Polyelectrolyte Multilayer Films: From Physico-Chemical Properties to the Control of Cellular Processes

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Abstract: Polyelectrolyte multilayer films have been well characterized for almost two decades and there is now a growing interest for the development of biomimetic films that could be used in vitro or in vivo to control cellular behaviors. In this review, the important properties of multilayer films designed for cell/surface interactions will be highlighted. The first part will deal with the physico-chemical properties of polypeptide and polysaccharide multilayer films, including their growth, swellability, stability and mechanical properties. In the second part, we will focus on important properties influencing cellular behaviors: i) film biodegradability, ii) film mechanical properties, iii) film bioactivity achieved by either the intrinsic properties of the film components or the insertion of small peptides, proteins, or DNA. In particular, films thicker than one micron are particularly well suited for loading bioactive molecules due to their reservoir capacities.

Keywords: Layer-by-layer, polysaccharides, biopolymers, bioactivity, cellular processes, adhesion, biodegradability, drug release.

INTRODUCTION

In the field of biomaterials, controlling the surface properties of the materials may be a means to influence cell behavior including recolonization, adhesion, migration or even differentiation. Therefore, various strategies have been developed to modify the materials' surface properties, such as Langmuir-Blodgett deposition and self-assembled monolayers [1]. For the past two decades, layer-by-layer (LbL), also called polyelectrolyte multilayer (PEM) coatings, has emerged to become a new and general way to modify and functionalize surfaces whose applications range from optical devices to biomaterial coatings [2, 3]. The technique is based on the alternate deposition of polyanions and polycations [4, 5]. In recent years, the use of polyaminoacids, natural polyelectrolytes and biopolymers has emerged [6-9]. On account of their biocompatibility and non toxicity, these latter films constitute a rapidly expanding field with great potential applications: preparation of bioactive and biomimetic coatings [7, 9, 10], preparation of drug release vehicles [8, 11], buildup of cell adhesive or anti-adhesive films [6, 9]. With LbL, natural polymers that are already widely used for biomedical applications including hydrogel preparation, soft tissue repair, [12, 13] drug delivery [14] and viscosupplementation [15] can now be prepared in the form of thin films and deposited onto various types of substrates.

There are relatively few reviews on LbL films for biomedical applications [16-18] as many reviews focus on different aspects of the PEM films [2, 3, 19-22]. Although there are several forms of LbL films (films, capsules and membranes), we will focus here on LbL supported films. The field of biosensors, which was already considered in a recent review [17] will not be part of the present review. In addition, we will focus on studies dealing with bioactive molecules that were effectively shown to have a biological effect and as such, will not consider studies using model dyes and proteins. Another important point is that we will be mostly concerned with LbL films made of polyaminoacids, polysaccharides or natural polymers that can be used for biomedical applications.

One of the great advantages of PEM films is that their architecture can be designed with nanometer precision to meet different requirements such as thickness, controlled permeability, biocompatibility, targeting, etc. A second great advantage is that many types of substrates have already been coated by LbL films, including spherical objects [23, 24], porous matrices [25], metallic biomaterials surfaces [10] or highly curved surfaces [26].

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In the first part of this review, we will focus on the physico-chemical properties of PEM films, including the type of thickness growth, their swellability, stability, internal structure and mechanical properties. In the second part, we will show how these films can be employed for controlling various cellular processes. Such control can arise from their intrinsic properties, from their biodegradability or from their bioactivity after insertion of various sorts of peptides, drugs, and proteins.

PHYSICO-CHEMICAL PROPERTIES

Film Growth: Linear Versus Exponential

Described by Decher and co-workers [4], the first investigated polyelectrolyte systems exhibited a linear growth of both mass and film thickness with the number of deposited layers. Poly(styrene sulfonate) /poly(allylamine hydrochloride) (PSS/PAH) films are one of the most prominent examples of linearly growing systems [27-32]. These films present a stratified structure, each polyelectrolyte layer interpenetrating only its neighbouring ones. The growth mechanism involves mainly electrostatic interactions between the polyelectrolytes from the solution and the polyelectrolytes of opposite charge forming the outer layer of the film. Each new polyelectrolyte deposition leads to a charge overcompensation that is the prerequisite that the next layer of oppositely charged polyelectrolyte can be added. This leads to a change in the zeta potential [27].

More recently, using polysaccharides and polypeptides, Elbert and co-workers [6] and Picart and co-workers [33, 34] described a new type of polyelectrolyte multilayers which are characterized by an exponential growth of both film mass and thickness with the number of deposition steps. Poly(L-lysine)/alginate (PLL/ALG) [6] and PLL/hyaluronan (PLL/HA) [33, 34] were the first reported examples. Chitosan/HA (CHI/HA) [9] and PLL/chondroitin sulfate (PLL/CSA) [35] are other subsequent examples. Whereas the typical thickness of a linearly growing film constituted of 20 layer pairs is of the order of 100 nm, the thickness of exponentially growing films can reach 3 μm or more after the deposition of a similar number of layers. Fig. (**1**) presents a schematic of all the techniques that can be employed to characterize film growth. The growth of linearly and exponentially growing films can indeed be followed by quartz crystal microbalance (Fig. (**2**)). We reported that the construction of (PLL/HA) films takes place over two buildup regimes. One consists of the formation of isolated islands of the PEM that grow to a continuous film. The second regime that sets in is characterized by an exponential increase of mass with the number of added layers. Since then, other exponentially growing films have been reported [36, 37]. Two explanations for this exponential growth mechanism have been proposed: one relies on the diffusion

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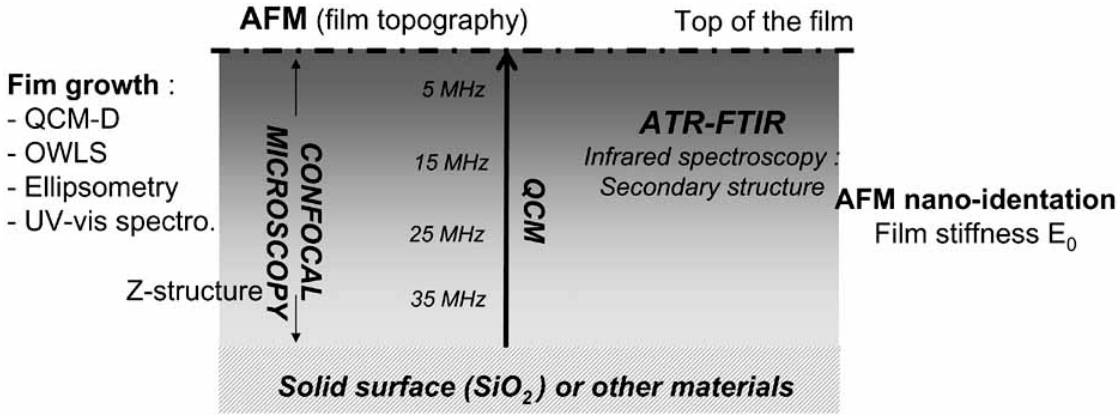

Fig. (1). Scheme of the different techniques used to investigate LbL film buildup: film growth can be followed by quartz crystal microbalance (QCM-D), optical waveguide lightmode spectroscopy (OWLS), UV-visible spectrometry and ellipsometry. Film chemical structure can be probed by Fourier Transform Infrared spectroscopy and z-structure can be observed by confocal laser scanning microscopy (CLSM) for films thicker than ~800 nm. AFM can be used both in topography mode to image film surface and in force mode to perform nano-indentations (AFM nano-indentation) to determine the film Young's modulus, E_0, which is a measure of the film's mechanical properties.

