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LbL Films as Reservoirs for Bioactive Molecules

D. Volodkin, A. Skirtach, and H. Möhwald

Abstract This review presents recent progress in utilizing polymeric films made by the layer-by-layer (LbL) technique (so-called multilayered films) as reservoirs for hosting and releasing bioactive molecules. This relatively new technique is distinguished by its high modularity and structural control at the nanometer level, giving polymeric surface films with tuneable physicochemical properties. A significant increase in research activities regarding the bioapplications of the multilayered films has taken place over the last decade. In this review, we address the bioapplications of LbL films and will focus on the loading and release of the film-embedded bioactive compounds and their bioactivity. Planar and free-standing 3D multilayered polyelectrolyte films (microcapsules) are considered. Special attention is paid to light-stimulated release, interaction of cells with the LbL films, and intracellular light-triggered delivery.

Keywords Bioactive · Layer-by-layer · Multilayered films · Polyelectrolyte self-assembly · Remote release

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1 Introduction

Layer-by-layer (LbL) polyelectrolyte self-assembly [1, 2], which is based on consecutive adsorption of polymers that have affinity to each other (Fig. 1), has emerged as a powerful and versatile strategy for engineering surface films aiming biofunctionalization. Not only electrostatic interactions, but also hydrogen bonding [3–5], host–guest interactions [6–11], and hydrophobic interactions [12] can be the main driving forces for assembly of the films. The fundamental physical mechanisms behind the LbL technique have been extensively studied [13–19] but are not fully understood so far. This relatively new technique possesses excellent characteristics such as fine film tuning in terms of thickness (nano- and microscale), stiffness, chemistry, stability, biofunctionality, and dynamics [20–22]. LbL films can be distinguished by the type of film growth: (1) linear with stratified structure interpenetration by each polyelectrolyte only into neighboring layers, or (2) exponential with free diffusion of at least one polyelectrolyte. The poly(styrene sulfonate)/poly(allylamine hydrochloride) (PSS/PAH) film is the most prominent example of the first category, and typical examples of exponentially growing films are films made with polyaminoacids and polysaccharides [23–26]. The nature of the polymer strongly affects the growth regime but is not the dominating factor; linearly growing films can switch their growth regime if interpolymer interaction is weakened by an increase of salt concentration (charge screening) or temperature [17, 27]. LbL films, especially exponentially growing ones, potentially have a high capability

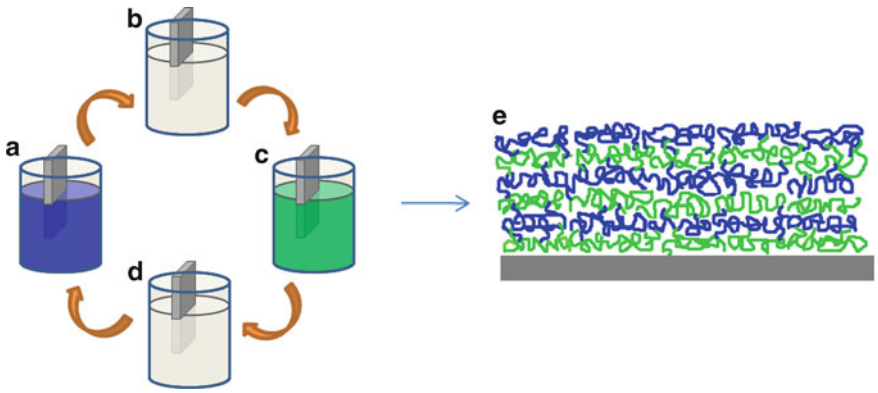

Fig. 1 LbL film deposition on a planar support. Immersion in polymer solutions of polycation (**a**) and polyanion (**c**). (**b, d**) Washing steps to remove nonadsorbed polymer molecules. (**e**) Structure of the LbL-assembled film

of loading (during LbL assembly or by postmodification) a variety of biomolecules in a controlled way, as well as controlled release characteristics. The physicochemical characteristics of the films, which are tuneable to a large extent, are the key to modulating the interaction with cells [28, 29].

The universal character of the LbL method has catalyzed the introduction of the method for a wide range of bioapplications. Proteins (enzymes) [30–33], polypeptides [34], polysaccharides [35], lipids [36, 37], nucleic acids [38–42], viruses [43], inorganic particles, and crystals [44] have been embedded in the films. Use of these compounds makes the films attractive for biorelated applications such as biosensors, drug delivery, tissue engineering, and biocoatings. Biological [45, 46] and nonbiological [21, 47–49] applications of LbL films are reviewed in the literature.

One of the main challenges in biotechnology and medicine is to develop a system able to provide a controlled release of bioactive compounds. This is attractive in view of the obvious advantages of controlled release, such as high efficiency and lower toxicity. LbL films containing bioactive molecules offer the ability to vary not only the amount of the molecules but also to trigger the release and thus enable control "on demand" with external and noninvasive stimuli such as a light. In this review, we aim to summarize the progress in applications of the polyelectrolyte LbL films as reservoirs and release carriers, highlighting planar and free-standing films and the mechanisms of loading and release, including release stimulated by irradiation with near-IR light.

2 Planar LbL Films

2.1 Loading of Free and Encapsulated Biomolecules

Bioactive films made by the LbL technique have been extensively studied by many scientific groups worldwide. The films can host not only bioactive molecules introduced as constituents of the film, but also carriers with encapsulated biomolecules, for instance liposomes (Lip) and polymeric capsules (Fig. 2). Stimuli-sensitive

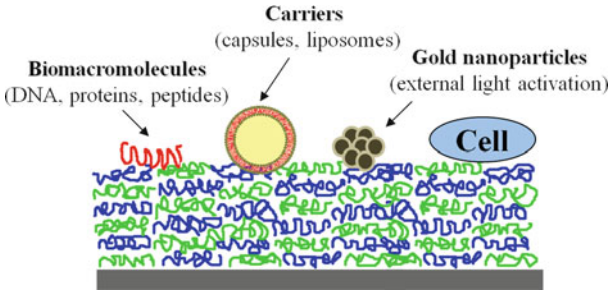

Fig. 2 LbL-assembled polymeric film, which can host different species and interact with cells

material can be immobilized in the film, giving the option to remotely activate the film to result in the release of active molecules. In this review, we focus on loading of the LbL films with biomolecules, release of the biomolecules (including light-stimulated release), and cell interaction with the film, which is an important issue in biomaterial science.

Bioactive macromolecules like peptides, proteins, and nucleic acids have been successfully embedded in planar LbL films. An important question is the retention of the bioactivity of the film-embedded biomolecules. The structural properties and stability of the LbL films formed from synthesized polypeptides of various amino acid sequences were recently reported [50]. The authors showed that control over the amino acid sequence enables control over non-covalent interpolypeptide interaction in the film, which determines the film properties. Haynie and coworkers showed by circular dichroism spectroscopy that the extent of adsorption of poly(L-glutamic acid) (PGA) and poly(L-lysine) (PLL) in the LbL films scales with the extent of secondary structure of the polypeptides in solution [51]. Boulmedais demonstrated that the secondary structure of the film composed of these polypeptides is the same as the peptide structure in the complex formed in solution [52], as found by Fourier transform IR spectroscopy (FTIR).

