Multiple Functionalities of Polyelectrolyte Multilayer Films: New Biomedical Applications

By Thomas Boudou, Thomas Crouzier, Kefeng Ren, Guillaume Blin, and Catherine Picart*

The design of advanced functional materials with nanometer- and micrometer-scale control over their properties is of considerable interest for both fundamental and applied studies because of the many potential applications for these materials in the fields of biomedical materials, tissue engineering, and regenerative medicine. The layer-by-layer deposition technique introduced in the early 1990s by Decher, Moehwald, and Lvov is a versatile technique, which has attracted an increasing number of researchers in recent years due to its wide range of advantages for biomedical applications: ease of preparation under “mild” conditions compatible with physiological media, capability of incorporating bioactive molecules, extracellular matrix components and biopolymers in the films, tunable mechanical properties, and spatio-temporal control over film organization. The last few years have seen a significant increase in reports exploring the possibilities offered by diffusing molecules into films to control their internal structures or design “reservoirs”, as well as control their mechanical properties. Such properties, associated with the chemical properties of films, are particularly important for designing biomedical devices that contain bioactive molecules. In this review, we highlight recent work on designing and controlling film properties at the nanometer and micrometer scales with a view to developing new biomaterial coatings, tissue engineered constructs that could mimic in vivo cellular microenvironments, and stem cell “niches”.

1. Introduction

In the field of implantable biomaterials and tissue engineered constructs, the bulk properties of materials are usually recognized as being important for the overall properties, such as mechanical strength, of the materials. Surface properties, however, are of utmost importance as they have an influence on subsequent tissue and cellular events, including protein adsorption, cell adhesion, and inflammatory response,[31] all these events being necessary for tissue remodeling. Considerable efforts are thus currently devoted toward the functionalization of the biomaterial surfaces commonly used in biomedical applications (typically metals, polymers, ceramics) in order to provide them with new functional biological properties and to render them more biomimetic. Here, biomimetic indicates an attempt to reproduce the self-organization of natural matrices. In particular, one of the major aims is to recreate the complex cell structure and environment at various length scales: from the cell membrane structure and pericellular coat (also called glycocalyx), which are important for signal transduction and the mechanical and chemical sensing of the cell,[32] to the extracellular matrix (ECM) composed of an entangled and hydrated network of proteins and glycosaminoglycans.[33] To this end, thin film coatings deposited on the “bulk” materials offer great potentialities. Designing thin films with nanometer scale control over their internal structures while preserving the bioactivity of embedded molecules and adjusting their delivery is thus a great challenge, in particular when the delivery must be performed under physiological conditions (limited pH range, fixed ionic strength, presence of physiological fluids and cells). Not only are thin films of utmost importance for the biomedical field, but also in many other fields such as electronics, optical devices, sensors, and catalysis. Several techniques have thus been developed to design thin films at the molecular level, including Langmuir–Blodgett (LB) and self-assembled monolayers (SAMs). As already indicated by Kotov and co-workers in his review,[34] both present a certain number of limitations and disadvantages. The most problematic are probably the limited amounts of biological molecules incorporated into LB films due to their limited stability, their monolayer nature, and the need for the presence of thiols on the substrate (e.g., for only noble metals or silane) in order to deposit SAMs. For biological applications, there is a thus a need for easier and more versatile deposition methods.

The layer-by-layer (LbL) method initially introduced by Moehwald, Decher, and Lvov 15 years ago consists in alternately depositing polyelectrolytes that self-assemble and self-organize
on the material’s surface, leading to the formation of polyelectrolyte multilayer films (PEM). The procedure is simple and in principle applicable to many different kinds of substrate. In the first 10 years of the development of this technique for biomedical applications, the proofs of concept were given and different types of films containing charged species were successfully prepared, including biological molecules (polypeptides, polysaccharides, DNA, proteins, viruses) and various kinds of nanoparticles (clay platelets, carbon nanotubes, etc.). In a second phase of the technical development, the behavior of the cells deposited on films began to be explored in 2001. At the same period, a different mechanism for film growth, namely exponential growth, was discovered, which opened up new opportunities for the design of thick films with reservoir capacities. Over the past five years, possibilities for the spatio-temporal control of cell growth have emerged and the first in vivo studies have been performed. It is only recently that more complex cell processes such as cell differentiation have begun to be explored and controlled by means of PEM films. Overall, the possibilities for using a wide range of polyelectrolytes and nanoobjects combined with the advantages offered by PEM coatings, such as spatial confinement and localized delivery, as well as protective effects on exposure to physiological media and external stresses, considerably enrich the biological applications for PEM films. Several reviews that include the biological field have been published in the last three years. They concern either the internal structure of the films[10–12] or the applications of PEM films at the nanoscale.[13] These applications can be for controlled erosion,[14] protein inspired nanofilms,[15] polyelectrolyte blends,[16] and biomedical applications including drug delivery, biosensors, biomimetism, and tissue engineering.[4] In this review, we focus our attention on the design of PEM films for biomaterial surface coatings and tissue engineering. We will attempt to provide readers with a global view of the biological applications for PEM films as a template for tissue mimetism and as biomaterial coatings that have been achieved within the last five years. This will include a survey of the physical and chemical properties that have emerged as being key points for controlling the nanostructure in relation to biological processes. It will also include the different possibilities for controlling cell behavior by means of film composition, bioactivity, mechanical properties, and spatio-temporal or three-dimensional organization (Scheme 1).

2. Multilayer Formation

The driving forces behind polyelectrolyte deposition using the LbL technique in order to form PEM have been already widely described and reviewed.[12] These include electrostatic as well as non-electrostatic interactions, including short-range interactions such as hydrophobicity,[17] hydrogen bonds,[18] Van der Waals forces, charge transfer halogen interactions,[19] and possibly covalent bonds formed by click chemistry.[20] Thus, both the intrinsic properties of the polyelectrolytes themselves (structure of the polyelectrolyte, charge density, chain stiffness) and the physical and chemical properties of the suspending medium (presence and type of salt, pH) are key parameters. Importantly, it is now acknowledged that the driving force behind multilayer formation is not only of electrostatic origin, but also the gain in entropy due to the release of counterions,[12,21] very similar to what is observed in the formation of polyelectrolyte complexes (PECs).[11]

### Scheme 1

Schematic view of the different levels of controls that can lead to PEMs with defined functionalities.
2.1. New Methods for Depositing and Anchoring

Various depositing methods have already been proposed for LbL buildup, including dip coating,[22,23] spin coating,[22,23] and spraying.[24] The most common to date is probably dip coating. Kotov and co-workers[25] recently developed a new dewetting method, which appears to be efficient, economical, and fast and could be used to create unique adsorption topographies, including fractal networks and aligned fibers. For future use and industrial applications of LbL films, the total time required for film preparation and the anchorage of the layer to the underlying substrate are probably important constraints. Therefore, particularly rapid methods such as spraying are being further developed.[26] Spray depositing was found to be effective even under conditions for which dipping failed to produce homogeneous films (e.g., extremely short contact times). Moreover, it was found that the rinsing step could be skipped, thus making it possible to speed up the whole buildup process.

Regarding anchorage to the underlying substrate, a recent development by Messesmith and co-workers[27] opens the way to possible to speed up the whole buildup process.

![Diagram](https://www.advmat.de)

**Figure 1.** Layer-by-layer assembly on polytetrafluoroethylene (PTFE). A) XPS spectra of bare PTFE (top), after the first cycle assembly of PEI-cathecol/HA-cathecol (PEI-C/HA-C, middle), and after three cycles (bottom). B) Surface composition of fluoride (F1s) as a function of the number of LbL deposition cycles of PEI-C/HA-C. C–E) Wetting of water on bare PTFE (C, Ψ = 106°), PTFE after three cycles of LbL assembly using PEI-C and HA-C (D, Ψ = 19.7°), and PTFE after three cycles assembly using PEI and HA (E, Ψ = 53.4°). Reproduced with permission from [27]. Copyright 2008 Wiley-VCH.

2.2. Growth Mode: Linear Versus Exponential

The first investigated polyelectrolyte systems were described by Decher et al.[5] These systems showed linear growth of both mass and film thickness with the number of layers deposited. Poly(styrene sulfonate)/poly(allylamine hydrochloride) (PSS/PAH) films are one of the most prominent examples of linearly growing systems.[28] These films have a stratified structure, each polyelectrolyte layer interpenetrating only its neighboring layers.

Films that grow exponentially have been described more recently by Elbert et al.[29] and Picart et al.[30] for poly(L-lysine)/alginate (PLL/ALG) and PLL/hyaluronan (PLL/HA) films. Initially, this type of growth was mostly observed in films based on polyaminoacids and polysaccharides[31–33] before being found to be much more commonly encountered. Either film roughness[34] or polyelectrolyte diffusion in and out of the film[9] were found to be at the origin of this growth (see Section 3.4), this latter mechanism being more and more recognized as being a key feature in film growth, even for films made of synthetic polyelectrolytes such as polyacrylic acid (PAA).[35,36] Polyelectrolyte diffusion can be easily visualized by confocal laser scanning microscopy (CLSM) when the films are thick enough (at least 1 μm). This was shown by observing PLLFITC[9] or end-labeled PAA[35] diffusion. A mechanism for this diffusion has also been proposed.[37] According to a theoretical model by Kankare, PLL/HA and CHI/HA films should always grow exponentially.[38] Recently, the exponential growth mechanism has seen some significant developments. Films containing inorganic sheets can thus also show exponential growth (Fig. 2).[39] In addition, the growth can be amplified by pH leading to even thicker films obtained in a very limited number of depositing cycles.[40] Better understanding of the different growth mechanisms is also emerging.[41] For instance, Porcel et al.[41] showed that a transition from exponential to linear growth occurs at a certain level in film buildup. It also appears that, even for synthetic polyelectrolyte films, exponential growth becomes dominant when NaCl concentrations increase[34] or when temperature is increased.[42] Interestingly, isothermal titration microcalorimetry investigations indicate that the linear growth regime is associated with exothermic complexation, whereas the exponential growth regime relates to endothermic complexation.[43]

Few studies have systematically investigated the influence of polymer molecular weight (MW) on the physical properties of PEM, whether for synthetic polyelectrolytes[44] or for natural polyelectrolytes. One of the reasons is the difficulty in obtaining monodispersed polymers, especially for natural polyelectrolytes which have very diverse production methods and are subjected to natural variation. Kujawa et al.[45] found that CHI/HA film thickness increased when the MW of these polysaccharides was higher. However, this effect was only attributed to a difference in film growth onset and not to actual differences in mass deposited per layer. Lynn and co-workers[15] used the (PAH/PAA) synthetic film model to underline the importance of MW in the buildup process. With lower MW PAA, they observed that growth approached that of exponentially growing systems. The use of low...
MW PAA instead of a higher MW (the one commonly used and commercially available) made it possible to diffuse it within these films, resulting in thicker and exponentially growing films. Such differences in diffusion were also observed by Porcel et al.,[46] who studied MW changes in the PLL/HA system. Changes in PLL and HA MW did not significantly affect the mass deposited per layer, but the use of high MW PLL did restrain PLL diffusion within the upper part of the film. Most of the investigations that focused on the influence of polymer length essentially studied the influence on the film growth curve and thickness, with other properties, mechanical, for example, rarely investigated.[47] Overall, both the nature of diffusing specie and its length are important.

3. Internal Structure of PEM Films

The close relationship between structure and the bulk properties of PEM films has created great interest in studying the details of their internal structure. One of the challenges in this area is to understand the driving force that makes multilayer buildup possible, another is to comprehend the arrangement of these polymers within the films and the dynamics of these phenomena, yet another is to identify the parameters that allow control over these properties. In 2006, von Klitzing[12] extensively reviewed the role of multilayer film structure in their buildup, density profile, water and ionic content, as well as mechanical properties and reaction to external stimuli. Here, we will focus on two important aspects of PEM internal structure: charge matching and polyelectrolyte secondary structure.

