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Polysaccharide-based polyelectrolyte multilayers

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ABSTRACT

In recent years, the layer-by-layer technique has grown in various fields. One of the emerging trends of bio-applications is the use of polysaccharides as main film components, which stems from their intrinsic physical, chemical and biological properties. These allow the simple formation, by self-assembly, of new kinds of mimics of extra-cellular matrices from plant and animal tissues. These assemblies, which possess specific properties arising from their hydration and internal composition, can indeed contain additional functionalities obtained by chemical modification of the biopolymers or film post-processing. They can be molded into different forms (films, membranes, and capsules).

1. Introduction

Layer-by-layer deposition of polyelectrolytes on to a solid substrate has become a popular tool for producing new types of thin coatings with controlled architecture. The formation of polyelectrolyte multilayer films (PEM) is usually acknowledged to be related to the formation of polyelectrolyte complexes in solution (also called "complex coacervates"). The method allows the polymers to self-assemble on a surface with a film growth that depends on the respective intrinsic properties of the polyelectrolytes as well as on the experimental conditions. Although many studies focus on synthetic polyelectrolytes, which offer numerous possibilities in the variation of buildup conditions, multilayer films containing polysaccharides have attracted considerable attention in the past 12 years. These polysaccharides are known to be crucial for the proper formation of native plant and human tissues in terms of structural and functional organization. Their constitutive side groups such as hydroxyl, carboxyl and sulfate, as well as their persistence length and the length of the polymeric chain (number of saccharide units), are all important for their interactions with water and proteins. In this review, the discussion focuses on polysaccharide-based PEM with specific emphasis on progress made in the last 3 years. In the past few years, much better knowledge of film growth, hydration and internal composition has been gained. Diffusion and dynamics in polysaccharide-based films have also been investigated and quantified. These films are indeed a powerful model for investigating the mechanisms of diffusion and water retention, as polysaccharides-based PEM films are amongst the most highly hydrated. Deposition of films on to a biodegradable core or on to a detachable substrate has made possible the formation of polysaccharide-based capsules or membranes. These new nanostructured biomimetic membranes may find applications as biodegradable drug delivery systems or functional patches. More recently, the formation of hydrid films made of polyelectrolyte blends has brought further understanding of the polyelectrolyte pairing and of the preferential incorporation of sulfated polysaccharides. Also, the possibility of chemically modifying the polysaccharides and successfully incorporating these new derivatives into PEM films has recently been shown by several groups. This is a powerful tool for giving PEM films additional functionalities and for designing multifunctional architectures. Overall, understanding how these PEM films self-assemble at the nanometer scale and how is it possible to tune their multiple properties (hydration, mechanical properties, bioactivity, spatial organization…) could be of help to us for our fundamental understanding of native tissues, as well as for biomedical applications. We believe that these PEMs could be used as a tool for biologists working on matrix biology, for biophysicists but also for biomaterials scientists. Indeed, the bottom-up approach of PEM films reduces the complexity of native extra-cellular matrices (ECMs), which are often investigated in a top to bottom approach (by successive degradation of the components of the matrix). PEMs can bring new insights into the interactions between polysaccharides and peptides, polypeptides, or proteins in a reconstituted thin matrix. Also, polysaccharide-based PEM films are a new kind of coating whose properties can be finely tuned and, in this sense, be employed for systematic studies of the

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influence of several adjustable parameters. Finally, thanks to their versatility and ease of deposition on to various kinds of support materials, polysaccharide-based PEM films will probably be a great opportunity for biomaterials scientists to modify the surface of biomaterials selectively, while maintaining their bulk properties unmodified. There is no doubt that fundamental understanding of these fascinating assemblies will serve many different aspects of research, from fundamentals to applications.

2. Why using polysaccharides in PEM films?

2.1. Definitions

Polysaccharides are a family of carbohydrates that play fundamental roles in many biological contexts. Their structure is made of sugar rings linked by glycosidic bonds and various side functions. Two elements are of utmost importance in the chemistry of polysaccharides. Firstly, the glycosidic bonds can be the target of glycoside hydrolase enzymes and can thus be biodegraded relatively easily. Secondly, the side groups can directly affect the polysaccharide's charge density, hydration and chemical reactivity, and can also be responsible for the formation of secondary structures. When charges are present, polysaccharides behave like polyelectrolytes. The negative charges are carboxylic groups (COO^-) with *p*Ka around 3–5 or sulfate groups (SO_3^-) with a *p*Ka of around 0.5–1.5 [1]. The positively charged groups are ammonium groups (NH_3^+) with a *p*Ka of around 7–10 [2]. PEM films self-assemble thanks to interactions between the negative and positive groups and thanks to the entropic gain associated with these associations. There are two main types of polysaccharides: those present in plant cells, cellulose being the main polysaccharide on earth, and those present in animal tissues, with glycogen the most important for energy storage.

2.2. Structure and functional roles of polysaccharides

Polysaccharides play many different roles *in vivo*. First, they are essential structural components of both plant and animal cells, and contribute to their unique self-assembling properties. For instance, cellulose can self-assemble into microfibrils that in turn associate into larger fibers through hydrogen interactions. Chitin is the main structural element of the crustacean exoskeleton (crab, shrimp, etc…) and cell walls of fungi. In human connective tissues, large quantities of glycosaminoglycans (GAG), which are long unbranched polysaccharides composed of a repeating disaccharide unit (hexose linked to a hexosamine), are found in the extra-cellular matrix. Chondroitin sulfate (CS) is the most prevalent, together with heparin sulfate (HS) and hyaluronan (HA). Of note, hyaluronan is the only non-sulfated GAG and heparin has the highest negative charge density of any known biological molecule. HA and CS are responsible for the unique hydration and mechanical properties of synovial fluid, cartilage and tendons. HA and CS are indeed highly hydrated polymers surrounded by respectively ~20 and ~30 water molecules per disaccharide unit in interaction through hydrogen bonds [3,4].