The properties of protein- and enzyme-containing films were reported by Ai [45]. The film-incorporated enzymes keep their catalytic activity and, moreover, have high tolerance to harsh conditions [45, 53–55]. Adsorption and embedding of fibrinogen in the multilayers from PSS and PAH preserve the secondary structure of the protein [55]; however, these polyelectrolytes can change the structure of bovine serum albumin and hen egg white lysozyme in the multilayers, and this effect is more pronounced for opposite charges of the last polymer layer and the protein. Proteins can strongly interact with the PSS/PAH film whatever the sign of the charge of both the multilayer and the protein [33], forming a monolayer in the case of the same charge sign and a thick layer for opposite charges, which suggests protein diffusion into the film.

Some controversies on the structural properties of the film-embedded polypeptides are revealed and also highlighted by Tang [46], which reflects the complex nature of the polypeptide interaction in the LbL films and could be also the case for the protein-containing films. An increased number of studies on this topic suggests means of developing polypeptide- and protein-based LbL films, despite the fact that interactions in these films are complex and involve multimode interactions such as electrostatics, hydrogen bonding, and hydrophobic interactions.

DNA has been embedded in LbL films by assembling with polycations like PLL, polyethyleneimine (PEI), and poly(dimethyldiallylammonium chloride) (PDAD) [38–42]. Zhang showed that the DNA released from the film with synthetic degradable polyamine is transcriptionally viable [41]. DNA molecules can keep their structure when incorporated in the film with synthetic polymers [39], making films with DNA suitable candidates for gene delivery.

Small drugs, as are most pharmaceuticals, can be directly loaded in the preformed films [56–58]. Schneider demonstrated that chitosan/hyaluronic acid (CHI/HA)

films have high capacity for the drugs diclofenac and paclitaxel. The drug loading can be modulated by varying the number of layers in the film, yielding a diclofenac concentration in the film of the order of 0.1–0.3 mg/mL.

One of the main challenges in the field of biocoating engineering by the LbL method is to increase the load of the films with biomolecules and, at the same time, shield the biomolecules from the surrounding medium. The strategy is the incorporation of reservoirs filled with biomolecules into the film architecture. Recently, phospholipid vesicles were used as such reservoirs due to their low permeability for even small species, biocompatibility, and controlled chemistry and size, which promises liposome utilization in both medical and nonmedical fields [59]. Surface immobilization of liposomes [60] was comprehensively studied, mainly on the basis of biospecific [61–63] and covalent bonding to the surface [64, 65]. However, liposomes are rather unstable and, in general, they undergo collapse or/and fusion when coming into contact with solid surfaces or polyelectrolyte films [36, 66–68]. Many strategies have been proposed in order to overcome liposome instability [69]. They include surface polymerization [70, 71], polymer coating [36, 66–68, 72–75], and LbL coating [76].

Stabilization by polypeptide (PLL) coating has been demonstrated [77–81] and the stabilized vesicles were successfully embedded in the LbL films in an intact state [77, 82–84]. Liposome stabilization by polyelectrolyte coating is attractive due to simplicity, noncovalent surface modification, and because a wide range of polyelectrolytes can provide the vesicles with versatile properties like targeting and stimuli-induced release. Figure 3 shows the vesicle-embedding process. Vesicles, composed of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), the sodium salt of 1,2-dipalmitoyl-*sn*-glycero-3-[phospho-*rac*-(1 glycerol)] (DPPG), and cholesterol (CL), with a diameter of around 130 nm were used. Unstabilized vesicles (i.e., without PLL coating) were fused onto HA/PLL films (Fig. 3a, d), but covered vesicles (Fig. 3c) were entrapped in the film by the LbL procedure (Fig. 3c, f) after removal of excess PLL, which adsorbs better than the coated vesicles (Fig. 3b, e). The study was aimed at optimization of liposome coverage with PLL [79, 80] and revealed an influence of many factors (polymer molecular mass, component ratio, mixing rate and order, temperature) on PLL–liposome complexation. Mixing of PLL and liposomes leads to formation of either single PLL-covered vesicles or aggregates. Differential scanning calorimetry experiments suggest that the adsorption of PLL does not induce phase separation in the lipid bilayer, as was previously observed for the polycation poly(*N*-ethyl-4-vinylpyridinium bromide) [72]. Another important finding is that PLL-covered vesicles keep their integrity in the solid state, even in aggregated form, and release carboxyfluorescein (CF) a little faster than native vesicles in the liquid state [80].

Interpolyelectrolyte and lipid–polyelectrolyte interactions play a crucial role in vesicle embedding [84], but the right selection of components can result in a fine structure of liposome-containing films (Fig. 4c, d). The amount of the vesicle-encapsulated material can be varied by a number of vesicle deposition steps (“interlayers”) or by the charge of the liposomes (Fig. 4e). The embedding

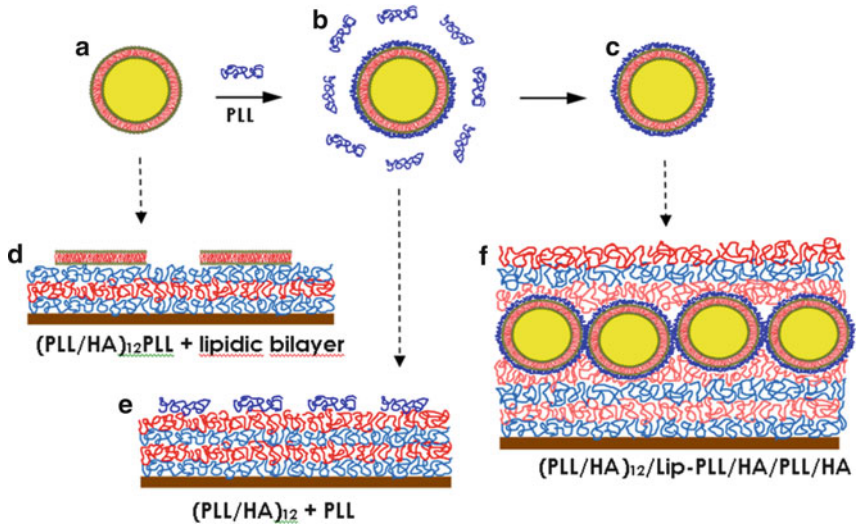

Fig. 3 Vesicle stabilization by PLL covering (**a, b**), followed by separation of well-covered single vesicles from excess of nonbound PLL (**b, c**). Native vesicles are ruptured upon adsorption on a $(PLL/HA)_{12}$/PLL film, forming a lipidic bilayer (**a, d**). Free non-bound PLL is preferably adsorbed on a $(PLL/HA)_{12}$ film rather than on PLL-covered vesicles (**b, e**). Liposome-containing film $(PLL/HA)_{12}$/Lip-PLL/HA/PLL/HA is formed by adsorption of PLL-covered liposomes (Lip-PLL) on a $(PLL/HA)_{12}$ film, followed by additional coating with HA/PLL/HA layers (**c, f**). Reproduced from [82]

matrix plays an important role, and exponentially growing PGA/PAH [77, 78] and PLL/HA [82–84] films were found to be suitable for embedding by the LbL technique. The main advantage of these exponentially growing films [34, 85] with respect to vesicle incorporation is the high water content, due to a gel-like structure formed by weak interpolymer interactions that make a "friendly environment" for the liposomes. We believe that these features allow successful embedding. To our knowledge, there are no studies aim at liposome embedding in linearly growing LbL films, which are mostly made from synthetic polymers and characterized by a low polymer hydration state. This stresses the unique properties of the exponentially growing films.