3.1. Charge Matching

The quantity and type of ions present within PEM are recognized as being critical parameters with respect to multilayer growth and physical properties in many PEM systems.[48] These effects are driven by the details in the interactions of these ions with the aqueous solvent and charged polymers. Ions from the Hofmeister series, a classification of ions going from cosmotropic (water structure making, highly hydrated ions) to chaotropic (water structure disrupting, weakly hydrated ions), were used in several studies to assess the importance of the ion type. It was found that thickness,[49] stiffness,[50] and swelling behavior[51] could all be modified by changing ion type.

The quantitative aspect was first underlined by Schlenoff et al.[52] who introduced the concept of intrinsic (polyelectrolyte–polyelectrolyte matching) versus extrinsic (polyelectrolyte–counterions matching) charge compensation. The film’s global electroneutrality is assured by the presence of counterions, which are displaced by oppositely charged polyelectrolytes upon complexation. Since then, most studies have focused on synthetic PAH/PSS films and have concluded that, as these films have a stoichiometry of about 1:1, they are thus mostly intrinsically compensated.[53] This indicates that they are relatively insensitive to doping by increased amounts of NaCl.[54] Techniques such as Fourier transform infrared spectroscopy (FTIR), neuron reflectometry, or fluorescence probes[55] have made it possible to probe the internal structures of the films and to directly quantify ion content. Jaber and Schlenoff[54] showed, using infrared active
perchlorate salt, that the residual amount of counterions in PAH/PSS and poly(dimethyldiallyl ammonium chloride)/PSS (PDDA/PSS) films is of the order of 3–6%. The free energy of association between the polymer segments was found to be higher for the former than for the latter (−10 kJ mol\(^{-1}\) vs. 5 kJ mol\(^{-1}\)). Recently, neutron reflectometry was also used to measure ion distribution in PAA/PAH films.\(^{[56]}\) A smaller number of studies concerned weakly charged and more hydrated natural polyelectrolytes such as polypeptides and polysaccharides. In a recent work, Crouzier and Picart\(^{[57]}\) compared various films made from PLL as polycation, and three different polysaccharides with increasing charge density and sulfate content (HA < chondroitine sulfate < heparin). Charge pairing was indirectly deduced by quantitative chemical analysis of the films by FTIR. Interestingly, and independently of the polyanion charge density, these films presented a ratio of 2:1 PLL over polyanion molar stoichiometry (on a monomeric basis). The net charge of the film directly depended on the charge density of the polyanion. For HA-based films, it was calculated that 46% of PLL positive charged were unpaired and compensated with Cl\(^{-}\) counterions (Fig. 3). On the contrary, PLL/heparin (PLL/HEP) films presented an excess negative charge, which was compensated by Na\(^{+}\) ions.

Understanding the role of counterions in multilayer films has also shown itself to be useful in more application-based studies. Salt etching PEM, which consists in detaching the soluble PEC caused by increased ionic strength within the film, has attracted attention. It makes it possible to adjust film degradability by controlling salt concentrations. Cohen and co-workers\(^{[58]}\) have reported this phenomenon in (PAA/PAH) films and made a proof of concept by forming a thickness gradient using this method. Such gradients could prove to be useful in the study of cell processes, as discussed in Section 5 of this review. Others have studied salt etching in films whose tightness is weaker,\(^{[59]}\) which resulted in more drastic degradation of the films.

### 3.2. Secondary Structure

From the wide variety of polyelectrolytes used to form PEM, polypeptides have the capacity to form secondary structures such as α helix or β sheets similar to what is seen in proteins.\(^{[60]}\) The secondary structure of PEM components can be considered to be an important parameter when designing films as a means of responding to biological questions.\(^{[61]}\) Studying the film’s secondary structure will also allow better understanding of self-assembly processes as well as allowing better control over its physical and chemical properties. Secondly, film structure may influence subsequent protein adsorption on the films due to specific interactions between secondary structures in the films and in the protein, or more complex natural processes such as calcium carbonate biomineralization.\(^{[62]}\)

Most studies have focused on the measurement of the secondary structure of polypeptide films made of polyaminoacids or polypeptides. Techniques such as UV spectroscopy, circular dichroism spectroscopy, and FTIR are usually employed to determine the structural properties of the films. Several parameters will affect the secondary structure of the film’s constituents, including the structural properties of the polyelectrolytes used for assembly\(^{[63–65]}\) as well as solvent properties. As previously mentioned, this includes ionic strength,\(^{[61,66]}\) pH,\(^{[61,66,67]}\) type of solvent,\(^{[68]}\) hydration,\(^{[69]}\) and temperature.\(^{[67]}\) Boulmedais et al.\(^{[63]}\) and Haynie and co-workers\(^{[64]}\) showed, for example, that adding a (PSS/PAH) layer on top of a [PLL/poly(l-glutamic)acid; PLL/PGA) film significantly changed the secondary structure of the film. The changes were different depending on whether PSS or PAH was deposited first. This phenomenon was seen as indirect proof that PSS and PAH diffuse within these films, interact with PLL/PGA and influence its internal structure. However, polypeptide films made with low MW-designed polypeptides showed little disruption in secondary structure, meaning they were more resistant to PSS/PAH diffusion.\(^{[64]}\)

### 3.3. Polyelectrolyte Blends

The nature and intrinsic properties of the polyelectrolytes used for PEM buildup will dictate their “bulk” properties. Thus, controlling the chemical composition of PEM also makes it possible to tailor their physical properties. With this as the goal, constructing PEM using polyelectrolyte blends appears to offer...
new possibilities for the modulation of film thickness, film morphology, and secondary structure,\textsuperscript{70,71} degradation rates, protein adsorption,\textsuperscript{156} or even mechanical properties. In 2008, a review by Caruso and co-workers\textsuperscript{72} emphasized the advantages of these new types of film and shed light on the first development made in this domain. Examples of applications in the fields of biology or nanoporous thin film preparation were given. Here, only a brief description of their physical properties will be given.

When designing a PEM blend, controlling the chemical composition of the film is of uppermost importance, as it is the origin of the desired, tailored properties of the film. This is usually done using FTIR spectroscopy. In many cases, preferential incorporation of one of the two polyanions in the film has been reported. This was seen for PLL/PSS–HA film\textsuperscript{73} and more recently for \(\beta\)-1,3-glycan sulfate (GlyS) and ALG-based films.\textsuperscript{74} In the case of PLL/GlyS–ALG film, GlyS was found to insert preferentially into the film and it almost totally exchanged the ALG when brought into contact with a (PLL/ALG) multilayer. The preferential incorporation was explained by the higher charge density of GlyS polymers over ALG and by the presence of sulfate groups in GlyS that interact more strongly with the ammonium groups in GlyS than the carboxylic groups present on the ALG chains. For other films, the differences in chemistry between the two polyanions can be more subtle,\textsuperscript{76} but still show preferential incorporation. This highlights the delicate balance that dictates PEM formation. The details of polyelectrolyte arrangement within blend films are still unresolved and difficult to investigate. One of the unanswered questions is the reason why in films such as PLL/PSS–HA blends, the proportion of HA in the PLL/PSS–HA increases monotonously with mass proportion of the HA in solution, whereas thickness does not.\textsuperscript{77} This is probably the result of a particular molecular organization of the polyelectrolytes within the films.

### 3.4. Diffusion of the Film Components

Interdiffusion, exchange of polyelectrolytes within LbL assemblies as well as diffusion of external molecules within films can be dominant processes that need to be well understood in order to rationally design films with well-defined structural properties. Diffusion phenomena are also of prime importance for loading and releasing bioactive molecules.

The vertical diffusion of one of the polyelectrolyte constituents of the film, which is related to the growth of exponentially growing films, was visualized by confocal microscopy.\textsuperscript{79} An estimate of the diffusion coefficient was obtained based on optical waveguide light mode spectroscopy (OWLS) data.\textsuperscript{80} Interestingly, in certain PEM couples, it appears that both polyanions and polycations can diffuse, as is the case for PLL/PGA films.\textsuperscript{75} Quantification of the translational (e.g., lateral) diffusion coefficients has been obtained based on fluorescence recovery after photo-bleaching (FRAP)\textsuperscript{76,77,78} or after pattern photo-bleaching (FRAPP) measurements.\textsuperscript{78} The diffusion coefficient is very low (<5 \(\times\) \(10^{-9}\) \(\text{m}^2\text{s}^{-1}\)) for systems that grow in a linear manner, such as PSS/PAH and PSS/PDDA, and it was found for these systems that the charge density along the polymer is the most important parameter controlling the formation of polymer complexes.\textsuperscript{77} Mean PLL diffusion coefficient in PLL/HA films that grow exponentially is about three orders of magnitude larger (i.e., \(\approx 0.2\ \mu\text{m}^2\text{s}^{-1}\)), but three different types of PLL molecules have been evidenced: highly mobile chains at the film surfaces (\(D \approx 1\ \mu\text{m}^2\text{s}^{-1}\)) and two kinds of embedded chains: mobile chains (\(D \approx 0.1\ \mu\text{m}^2\text{s}^{-1}\)) and almost immobile chains (\(D < 0.001\ \mu\text{m}^2\text{s}^{-1}\)).\textsuperscript{78}

Related to the diffusion of a film constituent is the exchange of polyelectrolytes within the film, when the film is in contact with a solution of a third type of polyelectrolyte. Exchange can be followed easily using FTIR spectroscopy due to the specific chemical signature of each polyelectrolyte. This was first evidenced by Boulmedais et al.\textsuperscript{79} who showed that PSS chains could diffuse within a PLL/PGA film and exchange with PGA chains. These observations were corroborated by further experiments using PAH in solution on PGA/PAH films containing multivalent ions\textsuperscript{80} and on PLL/HA films.\textsuperscript{81} In this latter case, PLL was quantitatively exchanged with the PAH chains and transformed into a PAH/HA film, whereas a PAH/HA multilayer remained stable in the presence of a PLL solution. Of note, it was shown by Jomaa and Schlenoff\textsuperscript{82} that self-exchange of isotopically labeled polycarboxylic acid within a polyelectrolyte multilayer is reversible (Fig. 4) whereas similar exchange with PSS, which forms non-labile PECs, is slow and irreversible but is facilitated by polyvalent ion pairing interactions of a third polyelectrolyte. The half-time of the exchange of PSS alone was 60 min and it decreased to 3 min in the presence of PMA in solution (even at concentrations that were four times lower).\textsuperscript{82}

Diffusion and exchange were found to depend on steric and thermodynamical parameters. Diffusion is sterically limited as molecules have to have the space to move through the entangled polymers network. Zacharia et al.\textsuperscript{83} found, for example, that higher MW PEI undergoes displacement much more slowly than low MW PEI. Also, many parameters such as salt type and concentration, polymer charge density or the nature of the chemical groups present on the polymers, will greatly affect the polymer/polymer affinities, in turn affecting diffusion and exchange phenomenon. To illustrate, the interdiffusion and exchange of a series of polyanymes with pre-assembled multilayer films was investigated.\textsuperscript{83} They found that fully charged polycations in dilute aqueous solutions were unable to diffuse through the multilayer film whereas partially charged polycations did have the necessary mobility. There was the same critical degree of ionization in solution (almost 70% in the exchange experiments with polyhexyl viologen (PXV), below which interdiffusion was possible. The kinetics of interdiffusion were significantly impacted by the polion degree of ionization and MW.

Of note, interdiffusion could be hindered by incorporating “blocking layers” or barriers by depositing dense films such as PSS/PAH,\textsuperscript{84} cross-linked films\textsuperscript{80} or degradable polymer layers consisting in poly(lactic-co-glycolic acid).\textsuperscript{85}

Interestingly, diffusion could also be employed for ordering viruses at the film surface. Belcher and co-workers\textsuperscript{86} investigated the depositing of rigid M13 viruses in LPEI/PAA films and evidenced that the interdiffusion present in these superlinearly growing systems favored virus ordering at the film surface. In addition, depending on the pH of the virus solution, close-packed structures, loosely packed structures and sparsely ordered structures were formed. Virus ordering could be controlled by manipulating the deposit pH of the underlying polyelectrolyte.
multilayers.\textsuperscript{87} Using FRAP, the diffusion coefficient of the viruses was estimated at $D = 0.1 \text{ m}^2 \text{s}^{-1}$ for FITC-labeled viruses assembled on polyelectrolyte multilayers. Controlling diffusion phenomena is thus of prime importance and can be employed for charging and subsequently releasing molecules inside the film, depending on pH and ionic strength for instance.