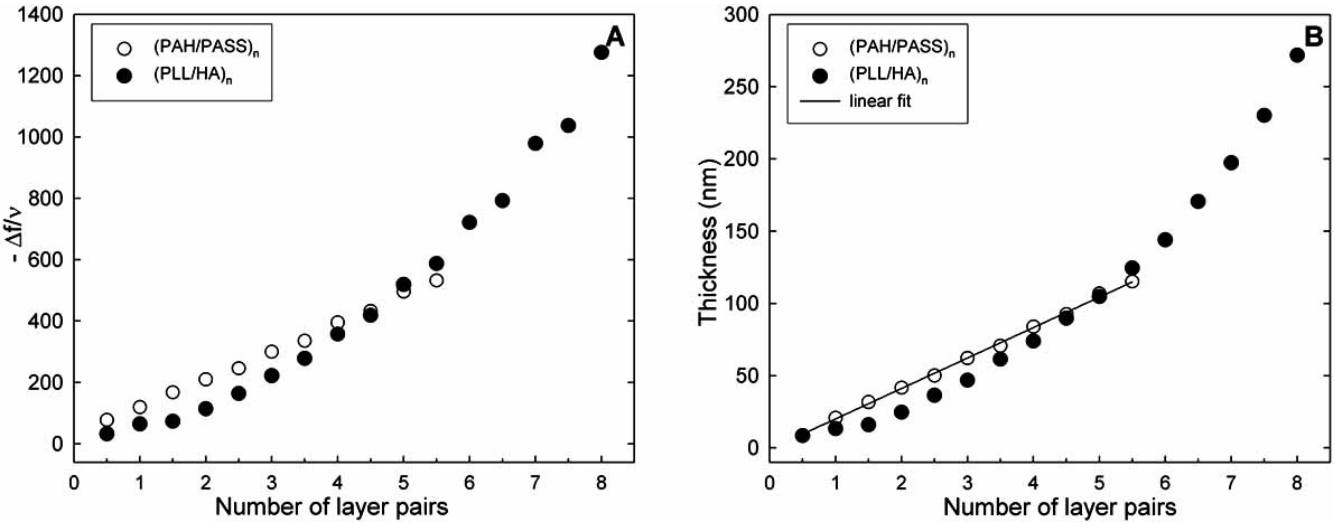

Fig. (2). Linear versus exponential growing films**.** Examples of linear and exponential thickness growths on SiO_2 crystals followed by quartz crystal microbalance (QCM-D). The frequency shift ($-\Delta f/\nu$) measured at 15 MHz (left) and the thickness deduced from the fit of the QCM data at the four frequencies and dissipations (right) are given for a linearly $(PSS/PAH)_n$ growing films (○) and exponentially $(PLL/HA)_n$ growing films (●) (*n* being the number of layer pairs). In (right), the linear fit on the (PSS/PAH) data is also shown (black line).

of polyelectrolyte "in" and "out" of the film during each "bilayer" step [34, 36] whereas the second one relies on the increase in film surface roughness as the film builds up [28, 38]. However, no change in surface roughness was observed for the exponentially growing films made of polypeptides [33, 36, 39]. An in depth study of the (PLL/HA) system shows that the growth mechanism relies on the diffusion "in" and "out" of the whole structure during each "bilayer" deposition step of one type of the polyelectrolytes constituting the films [34, 36]. PLL and CHI diffusion could be visualized by confocal laser scanning microscopy (CLSM) for (PLL/HA) and (CHI/HA) films using fluorescently labeled polyelectrolytes (respectively PLL^{FITC} and CHI^{FITC}). Diffusion of PLL was also observed by CLSM for (PLL/CSA) films [35]. Most, but not all, of the reported exponentially growing films contain PLL or CHI as polycation.

It was also observed that a polyanion/polycation system that grows exponentially under certain conditions can grow linearly when deposition conditions are changed. One such example is salt concentration where low concentrations corresponding to linear growth and high salt concentration corresponds to an exponential growth. This was demonstrated for chitosan/dextran (CHI/DEX) films by Serizawa *et al.* [7] and for (CHI/HA) films by Richert *et al.* [9]. This simplest explanation is that, by reducing the polyelectrolyte solution's salt concentration during buildup, films become thinner (for a given number of deposition steps) and more dense thereby hindering polyelectrolyte diffusion into the film. Interestingly, films containing collagen were found to grow linearly [40, 41]. It was also shown that, for the linearly growing films like those containing collagen, collagen adsorbed on top of the film did not diffuse into the material [40]. Table **1** summarizes all the different systems investigated, the buildup conditions and the type of thickness growth (linear or exponential). In Fig. (**3**), structures of polysaccharides commonly used in layer-by-layer buildup are represented.

Film Hydration and Swellability: Sensitivity to External Parameters such as pH and Ionic Strength

Film hydration can be estimated by measuring the film refractive index using techniques such as optical waveguide lightmode spectroscopy [33] or ellipsometry (by measuring respectively dry

Table 1. Studies involving natural based multilayer films, either with poly(L-lysine), chitosan, or collagen as polycations. Experimental conditions and type of thickness growth are given (linear indicates that the thickness increases linearly with the number of deposited layer pairs whereas exponential means that it grows exponentially)

	Study	Conditions	Type of growth
PLL as polycation			
PLL/ALG	Elbert *et al*. [6]	PBS	Exponential
PLL/HA	Picart *et al*. [33]	0.15 M NaCl pH 6.5	Exponential
PLL/CSA	Tezcaner *et al*. [35]	0.15 M NaCl pH 6	Exponential
PLL/HEP	Boulmedais *et al*. [109]	0.15 M Nacl + Hepes buffer, pH 7.4	Exponential
CHI as polycation			
CHI/HA	Richert *et al*. [9] Kujawa *et al*. [54]	0.15 M NaCl pH 5	Exponential
CHI/HEP	Fu *et al*. [98]	0.15 M NaCl pH 3 to 3.8	Linear
CHI/mucin	Svensson *et al*. [110]	Acetic acid, no salt pH=4	Linear
CHI/Dextran sulfate CHI/HEP	Serizawa *et al*. [7]	NaCl at different concentrations	Linear for NaCl < 0.5 M Exponential for 0.5M et 1M NaCl
COLLAGEN as polycation			
COL/HA	Zhang *et al*. [40]	0.05M NaCl, pH = 4	Linear
COL/HA	Johannson *et al*. [41]	M Acetate buffer pH 4	Linear

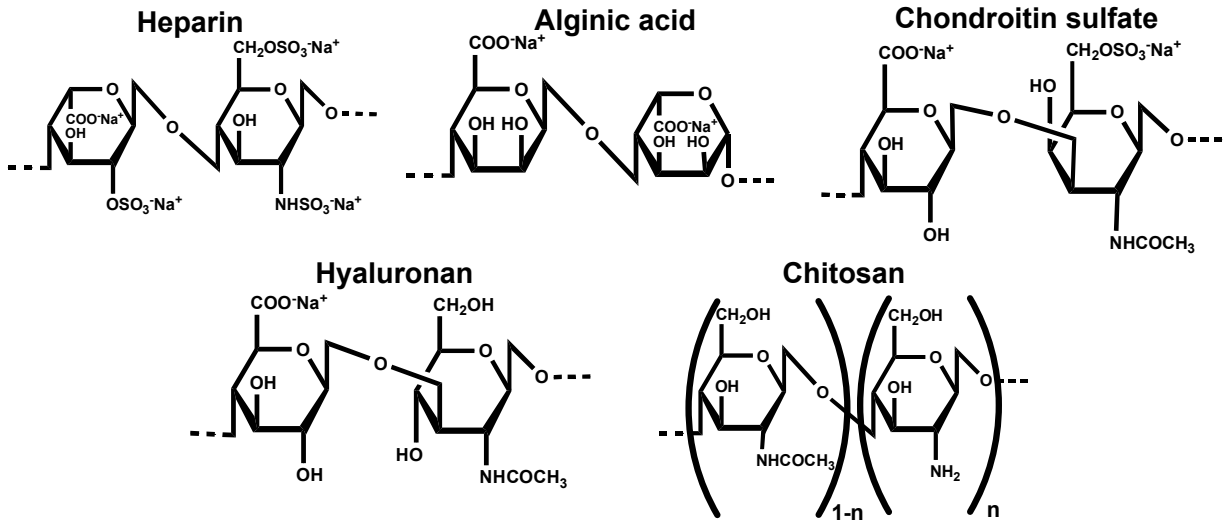

Fig. (3). Schematic of the molecular structures of the natural polysaccharides that are commonly employed in LbL buildup: heparin, alginic acid, chondroitin sulfate, hyaluronan, chitosan, n being the degree of deacetylation.

and hydrated film thickness) [42]. Refractive index of synthetic polyelectrolyte multilayer films, such as (PSS/PAH) films, was measured *in situ* by OWLS to be approximately 1.5 in physiological conditions [32]. This indicates that such films are relatively dense and contain only around 25% of water (a simple approximation of the water content is based on the following formula : n_{PEM} = 1.3340x*a* + (1-*a*)x1.56, 1.334 being the refractive index of a 0.15 M NaCl solution, 1.56 being the refractive index of a pure polymer film [42], and *a* being the fraction of water). Similar measurements have been done using PLL, poly(D-lysine) (PDL), or even chitosan as polycation in combination with polyanions such as gelatin [43], poly(L-glutamic) acid (PGA) [44], or hyaluronan [9]. In general, films made of polypeptides and polysaccharides in comparable ionic strength conditions are more hydrated than films made of synthetic polyelectrolytes such as (PSS/PAH). This observation is based on refractive indices that are ≈1.36-1.38 for polysaccharide films [9, 33] and ≈1.42 for (PGA/PLL) films [36] which would correspond to water contents ranging respectively from 95% to 60%. Of note, the majority of synthetic polyelectrolytes have hydrophobic chain backbones, which determine film properties prior to complex formation. On the other hand, natural polyelectrolytes have hydrophilic groups on the backbone. This refractive index for (PLL/HA) films is of the same order of magnitude of that found by ellipsometry [42] for wet films (1.35), and has to be compared to the refractive index for dried films (1.56). This indicates that the film swells by about 830% (initial conditions for film assembly

were pH 9 and 0.1 M NaCl). The high swelling capacities of polysaccharides, and in particular for hyaluronan [45], renders the buildup of much thicker films possible, up to several hundred of nanometers [9] or even several micrometers after deposition of 20 to 30 layer pairs [34]. As water content is an important parameter in film structure, the temperature of film buildup or of post-treatment storage can also play a role in the internal secondary structure of the film. This was nicely demonstrated by Bouldemais *et al.* for (PLL/PGA) films heated up to 89°C [46].