The fact that no apparent fusion of the vesicles is revealed by the atomic force microscopy (AFM) does not prove the liposome structural integrity (Fig. 4c, d). Analysis of the profiles of the embedded vesicles show that they are immersed in the film, suggesting the immersion by two different modes of the capping film layers: (1) exponential between the vesicles, and (2) linear on the vesicle top [82]. Evidence of vesicle stability is proved by a direct release study of the vesicle-encapsulated CF marker, as shown in Fig. 4f [82]. Similar results were found for DPPC vesicles filled with ferrocyanide ions [77]. No considerable release of the markers, at least during the first few hours after embedding, points to vesicle integrity.

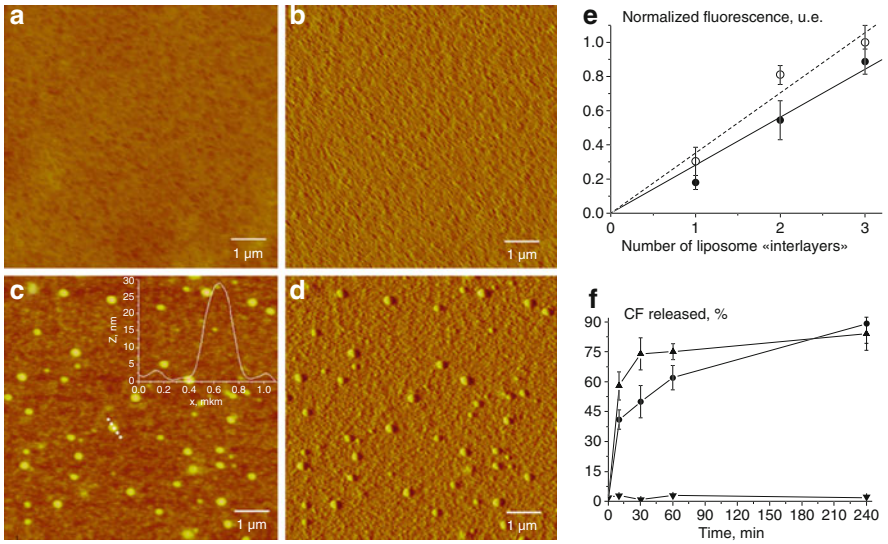

Fig. 4 AFM images of $(PLL/HA)_{12}$ film (**a** height and **b** deflection images) and liposome-containing film $(PLL/HA)_{12}$/Lip-PLL/HA/PLL/HA (**c** height and **d** deflection images). The *inset* in (**c**) corresponds to the height profile of the vesicle along the dotted line (*x*-axis in μm, *z*-axis in nm). Reproduced from [82]. (**e**) Fluorescence of solutions obtained by solubilization of liposome-containing films versus the number of vesicle "interlayers" in the films. Liposomes contain 10% (*filled circles*) and 30% (*empty circles*) of DDPG. Each value is the average of at least three independent experiments with its standard deviation (*error bars*). The *straight lines* are a linear fit through zero. Reproduced from [84]. (**f**) Time evolution of the cumulative CF release from vesicles embedded inside a $(PLL/HA)_{12}$/Lip-PLL/HA/PLL/HA film architecture, when the film is maintained at ambient temperature (*inverted triangles*) or heated and maintained at 45 °C (*triangles*). These release kinetics are compared to the release kinetics obtained for the same PLL covered vesicles in aqueous solution at 45 °C (*circles*). Each value is the average of at least three independent experiments. Reproduced from [82]

2.2 Release Capability

There are two mechanisms for release of active molecules from planar polyelectrolyte LbL films. The first is based on weakening of the molecule–polymer interaction, resulting in a release. In the second mechanism, the LbL film plays the role of a matrix housing a reservoir with encapsulated biomolecules. In this case, the drug release is determined not by interaction with polymer(s) but by the reservoir capacity. An example of the latter is liposome-containing films in which the release is affected by the lipid membrane permeability. Both mechanisms can take place. For instance, Burke showed that release as well as loading of small hydrophilic dye molecules from PAH/HA films depends not only on the dye–polymer interaction but on the dye aggregation ability and film swelling state [86].

The first mechanism is more widely reported in the literature and is based on various kinds of stimuli. The main principle of this release approach is that the

drug molecules interact with the polyelectrolyte more strongly at loading conditions than at conditions when the drug should be released. pH and ionic strength can affect the ionization of the polyelectrolyte side groups and thus change the interaction of probe molecules with the polyelectrolyte film network [86–88]. Large molecules like proteins were also shown to exhibit pH-dependent release behavior. Müller demonstrated that, for PEI/poly(acrylic acid) films, an adsorption and release of lysozyme and human serum albumin could be switched by changing the pH setting, thanks to electrostatics [88].

A switching to different physicochemical conditions results in both changes in the drug–polyelectrolyte interaction and changes in the interpolylectrolyte interactions. The latter leads to film decomposition or erosion, which is the second release mechanism. The polymers in the LbL films form a diffusion barrier for the releasing molecules. Sukhishvili and Granick have studied the stability of the multilayered films with variation of pH and ionic strength. The films were formed by hydrogen bonding, and critical values for the film disintegration have been found by FTIR [5]. Decomposition of the polyelectrolyte film structure has been also achieved utilizing hydrolytically degradable polymers [41, 42, 89, 90]. Thin degradable films and coatings that sustain the release of DNA from surfaces under physiological conditions could play an important role in the development of localized approaches to gene therapy [41, 42]. Chuang and co-authors have designed active antibiotic-releasing films with heterostructure by alternating deposition of hydrolytically degradable poly(*beta*-amino ester), HA, and the antibiotic gentamicin [90]. The design allows for direct loading of gentamicin without having to premodify it.

Release of liposome-encapsulated CF from HA/PLL films has been observed at temperatures above the lipid transition temperature (Fig. 4f). Below this temperature, the vesicles were stable at least for a few hours. The polyelectrolyte network destabilizes the embedded vesicles, which show higher lipidic bilayer permeability upon heating than do vesicles in solution [84]. No change in film properties upon heating has been reported as proof of the polyelectrolyte destabilization effect.

If the LbL film has relatively high thickness, the release rate can be driven by molecule diffusion through the film. Schneider demonstrated that drug diffusion through the film proceeds for several hours, as achieved by placement of the CHI/HA film loaded with diclofenac and paclitaxel in 0.15 M NaCl (pH 7.4) into phosphate-buffered saline solution. Vodouhe showed that the PLL/HA films passively loaded with taxol can regulate cell adhesion and viability by deposition of capping layers of PAH/PSS, which effects both drug release and cell adhesion [57].

