

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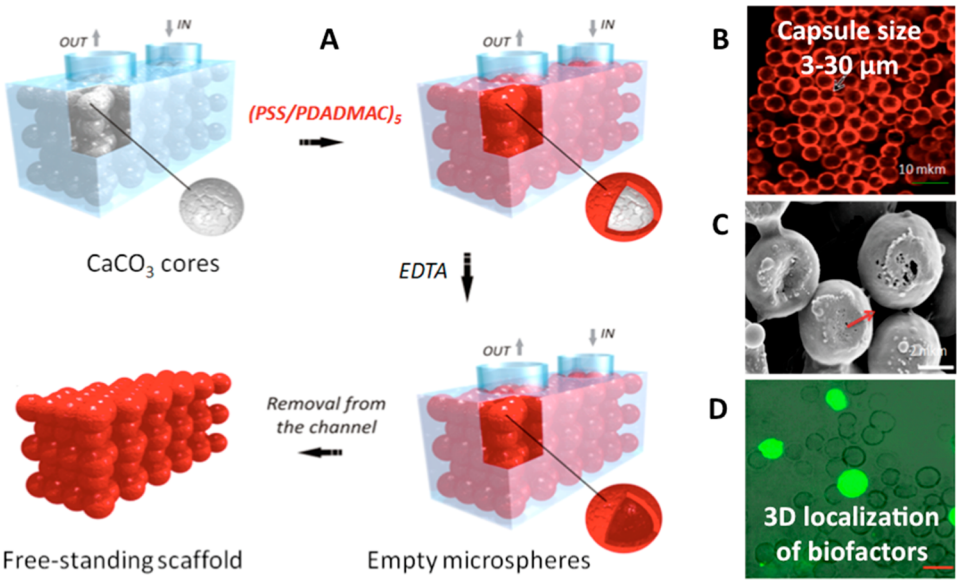

Figure 5. (A) Schematics of fabrication of porous scaffolds made of interconnected multilayer capsules. The scaffolds are assembled in microfluidic channels by packing $CaCO_3$ crystals, followed by crystal LbL coating, elimination of the carbonate crystals, and opening the chamber to remove the scaffold that has the same dimensions as the channel used. (B and C) CLSM and SEM images of the scaffold. (D) CLSM combined image of the scaffolds assembled using 20% of the $CaCO_3$ crystals preloaded with BSA to reach a controlled localization of the biomolecule-loaded capsules. Adopted from ref 69.

number of biofunctionalities such as biological inertness, reduced immunogenity, and temperature-dependent behavior.

The general concept proposed in this chapter opens a path for carrying out coupled reactions in well-defined and structured systems, where parameters such as the microenvironment of the enzyme, the spacing between compartments, and the properties of the separating compartments can be adjusted. Recent progress in the synthesis of pure vaterite $CaCO_3$ crystals with controlled pore sizes[66] and an understanding of the mechanism of protein loading via most probably the most attractive loading approach (i.e., cosynthesis[67]) can open new perspectives on fine-tuning the internal structure and biological characteristics of the protein-based compartmentalized particles as organelle mimics.

FUTURE PERSPECTIVES VIA THE INTEGRATION OF THE ABOVE APPROACHES

The approaches described in the sections above represent mimics of the ECM and ECM−cell interface. These approaches are, first of all, of interest for a fundamental understanding of the high dynamics of soluble signals (biofactors) that actually drives the proper development/organization of a tissue.[68] However, the mimics and control over the ECM composition and signal dynamics are of direct applied interest in guiding tissue development and the growth of a tissue with function similar to that of the living body. The development of such a "real" tissue is possible only when the composition and signal dynamics are well controlled. Thus, a formulation of scaffolds (temporal supports to initiate and instruct tissue growth) made of ECM mimics described above would be indispensable in reaching a novel level of tissue engineering.

Multilayer capsules have been utilized as building blocks to assemble porous scaffolds made of interconnected capsules (Figure 5).[69] Microfluidics has been used to assemble scaffolds under highly controlled conditions by packing vaterite $CaCO_3$ crystals in microfluidic chambers followed by LbL loading in the chamber, elimination of the carbonate core by EDTA, and opening the chamber to remove the scaffold that has the same dimensions as the chamber. It is important that the packing of prefilled and empty crystals gives an option to localize the biofactors previously loaded into the crystals (Figure 5D). This is a simple approach to distributing biofactors in three dimensions, protecting them, and adjusting the presentation of the biofactors by light as described in Light-Triggered Presentation of Biofactors. This gives a unique opportunity to control biofactors in three-dimensional tissue that is typically possible only by invasive micropipetting. It is of note that a thick shell of the interconnected capsules ensures the integrity and stability of the scaffolds. This is because of polymer diffusion into pores of the mesoporous $CaCO_3$ forming a thick shell.

Multilayer complex core−shell structures such as multishells, multicores, and other types of multicompartmentalized microparticles have been reported using porous agarose beads.[70] These structures show high promise for tissue engineering applications such as scaffolds. (Poly-L-arginine/dextran sodium salt)$_x$ capsules assembled on vaterite $CaCO_3$ crystals have been mixed with collagen scaffolds to make the scaffolds more functional, with no significant effect on the physical−chemical properties of the scaffolds.[70] $CaCO_3$-based capsules can be integrated into alginate scaffolds, giving so-called capsules-in-bead scaffolds with an option to separate bioactive compounds well in 3D space.[71] Not only spherical multilayer capsules but also multilayered tubes made of biopolymers can be produced for tissue engineering purposes such as scaffold fabrication.[72] $CaCO_3$ can be used as a shell material for a soft core to assemble inorganic shell microcapsules to be employed as scaffolds for hard tissue engineering.[73]

The current trends in the mimetics of biological processes indicate that significant effort will be put into the future development of scaffolds with externally controlled properties. This will allow the adjustment of scaffold properties in real time and thus the control of tissue development, especially on

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the level of a microtissue. This is, however, a big challenge because of difficulties in adjusting scaffold properties upon request in 3D. This is not a problem in 2D and a number of approaches have been developed, but in 3D, the external triggers (light and electromagnetic fields) are likely the only possible approaches to adjusting scaffold properties in real time. We believe that the integration of the approaches described above and developed in Möhwald's department will help researchers to tackle this issue.

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Notes

The authors declare no competing financial interest.

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