These polysaccharide based films were often, if not always, found to inhibit cell attachment [6, 9, 47], except when films were stiffened by covalent cross-linking [47]. Therefore, a trend that seems to emerge from all these cell lines and primary cell studies is that nanometer thin and dense films formed by few layer pairs, which are also very stiff, are more favorable for cellular adhesion than thick and highly hydrated films that are much softer [48].

A detailed study of the hydration and swelling properties of (PLL/HA) films indicates that the most important parameters are i) the assembly pH (that can be varied from 5 to 9 for these particular films) and ionic strength as well as ii) the swelling medium [42]. Thus, depending on the combination of these parameters, very different film properties can be achieved. Polysaccharides like HA have a random coil conformation but can form hydrogen bonds with water [2]. They can also exhibit hydrophobic interactions, [15], which are influenced by ionic strength. Very interestingly, the pKa of polyelectrolytes in the film demonstrate that both PLL and HA experience a significant shift in their p*K*a(apparent) values upon adsorption, compared to the accepted values (in dilute solution) of 9.36 $\pm$ 0.08 and 3.08 $\pm$ 0.03, respectively, in the presence of 1.0 mM NaCl. The p*K*a(apparent) values of both PLL and HA remained relatively constant after the first 3-4 deposited layers (at pH 7, it is of 4.8 for HA and 6.8 for PLL). Such decrease in the acid strength of HA and base strength of PLL is similar to that reported for other polyelectrolyte pairs [49]. It has been previously speculated and experimentally shown that the charge on the multilayer film surface strongly influences the acid-base equilibria of adsorbing polyelectrolyte chains [50]. According to Barret *et al.* for (PLL/HA) multilayer films, the overall trends in the p*K*a(apparent) shifts upon adsorption are influenced by the ability of both of these polymers to adopt some degree of secondary conformational order with changes in the local pH and ionic strength environment [42, 51-53]. In the intermediate pH range, HA is known to have some degree of chain stiffening in solution due to local hydrogen bonded regions, whereas PLL chains are reported to experience a random coil to α-helix transition at pH = 10.5 [53]. The same authors also investigated the swelling of (PAH/HA) films and found that these films exhibit a high dependence of swelling on the assembly solution pH. The swelling ratio varied between 2 at physiological pH = 7 to more than 8 at very acidic pH = 2 and was more pronounced than at basic pH = 10 (swelling ratio about 5).

Influence of the Molecular Weight of the Polyelectrolytes on the Film Buildup

Little is known about the influence of polyelectrolyte molecular weight for polyaminoacid and polysaccharide films. This is probably due to the polydispersity of the molecular weight of the polyaminoacids and polysaccharides and the limited range of molecular weights. In addition, this range depends on the manufacturer, on the source of polysaccharides (for instance, for hyaluronan, it can be produced by bacteria or extracted from rooster comb), of its purity, all of these parameters influencing the price of the polysaccharide. Kujawa *et al.* investigated the properties of CHI/HA films built with CHI and HA of two different molecular weights (30 kDa and 160 kDa for CHI and 31 kDa and 360 kDa for HA) [54]. They showed that film thickness in the exponential growth phase depends on the molecular weights of the nondiffusing species HA and the diffusing species CH. That is, the higher the MWs of the polysaccharides, the thicker the multilayer for a given number of deposited layer pairs. Most importantly, the exponential growth rate was found to be similar for all four MW pairs. Their data suggested that the polysaccharide MWs affect only the onset of the steep (exponential) growth phase. Porcel *et al.* studied the influence of PLL and HA MWs on the linear growth phase following the initial exponential growth phase [55]. The film thickness increment per layer pair deposited remains unchanged despite differences between their freely diffusing domains; low MWs PLL could diffuse within the whole film whereas high MW PLL (360 kDa) diffusion was always limited to the upper zone of the film (about 4 μm in thickness).

Film Structure (Hydrophobic Domains)

Beside electrostatic interactions, other secondary interactions such as hydrophobic interactions have been observed. In order to investigate the role of these interactions in film buildup, Glinel *et al.* used anionic amphiphilic polysaccharides of varying hydrophobicity [56]. The amphiphilic polysaccharides were obtained by grafting alkyl chains on carboxymethylpullulan (CMP), an anionic flexible polysaccharide. As the backbone from the CMP is highly hydrophilic, the hydrophobicity resulted from the grafted alkyl groups. They investigated the growth of CMP modified polymers in combination with various polycations such as poly(ethylene imine) (PEI), PLL and CHI and found that, for all these polycations, films prepared with a highly grafted alkyl chain (18C10, meaning 18% of grafting for a C10 chain) showed an exponential growth and were largely thicker than those prepared with the unmodified CMP. Notably, film thickness could be varied depending on the grafting degree and exhibited a maximum for a grafting degree around 10%. Film thickness also increased with the increase in alkyl chain length. Interestingly, in a second study, the authors also provided evidence that the entrapment of a hydrophobic dye in hydrophobic nano-reservoirs resulted from the aggregation of decyl pendent groups grafted on CMP chains [57]. This entrapment was achieved either by diffusion of the dye in the film or by pre-complexation of the dye with the amphiphilic CMP. They showed that multilayer loading capacity of the multilayers, as well as their release behavior, could be tuned by varying the grafting degree of CMP chains.

Another type of secondary interaction consists in making use of host-guest interaction as a driving force for building films. Van der Heyden *et al.* prepared multilayer films using host-guest interactions between two derivatized chitosans, one with β-cyclodextrin cavities and the other with adamantyl moieties [58]. They showed that the stability of the self-assembly is conferred by multivalent complexation occurring at each step of the construction and that the assembly growth is mainly governed by the availability of the complexation sites offered by each layer. They also showed that the films made of host-guest interactions are reversibly responsive to solvent change.

Film Stability in Physiological Media

Although, in principle, multilayer films can be built under extremely different conditions in terms of pH and ionic strength, the final suspending medium may depend on the foreseen application. In particular, when used for cell culture studies or biomaterial coatings, films have to be stable in culture medium and in physiological conditions (i.e. a medium at ~0.15 M NaCl and pH between 5 and 7.4). These requirements may greatly limit the range of possible buildup conditions. On the other hand, if films are to be used for a subsequent release of a film component itself or of a bioactive molecule (see below), then, stability is not an issue or at least, is not as important as in the first case.

It stems from the aforementioned properties of polyaminoacid and polysaccharide films (characterized by weak electrostatic charge, high hydration and swellability, secondary interactions) that

they are more susceptible to stability problems than synthetic polyelectrolyte films. This is particularly true when the films are built in a medium which has a different pH and/or ionic strength from a physiological medium. Then, the films have to sustain stresses upon medium change and can potentially be disrupted due to too high internal stresses. Typical cases include films built at acidic pH like collagen/HA (COL/HA) films or (CHI/HA) films, for which COL and CHI are polycationic at acidic pH (4 for COL and less that ~ 5.5 for CHI). Johansson *et al*. found that (COL/HA) films are not stable when the pH is raised from 4 to 7 [41]. This could be explained by the protonation/deprotonation process for the polyelectrolytes involved in the interaction. At pH 4.0, most acid functionalities are protonated, whereas they are deprotonated at pH 7. For collagen, the number of negatively charged acids on collagen approaches the number of protonated amines or the isoelectric point. The protonation/deprotonation processes induces the changes in the three-dimensional structure of the polyelectrolytes, which affects the electrostatic forces that existed between the polyelectrolyte layers. This dissolution was found to be irreversible. For (CHI/HA) films, we found that the stability depends on the molecular weight of the chitosan: whereas films built with high molecular weight chitosan are stable in physiological medium, [9] films built with chitosan oligosaccharides (MW 5000 g/mol) are exhibiting a change in structure when introduced into the culture medium [59]. We observed that this change in structure was mostly due to the presence of divalent ions (Ca^{2+}, Mg^{2+} in the culture medium) and not to the change in pH. In fact, divalent ions are known to complex chitosan [60] and alginate [61]. These observations are not only valid for polysaccharide multilayer films but also for hydrogen-bonded films built at very low pH [62] and for (PLL/PGA) films built at low pH [63].

Even when films are not built in acidic or basic conditions, they may be destabilized in physiological medium. This was observed for films containing PEI as polyanion and a mixture of heparin (HEP) and acidic fibroblast growth factors whose degradation could be observed in PBS at 37°C [64]. In contrary, films built with basic fibroblast growth factor and chondroitin sulfate [65] were stable in PBS. However, as an interplay of different interactions contribute to the overall film stability, it is difficult to establish a common rule and each type of film need to be tested individually. It has also to be noticed that the presence of cells, which are able to exert strong stresses on their matrix [66], can also affect the film stability. We will see below that such problem of stability in physiological medium and of mechanical resistance can be overcome by chemically cross-linking the films.