Alternative stimuli to induce the release of film-embedded biomolecules have been introduced. Disintegration of a DNA/Zr^{4+} film, triggered by an electric field, has been shown by Wang [91]. PLL/heparin films were built on an indium tin oxide semiconductor substrate and electrochemically dissolved [92]. Enzymes can work as agents to decompose polymers in the LbL films, yielding so-called enzymatic degradation [93]. Pepsin erosion of alginate (ALG)/CHI films has been recently reported [94]. Serizawa demonstrated very effective degradation of DNA/PDAD films by DNase I [95]. The use of stimuli-sensitive polymers, or modification of the

polymers used to form the LbL films with stimuli-responsive moieties, opens ways to establish surface coatings that are sensitive to light irradiation [96] or temperature change [97].

Special attention should be paid to noninvasive release of bioactive molecules from the films because it gives an option of delivery of bioactive molecules to a cell without changes in surrounding medium. Cells are generally very sensitive to changes such as variation in pH, ionic strength, etc. Our recent studies with the LbL films made from biopolymers HA and PLL demonstrate film reservoir properties, i.e., nano- and microcarriers such as liposomes or polymeric capsules as well as biomolecules (DNA, proteins, peptides, etc) can be incorporated in the film [82, 84, 98, 99]. The HA/PLL film has high loading capacity due to the polymer doping at the film surface (Fig. 5), which results in accumulation of a large amount of adsorbing material. This is many times less for PSS/PAH film, which has low polymer mobility [98, 100]. Microcapsules, gold nanoparticles, and DNA can be embedded in the HA/PLL film and located on the film surface [98, 100] (Figs. 5 and 6). Diffusion of embedded molecules (DNA) into the film can be triggered by heating [98, 100]. The amount of material incorporated into HA/PLL films can be larger than the mass of the polymers (HA and PLL) in the film, which is attributed to polymer transport.

Microcapsules can be adsorbed on the hydrogel HA/PLL film by direct contact of the film and the capsules [98, 100] (Fig. 6a). The opposite charges in polyelectrolyte multilayers of microcapsules are almost compensated for overall, but the strong attachment of microcapsules to the film (no capsule removal observed upon intensive film washing) can be attributed to some uncompensated negative charges on the last

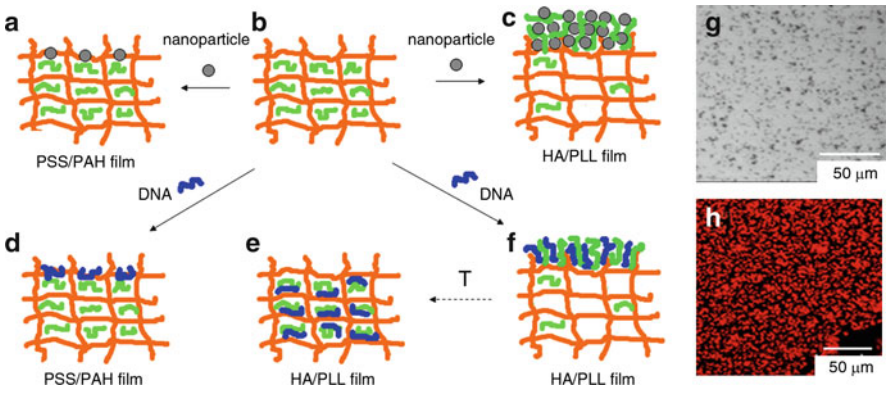

Fig. 5 Principal scheme of interactions of LbL films (**b**), namely, PSS/PAH and HA/PLL, with gold nanoparticles (**a, c**) and DNA (**d, f**). The nanoparticles and DNA interact only with the surface PAH groups of the PSS/PAH film (**a, d**). However, they can accumulate in large quantities as a result of the interaction with PLL "doping" from the whole interior of the HA/PLL film (**c, f**). Diffusion of DNA into the HA/PLL film can be triggered by heating to 70 °C (**e**). Optical and confocal fluorescent microscopy images of gold nanoparticles (**g**) and DNA-EtBr (**h**) adsorbed onto the $(PLL/HA)_{24}$/PLL film, respectively. Reproduced from [98]

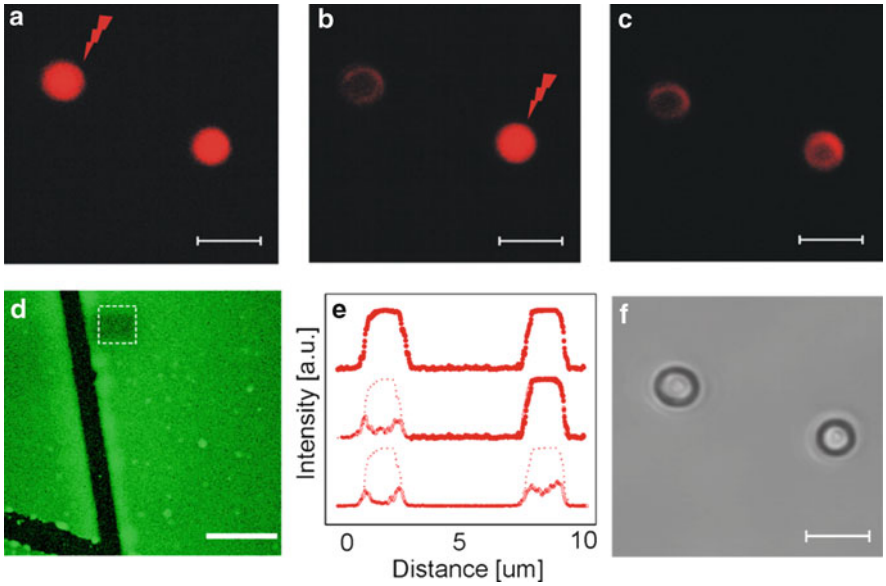

Fig. 6 Adsorption of microcapsules onto the $(PLL/HA)_{24}$/PLL films. (**a–c**) Confocal fluorescent microscopy images of the capsules exposed to the near-IR light irradiation. (**d**) CLSM image of the film surface (the film is prepared with PLL-FITC; *black lines* are scratches made by a needle for easier film imaging). (**e**) Cross-sectional profile of the capsules after step-by-step laser exposure (the sections from *top to bottom* correspond to the images **a–c**, respectively). (**f**) Optical microscopy images of the capsules after light irradiation. *Scale bars*: (**a–c, f**) 4 µm, (**d**) 25 µm. Reproduced from [100]

layer. It seems to be that the immersed capsules adapt to the "best" position in terms of interaction with the doped PLL molecules; the capsules can be immersed into the film as liposomes [82].

An important feature is film activation with micrometer precision by external stimulation with "biofriendly" near-IR light, which results in controlled release of film-embedded material [98, 100]. Laser activation of film-supported microcapsules shows remote release of encapsulated dextran by selective stimulation of the capsules with near-IR light (Fig. 6). Destruction of the HA/PLL film functionalized with gold nanoparticles occurs at irradiation with a light power of over 20 mW. Microcapsules modified with nanoparticles keep their integrity under the same conditions but become more permeable.

The HA/PLL film with adsorbed gold nanoparticles and DNA possesses remote-release features by stimulation with near-IR light of over 20–30 mW (Fig. 7). DNA release from the film modified with gold nanoparticles is thought to be caused by local destruction of the polymer network in the film, followed by blocking of PLL–DNA bonding and, as a result, release of DNA molecules from the film [98, 100]. Laser activation of the films can be used for affecting, releasing, or removing the upper coatings of the films, depending on the power of the laser.