Importantly, these polysaccharides are part of the pericellular coat (also called glycocalyx). This coat, which can be up to several μm in thickness [5], plays a major role in the interactions between a cell and its environment by mediating cellular adhesion and the diffusion of biomacromolecules such as growth factors [6].

Second, polysaccharides have a key functional role. Starch and glycogen are energy storage molecules. Glucose is soluble in water, hydrophilic and takes up space, whereas it is insoluble in the form of starch and can be stored much more compactly. In starch, the glucose molecules are bound by the easily hydrolysable alpha bond, in order to render the sugar reserve rapidly available [7]. In animal reserves, similar structures and bonds are found in glycogen. In the ECM of tissues, HS is known to interact with a large range of proteins and to be involved in the regulation of their bioactivity [8]. In particular, high affinities with several morphogens and growth factors, such as the basic fibroblast growth factor (bFGF) and bone morphogenetic growth factors (BMPs) [9] have been reported with heparin (HEP). These molecules are indeed key players in the maintenance of tissues and affect numerous cellular processes including cell proliferation and differentiation. The ECM thus work as a storage reservoir, by locally concentrating them, presenting them at the cell-surface and protecting them from enzymatic degradation, while releasing them at the appropriate time [10].

In addition, specific cell-surface receptors for polysaccharides have been identified. For instance, in the case of HA, CD44 or RhAMM are known to be involved in HA-mediated cell adhesion, proliferation, and survival [11].

Finally, due to their high level of hydration, HA, chondroitin sulfate A (CSA) or alginate (ALG) are also known for their protein-repellent properties [12]. This effect was attributed to the presence of the hydration shell around the polysaccharides, and precluded the protein/polyelectrolyte interactions.

2.3. Advantages and limitations of polysaccharides

The exceptional structural and functional properties of polysaccharides reviewed above are the rationale for their use as biomaterials as well as for surface modification of biomedical devices. Being natural constituents, they are ideal building blocks for creating systems mimicking the structural and biochemical properties of the *in vivo* cellular environment. Also, these polymers are naturally degraded by different kinds of enzymes present *in vivo* giving polysaccharides a great advantage over synthetic polyelectrolytes. For instance, (CHI/HA) film biodegradation *in intro* and *in vivo* has already been evidenced [13]. These superior advantages for biomedical applications as compared to synthetic polyelectrolytes explain why they have been increasingly employed as film constituents since the early 2000s.

However, certain difficulties are encountered when working with polysaccharides. First, although the commercial availability of polysaccharides has increased with the increasing demand, in particular for HA, wide variations in quality from one provider to another and from batch-to-batch can be an issue. Most polysaccharides are extracted and purified from natural tissues and are thus dependent on natural variations. Polydispersity is another concern, which renders systematic studies on the effect of molecular weight variations in a limited range quite difficult. An exception should be noted for HA, for which there has been recent developments in production technology, such as recombinant production [14]. In addition, the commercial availability and quality (monodispersity) of HA lots have greatly improved in the past few years [15]. Chemical modification can be a real challenge because of the high hydration shell and poor solubility in organic solvents. In addition, the variety of reactive groups present on the different polysaccharides (OH, COO^-) is limited. Their flexibility is also lower than that of synthetic polyelectrolytes as they have a higher persistence length. Due to their lower solubility, pH and ionic strength (I) can only be varied to a lower extent than for their synthetic counterparts. Thus, the overall degree of freedom in the choice of buildup conditions seems more limited. The advantages and limitations of using polysccahrides in PEM films for biomedical applications are summarized in Table 1.

2.4. Polysaccharides used in PEM films

In the past 10 years, there have been many attempts to assemble polysaccharides into PEM films. As mentioned above, most polysaccharides are negatively charged and are thus used as polyanion constituents, unless they are chemically modified to render them polycationic. This was indeed the case for amide modified HA [16] and for quaternized chitosan [17]. Plant derivatives such as carboxymethyl

Table 1
Comparison between the advantages and disadvantages of synthetic polyelectrolytes and polysaccharides used for the buildup of multilayer films in the context of biomedical and biomaterials research.

	Synthetic polyelectrolytes	Polysaccharides
Advantages	• Large choice of chemistries, structure and charge densities • Flexibility • Large working range of pH and ionic strength • Easy chemical modifications • Abundant and usually cheap • Available with highly controlled quality	• Natural polyelecrolytes (biomimetism) • Interesting structural properties: interactions with water, self-assembly, hydrogel formation • Functional properties: specific cell receptors, interactions with bioactive molecules (growth factors…), present in the pericellular coat • Biodegradability and biocompatibility (for most of them)
Limitations	• Most often non-biodegradable • Potentially harmful degradation products • No particular bioactivity	• Limited availability with well defined properties (purity, polydispersity); often purified from natural tissues • Chemical modifications can be particularly difficult due to the poor reactivity of the group, low charge density and poor solubility in solvents (need for "biofriendly" processes) • Limited pH and ionic strength working range due to solubility issues

cellulose [18], pectin [19] and its derivatives polygalacturinic acid and furcelan [20] have been successfully used. Animal-derived polysaccharides such as HA [21], CS [22], HEP [23] and mucin [24] are also increasingly investigated for their ability to form PEM films with specific properties. The choice of a polycationic polysaccharide is very limited. In fact, only chitosan (a de-acetylated form of chitin) is currently available and used in PEM films. Thanks to its numerous interesting properties, including wide availability, biocompatibility, wound-healing and antibacterial properties [25], chitosan is probably by far the most widely used polysaccharide in LbL films.