Importance of Film Mechanical Properties

Characterizing and modulating film mechanical properties have become a challenge of the last few years. In particular, for multilayer films made of polysaccharides and polyaminoacids, which are rather soft as compared to films made of synthetic polyelectrolytes, it is important to ensure that i) they will be stable in different suspending media and that ii) they can sustain different stresses (like shear stress). Some recent studies showed that film mechanical properties can be modulated in different ways.

Mechanical Properties Based on the Structure of the Polyelectrolyte

Schoeler *et al*. investigated the buildup of films containing PAH as polycation and two ionic polysaccharides, iota and lambda-carrageenan, of similar composition but different conformations [67]. Iota carrageenan is in helical conformation at room temperature whereas lambda carrageenan is in random coil conformation. The mechanical properties of these two film types were found to be very different, which suggests that the structure of the film can strongly influence its mechanical properties. Also, the presence of different sugar molecules (like lactose or mannose) is sufficient to create noticeable differences in film stiffness and to modulate cell adhesion [68].

Composite Films Incorporating Nanoparticles or Mixtures of Polyelectrolytes

To increase film mechanical properties, different strategies have been proposed and some of them have been applied to natural based films. One of the strategies is to incorporate colloids (of nanometer size) in the films [69, 70]. Although not directly applied to natural based films, one can observe that the (CHI/HA) and (PLL/HA) films containing adenovirus particles (undeformable particles of 70 nm in diameter) are a good surface for cell adhesion, whereas films that do not contain particles are non-adhesive [9, 47]. A second strategy relies on the incorporation of mineral materials like clay (montmorillonite) in the films. Kotov *et al*. recently investigated the formation of membranes made of CHI and montmorillonite [71] but found that they were less stiff than what would have been expected, based on the CHI intrinsic chain stiffness.

Another strategy consists in "capping" the "soft" films with a layer of a stronger polyelectroyte, like PSS. This was applied by Vodouhe *et al*. who observed that initially non-adhesive (PLL/HA) films could be rendered adhesive by the addition of a single PSS layer [72]. Presumably, there is an exchange between the HA molecules outside the film and the incoming PSS molecules. Another possibility would be to prepare films containing mixture of polyelectrolytes, such as hyaluronan and PSS [73] in combination with PLL, one of the mixture components being a strong polyelectrolyte.

Modulation of Mechanical Properties by Film Covalent Cross-Linking

Finally, it is possible to create covalent cross-links within the films by making use of the existing charged groups in the films. In our group, we developed a protocol based on the carbodiimide chemistry for cross-linking carboxyl groups with amine groups and thereby creating covalent amide bonds [47] (Fig. (**4**)). This protocol was applied to several types of polyelectrolyte pairs, including (PLL/HA) and (CHI/HA) films [59, 74]. The effective cross-linking was checked by Fourier Transform Infrared Spectroscopy in attenuated total reflection mode (Fig. (**5**)). For (PLL/HA) films, several peaks decreased and in particular the carboxylic peaks (at 1606 and 1412 cm^{-1} respectively) whereas at the same time the intensity of other bands increased. This is the case for the amide I and II bands (in the 1620-1675 cm^{-1} region and in the 1430-1470 cm^{-1} region respectively). The decrease of the carboxylic peaks of HA and the concomitant increase in the amide bands demonstrates the reaction between corresponding chemical groups and ammonium groups of PLL and of CHI. In the case of (CHI/HA) films, the disappearance of the characteristic saccharide peaks and the appearance of ester bands at around 1240 cm^{-1} and at 1740 cm^{-1} also suggest the formation of ester bonds (data not shown). Such bonds involve hydroxyl groups of polysaccharide and carboxylic groups or acid anhydride formed by the reaction between two carboxylic groups [75]. Very interestingly, film cross-linking has two important consequences that are probably related: i) a change in the film mechanical properties and ii) a drastic change in the film adhesive properties. These latter properties have been investigated by means of AFM nano-indentation experiments [74, 76, 77]. We found that the Young's modulus (E_0), which represents the mechanical stiffness of the films, is of ~ 5 kPa for native films whereas E_0 can be varied over two orders of magnitude from 5 to 500 kPa for cross-linked films (depending on the carbodiimide cross-linker concentration). This was the first time that the Young's modulus of such thin films could be systematically varied and tuned. It would also be interesting to investigate how far a cell can sense through the film, i.e. the minimum film thickness for which the cells sense the film rather than the underlying glass substrate. Such question has not been answered

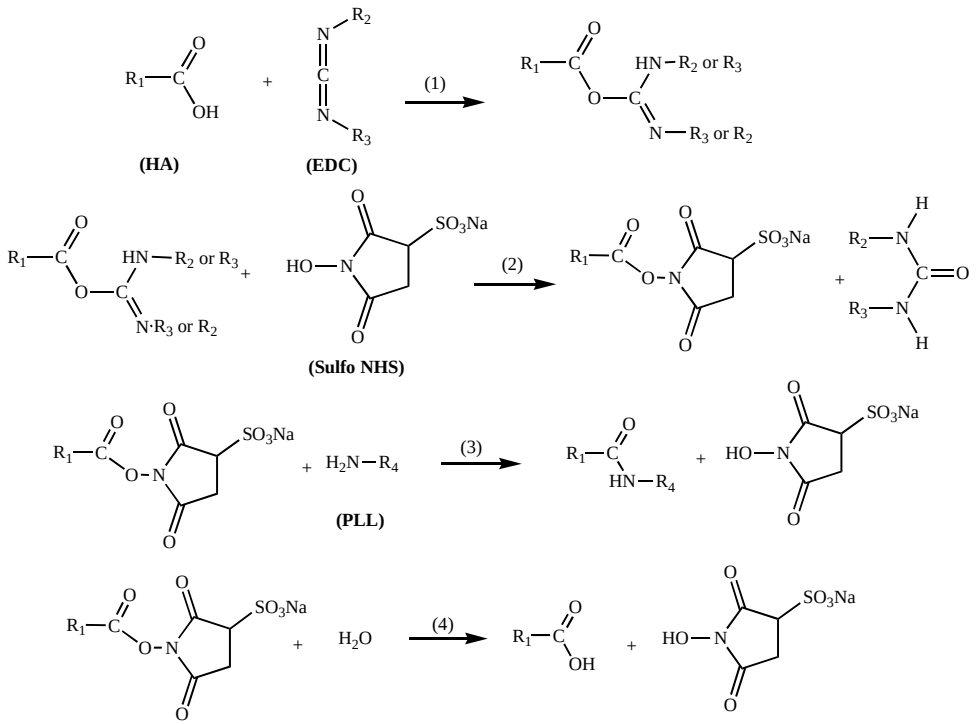

Fig. (4). Cross-linking of LbL films by means of EDC in combination with sulfo-NHS. EDC reacts with a carboxylic group and activates it (**1**). The activated complex is converted into an active ester with sulfo-NHS (**2**). The active ester reacts with primary amine to form an amide bound (**3**). The unreacted sites are hydrolyzed to give a regeneration of the carboxylic groups (**4**). Reproduced with permission from ref. [47]. Copyright 2004 American Chemical Society.

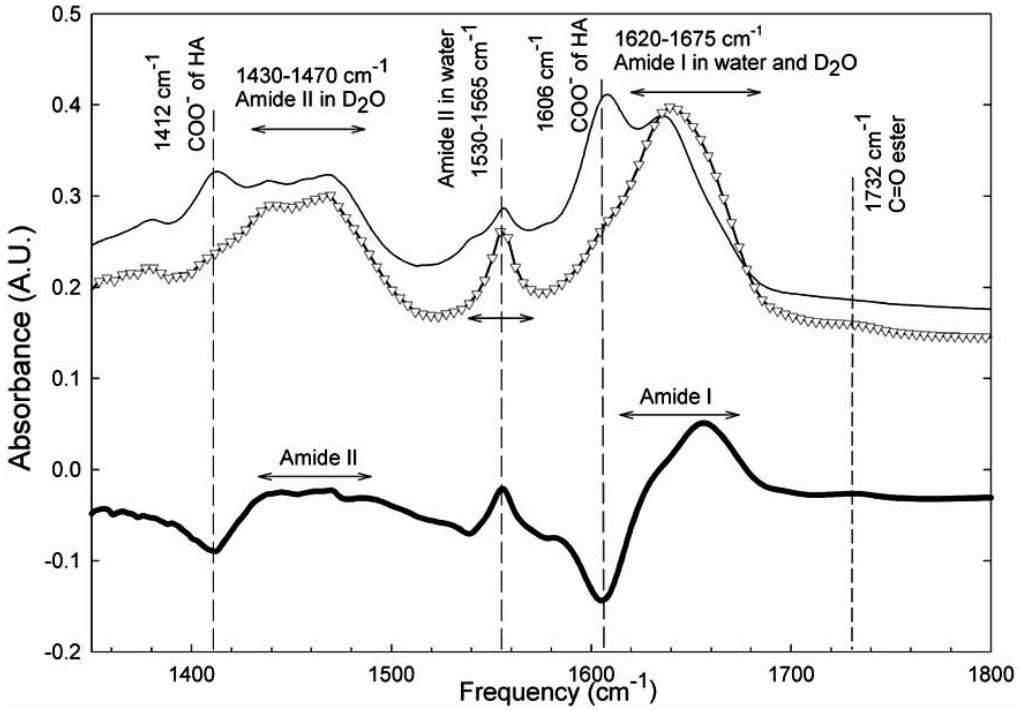

Fig. (5). Film cross-linking by EDC/sulfo-NHS followed by infrared spectroscopy in attenuated total reflection (ATR) mode. ATR-FTIR spectra of a native and a cross-linked $(PLL/HA)_8$ film before (—) and after the cross-linking procedure and the final rinsing step (–∇–). The difference between the two spectra (before and after cross-linking) is also represented (thick black line). (Adapted from [47]).