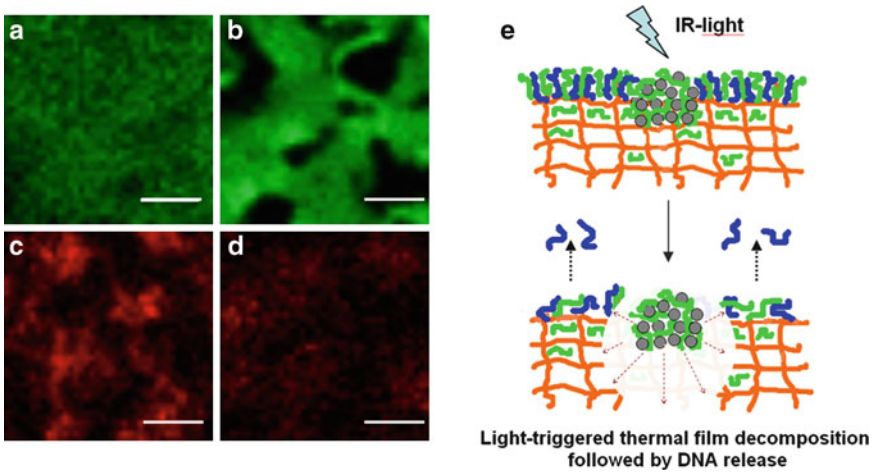

Fig. 7 $(PLL/HA)_{24}/PLL$ film with embedded DNA before (**a, c**) and after (**b, d**) irradiation with IR light. PLL in the film is labeled with FITC (**a, b**), and DNA is labeled with EtBr (**c, d**). *Scale bars*: 5 μm. (**e**) Suggested mechanism of DNA release induced by the distortion of the DNA-doping PLL interaction as a result of partial thermal film decomposition in vicinity of nanoparticle aggregates. Reproduced from [98]

3 Free-Standing LbL Films (Microcapsules)

Up to now, several techniques have been applied to encapsulate material of interest into various types of micro- and nanoparticles to achieve different drug administration routes and release characteristics. Some of these techniques are based on liposomes (vesicle-based) and some on polymeric particles (matrix systems, microgel beads or particles prepared by interfacial polymerization) [101]. The LbL technology as alternative method has attracted high interest for the production of microparticulate structures for delivery applications. This concerns first of all the colloidal particles made and/or modified by the LbL technique. The main principles of LbL deposition on colloidal particles [102, 103] are similar to those of film formation on planar surfaces. A very attractive and extremely fast-developing area using colloidal templating is the construction of multilayered polyelectrolyte capsules. The concept of capsule formation by the LbL technique involves alternating polyelectrolyte adsorption on a colloidal template, followed by decomposition of the sacrificial core [102]. This leads to the formation of hollow structures that replicate the templating particles in terms of size and shape. Figure 8 shows the principle scheme of capsule formation by the LbL approach. Fabrication and properties of the multilayer capsules are reviewed elsewhere [47, 104].

A broad variety of sacrificial colloidal cores have been used for hollow capsule fabrication. They are inorganic or organic particles from tens of nanometers and up to tens of micrometerss, like melamine formaldehyde (MF), polysterene spheres, $CaCO_3$ and $MgCO_3$ particles, protein and DNA aggregates, small dye

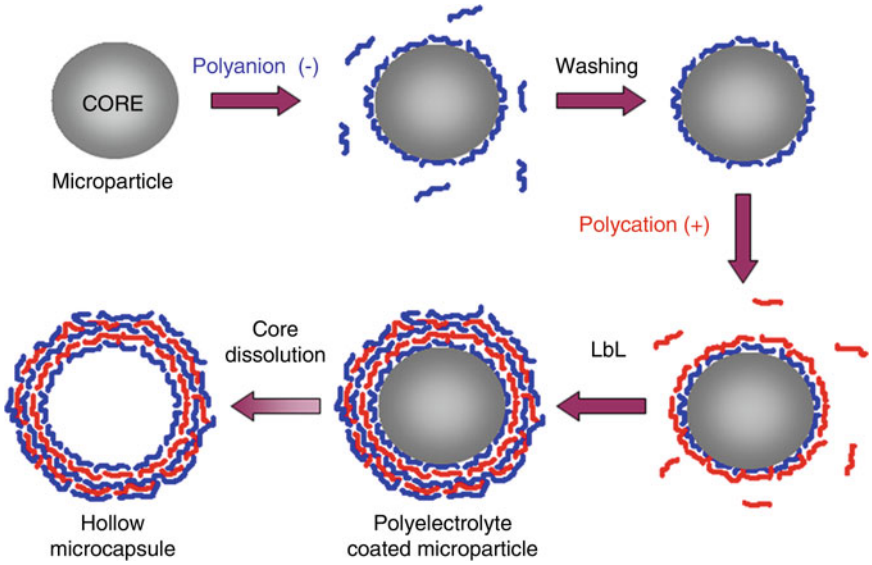

Fig. 8 Hollow capsule fabrication by the polyelectrolyte LbL self-assembly. The core is alternately coated with polycation and polyanion, followed by core dissolution and capsule formation

(drug) crystals, and even biological cells [45, 104–113]. MF particles were firstly employed and further intensively studied for capsule templating. However, the incomplete elimination of MF-oligomers during core dissolution strongly limits utilization of the MF cores for biological applications [114]. The oligomers are biologically incompatible. Silica oxide particles could be completely eliminated by dissolution of hazardous HF, which also limits the use of this kind of template. The cores composed of polylactic acid are biodegradable; however, the formed capsules are very polydisperse and possess a high tendency to aggregate [115].

Finally, inorganic particles from $CaCO_3$ were found to be the most suitable sacrificial cores for polyelectrolyte capsule templating [111–113, 116, 117] due to their fine structure, biocompatibility, low cost, and simple and mild decomposition (HCl or EDTA). Monodisperse porous spherical particles composed of $CaCO_3$ (Fig. 9d) were prepared and used to form polyelectrolyte capsules of a matrix type by the LbL technique [111, 112]. The polymer adsorption takes place not only on the particle surface but also within the porous interior. Dissolution of the $CaCO_3$ core leads to formation of polymer gel particles with dimensions equal to the size of the initially employed $CaCO_3$ cores (Fig. 9a–c). The capsule image obtained by scanning electron microscopy (Fig. 9e) demonstrates the sponge-like structure of the matrix capsules. Two different ways to encapsulate biological substances (proteins) in the matrix capsules were elaborated: [113] active loading (entrapment is achieved during fabrication of the microcapsules), and passive loading (encapsulation is achieved in the preformed capsules; the material of interest is taken into or generated in situ within the capsules). Protein molecules are distributed within the whole particle