As an alternative to chitosan, the cationic polypeptide PLL has been widely used in combination with polysaccharides in PEM film [21,26]. Poly(L-lysine) (PLL) can be considered as a model protein as lysine is, together with arginine, the major positively charged amino-acid found in proteins. To a certain extent, such polypeptides can mimic the natural polysaccharide–protein interactions occurring *in vivo*, for instance proteoglycan assemblies. Nonetheless, other synthetic cationic polyelectrolytes such as poly(ethylene imine) (PEI) [18] and poly(allylamine hydrochloride) (PAH) [27] are often used as polycations.

3. Film growth, hydration and internal composition

Polysaccharide-based PEM films were at the origin of the discovery of a new growth mode, namely the exponential growth of film thickness based on the diffusion of at least one of the polyelectrolyte species in the films. Polysaccharide multilayer films containing ALG and HA in combination with PLL (PLL/ALG) [26] and (PLL/HA) [21] were the first exponentially growing films to be reported. These greatly contrasted with the widely studied and linearly growing poly (styrene sulfonate)/poly(allylamine hydrochloride) (PSS/PAH) synthetic films. This type of growth was initially mostly observed in films based on polysaccharides and polyaminoacids [21,26,28] but it is nowadays widely recognized that many different types of systems, including synthetic PEM films, can grow exponentially [29]. Polyelectrolyte diffusion was found to be a key feature of this type of growth. Evidenced for the first time on (PLL/HA) films [21,29], some chains labeled with fluorescein isothyocyanate (FITC) were found to remain free in the films. These were able to diffuse toward the upper part of the films upon deposition of the HA layer. A significant contribution to film growth thus came from these free chains that were able to interact with the incoming HA chains. Watery and very thick (PLL/HA) films then became a model for deeper understanding of these phenomena. Later studies brought a better view of the diffusion process, in particular on its limitation to a certain zone [30]. Better understanding of the different growth mechanisms is also emerging. [31] For instance, Porcel et al. showed that a transition from exponential to linear growth occurs at a certain level in film buildup. [30] It also appears that, even for synthetic polyelectrolyte films, exponential growth becomes dominant when NaCl concentrations increase [32] or when temperature is increased [33]. 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. [34]

Simulations are also only in the early stages. According to Holm et al. in their recent review [35], no simulation study has been able to reproduce the exponential growth of PEMs. The interesting predictions made by Hoda and Larson [36] about exponential growth require a test *via* numerical simulations. One of the reasons for the lack of results may be that, so far, numerical studies have never explored the influence of a mismatch on the degree of charges between two different polyelectrolytes. As indicated earlier and as will be shown below, this is, however, quite probably often the case for exponentially growing films.

3.1. Hydration and swellability

Usually, the known properties of polysaccharides in solution will directly influence the properties of the PEM films formed. Hydration is a striking example. Neutron scattering is a major technique to study the distribution of salt ions and water in synthetic polyelectrolyte multilayer films [31]. However, due to the inherent difficulty in drying the polysaccharide-based films without changing their structure and due to the fact that deuterated polysaccharides are difficult to prepare, there are to our knowledge no data available on polysaccharide-based PEM films probed by neutrons. For such films, hydration is usually probed by comparing hydrated adsorbed masses measured by Quartz Cristal Microbalance with Dissipation monitoring (QCM-D) and dried masses measured by optical methods such as Surface Plasmon Resonance (SPR), ellipsometry or Fourier Transform Infrared Spectroscopy in Attenuated Total Reflection mode (ATR–FTIR). Water content is reported to be very high, ranging from 70 to 90% in (PLL/pectin) films [19,37], 60% in (PLL/CSA), ~90% in (PLL/HA) films [38], 80% for (CHI/HA) [39] and for starch based films [40], 93% for (PLL/furcellaran) [20] and over 90% for (CHI/mucin) films [41]. In relation to this high hydration, film refractive indices are usually low (~1.38–1.40) for polysaccharide-based films [42]. Hydration is a critical parameter for PEM films, impacting film thickness, swellability and diffusion of the film's components. Film hydration can vary during film buildup. Indeed, when two oppositely charged polymers complex together, the counterion–polymer and water–polymer bonds are disrupted, which can lead to dehydration of the multilayer film [19]. Several chemical side groups appear to interact with water molecules *via* hydrogen bonds. For instance, the ester groups present in pectin are known to be good acceptors of hydrogen bonds and can thus trap water molecules in proximity to the polymer. Films made from pectin with decreasing ester content have been found to be less hydrated and thus thinner and more dense [19]. Acetamido groups present on CS and HA may also be partially responsible for the high hydration of (PLL/CSA) and (PLL/HA) films. Interestingly, although HA and CSA have differently charged chemical functions and charge densities, they both have the acetamido group and both yield thick, hydrated

films in combination with PLL [38]. Heparin, with a similar structure but without this acetamido group, yields thin, dense films.

The film swelling properties (*i.e.* their ability to change volume and thickness as the environmental conditions are changed, such as pH, ionic strength, hydration) has been found to depend on buildup conditions. In the initial studies on HA-based PEM films, the buildup was performed in physiological conditions with no intermediate drying stems. The films are thus already highly hydrated and do not further swell in physiological solution (pH 6.5–7.5 and ionic strength of 0.1–0.15 M NaCl). The film was found to shrink to ~50% upon dehydration in ethanol baths of increased concentration [43]. Film cross-linking also induced a slight film swelling (~10%) as cross-linking is performed at a slightly lower pH (5.5) [44]. On the contrary, when the films are built by alternate dipping in the polyelectrolyte solution followed by intermediate drying steps, the swelling of the film between the dried and wet states can be very high (hundreds of %). According to Barrett et al. [45] who used intermediate drying steps, the swelling of (PAH/HA) films shows a high dependence on the assembly solution pH. The swelling ratio varied between 2 at physiological pH (pH = 7) to more than 8 at very acidic pH (pH = 2) and was more intermediate at basic pH (pH = 10) with a swelling ratio of about 5 (*i.e.* 500%).