yet but it was demonstrated that one micrometer thick films are sufficient to "isolate" the cells from the underlying substrate [78].

CONTROL OF CELLULAR PROCESSES

Beside the own properties of the polyaminoacids and polysaccharides that constitute the films, it is possible to take advantage of the high swelling properties of these films and of their large thickness for using them as a reservoir for bioactive molecules or drugs. It is precisely because these films have a low degree of ionic cross-links and a large porosity that they can be employed as reservoir. Therefore, not only can small molecules be loaded in the films but also proteins (Fig. (**6B**)) like myoglobin, which was found to dif-

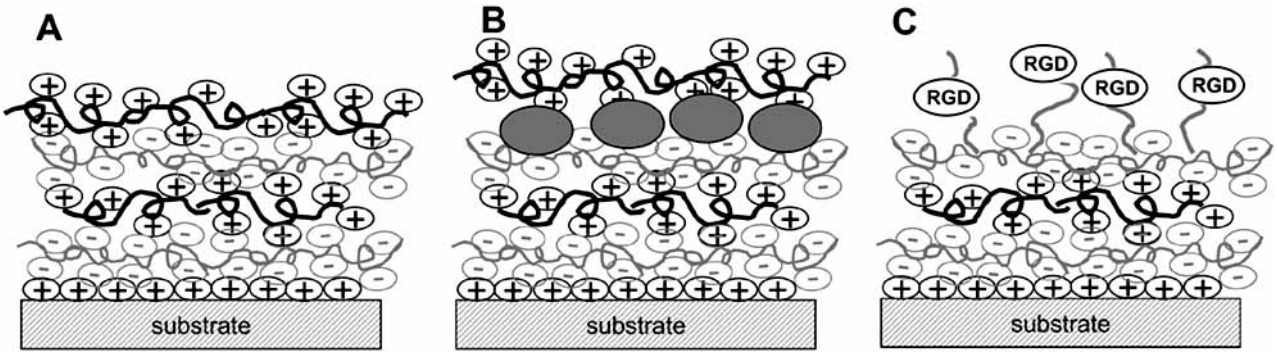

Fig. (6). Schematic of a polyelectrolyte multilayer film (**A**), of a film functionalized by direct adsorption of proteins or peptides in the film (**B**) and of a film functionalized by coupling a peptide to a polyelectrolyte and then adsorbing it as a regular layer (**C**).

fuse within (CHI/HA) films [79]. An interesting aspect is that the large thickness of these films can be precisely employed for increasing the loading capacity of the reservoirs, as will be illustrated below. Alternatively, if the polyelectrolyte is coupled to a peptide, the functionalized polyelectrolyte can be adsorbed as a regular polyelectrolyte layer (Fig. (**6C**)). In addition, the film mechanical properties can also be exploited to modulate cellular processes.

Intrinsic Properties of Polyelectrolyte Multilayer Films

CHI/DEX films were found to exhibit anti-coagulant properties only when dextran is the outermost layer of the film and when the films are built in 0.5 M NaCl or 1 M NaCl. On the other hand, CHI/HEP films built in 1 M NaCl also exhibited strong anticoagulant activity whatever the outermost surface of the film [7]. Thus, such multilayer films have a good potential for the surface modification of medical implants in contact with blood. The thromboresistance of a $(CHI/HA)_4$ coated NiTi substrate was also demonstrated by Thierry *et al*. [10]. These films were found to significantly reduce platelet adhesion by 38% after one hour exposure to platelet rich plasma. On the contrary, the adhesion of polymorphonuclear neutrophils increased slightly onto the coated surface, compared to bare metal.

Film Biodegradability

Enzymatically Biodegradable Films

The biodegradability of thin polymer films coated on material surfaces is one of the most important requirements for biomedical applications of these polymers. Due to their intrinsic properties and to the presence of specific enzymes *in vivo*, natural based multilayer films can be degraded in the presence of enzymes and thus release their content. Serizawa *et al.* were the first to demonstrate the alternating enzymatic hydrolysis of a LbL assembly formed from chitosan and dextran. [80]. Chitosanase, an enzyme that hydrolyzes chitosan, was applied in this process. More recently, we showed that (CHI/HA) films can be degraded by enzymes such as hyaluronidase, lysozyme and α-amylase, which are present in saliva and plasma [81]. Film biodegradability could be tuned by varying the extent of cross-linking using carbodiimide at various concentrations [59]. In addition, THP-1 macrophages can degrade (PLL/HA) native films whereas the cross-linked films are not degraded over the same time period [82]. Fig. (**7**) shows the biodegradability of (PLL/HA) and (CHI/HA) films in contact with THP-1 macrophages after one day in culture.

Biodegradability of *in vivo* films have not been sufficiently explored. In our group, we investigated the biodegradation of (CHI/HA) films cross-linked to different extents in two different locations: the rat oral cavity for possible applications in the dental field [83] and the mouse peritoneal cavity for possible tissue implantations [59]. We observed that the native films are very rapidly degraded due to the presence of saliva enzymes and probably due to strong mechanical stresses exerted on the films. On the other hand, about 60% of the cross-linked films remained intact after three days in the oral cavity. When implanted in the peritoneal cavity, the (CHI/HA) films induced an inflammatory response that directly depends on the extent of cross-linking: the more cross-linked the film is, the more macrophages it attracts. We observed that highly cross-linked films were also the most resistant to degradation [59]. It should be mentioned that the potential presence of toxins (for example endotoxins in polysaccharides such as alginate) might have a significant influence on biocompatibility and activation of human macrophages. This was demonstrated for alginate gels used as wound dressings [84]. So far, however, there is no data available on polyelectrolyte multilayer films for testing whether the presence of toxins can have an effect on the macrophages activation.

Hydrolytically Degradable Films

Deconstruction of layer-by-layer films can be also achieved for controlled release applications. Hammond *et al*. constructed hydrolytically degradable LbL films by depositing a degradable poly(α-amino ester) and a series of model therapeutic polysaccharides

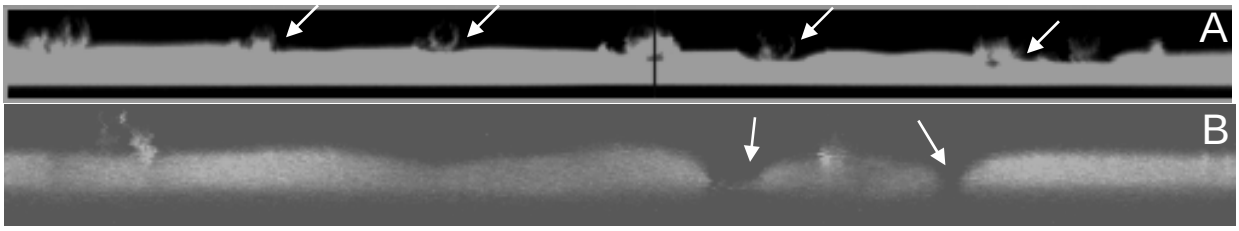

Fig. (7). Biodegradability of polysaccharide multilayer films. CLSM study of the biodegradability of a native $(PLL/HA)_{24}$-PLL^{FITC} (**A**) and of a cross-linked $(CHI/HA)_{24}$-CHI^{FITC} films (cross-linked at 5 mg/mL EDC concentration) (**B**) that have been in contact with THP-1 macrophages for 24 hours at 37°C. Cross-sections of the films are shown (image size is 230 μm x 12 μm for **A** and 230 μm x 22 μm for **B**). Arrows indicate the visible degradation sites. The original film thicknesses are ~ 4 μm for $(PLL/HA)_{24}$ –PLL^{FITC} films and ~ 6 μm for $(CHI/HA)_{24}$ –CHI^{FITC} films. (Adapted from [82] and [59]).