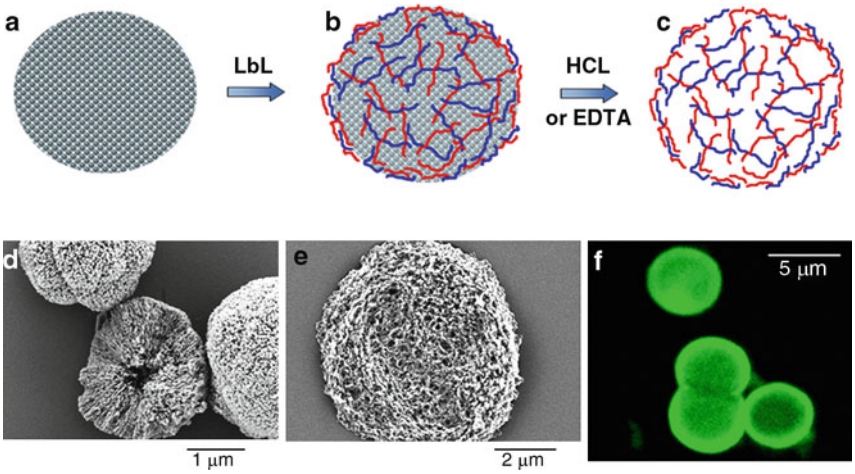

Fig. 9 (**a–c**) Preparation of matrix-type polyelectrolyte capsules templated on $CaCO_3$ microparticles. (**d, e**) Scanning microscopy images of the $CaCO_3$ microparticles and the matrix-type capsules, respectively. (**f**) Confocal laser scanning microscopy image of the capsules loaded with fluorescently labeled bovine serum albumin. Adapted from [111, 112]

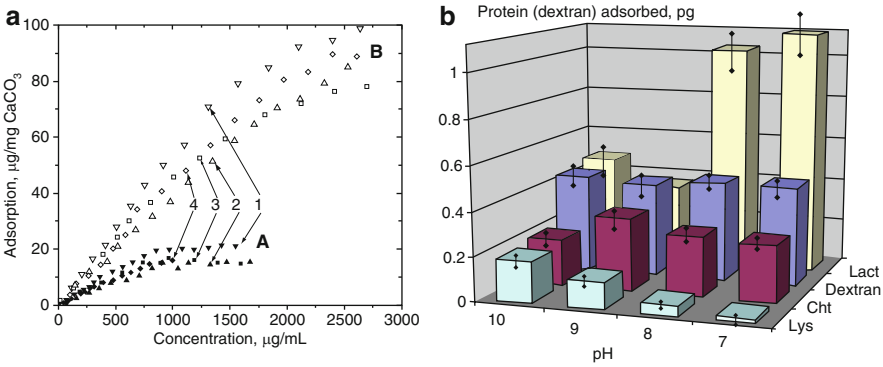

Fig. 10 (**a**) Adsorption isotherms of BSA (*1*), chymotrypsin (*2*), lysozyme (*3*), and PAH (*4*) adsorbing within $CaCO_3$ microparticles. The *curves of group A* describe the adsorption onto preformed $CaCO_3$ microparticles, and those of *group B* the adsorption and capture during the growth of spherical $CaCO_3$ microparticles (coprecipitation). (**b**) Amount of proteins and dextran adsorbed per $CaCO_3$ microparticle as a function of pH. Incubation time 1 h. Reproduced from [111]

interior according to the polymer distribution in the capsules because the loading of the protein is driven by the interaction with free polyelectrolyte chains (Fig. 9f). The $CaCO_3$ particles and matrix polyelectrolyte capsules have high protein capacity (up to 100 mg of embedded protein per 1 g of $CaCO_3$) [111, 116, 117], and the protein uptake in the particles was shown to be regulated by electrostatic interactions [111]. Figure 10 presents the protein adsorption isotherms (Fig. 10a) and protein uptake

(Fig. 10b) for the $CaCO_3$ cores. About a half of the protein (lactalbumin) molecules initially adsorbed in the $CaCO_3$ particles are kept in the formed capsules [111]. Peptidase α-chymotrypsin encapsulated into the matrix-type capsules keeps 85% of the initial enzymatic activity [116]. The capsules fabricated with biocompatible polymers like ALG and templated on biocompatible $CaCO_3$ cores are promising species for controlled delivery [113].

The capsules with a defined shell (so-called hollow multilayered polyelectrolyte capsules) are free-standing LbL films with a peculiar advantage – the ability to uptake the material inside the capsule, in other words to encapsulate it and to control its release by changing the LbL film permeability properties. Macromolecules like proteins have been successfully loaded into polyelectrolyte LbL capsules through pH-controlled and water/ethanol mixture-controlled methods [118, 119]. Alternative stimuli could be applied, e.g., a magnetic field [120]. This approach consists of destabilization of the LbL membrane, which then becomes more permeable. The capsule is loaded with molecules of interest under destabilized conditions and then the conditions are changed back to the initial ones (under which the LbL shell is stable).

Protein aggregates [107, 109, 121] or dye crystals [122–126] can serve as templates for LbL polyelectrolyte adsorption. Chymotrypsin aggregates encapsulated by PSS and PAH deposition contain a high protein amount and the enzyme keeps its bioactivity [107]. The aggregates prepared in this manner have high incorporation efficiency and a protein content of 50–70% [109]. An encapsulated catalase has been shown to be stable against protease degradation [121].

3.1 Cargo Release

The mechanism of drug release from multilayered capsules is totally different to that from planar drug-containing surface films. For the capsules filled with encapsulated drug, the release rate depends on capsule membrane permeability, which can be manipulated by stimuli that affect the structure and stability of the interpolyelectrolyte interactions (pH, ionic strength, temperature, etc.). The same stimuli could be applied for flat films and capsules; however, the leakage of the capsule contents is more sensitive to stimuli due to osmotic pressure created by the encapsulated material. DNA/spermidine or ALG/PLL capsules were shown to be decomposable at increased NaCl concentration, thus allowing the release of cargo [127]. The reversible character of the membrane destabilization in shell-like polyelectrolyte microcapsules allows keeping the material in capsules and then releasing it by changing the shell permeability through modifying external factors [128]. The remote activation properties and capsule targeting have been reviewed by Sukhorukov [129].

The matrix polyelectrolyte capsules have high protein-loading capacity, and both the loading and, in principle, the release are driven by electrostatic interaction with polyelectrolytes [111]. Moreover, the loading and release can be controlled by the number of polyelectrolyte adsorption steps [112] as well as by the pore size of the $CaCO_3$ cores [116].