3.5. Diffusion of Molecules Inserted into Films (Dyes, Growth Factors)

Delivery of bioactive molecules (ions, drugs, proteins) often relies on diffusive processes that will depend on the film’s internal structure and nanometer scale porosity. Such processes are also extremely present in real tissues for the delivery of nutrients.\textsuperscript{88}

For instance, in the case of polyelectrolyte multilayer capsules, diffusion is a method for loading molecules within the capsule’s interior and investigating their permeability using fluorescent dextrans.\textsuperscript{89} The diffusion of small model molecules, such as dyes, in planar LbL films is often investigated. Kharlampieva and Sukhishvili\textsuperscript{90} found for hydrogen-bonded multilayers that dye loading capacity was largely affected by the nature of the polyelectrolyte on top of the film (of opposite charge to that of the dye). An extraction of the dye through the film by polymers in solution contributed significantly to dye release in the case of electrostatically assembled films. Control of entrapment and the diffusion of silver (Ag) ions have attracted great interest due to the anti-bacterial properties of these ions (see Section 6.3).

Thick films that are hydrated and/or porous are particularly suitable for being used as reservoirs for active compounds. Using PLL/HA film as a matrix, Vodouhe et al.\textsuperscript{91} observed, using CLSM, that paclitaxelGreen 488 molecules diffused throughout the whole (PLL/HA)$_n$ film section and that the fluorescence was homogeneously distributed over the whole thickness of the film (12 $\mu$m). A similar strategy was employed by Schneider et al., who loaded cross-linked PLL/HA films with the anti-inflammatory drug sodium diclofenac and with paclitaxel. The amount of drug loaded could be adjusted by varying the film’s thickness.\textsuperscript{92} Paclitaxel-loaded films were efficient in killing cells, as less than 10% of the cells were still alive after 3 days of culture.\textsuperscript{93} Control of film porosity at the micrometer and nanometer scales is an important step toward controlling diffusion. Rubner showed that it is possible to adjust the amount of ketoprofen and cytochalasin D released from films by varying the number of layers in the porous regions of films, and the release rate depended on film pore size.\textsuperscript{94}

Larger molecules, such as proteins, can diffuse within or at the surface of films, depending on the film’s structural properties. Thus, albumin was found to diffuse in and on PSS/PAH films.\textsuperscript{95} Recently, the diffusion of Staphylococcal nuclease (SNase) was investigated by neutron reflectometry.\textsuperscript{96} It was shown that the SNase is partially penetrating (or diffusing) into a PSS terminating polyelectrolyte multilayer upon adsorption. This penetration is preferred when the positive charge of the protein is high.

Release of bioactive molecules or of film components can be triggered by a wide variety of stimuli, such as ionic strength, light, temperature, and sensitivity to hydrolysis. The various methods developed for controlling each of these parameters have been reviewed in detail by De Geest et al. in their recent review\textsuperscript{97} on polyelectrolyte capsules and by Kotov and co-workers.\textsuperscript{4} It appears that film porosity, a certain level of hydration and diffusion are important to allow for molecule mobility.

4. Modulation of Mechanical Properties

In recent years, designing PEM with adjustable mechanical properties has become a major challenge for applications in chemistry, physics, and biology. The characterization of their viscoelastic properties is thus crucial and several methods that are specific to thin films have been employed. A common one is to
perform nanoindentation experiments by means of atomic force microscopy, possibly using a colloidal probe as indenter.\[98,99\] Several other methods, relying on different physical principles, are well suited to characterizing thin films, some of them in a liquid state. These include quartz crystal microbalance\[50,100\] piezo-rheometry,\[101\] resonance frequency,\[102\] bulging tests,\[103\] the pendant drop technique,\[104\] osmotic pressure,\[105\] or the capillary wave technique\[106\] in the case of free-standing films. The stiffness of PEM can be modulated from a few kPa to several GPa depending on the structural properties of the polyelectrolytes, but also on the degree of cross-linking inside the film. In fact, both ionic cross-links, affected mostly by pH and ionic strength as well as covalent cross-links induced by chemicals or photo-activation will impact film stiffness. It can also be observed that the stiffness of a native multilayer film is also related to the buildup regime in the LbL process. Films that grow exponentially are generally considered to be softer than those with a linear buildup.\[101\] For instance, PLL/HA native films are rather viscous and Young's modulus \( (E_0) \) for cross-linked films reaches a maximum of 500 kPa, whereas PSS/PAH microcapsules in water are 300 MPa.\[107\]

4.1. Structural Mechanical Properties

A first means of modulation consists in modifying the film's internal structure by using polyelectrolytes with different conformations such as carrageenans. Schoeler et al. and Schönhoff et al. thus characterized the stiffness of PEM containing PAH as the polycation and two different anionic sulfated polysaccharides, \( \iota \)-carrageenan, which forms helical structures, and \( \lambda \)-carrageenan, which has a random coil conformation. Using AFM indentation, they found that films prepared with \( \iota \)-carrageenan were about three times stiffer than those with \( \lambda \)-carrageenan, highlighting the strong influence of polyelectrolyte structures on the film's rigidity.\[108\] In a similar way, grafting phospholipid,\[109\] or sugar molecules, such as lactose or mannose\[110\] on to one of the polyelectrolytes can significantly influence the film's stiffness. However, in contrast to pH, ionic strength may also soften as more salt was added.\[125\] Nolte et al.\[126\] and Tang et al.\[111\] demonstrated that PEMs undergo substantial swelling and softening as more salt was added.\[125\] Nolte et al.\[126\] and Tang et al.\[111\] demonstrated that PEMs undergo substantial swelling and softening in the presence of ambient water.

4.2. Influence of the pH

One of the many attractive features of PEM is the extent to which their mechanical properties can be tailored by varying the conditions used to assemble the films. Because of the pH-dependent dissociation of the weak acidic and alkaline functional groups on the chains, films prepared from weak polyelectrolytes (only partially charged at moderate pH near their pK) are strongly modulated by the pH and ionic strength environment. Mermut et al.\[120\] thus showed that Young’s modulus of films made of PAH and an azobenzene-containing polyelectrolyte was reduced from 6.5 to about 0.1 MPa when the assembly pH increased from 5 to 9. Several other groups, in particular the Rubner group, investigated the remarkable nanoscale control that can be exercised over the properties of (PAH/PAA) films (i.e., stiffness, thickness, roughness, wet-ability, and swelling behavior), by varying the pH conditions used to assemble the films.\[121,122\] In brief, PAH/PAA films assembled at a relatively neutral pH are significantly thinner and about two orders of magnitude stiffer than those assembled in acidic conditions.

4.3. Influence of Ionic Strength

Ionic strength has been shown to be a powerful parameter for controlling PEM properties, particularly their permeability and rigidity. However, in contrast to pH, ionic strength may also attenuate intermolecular interactions in a film composed of strong polyelectrolytes by screening the electrostatic charges. Looking for enhancement of drug delivery using PEM, Fery et al.\[123\] examined the formation of nanopores in PAH/PAA multilayer films through salt-induced structural changes and showed considerable softening of PAH/PSS when the salt concentration increased.\[124\] Working with PEM containing PSS and poly(diallyldimethylammonium), Schlenoff’s group observed similar behavior, with the films swelling, smoothing, and softening as more salt was added.\[125\] Nolte et al.\[126\] and Tang et al.\[111\] demonstrated that PEMs undergo substantial swelling and softening in the presence of ambient water.

4.4. Chemical Cross-linking

It is also possible to adjust the mechanical properties of PEMs by chemical means, for instance by creating covalent cross-links within the films. As an example, it is already known that high temperature (130 °C) can induce the formation of amide or imide bonds within films.\[127\] A protocol based on the carbodiimide chemistry for cross-linking carbonyl groups with amine groups in “mild” conditions (room temperature, salt-containing medium), thereby forming covalent amide bonds (also called peptide bonds), was proposed by Richert et al.\[128\] and Schuetz and Caruso.\[129\] Of note, the carbodiimide used [1-ethyl-3-(3-dimethylaminopropyl)]carbodi-
The error bars represent the standard deviation of 6–16 measurements of An exponential asymptotic fit to the data is also represented (thick line). The error bars represent the standard deviation of various approach velocities. Reproduced with permission from [134]. Copyright 2006 Wiley-VCH.

4.5. Photo-cross-linking

A few recent approaches to adjusting the mechanical properties of PEMs are based on photo-cross-linking. One major advantage to photo-cross-linking is that it offers the possibility of patterning PEMs. Yang and Rubner[138] extensively demonstrated the proofs of concept for this type of cross-linking. In recent studies, they synthesized a photo-cross-linkable weak polyanion poly(acrylic acid-ran-vinylbenzyl acrylate) (PAArVBA) and associated it with PAH to make films. Native and photo-cross-linked films were found to exhibit similar thickness trends but, while both films swelled, the cross-linked sample reached a plateau at 20% of the dry thickness, whereas the native sample continued to swell to roughly double the height of the cross-linked films.[139] Park et al. used similarly photo-cross-linkable benzophenone modified PAH or PAA in association with PSS. They showed in particular that the release of rhodamine B from these films could be controlled by the degree of induced cross-linking.[140]

Pozos-Vásquez et al. also reported on the preparation of polyelectrolyte films based on PLL and HA derivatives modified by photoreactive vinylbenzyl (VB) groups. The VB-modified HA incorporated into the films was cross-linked on UV irradiation and the force measurements taken by atomic force microscopy proved that the rigidity of the cross-linked films was increased up to fourfold.[141]

5. Control of Cellular Adhesion and Proliferation

The numerous possibilities for adjusting the chemical, physical, and mechanical properties of PEM films have fostered studies on the influence of these parameters on cell behavior.

Adhesion is the first cell event that occurs when a cell comes into contact with a material’s surface.[142] Importantly, adhesion influences subsequent cell events such as proliferation and differentiation.[143] Thus, the fine tuning of these initial events is a considerable challenge in the field of biomaterials. For bioengineers working on 3D biomaterials, cell adhesion is already recognized as being a multiparametric event that can be influenced by numerous cues such as specific chemical recognition via peptide sequences present in the ECM proteins, surface topography (roughness, presence of microstructures), surface hydrophobicity, and mechanical properties.[144] Thus, naturally, several attempts have already been made to investigate whether each of these individual parameters can be controlled in PEM films and affect cellular adhesion. Besides being used for specific applications, PEM films may also act as a new type of biomimetic material, shedding light on fundamental biological processes.

Here, we will focus on five different strategies used to control cell adhesion and proliferation: purely synthetic films which promote adhesion via non-specific interactions, anti-adhesive (or non-fouling) surfaces that prevent protein adsorption and cell adhesion, PEM made from ECM components used as building blocks, PEM coated with ECM proteins and PEM functionalized with specific ligands.

5.1. PEM Films Based on Synthetic Polyelectrolytes

Synthetic polyelectrolytes such as PSS (a strong polyelectrolyte), PAA, or PAH have been widely used in cell/film studies. In this case, initial cell adhesion is mostly mediated through electrostatic
interaction and, more indirectly, via serum proteins adsorbed on to the films. The main advantages of using synthetic polymers are the possibility of specifically adjusting certain parameters and how easy they are to modify chemically.

The most frequently studied synthetic PEM is, by far, linearly growing and dense PSS/PAH films. Cell types such as endothelial cells,[143] fibroblasts,[146,147] osteoblastic cells[148] and hepatocytes[149] have been cultured on these films. As a general rule, adhesion and proliferation on these films are very good, which may be attributed partly to the presence of sulfonate groups. The importance of such groups was evidenced in hepatocytes by using films made from PDDA and PSS.[150] The hepatocytes adhered only on the films terminated with a PSS layer and not on PDDA ending films. However, other cell lines, such as fibroblasts, were less sensitive and adhered on both the PDDA and PSS. Of note, certain serum proteins present in the cell culture medium, such as bovine serum albumin (BSA), adsorb on to the PSS-ending films, although only weakly.[151] PAH/PAA films are also widely studied. PAA-ending films were found to be resistant to the adsorption of BSA, fibrinogen or even to lysozyme, which is oppositely charged to PAA.[152] This was explained by the low charge density of PAA but also by its strong hydration that creates an exclusion volume above the PAA layer.