3.2. Internal composition

Film hydration is related to the affinity between the charged groups of the two polyelectrolytes. A polyelectrolyte couple with high affinity will chase water during complexation and form very dense and highly cross-linked networks, resulting in films close to a "glassy state" (*i.e.* "frozen" chains without mobility) [46]. The interactions between the polyelectrolytes in terms of stoichiometry and affinity are thus critical parameters. A question arises from these facts: how does polyelectrolyte chemistry, and in particular charge density, affect the film composition and ionic pairing of the polymers?

For polysaccharide-based films, the question was assessed by two different approaches. Ring et al. investigated systematic changes in the degree of de-acetylation of pectin in (PLL/pectin) films [19,37]. The ratio of PLL/pectin monomers was slightly below 1, between 0.83 and 0.97 depending on the degree of pectin de-esterification. In our group, we chose a series of structurally similar polysaccharides that bear an increasing charge density. To this end, HA, CSA and HEP, with their increasing sulfate contents (from 0 to 2.5) and charge density, were selected. The internal composition for (PLL/HA), (PLL/CSA), (PLL/HEP), and (CHI/HA) were probed by FTIR [38] [39]. The PLL/polyanion monomer ratio was around 0.5 for all the films investigated. Although the polycationic/polyanionic ratios were different in the two studies, many similarities emerged. First, the composition ratio did not seem to drastically depend on the charge density of the polyelectrolytes. It seems that sterical hindrance is an important driver for polysaccharide assembly, which is in a certain way independent of the charge position or density. Second, because of this approximately constant ratio, the charge is directly influenced by the charged density of the polyelectrolytes used. Thus (PLL/pectin), (PLL/HA) and (CHI/HA) films were found to be positively charged, (PLL/CSA) is almost neutral whereas (PLL/HEP) films are negatively charged. This excess of charges is compensated by mobile counterions to ensure the overall electroneutrality of the film. Third, growth mode was affected by charge density, more linear growth being observed for highly charged polysaccharides (highly de-acetylated pectin or heparin), whereas growth was exponential for the less charged polysaccharides (HA, CSA, highly acetylated pectin). Of note, ionic pairing in polysaccharide films has been investigated very little. In our recent work, we showed that sulfate groups are much more likely to interact with the ammonium groups of the polycation than the carboxylic groups [38]. This must be related to the preferential incorporation observed in blended films (see paragraph 7).

4. Physical and chemical parameters and molecular weight: influence on film growth

pH and ionic strength are two important parameters that can be varied to modulate film thickness. Their systematic influence on film growth has been studied mostly for PEM films based on synthetic polyelectrolytes [47]. As a general rule, film thickness increases when the pH is close to the pK_a of the polyelectrolytes and when I is increased. Only few systematic studies are specifically dedicated to polysaccharide-based PEM films. For polysaccharides, the working ranges for pH and I are usually limited to 3–10 for pH and 10^{-4} M to 1 M for I respectively, due to their solubility limits. Very interestingly, it appears that such studies have mostly been performed on CHI-containing films in association with different polyanions. Of note, the pH working range for CHI is usually limited to pH below 5.5 due to its low pK_b (close to 6). On the contrary, for HEP (the only polysaccharide being a strong polyelectrolyte), its total charge is almost independent on the pH (a single COO^- group versus 2 to 3 sulfate groups). In a pioneering work, Lvov et al. showed that the thickness of (CHI/PSS) films increased with I and that the adsorption kinetics depended on I [48]. Later on, Richert et al. [49] showed for (CHI/HA) films that film thickness increased with I over the range 10^{-4} M to 0.15 M NaCl, as higher I did not lead to proper PEM film formation. Post-assembly stability was also checked for such films [50].

Radeva et al. [51] evidenced, while maintaining the pH of CHI constant at 4, that the pH of the carboxymethyl cellulose (CMC) solution significantly influenced film growth: film thickness was higher when buildup pH was close to the pK_a of CMC. Recently, Boddohi and Kuiper [52] showed for (CHI/HEP) films that film thickness increased with ionic strength in the 0.1 to 0.5 M NaCl range. In addition, the influence of pH (from 4.6 to 5.8) was most prominent for the intermediate ionic strength (0.2 M NaCl), with thicker films formed at the highest pH (*i.e.* closer to the pK_b of chitosan).

The effect of temperature has been studied little. Kankare et al. observed for (PLL/HA) films that temperature had an important effect on film growth. Their data suggested a change in diffusion rate with a temperature rise from 0.5 °C to 55 °C.

Overall, although less studied, polysaccharide-based PEM films appear to roughly follow the trends observed for synthetic PEM films with respect to their dependence on pH, I and temperature. For the reasons mentioned above, systematic studies on the effect of molecular weight are more tedious than for synthetic polyelectrolytes, because of their higher polydispersity. We have already reported the effect of polyelectrolyte molecular weight variations [42], where no clear common trend could be deduced from the different studies.