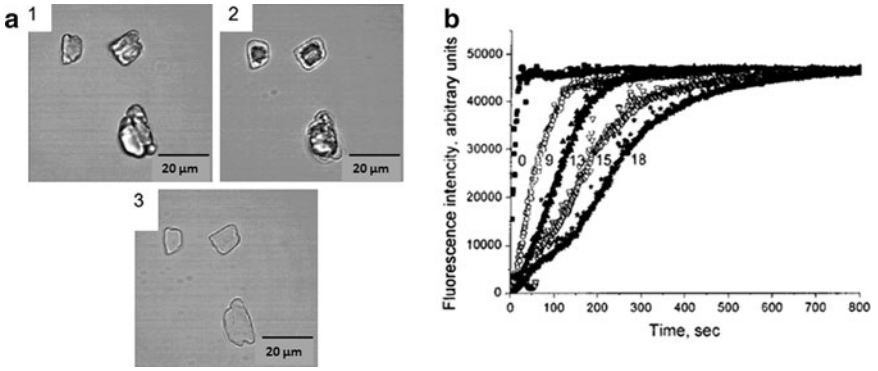

Fig. 11 (**a**) Optical microscopy images of the release from ibuprofen crystals covered with a $(CHI/dextran\ sulfate)_{15}$ shell: *1* before dissolution; *2* during dissolution; and *3* after removal of the crystal cores. The mean size of the encapsulated ibuprofen microcrystals is 15.3 μm. Reproduced from [112]. (**b**) Fluorescence increases with time, obtained by dissolving fluorescein crystals covered with shells of different thicknesses (9, 13, 15, and 18 polyelectrolyte-deposited PSS/PAH layers). The release from the native (uncovered) fluorescein crystals is shown as 0. Reproduced from [122]

The LbL film forms a barrier that prolongs the release of dye from crystals coated with the LbL films (Fig. 11). Crystals such as small drug microcrystals [122–126] and protein/enzyme crystals or aggregates [107, 121] can be encapsulated by polyelectrolyte LbL assembly and the release rate adjusted by changing the number of alternating polymer deposition steps or the polyelectrolyte nature. Antipov showed that nine bilayers of PSS and PAH can decrease the release time of the coated fluorescein crystals from seconds to minutes at conditions under which the crystals become soluble [122]. A decrease in the release rate was shown for furosemide crystals coated with a combination of PSS, PDAD, and gelatine [123]. Significantly longer release kinetics were reported by Qiu for ibuprofen encapsulated by coating with biopolymers (polysaccharides dextran sulfate and CHI) [125]. Enzymatic degradation of indomethacin crystals covered by the ALG/CHI shell led to drug release [94], again showing that disintegration of the LbL film formed on the drug crystal surface is the main mechanism for drug release.

Although the polyelectrolyte LbL membrane works as a barrier to prolong drug release, in some applications the release time is already long. Annealing of capsules upon heating can dramatically reduce the film permeability [130, 131]. Thus, polyelectrolyte microcapsules made by the LbL technique are drug delivery carriers with a wide range of release rates. Together with the advantage of being able to control the capsule size within a range of less than 1 μm and up to tens of micrometers, this makes the capsules very promising delivery carriers.

The remote release of encapsulated materials is desired for bioapplications in order to minimize drug toxicity, to control the properties of biosurfaces and interfaces, and to study intracellular processes [132]. Remote release can be more convenient for a patient because external stimuli like a magnetic field, light, and ultrasound are

not harmful. Light-stimulated remote release is of special interest because of the possibility for external control of the light intensity and modulation, and because of its noninvasive character. Numerous examples demonstrate promising applications of this concept using liposomes as carriers [133–137]. For example, liposomes decorated with gold nanoparticles by electrostatic complexation are prospective vehicles for pulsed-light-stimulated release [133]. Irradiation by "biofriendly" near-IR light can induce fast release of vesicle contents within a few seconds of light treatment [133]. Remote release from polyelectrolyte LbL microcapsules functionalized with metal nanoparticles has been demonstrated using laser light to burst open or deform the capsules [138]. Both the magnetic and optical responses of microcapsules modified by iron oxide and gold nanoparticles, respectively, were demonstrated by Gorin and co-authors [139].

The engineering of stable liposome-containing LbL films to allow release of active content in response to external stimuli opens a new route to the preparation of biocoatings for delivery on demand. This is possible thanks to the versatility of the LbL films as an instrument for making functional surfaces sensitive to biorelated stimuli [140].

3.2 Intracellular Light-Triggered Delivery

Intracellular trafficking of small peptides and the behavior of various biomolecules can be studied using remote-controlled release from microcapsules by laser irradiation. Polyelectrolyte multilayer microcapsules can be used for encapsulation of these biomolecules. Thermally shrunk microcapsules were shown capable of intracellular delivery of various biomolecules [132]. Intracellular release can be conducted using a laser source that is biologically friendly by using a near-IR laser source; the mechanism of interaction is the localized heating of nanoparticles by laser. One requirement of these experiments is to have minimum absorption by cells and tissue, and maximum absorption by nanoparticles. In regard to lasers, one can choose the wavelength of the laser to be in a desired region in the biologically friendly window (the near-IR part of the spectrum). Absorption by nanoparticles can be tuned to the near-IR region by making aggregates. Aggregation leads to an increased absorption in the near-IR region [141] due to dipole and higher order multipolar contributions, as well as to interaction between the nanoparticles. Also, the concentration of metal nanoparticles is important because (1) when the distance between the two adjacent nanoparticles is of the order of their size, the thermal effects produced by adjacent nanoparticles add up; and (2) the interaction of nanoparticles situated close to each other leads to an increase of absorption at the lower energies or higher wavelengths compared to the surface plasmon resonance band of standalone nanoparticles. To reduce the total amount of heat generated in the vicinity of nanoparticles, it is desirable to control the spatial distribution of nanoparticles [142].

Both remote activation and release of encapsulated materials inside living cells have been recently reported [143, 144]. Experiments on remote activation were

conducted using microcapsules containing silver nanoparticles in their walls. Remote release experiments have also been reported for capsules containing gold–gold sulfide nanoparticles in their walls.

Conditions for remote release experiments are extremely important. It was recently reported that at a low incident intensity of laser light, the release can be carried out nondestructively for cells [145]. This case is suitable for intracellular delivery, which is targeted for studying intracellular functions. Another extreme case is that at a high incident intensity of laser light (close to 100 mW), cells can be killed. This approach is suitable for cancer therapy. Figure 12 presents both of these cases: release from microcapsules was carried out into the cytosol of cells and the effects of both low and high intensity light were studied in detail [145].

The methods of remote release described here can be used for studying numerous processes relevant for biology, e.g., the cell-surface presentation of small peptides, transport of biomolecules, and cell functions in general. Also, such methods can be used to study the properties and release from other systems, e.g., liposomes [133, 146, 147].