(HEP, low molecular heparin and chondroitin sulfate). These films exhibited a pH dependent degradation and release behavior [85]. Then, they further developed complex layer-by-layer films made of alternating blocks allowing or blocking molecule diffusion. Using radiolabeled polyelectrolytes, they could show that covalently cross-linked barriers effectively block interlayer diffusion, leading to compartimentalized structures [86]. They observed that very large numbers of ionically cross-linked barriers could not block interlayer diffusion. Using these diffusing and non-diffusion strata, they designed degradable films capable of parallel and serial multiagent release. A similar strategy was employed by Garza *et al*. by using a mixed architecture made of (PLL/HA) reservoir alternated with a hydrolysable poly(lactic-co-glycolic acid) (PLGA) barrier [87]. Bone marrow cells seeded on these composite architectures ending by a (PLL/HA) reservoir rapidly degraded it and internalized the PLL chains confined in this reservoir. Cells locally degraded the PLGA barrier and internalized the PLL localized in a lower (PLL/HA) compartment after 5 days.

Importance of Film Stiffness in Cell Adhesion

Most of the polyaminoacids and polysaccharides multilayer films containing more than six layer pairs exhibit poor or even anti-adhesive properties, presumably due to their high hydration and low elastic modulus, E_0. This is valid for (CHI/HA) films on top of which adhesion of primary chondrocytes was extremely low (less than 4% of the control) for both CHI and HA ending films, [9], for (PLL/HA) films that are very "cell resistant" toward chondrosarcoma cells adhesion [47, 88]. Also, (PLL/ALG) films were found to inhibit fibroblast adhesion and be non-flowling [6]. Surprisingly, however, photoreceptor cells could adhere and remain viable when plated on (PLL/CSA) and (PLL/HA) films [35]. It might be that these cells, as well as neuron cells, prefer softer matrices than other cell types. For (PLL/PGA) films, the adhesion depends on the number of layer deposited and on the type of ending layer (whether positively or negatively charged). When only few layers are deposited on a relatively stiff cushion (like on a PEI-$(PSS/PAH)_2$ precursor layer), the films are usually adhesive, with however differences depending on the surface chemistry [44, 89]. As a general rule, films made from synthetic polyelectrolytes like (PSS/PAH) are much stiffer, thinner and more favorable for cell attachment [44, 63, 89, 90].

A few years ago, a cross-linking protocol performed in aqueous solution was developed for multilayer films containing carboxylic and amine groups [47] and was found to have a profound effect on cell adhesion. This protocol has the advantage of being applicable to a wide range of films. Very interestingly, cross-linking the films has several consequences on their physico-chemical properties: the diffusion of polyelectrolytes is limited [47], film biodegradability can be tuned [81], and their mechanical properties are also changed [78]. With respect to the cell behavior on top of cross-linked films, we have already shown that the "switch" from non-adhesive to adhesive upon cross-linking is a common property for many films types, including (PLL/HA) [47, 74, 82], (CHI/HA), [9, 77], PLL/poly(alginic acid), PLL/poly(galacturonic acid) and (PLL/PGA) [91] and was observed for a large variety of cell types (chondrosarcomas, [78] chondrocytes, [82] osteoblasts, [91] neurons [82], smooth muscle cells, [74]). We also observed that proliferation is enhanced on the cross-linked films as compared to the native ones or as compared to films grafted with an adhesive peptide (a 15 amino-acids peptide containing the RGD sequence) [91]. Very interestingly, film stiffness can also be varied over a wide range of Yong's moduli by simply adjusting the cross-linker concentration [76]. Both cell adhesion and cell spreading area were found to increase monotonically with EDC cross-linker concentration [78]. Recently, we investigated the differentiation of myogenic cells on films cross-linked at various EDC concentrations and found that the differentiation process, including the initial morphology of the myotubes formed and the subsequent striation of myotubes at later stages are greatly influenced by film mechanical properties [92].

Surface Mediated Transfection

Polyelectrolyte multilayer films can also be used for controlled delivery of DNA complexes, where the immobilization of DNA offers the potential to enhance gene transfer by maintaining an elevated concentration of DNA within the cellular microenvironment. This was elegantly demonstrated by Jessel *et al*. with two different types of vectors in a (PLL/PGA) architecture while using β-cyclodextrins as core molecule for the DNA [93]. A vector expressing a nuclear transcription factor (SPT7) and one expressing GFP as cytoplasmic protein (EGFP) were embedded in the film at different depths using β-cyclodextrin to complex them. The time sequence of the transfection could be controlled depending on the sequence of embedding of the vectors. The authors showed that these functionalized films could act as an efficient gene delivery tool to transfect cells. Larger molecules like adenovirus (Ad) particles or even proteins like growth factors can be adsorbed onto or embedded in natural based films [94]. The Ad particles, which are 70 nm in diameter, were found to adsorb on (PSS/PAH) film surface and to be partially embedded in the multilayer films. They were even found to diffuse within (PLL/HA) films. The bioactivity of the particles was preserved when adsorbed in (PLL/HA) (18% remained infectious) and (PLL/PGA) films (24% remained infectious). Interestingly, whereas the Ad particles enveloped by (PLL/PGA) and (PSS/PAH) films (two to six layer pairs) remain inaccessible to cells, in (PLL/HA) and (CHI/HA) films, the overlay Ad with the same number of layers is responsible for a progressive and less important decrease in cell transduction. The authors postulate that Ad diffusion through the multilayers make it more accessible for cellular uptake and/or more available for cell infection. The maximal cell transduction efficacy could be achieved when Ad is well embedded and overlaid in two levels in (PLL/HA) and (CHI/HA) films.

The hydrolytically degradable synthetic polymers were also used by Lynn *et al*. to serve as local transfection agents, when films also incorporated plasmid DNA [95]. The placement of film-coated slides in contact with COS-7 cells growing in serum containing culture medium resulted in gene expression in cells localized under the film coated portion of the slides. The average percentage of cells expressing EGFP relative varied between ~5 and ~38%. Their study also suggested that the presence of the cationic polymer might contribute to the internalization and expression of the plasmid.

The physico-chemical properties of films containing DNA also depended on both the pH and the ionic strength of the polyelectrolyte solutions used [96]. Films eroded gradually upon incubation in a physiological buffer at 37°C for a period of about 30h. The authors indeed showed that the DNA was released in a relaxed, open circular topology and that the released plasmid promoted the expression of EGFP in COS-7 cell line.

Anti-Bacterial Films

Natural polyelectrolytes can be potentially employed in multilayer films due to their intrinsic bioactivity. For instance, use of chitosan as anti-bacterial dressings has received considerable attention in recent years [97]. The exact antibacterial mechanism of chitosan is still unknown but different mechanisms have been proposed: interaction between positively charged chitosan molecules and negatively charged microbial membranes causing leakage of intracellular constituents. Heparin with its anti-thrombogenicity and strong hydrophilicity prevents adhesion of bacterial cells and is an excellent candidate for anti-adhesive coatings. Hyaluronan can be

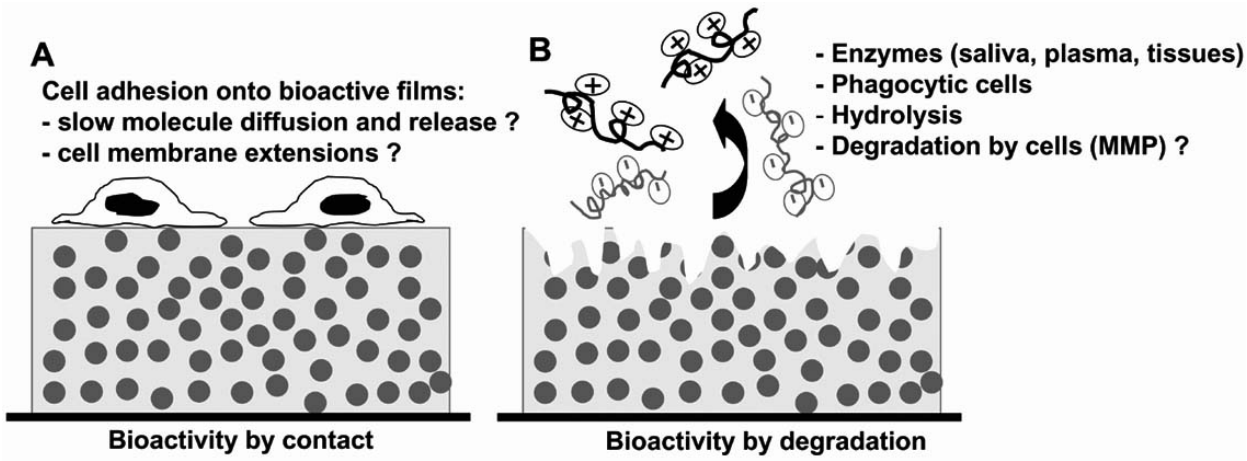

Fig. (8). Schematic of different mechanisms for achieving film bioactivity. **A**) Bioactivity by cell contact with a bioactive film. The question whether there is a slow diffusion of bioactive molecules out of the film (release) or the presence of cells extensions to "sense" the film is still open. **B**) Bioactivity by degradation. Enzymes, phagocytic cells and hydrolysis can all provoke film degradation and induce release of the film components; matrix metallo-protease (MMP) might play a role in film degradation, although this has never been demonstrated.

used for its high water retention capacity. Thus, anti-bacterial properties of films containing these polysaccharides have been explored.