5. Diffusion and dynamics in polysaccharide-based films

Diffusion of one of the film's components has in many cases been related to exponential film growth. The first visual proof of PLL^{-FITC} diffusion in a (PLL/HA) film [29] was obtained from confocal laser scanning microscopy (CLSM). Later on, CHI^{-FITC} was also found to diffuse in (CHI/HA) films [49]. Several studies then focused on measuring diffusion coefficients in order to obtain better insight into the structure and dynamics of the film. Diffusion can either be measured "in plane" by fluorescence recovery after photobleaching (FRAP, using a fluorescence microscope) or by FRAPP (Fluorescence Recovery After Pattern Photobleaching) or "out of plane" for diffusion in the z-direction. In the latter case, Fluorescence Resonance Energy Transfer (FRET) between a fluorescently labeled polyelectrolyte embedded in the film and an added fluorescent layer of the same polyelectrolyte can be used. The pair of dyes must be carefully chosen so that energy transfer can occur. Alternately, CLSM can also be used but it is limited to thick films (a few μm in thickness). Evanescent wave techniques such as Fourier Transform Infrared Spectroscopy in Attenuated Total Reflection mode (ATR–FTIR) are also an appropriate tool but the z-resolution is limited by the

penetration depth of the evanescence wave (typically 500 to 900 nm depending on the type of crystal used).

The "in plane" diffusion of PLL^{-FITC} in (PLL/HA) films measured by FRAP–CLSM was found to be 0.1 μm^2/s with 40% of the PLL chains mobile [53]. A better view of PLL^{-FITC} dynamics was recently obtained by FRAPP. The diffusion behavior was found to be different for PLL chains deposited on top of the film or PLL chains embedded in the film, even under just one HA layer. For embedded chains, two populations were found: a mobile one with a diffusion coefficient D of the order of 0.1 μm^2/s and a population that appears immobile ($D<0.001$ μm^2/s). For chains deposited on top of the multilayer, a third and rapidly diffused population appeared ($D=1$ μm^2/s). These results showed that there are different types of diffusive PLL chains in films.

In a FRAPP study by Von Klizing et al. [54], lateral diffusion coefficient measurements on poly(dimethyldiallyl ammonium chloride) (PDDA)/HA films using PAH^{-FITC} as the probing layer were estimated at 5×10^{-4} μm^2/s, *i.e.* much below the values given in the aforementioned studies. These discrepancies may be explained by a more dense structure for PDDA/HA films as compared to PLL/HA or by the fact that the PDDA/HA films were dried after buildup, which may increase their ordering. However, further experiments are needed to test these hypotheses.

In a recent work, Tilton et al. probed the dynamics of chitosan in (CHI/HEP) films [55]. Using different fluorescently labeled chitosan, they measured the "out of plane" (z-direction) diffusion using FRET. By modeling their experimental data of FRET efficiency, they deduced values of 1 to 8×10^{-8} μm^2/s depending on the salt concentration and pH of the CHI (higher for higher I and pH). Crouzier et al. [38] recently reported values of 3×10^{-3} μm^2/s for the lateral diffusion of PLL^{-FITC} in (PLL/HEP) films (~80 nm in thickness), which was 20 times less than in HA-based films. Here again, further studies are required to fully understand the origin of such a high (10^5 fold) difference between these studies on quite similar films.

Diffusion of proteins or of smaller molecules such as ions can be used for electrochemistry studies on redox molecules. For example, myoglobin was loaded into (CHI/HA) films by means of post-diffusion. The influence of several factors, such as layer number, the pH of the loading solution, as well as its ionic strength, were systematically studied (Fig. 1) [56]. The cyclic voltammetric peak pair of the myoglobin Fe^{III}/Fe^{II} redox couple for $(CHI/HA)_n$-myoglobin films on pyrolytic graphite electrodes was used to investigate the loading behavior of (CHI/HA) films with regard to myoglobin. Another report

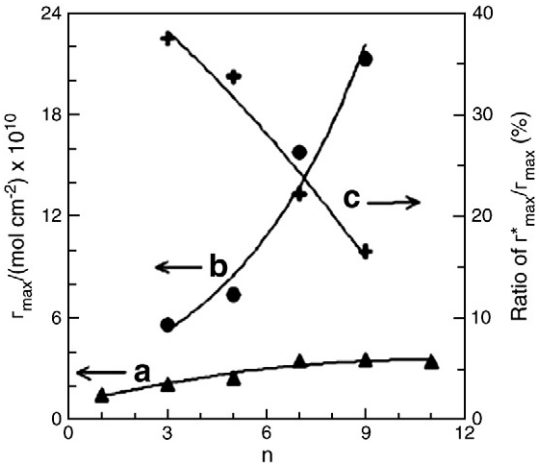

Fig. 1. Influence of the number of layer pairs (n) of $(CHI/HA)_n$-myoglobin films on (a) the maximum surface concentration of electroactive myoglobin (Γ_{max}) measured by cyclic voltametry in pH 7.0 buffers at t_{max} and 0.2 V s^{-1}, (b) the surface concentration of myoglobin (Γ_{max}) measured by QCM at the same t_{max}, and (c) the ratio of $\Gamma^*_{max}/\Gamma_{max}$. (From Lu and Hu, *J. Phys. Chem. B, 110,* 23710–23718, 2006) ; copyright American Chemical Society 2006.

dealt with the study of the confinement of ferricyanide $(Fe(CN)6)^{3-}$ ions into (PAH/ALG) and (PAH/CMC) films. Their loading was higher when the surface charge of the film was positive [57]. On the contrary, positively charged hexa-ammine ruthenium ions $Ru(NH3)_6^{3+}$ could not penetrate the films, which suggested that PAH was in great excess in such films. Of note, a similar trend concerning the presence of a great excess of polycations, *i.e.* a significant non stoichiometry, was also found in other polysaccharide multilayer films containing PLL [38]. In a later work by Anzai et al. [58], a significant effect of the type of polycation (whether PEI or PDAMAC) was found on the redox properties of the $(FeCN_6^{3-})$ ions.