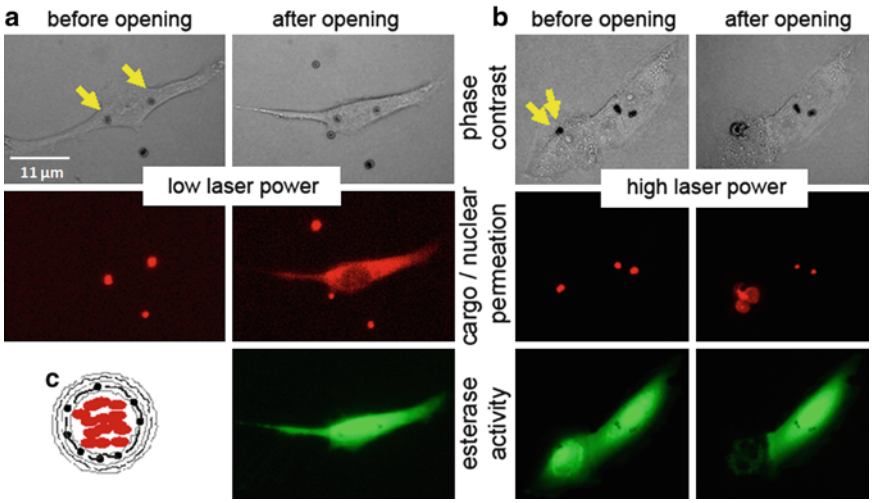

Fig. 12 Cargo release and viability/cytotoxicity experiments with capsules filled with red Alexa Fluor 594 dextran as cargo and AuS_2 particles embedded in their walls. Capsules were illuminated with (**a**) low laser power (2.3 mW), the minimum power needed to open the capsules, and (**b**) high laser power (31 mW), the maximum power output reachable with the laser diode used in these experiments. Phase contrast images (*top row*) show cells that have incorporated capsules (*arrows*) before and after laser illumination. Fluorescence images in the *middle row* show the cargo release and the nuclear permeation in cases where capsules trapped in cells were excited with low laser power and high laser power, respectively. In the case of high power illumination, permeation of the cell membrane leads to loss of fluorescent cargo by diffusion out of the cell. Fluorescence images on the *bottom row* indicate decrease of esterase activity in cells where capsules were excited with high and low laser power, respectively. (**c**) Geometry of capsules with Alexa Fluor 594 dextran (*ellipsoids*) in their cavity and AuS_2 particles (*black circles*) embedded in their walls. Reproduced from [145]

4 LbL Films Govern Cellular Response

The interaction between the substrate to which cells are attached and the various components of a cell is a major factor in deciding the fate of the cell upon that particular substrate. This is especially true for anchorage-dependent cells, which show specific responses (chemical signals, mechanical alterations, or biological factors) to the substrate on which they are grown. Such adherent cells must attach to and spread on the surface in order to function. In tissue, this surface is the extracellular matrix (ECM), a scaffold formed by the assembly of charged macromolecules – several large proteins (e.g., fibronectin, laminins, collagens) and glucoseaminoglycans [148]. The ECM provides a microenvironment that organizes cells into a tissue. The ECM is not only a physical support of cells but a dynamic system with a high traffic of bioactive molecules that inducing certain cellular events. LbL deposition provides unique control over polymer architecture with nanometer precision and is a tool that can be used to emulate the properties of natural ECM. Protein interaction with polyelectrolyte multilayers has been evaluated and quantified [33, 45, 55, 88, 149]. The interaction strongly depends on the sign of the charge of both the film and the protein, as well as on the hydrophilicity of both. Understanding of the interaction between protein molecules and multilayers is the key to engineering an artificial ECM with tailored cellular response.

The interaction of LbL films with cells has been intensively studied [42, 150–156]. The first work devoted to cell interaction with the LbL films was performed at the beginning of this decade [157]. LbL films made from synthetic polyelectrolytes, biopolymers, components of the ECM, and polymers grafted with specific ligands have all been used to study cellular behavior. Picart and Boudou summarized LbL film functionality with respect to the behavior of cells in contact with the films [28, 29]. The physicochemical characteristics of the film can be tuned to a large extent, which gives the option to modulate cell interaction with the film [29, 158, 159]. Cell interactions with multilayered films can be tuned by the number of layers [155], the type of the outermost polymer layer, and the presence of proteins [155, 159, 160]. No adherence of chondrosarcoma cells was found on PGA-terminating films, whereas adhesion to PLL-ending films was significant [155]. This is related to the fact that PGA-ending films prevent the adsorption of serum proteins. Interestingly, the adhesion force decreased when the film thickness increased. Additionally, the films can offer delivery of incorporated active substances, for instance DNA [42, 150].

Surface coating by LbL films can alter the mechanical surface properties, thus opening a way to control cell adhesion. Coating with the film can lead to weakening of cell interactions with the underlying surface making it relatively bioinert [154]. The mechanical properties of LbL films play an important role in various cellular processes including cell attachment, proliferation, and differentiation. Ren studied the effect of PLL/HA film crosslinking on the film stiffness and on skeletal muscle cell adhesion and proliferation [153]. It has been demonstrated that crosslinked films (Young's modulus, $E_o > 320\,\text{kPa}$) promote cell adhesion and proliferation, but soft films ($E_o \sim 3\,\text{kPa}$) do not. It is reported that the crosslinking does not

significantly affect the thickness and morphology of the films. Similar findings have been demonstrated for chondrosarcoma cells [156]. LbL films enable control over mechanical properties in a wide range (kPa to GPa) by tuning the film thickness, polymer nature, environmental conditions like pH and salt, introduction of chemical crosslinking, rigidification with stiff material, etc. [158, 159, 161, 162]. Kocgozlu has recently shown the importance of film elasticity for the regulation of replication and transcription activities in a wide range of elastic moduli from 0 to 500 kPa [163]. Mechanical properties of the surface are crucial for cellular response and the wide range of stiffness of the LbL films covers typical values found in natural tissue [164].

LbL films possess multifold properties and very important characteristics that enable their use in bioapplications such as implant biocoatings and functionalization. Drug delivery from permanent or long-term implanted biodevices remains a challenging area in medicine. Direct drug delivery from an implant surface could be, in principle, the main task but the surface-located drugs have another function. They are required to trigger a desired cell response around the implanted material (wound healing, bone growth) and to minimize or prevent biomaterial-associated complications accompanying implantation (e.g., bacterial colonization). Immobilization of bioactive molecules on the implant surface by the LbL approach provides very effective local delivery of often very expensive and toxic pharmaceuticals. Nonspecific protein adsorption could be minimized by surface modification with LbL films and, at the same time, beneficial molecules like proteins could become selectively adsorbed on the biointerfaces by the LbL technique [45, 46]. Minimization of thrombogenicity is a main challenge for biomedical devices that are in contact with blood. Thierry showed that coverage of metal endovascular stents with HA/$(CHI/HA)_4$ multilayers reduces platelet adhesion by 38% [165].

Potential applications of the LbL films as antibacterial coatings have also been reported. Multilayers of albumin/heparin can significantly reduce bacterial adhesion [166]. In our recent study, we have shown that a PLL/HA multilayer film loaded with liposome aggregates as reservoirs containing silver ions has strong bactericidal activity [167]. A contact of 120 min with an $AgNO_3$ coating (with 120 ng/cm^2 $AgNO_3$ concentration) induces a 4-log reduction of the *Escherichia coli* population [167]. Chuang has demonstrated that degradable LbL films can effectively release gentamicine [90].

5 Conclusion

The LbL technique is now becoming one of the central tools in biomedical engineering, i.e., for biomaterial modification and drug delivery. The advantages of being able to control material deposition, immobilize almost any molecule from large polymers to small substances, and modify almost any surface, including sophisticated shapes or miniaturized supports, make the technique one of the most dominant in the field of surface nanotechnology. This review demonstrates that polyelectrolyte

LbL films are capable not only of immobilizing bioactive substances but also of opening various routes to control their release, e.g., by remote release with noninvasive stimulus, which is a challenge in medicine. Remote light activation of LbL films can serve future bioapplications in which high loading capacity together with remote-release functionalities are demanded. Additionally, LbL films provide a surface of biomaterials with physical and chemical properties that are desirable for controlling the interaction with living cells. 3D structures (free-standing LbL films or capsules) have been developed and open new perspectives for the formulation of microparticulate material with defined size and surface chemistry, enabling different administration routes and release capabilities.

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