Usually, proteins adsorb preferentially on to films of opposite charge.[152,153] However, it now seems to be accepted that protein adsorption cannot account for the significant differences in cell adhesion.[122,154] For PAH/PAA films, the non-adhesiveness of the films built at pH 2 and the high adhesion observed for films built at pH 6.5 were instead attributed to the ability of the former to swell.[122]

Synthetic polymers were also employed by Salloum et al.[155] for investigating the combined effects of increasing surface charge and hydrophobicity on vascular smooth muscle cell (SMCs) adhesion. On the most hydrophobic surfaces, the A7R5 SMCs spread and were not very motile, whereas on the most hydrophilic surfaces, these cells adhered poorly and displayed characteristics of being highly motile.

If synthetic polymers are to be used for in vivo implantations, their possible toxicity must also be evaluated. The biocompatibility of a single PEI layer was tested on both fibroblastic and osteoblastic cells. Pure titanium (Ti) and nickel–titanium (NiTi) alloy were coated with PEI and morphology, adhesion, and viability were assessed for up to 7 days after seeding. The results show that the cells were less viable and proliferated less on PEI-coated titanium than on the control, suggesting that PEI is potentially cytotoxic.[156] On the other hand, PSS/PAH films deposited on human umbilical arteries showed good grafting behavior and no inflammation in a rabbit model after 12 weeks of implantation.[157] Systematic studies for each specific case are thus required.

5.2. Non-fouling PEM Films

Non- or low-fouling surfaces are interesting in biomedical applications for two reasons. They can be an effective way of controlling cellular and bacterial adhesion by enhanced resistance to serum proteins. Rendering a surface non-fouling can also serve as immuno-“camouflage” to prevent immune rejection of implanted biomaterials or to enhance the efficiency of injected drug delivery vehicles. The proteins adsorbed on a material's surface may in fact be denatured, or the material's surface itself may present epitopes that could be recognized as foreign by the immune system and will thus induce immune reactions.[158] Derivatives of polyethylene glycol (PEG), a highly hydrated polymer, are very effective in rendering surfaces non-adsorbent to proteins and have been widely used since the 1990s to modify various substrates.[159] Compared to the standard chemical grafting techniques used for PEG surface functionalization, multilayer film depositing has the advantage of being rather independent of the nature or topology of the material. Thus, PEM films have been constructed using PEG-grafted polymers[160] or by depositing a PEG layer on top of the films,[161] yielding a non-fouling multilayer film. In a more biomimetic approach, phosphorylcholine (PC) and ethylene oxide (EO)3 groups, which are naturally non-fouling components of erythrocyte membranes, have been grafted on to a (PSS/PAH) film, thereby lowering protein adsorption.[162] The non-fouling properties were attributed to the zwitterionic properties of the molecules. Films containing natural polysaccharides are often non-fouling and non-adhesive for cells (see Section 5.3) due to their high water content and softness.

One of the advantages of non-fouling PEM is their ability to create a so-called “blank slate,”[163] e.g., a non-adhesive surface that can be subsequently modified by covalently grafting adhesion peptides. Cooper-White and co-workers[163] presented cytophobic PEM made from high MW hyaluronic acid and CHI which was resistant to serum protein adsorption. Upon covalent grafting of collagen (COL) IV on top of the films, the films switched to cytophilic. A similar strategy based on “click” chemistry was recently proposed by Caruso and co-workers.[20] Alkyne or azide groups incorporated into the polymer are used to create covalent linkages between polymer layers or between polymers and other molecules under mild conditions.[20] In this study, they designed low fouling PEG acrylate multilayers on to which they “clicked” an RGD peptide. Monkey kidney epithelial cells adhered and grew only on the RGD-functionalized PEG films.

5.3. Extracellular Matrix Components as Building Blocks

A step closer to recreating the original matrix into which cells develop in vivo is to use ECM components as building blocks for the films. One advantage of these natural components is their bioavailability and their possible biodegradability, as specific enzymes are present in tissue and biological fluids. Thus, besides being used as natural mimics, they can be potentially employed as biodegradable delivery systems.

PEM made of ECM proteins such as COL[164,165] or gelatine (GEL)[166] and of glycoaminoglycans such as HA,[160] CS,[33,167] and HEP[168,169] have been reported. Other polysaccharides, which are not present in the human body but can be found in algae, crustacean shells, or fungi, are also used. This is the case for ALG[29,170] dextran sulfate (DEXS)[171] and CHI[32,170,172] which are already being used in tissue engineering[173,174] and have also been the subject of several studies. However, it is difficult to find a
general rule concerning cell behavior for such films, as this depends both on the properties of the film (thickness, hydration, mechanical properties) and cell type. For instance, PEM made from highly hydrated polysaccharides from a combination of polysaccharides and polyaminoacids often yield gel-like films. Some cells are known to adhere poorly to hydrated surfaces and materials that are too soft. This was indeed observed for chondrosarcoma cells, chondrocytes, and osteoblast adhesion on to films such as PLL/HA, CHI/HA, PLL/ALG, or PLL/PGA. However, for certain cell types, softness is preferred. For instance, neuronal cells were found to adhere to COL/HA films. The outer layer chemistry has been found to be important in some cases as COL ending films showed improved adhesion, but COL was not necessary for adhesion. Cortical neurons and hippocampal neurons were sensitive to a different surface chemistry. Similarly, Tetzner et al. showed that photoreceptor cells exhibit good viability on PLL/HA and PLL/CSA films. Shen and co-workers also reported that photoreceptor cells exhibit good viability on PLL/HA and PLL/PGA. However, for certain cell types, softness is preferred. For instance, neuronal cells were found to adhere to COL/HA films.

So far, it appears that there is limited understanding of the detailed molecular mechanisms of cell adhesion on to these biomimetic films. In particular, there is only a study that aimed to characterize the types of integrins involved in cell adhesion, PSS/PAH films being taken as substrate.

5.4. ECM Protein Adsorption on to PEM Films

Several studies have tried to increase cell adhesion on PEM films by coating the final layer with adhesion-promoting molecules such as fibronectin (FN), vitronectin (VN), or COL that are known to engage specific cell receptors. In this paragraph, we will report studies that deal with cell interactions on the ECM protein modified films. The first step of these studies is to characterize and quantify protein adsorption on the films. The underlying idea is to give an additional functionality to the films by enhancing cell interactions while preserving the “bulk” properties (biodegradability, mechanical properties, etc.). Wimmer et al. added a final FN layer to PLL/DEXS films and found that higher amounts of FN were adsorbed on positively charged PLL ending films. Human umbilical vein endothelial cells spread to a greater extent and more symmetrically on FN-coated films. They also concluded that the presence of FN is a more important factor than film charge or layer number in controlling the interactions between multilayer films and living cells. Goldstein and co-workers quantified FN adsorption on to PAH/HEP films at various pH solutions and found the highest FN adsorption at pH 8.4, which they attributed to a charge effect. However, adsorbed amount of FN was not the sole factor explaining the differences observed in cell adhesion strength. Semenov et al. showed recently that FN adsorbed on to cross-linked PLL/HA films promoted focal adhesion formation and was critical for maintaining densely grown mesenchymal stromal cell cultures over weeks for their differentiation.

A similar study was conducted on PAH/PSS films by McShane and co-workers. After coating the films with FN or GEL, they observed a general increase in the adhesion and proliferation of SMCs. However, they also noted that these properties depend on the number of layers in the PEM, meaning that not only outer layer chemistry but also film bulk nanostructure control cellular adhesion.

5.5. Grafting of Specific Peptides or Ligands for Recognition

Another strategy for selectively improving cell adhesion on PEM films is to graft peptides that are known to interact with specific cell adhesion receptors. Basically, this idea underlying this strategy is similar to the previous one except that the method is different: here, only short sequences of the ECM proteins are considered and have to be covalently grafted to one of the polyelectrolyte. This requires first a synthetic chemistry step. The most prominent example is that of the RGD sequence. Kessler and co-workers reviewed the considerable number of strategies that have been developed to immobilize RGD on polymers, RGD being a central integrin-binding region in FN and COL. Using PEM, a new strategy consists in grafting the peptide to one of the polyelectrolytes and then adsorbing the modified polyelectrolyte as a regular layer. PEM films exhibiting a poor adhesion are excellent candidates for such functionalization, which was applied using PAH–RGD and PGA–RGD for cell attachment. Recently, in an elegant work by Werner et al., it was shown that a laminin 5 derived peptide grafted to PGA could induce specific cell adhesive structures in epithelial cells called hemidesmosomes and activate β4 integrins (Fig. 6). Using a different strategy, HA/CHI films on to which cells adhered poorly were rendered adherent by functionalization with an RGD containing peptide using carbodiimide chemistry. The immobilized RGD was shown to have a beneficial influence on osteoblast adhesion and proliferation. Other peptides, such as α-melanocyte-stimulating hormone (α-MSH), with anti-inflammatory properties, have also successfully been integrated into multilayer films. Initially coupled with PLL, α-MSH was effective toward melanoma cells that were induced to produce melanocytin. Then, coupled with PGA and introduced into PLL/PGA films, it was efficient in annihilating the effect of a bacterial endotoxin that stimulated an inflammatory response in human mononuclear cells. The morphology of the monocytes was also affected by α-MSH as the cells formed many “fibber like” protrusions not visible on standard PLL/PGA films.

Importantly, however, the question has been raised as to whether these chemical modifications in the polyelectrolytes alter other physical chemical properties such as protein adsorption or mechanical properties, in turn influencing cell adhesion and proliferation. This is supported by recent findings by Thompson et al. and Schneider et al. who measured the mechanical properties of the films with or without modified polyelectrolytes.

5.6. The Role of Film Mechanical Properties

It is increasingly accepted that cell processes depend on the reciprocal and dynamic interactions of cells with their surrounding microenvironment, which include biochemical and mechan-
The same group recently showed that the adhesion and proliferation of human microvascular endothelial cells (HOEC) were independent of the substrata’s chemical composition. They found that, on unmodified PAH/PAA surfaces, hepatocyte attachment increased with PEM rigidity.[195] But this trend was canceled when the PEM substrata was modified with COL I or with COL I pre-mixed with the small proteoglycan decorin (Fig. 7). They also demonstrated that hepatic albumin secretion (a marker for liver-specific protein synthesis) over 2 weeks decreased with increasing substrata stiffness, correlating that hepatocytes formed stable, spheroid aggregates preferentially on protein-modified compliant surfaces, whereas cells detached from stiffer substrata after only a few days of culture. Such detachment was presumably due to the dominance of cell/cell over cell/substrate interactions (Fig. 7B).[195]

As previously described in Section 4.4, Picart et al. developed an alternate strategy for tuning film mechanical properties using a simple water-based chemical cross-linking method applicable to PEM films that contain amine and carboxylic groups. For PLL/HA films, fine-tuning the EDC cross-linker concentration in contact with the films made it possible to vary the film’s stiffness over two orders of magnitude.[134] Interestingly, chondrosarcoma cells, initially poorly adherent on native PEM, adhered, spread, and proliferated on the cross-linked films, with a strong dependence of all these parameters on the cross-linking extent. This adhesion “switch” appeared to be a property common to many film types, including PLL/HA,[128,130,196,197] CHI/HA,[198] PLL/P(/Q)H,[110,199] and PLL/poly(galacturonic acid).[199] This change in adhesive properties was observed for a wide variety of cell types, including chondrocytes,[196] chondrosarcomas,[110] macrophages,[132,196] neurons,[196] osteoblasts,[199] SMCs,[128] and skeletal muscle cells.[197]

Wittmer et al.[151] also focused their work on human liver tissue engineering applications. Using different cells and film types, they investigated the effects of the terminal layer, film cross-linking (using the EDC protocol previously mentioned), and COL adsorption. Cross-linked PLL/ALG and PLL/PGA films supported the attachment and function of adult rat hepatocytes, independently of the terminal layer, provided that the COL was adsorbed on to the films. PAH/PSS, cross-linked PLL/ALG, and cross-linked (PLL/P(GA))<sub>n</sub>–PLL films all supported the attachment, layer confluence, and function of human fetal hepatoblasts, with the latter films promoting the greatest level of function at 8 days. Overall, film composition, terminal layer, and rigidity are found to be key variables in promoting the attachment and function of hepatic cells, while film charge and biofunctionality are somewhat less important.