6. Microcapsules and membranes based on polysaccharide multilayer films

PEM films can be deposited on a non planar template, such as a microparticle, to create polyelectrolyte microcapsules. To this end, the core must be degraded after film deposition. Free-standing films (*i.e.* membranes) can also be prepared by detachment of the film from the supporting substrate. In both fabrication processes, the detachment or dissolution steps have already been performed in rather harsh conditions (HF acid or concentrated HCl) in the case of synthetic polyelectrolytes [59]. This may explain why only few examples of polyelectrolyte microcaspules containing polysaccharides have been shown, as alternative strategies for core dissolution needed to be established. One of the first types of microcapsule consisted of (CHI/chitosan sulfate) [60]. DeGeest et al. have already succeeded in preparing capsules containing dextran sulfate [61]. As interactions between the polyelectrolytes are usually weaker in the case of polysaccharides, there is now a search for milder conditions for core dissolution in aqueous conditions, and in particular for lowering the osmotic pressure that is produced during core dissolution. To this end, biodegradable cores such as $CaCO_3$ are preferred [62,63], as well as other kinds of template such as microgels [64]. LbL assemblies based on HA have only recently been the basis for microcapsule formation from a $CaCO_3$ template [62]. The conditions for core dissolution were carefully investigated and it was found that EDTA or citric acid are successful chelating agents. However, in some cases, chemical cross-linking prior to core degradation is necessary [62,64] in order to strengthen the PEM film.

Interestingly, a considerable effect of the molecular weight of HA as well as on the HA solution concentration was observed in the case of PAH/HA microcapsules. This was attributed to the diffusion of polyelectrolyte chains into the porous $CaCO_3$ pores (60 nm in diameter) for low MW HA. Higher HA concentrations led to a more dense and entangled structure that was more favorable for capsule formation.

PEM membranes based on polysaccharides are also rare. For the same reason as for capsule preparation, conditions that are too harsh can lead to film rupture. This was indeed observed for (PLL/HA) films after dissolution of the polystyrene substrate in THF (tetrahydrofuran) [65]. An alternative strategy was to chemically cross-link the films prior to their detachment in 0.1 M NaOH. In this case, the membranes were homogeneous. Kotov et al. investigated the mechanical properties of membranes made of CHI and montmorillonite (MTM), as CHI possesses higher strength than PDADMAC [59]. They successfully prepared such membranes, which were however found to exhibit much lower mechanical properties than their PDADMAC counterparts. These authors attributed the decrease in mechanical properties to the lower flexibility polymer (persistence length of ~5 nm for CHI) resulting in poor interfacial adhesion with the clay.

7. Hybrid films

Polyelectrolyte blends (reviewed by Quinn et al. [66]) have recently emerged as a new tool for modulating film thickness, film

morphology and secondary structure, degradation rates, protein adsorption [67] or even mechanical properties. A limited number of studies deal with polysaccharides as one of the components in the blend. Quantitative FTIR spectroscopy measurements using characteristic peaks for each polyelectrolyte allow their mass fraction in the film to be determined. When HA was mixed with either PSS (a strong synthetic polyelectrolyte) [68] or HEP (a strong polysaccharide) [69] and combined with PLL as the polycation, a preferential incorporation of the strong polyelectrolyte was always observed. Of note, a very steep increase in the HA effective mass content in the film was evidenced when the HA mass fraction in solution was within the 95–100% range. When a sulfated polysaccharide (β-1,3 glycan sulfate (GlyS)) was mixed with ALG (carboxylic groups), a similar trend was observed: GlyS was found to insert preferentially into the film [70] and it almost totally exchanged the ALG when brought into contact with a (PLL/ALG) multilayer (Fig. 2). 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 of PLL than the carboxylic groups present on the ALG chains. Isothermal titration calorimetry (ITC) experiments, which allowed the enthalpic contribution of the complex formation to be measured, supported this assumption. Interactions between PLL and HA are endothermic while those between PLL and HEP are exothermic [69]. The details of polyelectrolyte arrangements within blend films are still unresolved and difficult to investigate. Several questions remain unanswered. For instance, why are the (PLL/HA-HEP) blend films very thin (~100 nm) whereas they contain a small but non negligible % of HA? How are the HA chains organized in the films?

Interesting information and new film properties can be gained from another strategy, which makes use of adding synthetic polyelectrolyte layers on top or in-between polysaccharide layers. For instance, Francius et al. first deposited soft (PLL/HA) layers and capped them with hard PSS/PAH ones [71]. A considerable stiffening of the multilayer was observed by using the Atomic Force Microscopy (AFM) indentation technique (see paragraph 8). Salomäki and Kankare hybridized (CHI/HA) layers with synthetic polyelectrolyte PAA, added at different steps in the buildup [72]. They found that the exponentially growing films could be tuned to linear by codepositing PAA in multilayers. PAA was shown to destroy the soft diffuse matrix formed by CHI/HA. Interestingly, $(CHI/HA/CHI/PAA)_n$ films resembled (CHI/HA), which are hydrogel-like films with elastic behavior, but $[(CHI/HA)_3/CHI/PAA]_n$ exhibited a film bulk that resembled Newtonian fluid.

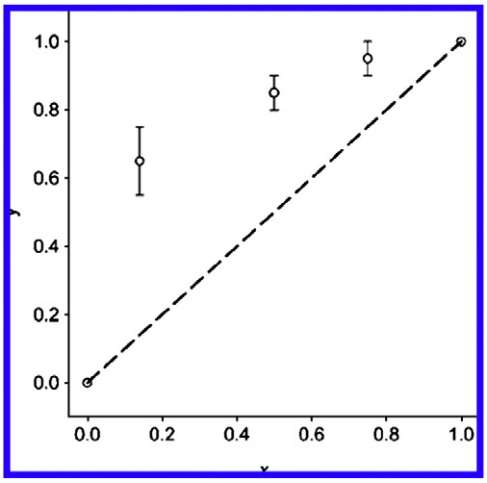

Fig. 2. Apparent mass fraction y of GlyS in the PEI-[GlyS(x)-ALG($1-x$)/PLL]$_7$ films as a function of x, the mass fraction of GlyS in the blend solution. The optimal value of y was determined by fitting to the experimental IR absorbance spectra. The dashed line represents the ideal situation for which the mass fraction of GlyS in the film would be equal to its mass fraction in the blend. The error bars correspond to the width of the flat region of the minimum in the $<\xi^2>$ vs. y curves. (From Ball et al., *Langmuir* 25:3593–3600, 2009, reproduced with permission, copyright ACS 2009).