Bacterial adhesion (*E. coli* Gram-negative strain) was investigated on certain types of natural based multilayer films containing chitosan and/or heparin $(CHI/HA)_{10}$ films (built in 0.15 M NaCl) are highly resistant to bacterial adhesion and lead to a ≈ 80% decrease in bacterial adhesion as compared to bare glass [9]. 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 observed differences 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) and also probably by an increased film rigidity. The $(CHI/HA)_{10}$ films built in the presence of 0.15M NaCl could thus be used as anti-microbial coatings for biomaterial.

In another study, (CHI/HEP) multilayer films were found to kill the bacteria adhered to the surface. *E. coli* initial adhesion was also greatly decreased on the multilayer film [98]. The assembly pH was found to be an important parameter in the design of efficient anti-adhesive and anti-bacterial film.

In order to enhance the antibacterial effect of multilayer films, the same authors prepared films containing silver nanoparticles (sizes 10-40 nm) and coated a polyethylene terephtalate graft with alternating layers of CHI/HEP, chitosan being complexed with silver nanoparticles with sizes (10-40 nm). The multilayer films containing nanosilver were not only effective as antibacterial but also as anticoagulant coating, while being non-toxic for the cells. The anti-bacterial properties of film functionalized with a peptide like defensin, as described below, have also been observed [83]. Finally, highly hydrated LbL films ending by a poly(ethylene) glycol (PEG) grafted PGA layer were found to resist serum adsorption as well as *E. coli* adhesion [99]. The effect was even more pronounced when three layer pairs of (PLL/ PGA-PEG) were deposited.

The different methods for achieving film bioactivity, either by direct contact with the cells, or by film degradation and release of the active constituent, are summarized in Fig. (**8**).

Bioactivity of Small Molecules (Peptides and Drugs)

A simple method to load a film with small molecules is to embed them by adsorption (Fig. (**6B**)). This was demonstrated for antibacterial peptides that are made up of about 40 amino acids, such as insect defensins. These peptides were immobilized in a (PLL/PGA) architecture and were found to be very efficient against *M. luteus* and *E. coli D22* adhesion, but only when PLL was the outermost layer of the films [100]. The authors suggested that the adhesion of bacteria is a prerequisite for the peptide to be active when it is embedded in the film. If the bacteria do not come into contact with the film and remain in close contact with the film for a sufficient period of time, as may be the case for films in which the final layer is PGA (negatively charged), the peptide will not be able to interact with and disrupt the bacterial cell membrane. This hypothesis was supported by CLSM and scanning electron microscopy observations.

For a different application, Thierry *et al*. found that the incorporation of sodium nitroprussid, a nitrous oxide donor that is widely used clinically to reduce blood pressure, within the (CHI/HA) coating further decreased platelet adhesion by 40%. The reservoir capacity of thick films was nicely demonstrated by Vodouhe *et al.* [72]. Using (PLL/HA) film as matrix, they observed using CLSM, that paclitaxelGreen 488 molecules diffuse through the whole $(PLL/HA)_{60}$ film section and that the fluorescence is homogeneously distributed over the whole film thickness. They successfully increased the amount of drug uptaken by increasing the paclitaxel solution concentration. They found that the effective concentration in the film is from 20 to 50 times higher than the initial solution concentration. For instance, when the solution concentration was 10 μg/mL, the effective concentration in the film was 500 μg/mL. Using this method, the drug content in (PLL/HA) films can be finely tuned in a large range concentration. 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 tuned by varying the film thickness [101]. The effect of paclitaxel loaded in the cross-linked (PLL/HA) films could be clearly seen over the three days culture period (Fig. (**7**)). After three days in contact with the bioactive films, less than 10% of the cells were still alive (Fig. (**9**)) [102]. Another way to deliver a drug is to couple it to the polyelectrolyte *via* a hydrolysable linkage. This so-called prodrug approach was employed by Thierry and al for loading and subsequently releasing paclitaxel from (CHI/HA-paclitaxel) films [103].

An alternative approach is to covalently graft a peptide to the polymer backbone and to adsorb the modified polymer as a regular layer. In this case, care has to be taken that the peptide remains sufficiently flexible and accessible to the cells. Often, a spacer molecule is coupled to increase the distance between the polymer backbone and the active site of the peptide. This approach was first

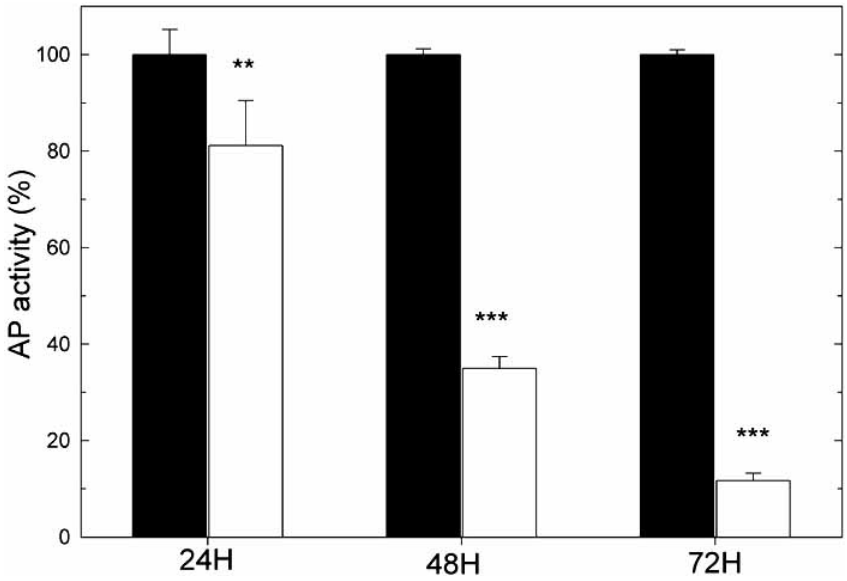

Fig. (9). Bioactivity of cross-linked polysaccharide multilayer films loaded with paclitaxel. Acid phosphatase (AP) activity for HT29 cells cultured on cross-linked $(PLL/HA)_{12}$ films loaded or not with paclitaxel, after different time periods of 24H, 48H and 72H in culture. The error bars represent the standard deviation. The value of 100% has been arbitrary set at 100% for CL films at each time period (* $p<0.05$; ** $p<0.01$; *** $p<0.001$ versus controls, which are the CL films at time 24H, 48H, and 72H respectively). (Adapted from [102]).

employed by Chluba *et al*. for grafting the alpha-melanocyte stimulating hormone (α-MSH) on poly(L-lysine) and subsequently testing its bioactivity on melanoma cells [104]. The authors showed that the long time activity of the hormone is maintained when embedded in multilayer architectures whereas its short time activity depends on the integration depth. The effect of this peptide grafted to poly(L-glutamic acid) and adsorbed on a (PLL/PGA) film was further shown in an *in vivo* study [105]. The bioactive coating was deposited on tracheal prostheses and systemic IL-10 production was only detected in rats implanted with prostheses functionalized by α-MSH. Beside α-MSH, a 15 amino acid peptide containing the RGD motif was grafted to PGA following the scheme presented in Fig. (**10**). It relies in first grafting a maleimide group on the carboxylic group of PGA. The biological effect of such $(PLL/PGA)_5$-PLL-PGA-RGD functionalized film is to increase the short time adhesion of osteoblasts cells and to allow these cells to proliferate over several days in culture (Fig. (**11**)), whereas un-functionalized films are very weakly adherent for cells [91].