These research papers, studying different cell types on different PEM films, highlight on the one hand the strong dependence of cell processes on both the mechanical and chemical stimuli defined by neighboring cells and extracellular matrices.[144] In particular, the decoupling (or independent adjusting) of the mechanical and chemical properties has already been achieved, using model synthetic gels such as polyacrylamide (PAAm) gels coated with COL at increasing densities.[195]

Very interestingly, with a similar approach to that already established for PAAm gels, Van Vliet and co-workers[194] also investigated how modulation of the mechanical properties of cell substrata via control of the assembly pH of the films may affect cell function. The stiffness of PAH/PAA films assembled at different pH was varied over several orders of magnitude, independently of the substrata’s chemical composition. They showed that the adhesion and proliferation of human microvascular endothelial cells strongly increased as the PEM became stiffer.[194] The same group recently adjusted independently the mechanical and chemical properties of films by modifying the film surface with COL I or a mixture of COL I/decorin. Primary rat hepatocytes were chosen for this purpose. These cells are widely considered to be ideal for constructing liver tissue models but are known to rapidly lose their viability (within a few hours or days) and phenotype functions upon isolation from the native in vivo microenvironment of the liver. They found that, on unmodified PAH/PAA surfaces, hepatocyte attachment increased with PEM rigidity,[195] but this trend was canceled when the PEM substrata was modified with COL I or with COL I pre-mixed with the small proteoglycan decorin (Fig. 7). They also demonstrated that hepatic albumin secretion (a marker for liver-specific protein synthesis) over 2 weeks decreased with increasing substrata stiffness, correlating that hepatocytes formed stable, spheroid aggregates preferentially on protein-modified compliant surfaces, whereas cells detached from stiffer substrata after only a few days of culture. Such detachment was presumably due to the dominance of cell/cell over cell/substrate interactions (Fig. 7B).[195]

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chemical properties of the substrata, and on the other hand, the difficulties for decoupling these two distinct properties. It effectively remains possible that substrata stiffness may not only define the initial microenvironment that influences cell adhesion, but also the subsequent capacity of cell-generated proteins and ligands to be produced and/or absorbed on to the PEMs as in an auto-amplification loop. These correlative factors need to be addressed further in the future.

6. PEM Coatings of Biomaterials: From In Vitro to In Vivo Studies

6.1. PEMs for Implantable Biomaterials

For applications in the fields of implantable biomaterials and tissue engineering, films are used as surface coatings with the aim of providing an additional functionality for the original materials or engineered tissue. Several aspects therefore appear particularly important:

- for coating the surfaces of biomaterials (metals, synthetic, or natural polymers, ceramics), it is first important to characterize the coating of the film on the materials and then to investigate the behavior of specific cell types, depending on the planned application. Table 1 summarizes all the studies on various materials as initial substrates. They are classified according to the kind of application: bone, vascular tissue, dental tissue, neuronal tissue, pancreas, tracheal prostheses, and general engineering applications. It appears that bone and vascular tissue engineering are the fields that have attracted the greatest number of studies. All the studies presented in this table were performed in vitro.

- Secondly, the design of the surfaces with a targeted functionality is a considerable challenge. It is possible that adhesive peptides or ECM proteins may enhance specific cell adhesion (see Section 5). The growth factors embedded in films can provide both a new functionality and a way to adjust cell differentiation. The studies concerning the beneficial effect of various proteins and growth factors are presented in Table 2.

- Finally, in vitro studies must be followed by in vivo studies to investigate the effects of the coatings in a more complex biological environment and for a specific purpose. The in vivo studies on PEM films began to emerge only four years ago and the number seems to have grown rapidly in 2009 (four articles already published this year). Table 3 shows all the in vivo studies to date. Figure 8 shows as an example a PEM-coated titanium prosthesis that had been implanted in rats. This further proves that PEM films are currently the subject of major developments into applications in the field of biomaterials.

6.2. Anti-coagulation

Natural polyelectrolytes can potentially be used in multilayer films due to their intrinsic bioactivity. DEX and HEP can be used for their anti-thrombogenicity. HA can be used for its high water retention capacity. CHI/DEX films were found to exhibit anti-coagulant properties only when dextran was the outermost layer of the film and when the films were built in 0.5 M NaCl or 1 M NaCl.[172] On the other hand, CHI/HEP films built in 1 M NaCl also exhibited strong anti-coagulant activity regardless of the outermost surface of the film.[172] This type of multilayer film thus has good potential for the surface modification of medical implants in contact with blood.

The thromboresistance of a (CHI/HA),-coated NiTi substrate was also demonstrated by Thierry et al.[200] These films were found to significantly reduce platelet adhesion by 38% after 1 h of exposure to platelet-rich plasma. On the contrary, the adhesion of polymorphonuclear neutrophils on to the coated surface increased slightly, compared to bare metal.

Hemocompatible albumin/HEP coatings were bound to be anti-coagulant and to reduce platelet adhesion drastically.[201] The coated PEM greatly prolonged the plasma recalcification time. The films were also tested for their ability to potentiate thrombin inhibition by anti-thrombin and its dependence on the layer arrangement.[202] The order of activity of the surface-bound HEP matched their order in solution; however, their activity was reduced to less than 10% due to binding. Increasing the number of layer pairs was found to increase the activity of the coatings, suggesting that heparin inside the assemblies was available for the interaction.

6.3. Antibacterial Properties

The use of CHI in anti-bacterial dressings has received considerable attention in the last decade.[173] The exact anti-bacterial mechanism of CHI is still unknown. One mechanism proposed is based on the interaction between positively charged CHI molecules and negatively charged microbial membranes causing leakage of intracellular constituents.
<table>
<thead>
<tr>
<th>Application</th>
<th>Substrate</th>
<th>Film Light</th>
<th>Cells</th>
<th>Main findings</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone</td>
<td>Ti</td>
<td>(PLL/DNA),</td>
<td>Osteoblasts</td>
<td>Deposition of calcium phosphate was enhanced on DNA coatings</td>
<td>[275]</td>
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<td></td>
<td></td>
<td>(PAH/DNA)</td>
<td></td>
<td>Formulation of carbonate apatite</td>
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<tr>
<td></td>
<td>Ti</td>
<td>CHI/GEL</td>
<td>Osteoblasts</td>
<td>Increased viability and proliferation</td>
<td>[276]</td>
</tr>
<tr>
<td></td>
<td>Ti</td>
<td>CHI/HA films</td>
<td>Osteoblasts</td>
<td>No adhesion for native CHI/HA film, enhanced adhesion in the presence of the peptide for CHI ending surface only</td>
<td>[189]</td>
</tr>
<tr>
<td></td>
<td>Ti</td>
<td>+ RGD X-linked at the surface</td>
<td>S. aureus</td>
<td>Decreased adhesion and enhanced anti-bacterial activity of CHI/HA films (w or w/o RGD)</td>
<td></td>
</tr>
<tr>
<td>Electrospun PCL fibers</td>
<td>Gelatin/PSS coated with bone like calcium phosphate</td>
<td>Osteoblasts</td>
<td>Enhancement of cytocompatibility</td>
<td>[277]</td>
<td></td>
</tr>
<tr>
<td>Bone marrow</td>
<td>PLA scaffold</td>
<td>PEI/GEL</td>
<td>Chondrocytes</td>
<td>Increased adhesion and growth</td>
<td>[279]</td>
</tr>
<tr>
<td></td>
<td>PDMS (microfluidic channels)</td>
<td>(PDDA/clay)/(COL/FN)</td>
<td>Murine bone marrow cells</td>
<td>Automated μfluidic perfusion system and increased cell attachment and spreading</td>
<td>[280]</td>
</tr>
<tr>
<td></td>
<td>PA hydrogel ICC scaffolds</td>
<td>PDDA/Clay platelets</td>
<td>Hematopoietic stem cells</td>
<td>Expansion of CD34+ stem cells and B-lymphocyte differentiation</td>
<td>[272]</td>
</tr>
<tr>
<td>Vascular engineering</td>
<td>PET filaments, thread and prostheses</td>
<td>(PSS/PAH), (PLL/PGA), (PLL/PA)</td>
<td>Osteoprogenitors</td>
<td>Change in surface wettability</td>
<td>[278]</td>
</tr>
<tr>
<td></td>
<td>PCL</td>
<td>CHI-CL-gPEG/HEP</td>
<td>Platelets</td>
<td>Increased proliferation</td>
<td>[282]</td>
</tr>
<tr>
<td></td>
<td>PTFE</td>
<td>PSS/PAH</td>
<td>Endothelial cells</td>
<td>Enhanced adhesion and spreading on the films</td>
<td>[283]</td>
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<td></td>
<td>Vascular artery</td>
<td>CHI/HA</td>
<td>—</td>
<td>Film coated in situ in the artery in physiological conditions</td>
<td>[200]</td>
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<td></td>
<td>Cryopreserved artery</td>
<td>PAH/PSS</td>
<td>Endothelial cells</td>
<td>Prevention of platelet adhesion</td>
<td>[284]</td>
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<td></td>
<td>NiTi</td>
<td>Photo X-linked ALG/HEP/ p-diazonium diphenylamine polymer</td>
<td>—</td>
<td>Adhesion and spreading of endothelial cells</td>
<td>[282]</td>
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<tr>
<td></td>
<td>Stainless steel</td>
<td>CHI/HEP, HA/HEP</td>
<td>—</td>
<td>Longer blood clotting time than pure steel substrates, Control of releasing rate of Sirolimus by the number of layers</td>
<td>[285,286]</td>
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<td></td>
<td>Polyethersulfone</td>
<td>HEP/ALB</td>
<td>Leucocytes</td>
<td>Reduced activation of coagulation</td>
<td>[287]</td>
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<td></td>
<td>Neuronal tissue</td>
<td>CHI/HEP</td>
<td>Platelets</td>
<td>Decrease in the number of viable bacteria</td>
<td>[204]</td>
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<tr>
<td></td>
<td>PCL</td>
<td>HEP/ALB</td>
<td>Leucocytes</td>
<td>Reduced blood level of complement fragment C3a</td>
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<td></td>
<td>Neuronal tissue</td>
<td>CHI/HEP</td>
<td>Neuroblastoma and glioma</td>
<td>Immunological activity of anti-TGFβ1 preserved</td>
<td>[288]</td>
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<tr>
<td></td>
<td>Neuronal tissue</td>
<td>HYAFT 11</td>
<td>—</td>
<td>Good attachment and neuronal differentiation</td>
<td>[112]</td>
</tr>
<tr>
<td></td>
<td>Free standing membrane</td>
<td>SWNT/PAA</td>
<td>—</td>
<td>Guidance of neurites outgrowth</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Neuronal tissue</td>
<td>Polyethyleneterephthalate</td>
<td>—</td>
<td>Efficient encapsulation of islets in thin films while preserving cell viability and insulin release function</td>
<td>[289]</td>
</tr>
<tr>
<td></td>
<td>Neuronal tissue</td>
<td>Islets of Langerhans</td>
<td>—</td>
<td>Immobilization of enamel matrix derivate (EMD) both on top and within PEM films</td>
<td>[291]</td>
</tr>
<tr>
<td></td>
<td>Dental applications</td>
<td>Ti and SiO₂</td>
<td>—</td>
<td>Specific formation of adhesive structures (hemidesmosomes) in the presence of the peptide</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Porous Ti</td>
<td>(PLL/PGA) and laminin-derived peptide</td>
<td>Epithelial cells</td>
<td>In vitro, the films enhance epithelial cell colonization and proliferation</td>
<td>[188]</td>
</tr>
</tbody>
</table>
Table 1. Continued.