More generally, highly diffusive films can be combined with "blocking layers" or barriers by depositing dense films such as (PSS/PAH), [73] or degradable polymer layers consisting in poly(lactic-co-glycolic acid) [74]. Such films are particularly interesting for drug delivery applications where different reservoirs (the diffusive part) would serve as storage for delivering different molecules embedded in separate compartments in the film. Indeed, the highly hydrated and weekly coupled polysaccharide-based PEM films such as (PLL/HA) are suitable for the incorporation of liposomes, which themselves can potentially be loaded with bioactive compounds. Recently, liposomes filled with an $AgNO_3$ solution were successfully incorporated in (PLL/HA) films to form antibacterial coatings. [75].

8. New functionalities obtained by chemical modification of the polysaccharides

As described previously, the properties and functionalities of PEM films depend greatly on the chemistry of the polysaccharides used. It is thus possible to modulate or add functionalities to a film by chemically modifying its constituents. In the last five years, several polysaccharide modifications have been developed to provide them with new functionalities [76].

8.1. Grafting of photo-sensitive groups

Although the grafting of photo-reactive groups on to synthetic polyelectrolytes and their subsequent use for LbL film formation has been developed for several years [77,78], this chemical modification has only recently been applied to natural polysaccharides for PEM formation. Of note, thick polysaccharide-based hydrogels formed by photo-cross-linking are already relatively common [79]. Pozos Vasquez et al. presented an alternative chemistry for synthesizing vinylbenzene (VB)-grafted hyaluronan derivatives (HA-VB) [80] *via* an ester linkage. These anionic derivatives were assembled with cationic PLL to prepare photo-cross-linkable PEM films. Shining UV light on to films will induce the formation of covalent bonds between the photo-reactive groups, and thus crosslink the film. The extent of the cross-linking and film stiffness was found to depend on the degree of VB grafting, as evidenced by nano-mechanical measurements. The synthesis of photo-cross-linkable polysaccharides is of particular interest for the future development of spatially patterned polysaccharide-based films.

8.2. Grafting of hydrophobic groups

Delivery of poorly soluble (highly hydrophobic) drugs is a challenge in the field of drug delivery. Whereas post-diffusion has already been used for loading small drugs [81], peptides [82] or growth factors [83], this method is not appropriate for loading high amounts of hydrophobic compounds. To explore the potentiality of PEM films as delivery reservoirs for hydrophobic molecules, Guyomard et al. synthesized anionic amphiphilic polysaccharides of varying hydrophobicity obtained by grafting alkyl chains on to carboxymethyl pullulan (CMP) [18,84]. As the backbone of CMP is highly hydrophilic, the hydrophobicity of these amphiphilic derivatives resulted only from the grafted alkyl groups and led to the formation of hydrophobic nanodomains in the films. Such films were probed for their ability to trap a hydrophobic dye, Nile Red (NR) (considered here as model hydrophobic drug). The authors demonstrated that the maximal amount of dye loaded in the films depended on the degree of grafting of the CMP derivatives. Later on, such films were used to deliver a non-water soluble natural antibacterial peptide, gramicidin A, and was shown to effectively kill a gram-positive bacterium, *E. faecalis* [85].

Recently, alkylamino hydrazide derivatives of HA were similarly used to form hydrophobic nanodomains in otherwise hydrophilic (PLL/

HA) films [86]. The insertion of NR into the films after pre-complexation with the derivatives was found to depend on the alkyl chain length, on the degree of substitution (Fig. 3) and on the HA molecular weight. Interestingly, the effective concentration of NR in the films was ~5000 times higher than the initial NR concentration in solution. The percentage of dye released was between 10% and 60% after 8 days, depending on the alkyl chain length of the alkyl chain grafted. Such films thus seem promising for the localized delivery of hydrophobic drugs at high concentrations.

8.3. New chitosan derivatives

A derivative of chitosan grafted with phosphorylcholine (PC) was recently synthesized by Winnik et al. [87] It was found to be nontoxic and soluble under physiological conditions, even with low levels of PC incorporation (~20 mol% PC per glucosamine unit). This modified PC-CHI may be further used as a new biomaterial due to its interesting protein-repelling properties and biocompatibility. Like CHI, PC-CHI is a polycation under acidic conditions and could form PEM films when combined with HA. By using QCM-D, the authors found that the resulting films behave as highly hydrated soft gels. This high water content for (PC-CHI/HA) films reflects the known ability of the PC group to undergo hydrogen bond interactions with water molecules. Logically, both storage and loss moduli for the (PC-CHI/HA) films were about one order of magnitude smaller than those of (CHI/HA) films.

Recently, two derivatives of CHI containing oppositely charged groups were synthesized by Komakowska et al. [17], one by cationic modification of chitosan and the other by grafting sulfonate groups on to carboxymethyl chitosan. Thus, "one-component" chitosan-based films were formed and characterized in physiological conditions (neutral pH, 0.1 M NaCl).