Bioactivity of Embedded Proteins and Growth Factors

One of the first pieces of evidence that protein embedded in polyelectrolyte multilayer films retain their bioactivity came from Jessel *et al*. [106]. In their study, Protein A was embedded at differ-

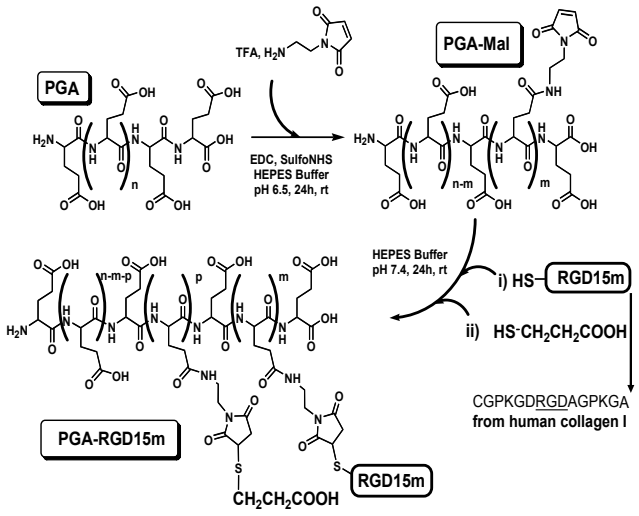

Fig. (10). Scheme of the synthesis of the 15 amino acid peptide that contained a -RGD- sequence (PGA-RGD15m). In a first step, the PGA was conjugated to the maleimide groups (PGA-Mal). Then, the conjugated PGA-Mal was mixed with the PGD15m peptide. Mercaptopropionic acid was used to neutralize the unreacted maleimide groups. The final PGA-RGD contains thus both the RGD function (~10%) and carboxylic sites and remains highly negatively charged (adapted from [91])

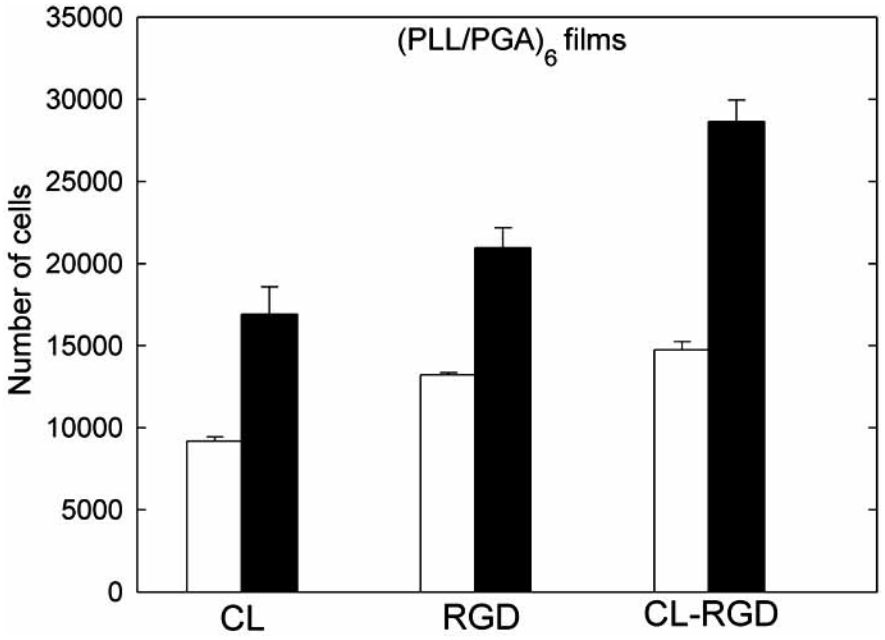

Fig. (11). Combined effect of cross-linking and of an RGD adhesion peptide on the proliferation of primary osteoblasts cells as measured by the alkaline phosphatase test at short time (white bars) and after eight days (black bars) in culture. (left) a cross-linked $(PLL/PGA)_6$ film compared to a RGD functionalized film (middle) and to a cross-linked film on top of which a PGA-RGD layer has been deposited (right). (Adapted from [91]).

ent levels in (PLL/PGA) films and was found to induce a time-dependent expression of TNF-α in THP-1 phagocytic cells. Interestingly, the cells were shown to come into contact with the protein by local degradation of the films. Notably, the cells were able to degrade the PLL but not poly(D-lysine), which forms a barrier between the cells and protein A. In this case, TNF-α production was significantly reduced.

Non phagocytic cells such as neurons have also been shown to respond to protein embedded in a multilayer architecture. In the study by Vodouhe *et al.* multilayer films (mostly ending by PSS) were functionalized with a growth factor, Brain Derived Neurotrophic Factor (BDNF) or a chemorepulsive protein, Semaphorin 3A (Sema3A) [107]. The quantitative amount of protein adsorbed was estimated by optical waveguide lightmode spectroscopy. The authors showed that the embedded proteins were stable in the multilayer architecture and that the protein was not released in the culture medium after two days in culture (or at least, the release level was below the detection level). Very interestingly, BDNF induced an increased neuronal activity and an increased neurite length, whereas Sema3A induced a decreased activity and neurite length. Thus, the structure of the films could be correlated to their effective biological activity.

Shen *et al*. introduced a new class of bioactive films using directly growth factors (acidic or basic fibroblasts growth factors, aFGF or bFGF respectively) as building blocks, either mixed with heparin and deposited alternately with PEI, or directly used as polycation and deposited with chondroitin sulfate A [64, 65]. An enhanced secretion of collagen type I and interleukin 6 (IL-6) by fibroblasts seeded on the five layer pairs of (aFGF/HEP)/PEI was also observed by immuno-histochemistry. When bFGF was directly built in multilayer films with CSA, the films containing bFGF had an improved bioactivity. *In vitro* incubation of the CSA/bFGF multilayers in PBS showed that about 30% of the incorporated bFGF was released within eight days. The fact that growth factors retained their biological activity is extremely interesting for biomedical applications.

Using films made of (PLL/CSA), Tezcaner *et al.* prepared functionalized multilayers by adsorbing bFGF or the insoluble fraction of the intercellular photoreceptor matrix (IPM) on or within the (PLL/CSA) polyelectrolyte multilayers [35]. They showed that bFGF and IPM adsorption on top of the $(PLL/CSA)_{10}$/PLL polyelectrolyte films increased the number of photoreceptor cells attached, and in particular bFGF adsorbed on the top led to a statistically significant increase in photoreceptor cell survival at day 7. Recent developments include the use of films containing growth factors, such as bone morphogenetic protein 2 (BMP2) and transforming growth factor 1 (TGF β1) for inducing the specific differentiation of embryonic stem cells to form bone tissue [108]. The authors used monocarboxylic β-cyclodextrins to favor the insertion of both growth factors and showed that both were required for inducing an effective differentiation. However, little is known about the exact amount of protein adsorbed or about the interaction mechanism of the growth factors and the embryonic like bodies.

CONCLUSION

Within the past decade, the development of layer-by-layer film for biomedical applications has emerged. It is no doubt that the biological applications of LbL, which are still at early stages, will see a rapid development in the next decade. This will require gaining better molecular insight into the structure of LbL films, which is a challenge since techniques usually employed for studying bulk hydrogel structure are not adequate and sensitive enough for investigating thin supported films. Therefore, it is envisioned that single molecule techniques such as fluorescence correlation spectroscopy, colloidal probe microrheology, nuclear magnetic resonance (and others) will be powerful tools for learning more about the diffusion of water and molecules in the films and about their local mechanical properties. In particular, the film porosity is an important parameter that has to be known and controlled for drug and growth factor release applications. The role of polyelectrolyte chain stiffness, which is an important parameter for polysaccharide molecules, could be studied. Similarly, hydrophobicity, which could play an important role in the insertion of amphiphilic growth factors or hydrophobic drugs could be tuned by grafting hydrophobic side chains or playing on the suspending medium parameters. Very importantly, new strategies for cross-linking, allowing at the same time to preserve the biofunctionality of biomolecules and to enhance film mechanical resistance, will have to be developed. This

may include the development of photo-crosslinked, or thermo-sensitive films (with a temperature transition close to 37°C), similarly to what has been developed for polysaccharide hydrogels. This also encompasses the preparation of composite films containing organic-inorganic molecules that could in addition contain functionalized colloids.

Smart systems will be developed, making use of the "reservoir" capacity of the polysaccharide multilayer films and of the "barrier" capacity of very interdigitated films. These reservoirs will have to be optimized in order to increase the loaded amount, to optimize the release, while at the same time allowing for cell adhesion. Also, LbL will help to answer fundamental biology questions and to modulate cell adhesion, proliferation and differentiation depending on the film properties. In this way, the films may also constitute a new surface for stem cells specification and/or differentiation. Surface transfection and tissue engineered matrices by means of LbL will be developed and optimized. Studies of the durability and tissue response *in vivo* will have to be conducted for films that are aimed at being implanted or in contact with human tissues. In summary, the LbL films made of natural polymers really seem to be very good candidates for biological applications.

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LIST OF ABBREVIATIONS

ALG	=	Alginate
BDNF	=	Brain derived neurotrophic factor
CHI	=	Chitosan
CSA	=	Chondroitin sulfate A
CLSM	=	Confocal laser scanning microscopy
CMP	=	Carboxymethylpullulan
COL	=	Collagen
DEX	=	Dextran
aFGF	=	Acidic fibroblast growth factor (resp. bFGF for basic)
HA	=	Hyaluronan
HEP	=	Heparin
PEI	=	Poly(ethylene)imine
PLL	=	Poly(L-lysine)
PAH	=	Poly(allylamine) hydrochloride
PEM	=	Polyelectrolyte multilayer
PSS	=	Poly(styrene) sulfonate
PLGA	=	Poly(lactic-co-glycolic) acid

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