<table>
<thead>
<tr>
<th>Application</th>
<th>Substrate</th>
<th>Film</th>
<th>Cells</th>
<th>Main findings</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMMA discs</td>
<td>(CHI/HA)</td>
<td></td>
<td></td>
<td>Enhanced resistance to enzymatic degradation of X-linked films</td>
<td>[133]</td>
</tr>
<tr>
<td>Ti</td>
<td>(PAH/PSS), (PLL/HA)</td>
<td>Fibroblasts</td>
<td>Enhanced adhesion and proliferation on (PAH/PSS) films</td>
<td>[146]</td>
<td></td>
</tr>
<tr>
<td>Tracheal prostheses</td>
<td>Ti</td>
<td>(PLL/PGA+aMSH)</td>
<td>Chondrosarcomas</td>
<td>More stable adhesion (focal contacts) on negatively charged or uncoated surfaces</td>
<td>[292]</td>
</tr>
<tr>
<td>Ti</td>
<td>(PSS/PAH), (PLL/PGA)</td>
<td>Endothelial cell</td>
<td>Enhanced spreading and proliferation of endothelial cells via activation of VEGFR2 receptors and downstream kinases</td>
<td>[293]</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Summary of the different studies using a bioactive protein loaded in LbL films (X-linked means cross-linked).

<table>
<thead>
<tr>
<th>Bioactive molecule proteins/growth factors</th>
<th>Film type</th>
<th>Main findings</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein A (PLL/PGA)</td>
<td></td>
<td>Expression of TNF-a in THP-1 phagocytic cells</td>
<td>[294]</td>
</tr>
<tr>
<td>BDNF and Sema3 [a]</td>
<td>(PSS/PAH)</td>
<td>Increased neuronal activity</td>
<td>[295]</td>
</tr>
<tr>
<td>aFGF/bFGF [b] (aFGF/heparin)/PEI</td>
<td>CSA/bFGF</td>
<td>Enhanced expression of collagen I and IL-6</td>
<td>[296]</td>
</tr>
<tr>
<td>bFGF or IPM (PLL/CSA)</td>
<td></td>
<td>Increased in the number of photoreceptor cells attached</td>
<td>[33]</td>
</tr>
<tr>
<td>BMP2/TGFb1 [c]</td>
<td>(PLL/PCA)</td>
<td>Specific differentiation of embryonic stem cells</td>
<td>[266]</td>
</tr>
<tr>
<td>BMP4/noggin (PLL/PGA)</td>
<td></td>
<td>Inhibition or induction of cell death in tooth development</td>
<td>[267]</td>
</tr>
<tr>
<td>BMP-2</td>
<td>(PLL/HA) X-linked films</td>
<td>Dose-dependent differentiation of myoblasts in osteoblasts</td>
<td>[268]</td>
</tr>
<tr>
<td>VEGF [d]</td>
<td>(PSS/PAH)</td>
<td>Pro-angiogenic prosthetic coating</td>
<td>[293]</td>
</tr>
</tbody>
</table>


Table 3. Summary of the studies investigating the properties of LbL films in vivo.

<table>
<thead>
<tr>
<th>Film type and experimental model</th>
<th>Main findings</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>(PLL/PGA) uMSH films deposited on tracheal prostheses</td>
<td>Fibroblastic colonization of the peripheric site and respiratory epithelium on the internal side</td>
<td>[274]</td>
</tr>
<tr>
<td>(CHI/HA) native and X-linked films in a rat mouth model</td>
<td>Much slower degradation of X-linked films</td>
<td>[133]</td>
</tr>
<tr>
<td>(CHI/HA) native and X-linked in a mouse peritoneal cavity</td>
<td>Partial degradation of CL films</td>
<td>[248]</td>
</tr>
<tr>
<td>(PLL/ALG)–PC coated ALG microcapsules</td>
<td>Adherent cells at the periphery of the capsule</td>
<td>[228]</td>
</tr>
<tr>
<td>PAH/DNA or Poly(ε-lysine)/DNA</td>
<td>Uniform tissue response</td>
<td>[297]</td>
</tr>
<tr>
<td>In rats</td>
<td>Induction of fibrous tissue capsules for all the conditions</td>
<td>[188]</td>
</tr>
<tr>
<td>(PLL/PGA)-laminin 5 derived peptide or CL films on porous titanium</td>
<td>Focal contact formation on CL films</td>
<td>[272]</td>
</tr>
<tr>
<td>(PDDA/clay platelet) films in inverted colloidal crystals</td>
<td>High vascularization around the construct</td>
<td>[272]</td>
</tr>
<tr>
<td>Bis-ureio-surfactant and DNA coating in rats</td>
<td>Presence of both mature and immature precursor cells</td>
<td>[298]</td>
</tr>
<tr>
<td>(CHI/HEP) film on stainless steel coronary stents in pigs</td>
<td>Increase of bone to implant contact after 1 week</td>
<td>[299]</td>
</tr>
<tr>
<td>Simulated body fluid DNA-based coating was found to increase both early and late peri-implant bone response</td>
<td>Improved anti-coagulation properties</td>
<td>[299]</td>
</tr>
</tbody>
</table>
Bacterial adhesion (E. coli Gram-negative strain) was investigated on certain types of natural-based multilayer films containing CHI and/or HEP. (CHI/HA)$_{10}$ films (built in 0.15 m NaCl) are highly resistant to bacterial adhesion and lead to a $\approx 80\%$ decrease in bacterial adhesion as compared to bare glass.\textsuperscript{[12]} On the other hand, (CHI/HA)$_{20}$ films built in $10^{-2}$ m NaCl were less resistant to bacterial adhesion (40% less than control on the CHI ending films and 20% less on the HA-ending films). The differences observed were explained by the lower thickness of the (CHI/HA) films built in $10^{-2}$ m NaCl (120 nm as compared to 300 nm for those built in 0.15 m NaCl). CHI/k-carrageenan films were found to have a greater effect in decreasing initial bacterial adhesion than a thinner CHI coating, which was explained by the greater hydration of the multilayer films.\textsuperscript{[203]} Adhesion of negatively charged enterococci was slightly enhanced on CHI-terminated multilayers, but the anti-bacterial effect was absent on k-carregan-terminated multilayers.

Heparin with its anti-thrombogenicity and strong hydrophilicity also prevented adhesion of bacteria. The anti-bacterial properties of films containing both polysaccharides have thus been explored. CHI/HEP multilayer films were found to kill the bacteria that had adhered to the surface. Initial E. coli adhesion was also greatly decreased on the multilayer films.\textsuperscript{[204]} The assembly pH was found to be an important parameter in the design of efficient anti-adhesive and anti-bacterial films.

In order to enhance the anti-bacterial effect of multilayer films, the same authors prepared films containing silver nanoparticles and coated with a polyethylene terephthalate graft with alternating layers of CHI/HEP, CHI being complexed with silver nanoparticles of 10–40 nm in size.\textsuperscript{[205]} The multilayer films containing nanosilver not only had effective anti-bacterial properties but also had an anti-coagulant coating, while remaining non-toxic for the cells. Other types of film, such as PGA/lysozyme, were also found to inhibit bacterial adhesion.\textsuperscript{[206]}

Other strategies rely on the use of anti-fungal peptides embedded in films,\textsuperscript{[207]} possibly incorporated by means of an amphiphilic polyelectrolyte pre-complex with an hydrophobic peptide\textsuperscript{[208]} or on Ag$^+$ ions as bactericides. In a first approach, Rubner and co-workers\textsuperscript{[209]} prepared hydrogen-bonded multilayers containing Ag nanoparticles synthesized in situ and that were deposited on both planar surfaces and magnetic colloidal particles. The duration of the sustained release of anti-bacterial Ag ions from these coatings was prolonged by increasing the total supply of zero-valent silver in the films via multiple loading and reduction cycles. Later, the same authors\textsuperscript{[210]} constructed thin films with two distinct layered functional regions: a reservoir for the loading and release of bactericidal chemicals and a nanoparticle surface cap with immobilized bactericides. This resulted in dual-functional bactericidal coatings with both a chemical-releasing bacteria-killing capacity and a contact bacteria-killing capacity. These dual-functional coatings showed very high initial bacteria-killing efficiency due to the release of the Ag ions and retained significant anti-bacterial activity after the depletion of the embedded Ag because of the immobilized quaternary ammonium salts. Another strategy consists in loading silver ions into liposomes and subsequently embedding liposome aggregates into PLL/HA films.\textsuperscript{[211]} The strong bactericidal effect observed was attributed to the diffusion of the silver ions out of the AgNO$_3$ coating, leading to a significant bactericidal concentration close to the membrane of the bacteria.

### 6.4. Localized Gene Therapy of DNA and RNA

The controlled delivery of DNA complexes from PEMs offers the potential to enhance gene transfer by maintaining an elevated concentration of DNA within the cellular microenvironment with an appropriate polyelectrolyte film carrier that will facilitate DNA introduction. There are already several very interesting reviews about gene delivery through Lbl method.\textsuperscript{[14,212]} Here, we will highlight recent studies that present new advances in this field.

Jessel et al.\textsuperscript{[213]} reported the fabrication of substrates containing β-cyclodextrin–DNA (CD–DNA) complexes embedded in a...
PEM film in which specific expression of nuclear or cytoplasmic proteins is selectively and sequentially produced. These CD–DNA complexes adsorbed on PEM films acted as an efficient gene delivery tool to transfect cells. Synthesis of new cationic polymers is one of important developments in the field of PEMs for gene delivery. Lu et al.\cite{214} reported a biodegradable polycation poly(2-aminooethyl propylene phosphate), which could form multilayers with plasmid DNA (pDNA) and lead to prolonged pDNA delivery up to 2 months. Reducible polycations such as those containing disulfide bonds are also of interest for triggering gene release from PEM films under reductive conditions in the presence of DTT for instance.\cite{215} Cai et al.\cite{216} reported multilayers of galactosylated CHI and pDNA. Because the galactose group is a specific ligand for the asialoglycoprotein receptor (ASGP-R) of hepatocytes, these films have a specific higher transfection rate on hepatoma G2 cells. An elegant strategy for bi-functionalization of PEM films was developed by Meyer et al.\cite{217} These authors show that it is possible to functionalize PEM films both for cell transfection and for activation via a peptide signaling pathway. Toward this end, they prepared films containing pDNA pre-complexed with PEI and a peptide molecule NBPSMH. This peptide, grafted to PGA was used as a signal molecule for melanoma cells B16-F1 and for its ability to enhance gene delivery in a receptor-independent manner.

Another development relies in the incorporation and functionality of other gene materials, such as viral vectors and silencing.\cite{218,219} Recently, Dimitrova et al.\cite{219} demonstrated, using hepatitis C virus infection (HCV) as a model, that siRNAs targeting the viral genome were efficiently delivered by PEM films. This delivery method resulted in a marked, dose-dependent, specific, and sustained inhibition of HCV replication and infection in hepatocyte-derived cells. Comparative analysis demonstrated that delivery of siRNAs by the films was more sustained and durable than siRNAs delivery by standard methods, including electroporation or liposomes. The anti-viral effect of siRNAs films was reversed by a hyaluronidase inhibitor, suggesting that active degradation of films by cellular enzymes is required for siRNA delivery.

7. Biomimetism Using PEM Films

7.1. Mimicking the Properties of Natural Materials

Mimicking natural materials and the cell microenvironment is some of the possibilities offered by LbL films. Mimics of artificial nacre made of organic/inorganic structures such as nanoplatelets or clays have been designed and studied by the Kotov group.\cite{111,220} Recently, it was observed that 1,3,4-dihydroxyphenylalanine (DOPA) molecules impart unusual adhesive strength to the LbL clay composite and the hardening mechanism found in the natural cement.\cite{221} Superhydrophobic surfaces also attract lots of attention and are inspired by the natural designs of the Lotus leaf or insect wings. Ji et al.\cite{40} prepared superhydrophobic films using films that grow exponentially with growth that was amplified due to the presence of silver ions. Other strategies rely on organic–inorganic hybrid films, such as PAH and ZrO\textsubscript{2} nanoparticle coatings\cite{222} in combination with the LbL technique with electrochemical deposition of dendritic gold aggregate coatings on gold threads\cite{223} or on a polyelectrolyte/sodium silicate combination.\cite{224} In these latter cases, a final fluorination step\cite{223} was usually applied in which a fluoroalkylsilane was deposited. More complex hydrophilic patterns on superhydrophobic surfaces were also designed to mimic the Namib Desert beetle.\cite{225}

7.2. Biomimetic Membranes Using Lipid and PEM Films

All cell membranes are composite materials made of a lipid bilayer and of various types of protein, either intrinsic proteins (e.g., inserted into the lipid bilayer) or extrinsic (e.g., anchored or adsorbed on to the inner or outer surface of the lipid bilayer). Recently, lipid membranes deposited on LbL films have begun to be manufactured and studied. A planar biomimetic model membrane made of a lipid bilayer (dimyristylophosphatidylycerine, DPPC) supported by PSS/PAH multilayers was studied by neutron reflectivity.\cite{226} The membrane was deposited only on a negatively charged polyelectrolyte surface. Similarly, Pilbat et al.\cite{227} recently showed that a DPPC bilayer can be deposited on PLL/PGA films and that further layers can be deposited on top of the lipid bilayer. The phase transition of the embedded DPPC was decreased. Interestingly, gramicidin D, a soluble peptide, was successfully incorporated into DPPC films, with the lipid films, either with or without gramicidin, being thermally stable.

An elegant application of a biomimetic membrane for coating ALG hydrogel microcapsules in order to improve tissue response when implanted in vivo was recently presented by Chaikof and co-workers.\cite{228} The mimetic membrane was made of two PLL/ALG layer pairs, an amphiphilic terpolymer anchored to the PEM film and a phospholipid (PC) film, which is made by fusion of unilamellar vesicles on to octadecyl chains of the terpolymer. The biocompatibility of the membrane-mimetic-coated microspheres was high (87.5%) and it resisted cell and fibrotic overgrowth. The biostability of the coating was evidenced by confocal observations after 4 weeks of implantation.

In a recent work, Kujawa et al.\cite{229} combined polysaccharides and lipids and characterized PEM films made of HA and phosphorylcholine chitosan (PC-CHI) by quartz crystal microbalance (QCM-D) and surface plasmon resonance (SPR). These films exhibited fluid gel-like characteristics that were attributed to their high water content (50 wt %). Composite films made of polyelectrolytes, lipids, and viruses have also been explored by Donath and co-workers.\cite{230} These authors showed that viruses can fuse successfully on a lipid-coated LbL composite. The virus can also be patterned on to a PAH surface and specifically recognized by antibodies against the Influenza virus used.

A very elegant application of these new composite PEM films/lipid membranes has been presented by Battle et al.\cite{231} An innovative engineered ion channel, gramicidin (bisgA), incorporated into the lipid membrane coating on a PSS/PAH microcapsule, was found to provide a functional capacity for controlling transport across the microcapsule wall. The microcapsules provided transport and permeation for drug-analog neutral species, as well as positively and negatively charged ionic...
species. This controlled transport could be adjusted by controlling the gating of incorporated bisgA ion channels.

8. Spatio-Temporal Control of Cells by LbL Films

In particular, cell/cell as well as cell/matrix interactions are regulated by the geometry of the cell microenvironment and by the cell positioning toward one another, which are particularly important during tissue development. Therefore, a biomaterial that would allow controlling heterotypic cell/cell interactions by controlling geometry of multicellular systems would be extremely relevant for the control of stem cells differentiation and organization. The capability to control the topographic organization of the films by micropatterning can provide new insights into the molecular mechanisms underlying cell/surface interactions and cell/cell interactions. PEM films are especially useful for spatially confining cells into adhesive areas or preparing heterotypic cellular co-culture.

8.1. Single Cell Culture on Micropatterned PEM Films

One of the first applications for patterning PEM films for cell adhesion studies was performed by Rubner and co-workers on H-bonded PEM films (PAA/PAAm). They showed first that a photosensitive polyelectrolyte can be used to control spatially film cross-linking via a photo-mask and to create an adhesive cell area upon irradiation, the non-irradiated area being simply washed away with a water rinse. In a second work, these authors showed that PAH could be stamped on to a PAA/PAAm non-adhesive film, thus rendering it adhesive for fibroblasts. They subsequently grafted RGD covalently at various densities on to PAH via the amine groups by controlling the stamping conditions. Cell activity and alignment was found to be controlled by the density of the adhesive ligand.

McShane and co-workers developed a method that is based on a combination of photolithography and LbL self-assembly. Depositing a precursor layer of PDDA was followed by depositing a photore sist, selective removal, depositing of the film and finally removal of the remaining photore sist. Standard polyelectrolytes were used in combination with secreted phospholipase A2 (sPLA2), gelatin and BSA as proteins, PLL as the polypeptide. Interestingly, the proteins and polypeptides resisted the dissolution of the resin in acetone. sPLA2 was found to have a potential for binding neurons. Later on, the same group cultured cells on gelatin and FN-coated micropatterns. They observed that SMCs initially landed on the gelatin-coated surface, not on the PDDA-coated surface in between. But further growth of the cells was affected by the shape of the patterns: strip patterns limited cell growth beyond the patterns, but square patterns could not. On the contrary, the SMCs cultured on FN-coated micropatterns initially landed on the PDDA-coated surface and then migrated to both FN-coated square and strip patterns. These findings show that different adhesive proteins have different effects on the initial attachment and later growth of SMCs. A further development of the technique is to combine two lithographic steps such as to create multicompontent heterostructures of bioactive coatings on a single substrate with great precision (a few μm).

An alternative microfluidics approach was used by the same group to pattern PEM on flat, oxidized poly(dimethylsiloxane) (PDMS) surfaces by sequentially flowing polycations through a microchannel network that was placed in contact with the PDMS surface. Cell-to-cell communication between cells on adjacent PEM lines was observed in the form of interconnecting tubes containing actin that were a few hundred nanometers in diameter and up to 55 μm in length. Further use of these types of microfluidic patterning was explored for the attachment of neuron-like cells (P19 cells) derived from on to PEM films. These cells are difficult to culture and require an underlying fibroblast monolayer when cultured on bare tissue culture substrates. Patterned PEM allowed their confined attachment and directed neuronal outgrowth.

Recently, Kidambi et al. presented a new strategy to pattern the surface of pre-deposited PEM films. PEM surfaces with PDDA as the topmost layer were chemically patterned by microcontact printing (μCP) oligomeric PEG molecules with an activated carboxylic acid terminal group (m-PEG acid). These surfaces, which resisted protein and cell adhesion, could be made adhesive by removing the PEG molecules, by exposing the surface to a salt solution, without affecting the PEMs underneath the SAM.

As previously mentioned, photo-cross-linking films for cell applications was introduced by the Rubner group. Recent developments have been presented by Tsai and co-workers. These authors showed that PAA/PAAm patterns generated by means of PAA conjugated to 4-azidoaniline could be switched to adhesive or non-adhesive depending on the type of final layer added, either PAH-g-PEG or PLL added to the top of the PAA/Pam non-adhesive pattern. Besides synthetic polyelectrolytes, natural polyelectrolytes such as HA have been recently coupled with photo-sensitive groups and inserted into LbL films. This will certainly open up new possibilities for micropatterning polysaccharide-based films, which are much more fragile than synthetic ones.

8.2. Heterotypic Cell Co-cultures Using Patterned PEM Films

Cell/cell and in particular heterotypic interactions exist in vivo in several tissues and are crucial for the development, repair, and metabolism of these tissues. PEM films can be employed as a tool to mimic cell/cell interactions in vitro using two different cell types. For instance, it is possible to take advantage of the fact that one cell type prefers a certain type of surface chemistry. PEMs can thus be used to produce defined cell-resistant and cell-adhesive surfaces depending on the topmost surface and the type of cells used.

An example given by Khademhosseini et al. consists in the patterning of HA and PLL regions. Regions coated with HA are non-adhesive for murine fibroblasts or embryonic stem cells, so these cells grow on the glass substrate. Then, the surface is coated with a PLL layer, which deposits on the HA layer and is adhesive for hepatocytes. More recently, the same group patterned only molecules from the extra-cellular matrix including HA, COL, and FN. HA had high resistant properties toward FN adsorption.
and high affinity to COL. Thus, the adsorption of COL switched the HA surface from being cell-repulsive to cell-adherent without cytotoxic effects, thereby making distinctly localized co-cultures possible.

Using a similar strategy, Kidambi et al.[244] co-cultured primary hepatocytes and fibroblasts. When primary hepatocytes were seeded on top of the patterned PEM surfaces, they attached and spread predominantly on the PSS surfaces resulting in primary hepatocyte patterns. Once the hepatocytes were attached, fibroblasts were subsequently seeded and attached to the PDDA surfaces. As a result, co-culture patterns of fibroblasts and primary hepatocytes were obtained on synthetic PEM surfaces. Importantly, the hepatocytes maintained hepato-specific function much longer than the patterned single culture of primary hepatocytes.

The same group further proved the interest of this concept by co-culturing primary neurons and astrocytes on PEMs (Fig. 9).[245] PEM films ending by PSS were adhesive for neurons but not for astrocytes. After microcontact printing a layer of PDDA on top of the films, the astrocytes adhered. The authors evaluated the effect of saturated free fatty acids on this co-culture system as compared with monocultures of neurons and astrocytes by measuring the level of reactive oxygen species (ROS; a widely accepted marker for oxidative stress). The elevation in ROS levels was seen to occur earlier in the patterned co-culture. The results obtained with a patterned PEM co-culture system could provide insights into neuron cell function and perhaps even the pathogenesis of neurodegenerative diseases.

Recently, a new method, namely the room temperature imprinting technique, which relies on mechanical compression of a film, was used to pattern PAA/PAH films.[246] Cell adhesion on to these films was found to depend on the size of the line and on the space between the lines.

Owing to the important development of the micropatterning techniques and on the control of other film properties, it is envisioned that micropatterned films will provide further functionalities in the future.

8.3. Temporal Degradation

Besides spatial control of film organization, a great amount of work deals with the temporal control of film durability and, in particular, controlled degradability. This has become of upmost importance in the field of drug delivery, in which PEMs have found many applications. The different stimulus pathways used to trigger deconstruction or dissolution have already been detailed by Tang et al.[4] and Lynn[14] for PEM films and De Geest et al.[97] for PEM capsules.

A new strategy relies on the preparation of films with adjustable biodegradability, depending on the D over L-lysine enantiomer ratio in the polyelectrolyte solution.[247] THP-1 macrophages produced TNF-α when they came into contact with protein A embedded in the film. The production of TNF-α started after a varying induction time and displayed a transition from no-production to full-production, which took place over a time period that depended on both the film's composition and the embedding depth. The same type of macrophages degraded cross-linked CHI/HA films in an adjustable manner that depended on the extent of film cross-linking.[248] Of note, the initial adhesion of these macrophages was also related to the degree of cross-linking.

A development of the widely explored hydrolytically degradable polymers was recently presented by Lynn's group.[249] It consists in synthesizing “charge-shifting” cationic polymers. Liu et al. demonstrated that the addition of citraconic anhydride to PAH yields an anionic, carboxylate-functionalized polymer (called polymer 2) that can be converted readily back to cationic PAH in acidic environments. The incorporation of polymer 2 into polyelectrolyte multilayers thus provides an approach to the manufacture of films that are stable at neutral pH but that erode over a period of several days in acidic media (e.g., a pH of 5).

As proof of concept, they demonstrated that ultrathin films roughly 100 nm thick manufactured using polymer 2 sustained the release of fluorescently labeled PAH for up to 4 days when incubated at pH 5.0. Furthermore, the synthetic approach was more recently applied to the release of DNA[250] using two different DNA constructs that exhibited separate and essentially non-overlapping release profiles.

9. Toward 3D Cell Architectures

Tissue organization in vivo relies on a complex 3D organization of cells and ECM molecules. There is thus a need to go toward the third dimension for building complex cellular architectures. Controlling cell adhesion and differentiation in 3D cultures is a challenge in itself for tissue engineering applications[251] and needs combination between advanced chemistry, biology, and microtechniques.[252]
9.1. Cell Encapsulation

PEM films can potentially be employed for this purpose as they can coat various cell types or even be used to build multilayered cell architectures.

One of the first examples originated from Diaspro and co-workers[253] who reported that single yeast cells encased within PSS/PAH polyelectrolyte shells were able to maintain their viability, functionality, and normal exchange of nutrients and waste. Lbl assembly has also been used to modify platelet surfaces with antibodies as a means of investigating targeted-delivery mechanisms within the walls of blood vessel substitutes[254] and for encapsulation of E. coli cells.[255] Later on, Mills and co-workers[256] coated mesenchymal stem cells with PLL/HA layers and showed that the cells maintained their shape and viability for up to 1 week. Using platelets as model cells, Tabrizian and co-workers[257] detected cytoskeletal changes in blood platelets coated with CHI/HA multilayers by QCM-D.

Applications for cell coatings in pancreas tissue engineering have also emerged recently. Krol et al.[258] applied a nanometer-thick PAH/PSS/PAH coating to cover and protect human pancreatic islets. Macroscopically, no significant changes in the morphology of the islets were observed and their functionality was proved by insulin release. However, Chaikoff and co-workers[259] found on the contrary that these films were cytotoxic to human pancreatic islets. As an alternative coating, they employed PLL-g-PEG in combination with biotin and proved cell viability in a coating composed of 8-layer pairs.

9.2. Layered Cellular Architectures

Yarmush and co-workers[260] introduced the first development of Lbl films to generate in vitro multilayered cellular architectures. They created hybrid alternating layers of hepatocyte—CHI/DNA layers—and several other cell types including hepatocytes, endothelial cells, and fibroblasts. Importantly, they found that hepatocytes maintain their polygonal morphology and that their secretion of specific functional markers (albumin and urea) was enhanced in the layered architecture, presumably due to the presence of a second layer.

A recent development in this technique was achieved by Akashi co-workers[261] who manufactured up to four cell multilayers of fibroblasts. They also showed the potential for the technique to manufacture xenogenic cell multilayers, which are composed, as in blood vessels, of human smooth muscle and endothelial cells. This work was indeed the first example of cell multilayers constructed by controlling the thickness of the ECM nanofilms on cell surfaces.

A different strategy was used by Guillaume-Gentil et al.[262] for creating free standing cell sheets based on Lbl films. Cells were first grown to confluence on thin polyelectrolyte films and subsequently detached under electrochemical control. In the presence of PLL-g-PEG/PEG–RGD, a final layer of cells was detached from the substrate by electrochemical polarization. For films such as PLL/HA and PLL/PGA films, various cell types spontaneously detached while reaching confluence.

A very elegant strategy was recently proposed by Swiston et al.[263] for creating multilayer patches attached to lymphocyte and T-cells. The multilayer consisted in three stacks of first a labile releasable region, then a functional region (called payload) that could contain any kind of molecule, and finally a cell-adhesive region. The film was first deposited on a micropatterned substrate to allow for spatial control of the film, then the cells were allowed to adhere to it. Finally, the film was detached using a physical and chemical stimulus. In their example, HA ending films were chosen as they made possible a specific attachment of these cell types, presumably via CD44 receptors. Of note, these films combined three types of functional properties: spatially organized, specifically triggering cell adhesion, and releasable from the substrate. These films may find applications in immune system engineering such as bioimaging or lymphocyte-directed drug delivery.

9.3. Cell and Stem Cell Differentiation

Cells with capacities to differentiate and, in particular, stem cells, are currently the subject of several studies thanks to their potential applications in tissue repair in situ and tissue engineering. Stem cells in vitro or in vivo are in “niches,” a term that refers to the stem cell microenvironment.[264] These niches are found during embryonic development (embryonic stem cells) but also in the human body (adult stem cells), where the stem cells can be activated by several signals to either promote self-renewal or differentiation to form new tissues. Important characteristics within the niche are cell/cell interactions between stem cells, as well as interactions between stem cells and neighboring differentiated cells, interactions between stem cells and adhesion molecules, ECM components, growth factors, cytokines, and physiochemical nature of the environment.

Scientists are studying the various components of the niche and trying to replicate the in vivo niche conditions in vitro. This is because for regenerative therapies, cell proliferation and differentiation must be controlled in flasks, so that sufficient quantity of the proper cell type is produced prior to being introduced back into the patient for therapy. Other cells, such as skeletal muscle cells are called pluripotent in that they can differentiate in to different cell types depending on the signals received.[265]

Studies concerning stem cell adhesion, proliferation and differentiation on PEM films are just emerging. Given the possibilities for spatially controlling film topography (see Section 8), providing cell co-cultures, loading films with chemical factors such as morphogens (see Table 2) and modulating film mechanical properties, PEM films appear to have great potential for applications in stem cell-based tissue engineering.

Dierich et al.[266] were the first to show the use of PEM films for the differentiation of embryonic bodies (EBs) into cartilage and bone. A poly(l-lysine succinylated)/PGA film, into which BMP2 (bone morphogenetic 2) and TGFβ1 (transforming growth factor 1) had been embedded was chosen for this purpose. They found that both BMP2 and TGFβ1 needed to be present simultaneously in the film to trigger proteoglycan production and drive the EBs to cartilage and bone formation (Fig. 10). This constituted the first example of a multilayer whose biological activity was based on a synergy effect between two active compounds.
The same authors subsequently investigated the effect of a growth factor, BMP-4, and its antagonist, Noggin, embedded in a PLL/PGA film on tooth development. They showed that these films can induce or inhibit cell death in tooth development and that the biological effects of the active molecules are conserved. The functionalized PEMs could thus act as efficient delivery tools for activating cells. This approach shows promise as it could be used to finely reproduce architectures with cell inclusions as well as to provide tissue organization.

A recent study by Crouzier et al. gave the proof that controlled amounts of a morphogen such as BMP-2 can be trapped in a thick film made of (PLL/HA) and retain their activity. The amount of BMP2 trapped could be adjusted by varying both the number of layers in the film and the initial BMP2 concentration in solution. Interestingly, myoblast cells grown on unloaded films differentiated into myotubes in a manner that depended on the stiffness of the PLL/HA film. The same cells grown on BMP2-loaded films differentiated into osteoblasts, the expression of alkaline phosphatase (a marker for osteoblastic activity) depending on the amount of BMP2 loaded in the films (Fig. 11).

A first step in the development of nanomaterials for neural interfaces was provided by Jan and Kotov, who demonstrated the differentiation of environment-sensitive neuronal stem cells (NSCs), both as neurospheres and single cells, on a (PEI/single wall carbon nanotube) multilayer film. NSCs behaved similarly to those cultured on the standard and widely used poly(L-ornithine) substratum in terms of cell viability, development of neural processes, and appearance and progression of neural markers.

In the vascular field, an application of PEM in the endothelialization of vascular grafts has been investigated. Berthelemy et al. found that endothelial progenitors differentiated much faster on PSS/PAH multilayer films than on any other ECM that had been used up until then, including a layer of jugular venous endothelial cells. The differentiation occurred in 2 weeks as compared to 2 months for classical coatings and the cells formed an endothelium-like confluent cellular monolayer. This system is of course extremely relevant for future therapeutic approaches, but it could also provide a very interesting template for further studies on the mechanisms of endothelial cell differentiation.

In a recent study describing further efforts for providing stem cells with a biomimetic niche environment, Nichols et al. built an elegant scaffold with an inverted colloidal crystal topography reminiscent of bone marrow architecture, which was further coated with albumin/PDDA films (Fig. 12). Bone marrow stromal cells were first allowed to attach to the scaffold. Subsequently, CD34+ hematopoietic stem cells were seeded in the scaffold to create a three dimensional co-culture. By allowing CD34+ stem cells to self organize within this scaffold in the presence of stromal cells, the authors could recreate ex vivo part of the complexity occurring in vivo. The authors demonstrated that the scaffold supports CD34+ cell expansion and B lymphocyte differentiation with production of antigen specific IgG antibodies. Finally, implantation of these bone marrow

Figure 10. Von Kossa staining of EBs differentiated on the surface of a (PLL–PGA)10–PLL–(PGA–PLLs)5 film (A) or a (PLL–PGA)5–PLL–cCD–TGFβ1–cCD–PLL–(PGA–PLL)s–BMP2–PLL–(PGA–PLLs)5 film (B,C) (PLLs means PLL succinylated). Reproduced with permission from [266]. Copyright 2007 Wiley-VCH.

Figure 11. Immunochemical and histochemical staining of troponin T (a marker for myotube) (A–E) and alkaline phosphatase (a marker for osteoblasts) (A’–E’) of C2C12 on BMP2 loaded films for increasing BMP-2 initial concentrations: A) 0; B) 0.5 μg mL⁻¹; C) 1 μg mL⁻¹; D) 10 μg mL⁻¹; E) 50 μg mL⁻¹ (scale bar 150 μm). Reproduced with permission from [268]. Copyright 2009 Wiley-VCH.
constructs on to the backs of severe combined immunodeficiency mice proved successful and led to the generation of human immune cells. In addition to providing a structure that could be used for amplifying a large part of hematopoietic tissue, this three-dimensional matrix may also be useful for investigating the complex interactions occurring in bone marrow.

10. Conclusions and Perspectives

Over the last five years, there has been considerable development in the fields of mimicking self-assembly of real ECM components, developing PEM films in biology and from biomaterial surfaces, biomimetics, and tissue engineering. An important aspect is the dynamic nature of mono- or multicellular systems (interactions between cells or cell/matrix) that occur over several hours, days, and weeks. Based on this survey, it appears that better defined applications and multifunctionalization using several strategies simultaneously have emerged only recently. Thus, in addition to being used for tissue engineering applications, PEMs may serve as new biomimetic matrices for controlled physical, chemical, and biochemical properties (including chemistry, mechanics, and bioactivity) for investigating cell/material or cell/cell interactions from a fundamental mechanistic point of view. Such PEM coatings may offer new tools to biophysicists who need well-controlled and well-characterized biomimetic matrices, and these coatings offer new potentialities when compared to classic synthetic materials such as PAAm gels. The potentialities for manufacturing multifunctional coatings that combine, for instance, spatial organization and bioactivity, or adjustable stiffness and chemistry, or adjustable stiffness and bioactivity, are apparently unlimited. The design and pertinence of such multifunctional architectures will rely on a strong multidisciplinary approach and will require collaboration between engineers, physical chemists, organic chemists, biochemists, and cell and stem cell biologists.

Regarding the control over the internal structure of films, much work has been done on the widely investigated PSS/PAH system, considered so far as a "model." To date, few studies concern more biologically relevant and biomimetic films or films that grow exponentially, although more and more films are found to exhibit this type of growth, initially considered to be rather peculiar. A somewhat provocative line of thought could wonder whether films that grow linearly are not, on the contrary, an exception to the rule. There is a need to better understand and develop tools for investigating the internal structure of thick PEM films under physiological conditions (e.g., in liquids), both from experimental and theoretical points of view. These include obtaining access to vertical structures at the nanometer scale, over several hundred nanometers and not only in the evanescent zone, and measuring their nanometer scale porosity in liquids. All these properties, which will in turn influence the diffusion of molecular species within films, must be investigated to increase our knowledge of the fundamental processes underlying film structure but also to control diffusive processes in drug delivery and cell systems.

Finally, PEM coatings for applications in tissue engineering will undoubtedly experience considerable development in the next decade. Targeted applications for PEMs coatings will be followed by in vivo clinical studies. The requirements and constraints for each specific application, whether biodegradation, biostability, adhesion on to the "base" material, film aging, insertion of a specific growth factor in adjustable amounts will be addressed case by case in collaboration with clinicians and, in a next step, with industrialists. Toxicology studies on PEM coatings and the response of the immune systems and phagocytic cells are needed. Nowadays, there is an increasing amount of research into fundamental biology and the applications for stem cells. The few studies that have been carried out on stem cells using PEM coatings indicate that PEM may have most of the aspects that are important for modulating the outcome and organization of stem cells, opening up perspectives for translating these films into relevant clinical applications. Supra-cellular organization based on PEM films is indeed an original and innovative means of forming 3D mimetic architectures.

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