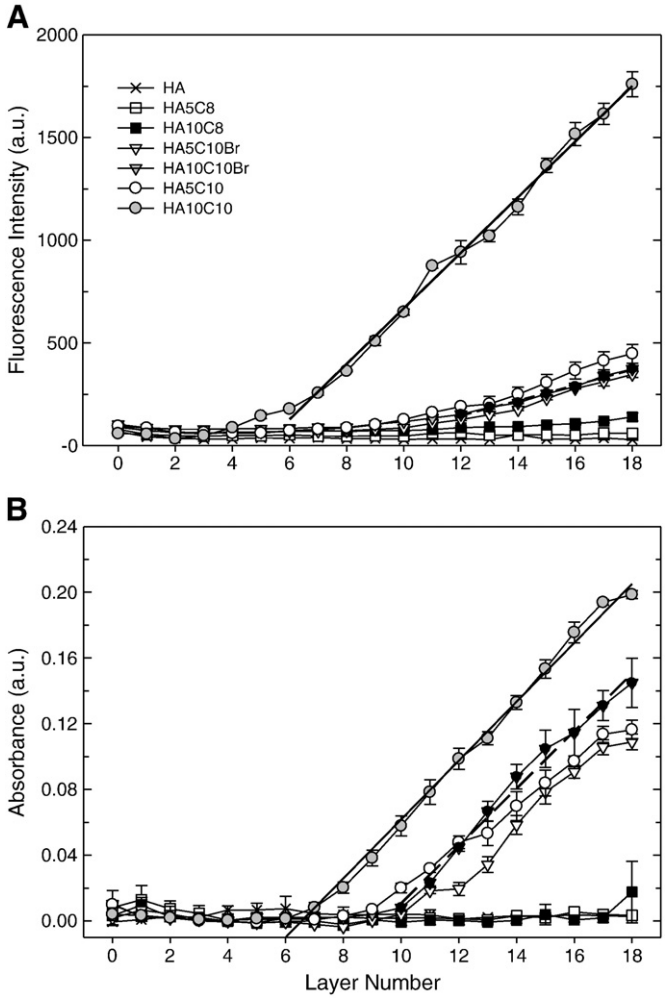

Fig. 3. (A) Nile red incorporation in the (PLL/alkylated HA-200 derivatives)$_{18}$ films followed by a fluorescence microplate reader after each deposition step of the polyanion.(HA-200 means HA with a molecular weight of 200 000 g/mol; the alkylated derivates HA_GC_L. G being the grafting ratio and L the chain length); (B) Absorbance measurements at 590 ± 2.5 nm performed on the same films. Linear fits for the HA10C10 (plain lines) and HA10C10Br derivatives (branched derivatives, dotted lines) are shown in (A) and (B). For the fluorescence curves, the slopes are respectively of 135.3 (calculated for $i \geq 6$) and 37.4 (calculated for $i \geq 10$) for these derivatives; for the absorbance curves, the slopes calculated over the same range are similar with respective values of 0.0179 and 0.0175. (From Kadi et al., *Biomacromolecules* 10:2875–2884, 2009, copyright American Chemical Society, reproduced with permission).

9. Mechanical properties of polysaccharide-based PEM films

Recently, the design of new functional surfaces with precise control over their mechanical properties has emerged as being of utmost importance due to the cells' sensitivity to substrate mechanical properties [88,89]. In this context, designing PEMs with tunable stiffness has become a major challenge and characterization of their viscoelastic properties is crucial. A commonly used technique consists in using an AFM to perform nano-indentation experiments in liquid [90–92]. QCM-D [93,94] and piezo-rheometry [94] have also been proposed to quantify the stiffness of such thin films in liquid conditions. As a general rule, polysaccharide multilayer films are much softer than their synthetic counterparts [95], but several possibilities exist for modulating their stiffness.

9.1. Influence of the structure of polyelectrolytes

The first modulation method consists in modifying the film's internal composition and structure. Schoeler et al. and Schönhoff et al. thus investigated the buildup of films containing PAH as the polycation and two anionic carrageenans, ι-carrageenan, which forms helical structures, and λ-carrageenan, which is in a random coil conformation [96,97]. Inspecting the morphology and internal structure of the films revealed that the helical conformation of ι-carrageenan in solution was maintained during the multilayer buildup. Furthermore, the presence of such ordered structures resulted in thicker and smoother films, with a Young's modulus 3 times higher than the unstructured λ-carrageenan based ones.

A second strategy for stiffening PEM films is to complexify the film's architecture (see paragraph 7) by inserting layers of synthetic polyelectrolytes, which are more charged and more flexible than polysaccharides. A considerable stiffening of the films was observed either by deposition of synthetic (PSS/PAH) layers on top of a (PLL/HA) film [71,98] or by insertion of PAA layers into a (CHI/HA) film [72].

Another way of adjusting the rigidity of PEM films consists in incorporating rigid nano-objects (nanoparticles, clays…). Inspired by inorganic–organic composite materials such as seashells and lamellar bone, this technique has already been widely used in the case of synthetic PEM but only rarely applied to polysaccharide multilayers. Podsiadlo et al. observed that a composite multilayer film containing cationic CHI and anionic nanoparticles of montmorrillonite has a 3-fold increase in Young's modulus compared to pure CHI [59].

9.2. Chemical cross-linking

The mechanical properties of PEM films can also be modulated by chemically cross-linking the polyelectrolytes. Richert et al. initially reported the fabrication of PEM films based on polysaccharides and/or polypeptides whose rigidity was varied by cross-linking the carboxylic groups of the polyanion with the amine groups of the polycation using carbodiimide chemistry [43]. Covalent amide bond formation was evidenced by FTIR. Using AFM nano-indentations, considerable stiffening of both (PLL/HA) and (CHI/HA) films was measured and the

Young's modulus was up to 2 orders of magnitude higher than for the native (uncross-linked) films [81,90]. Moreover, cross-linking PEM films significantly improved their resistance to biodegradation, both *in vitro* and *in vivo* [99]. The latest development focuses on systematic variations in the carbodiimide concentration so as to tune film stiffness over a wide range [100,101]. Interestingly, combining quantitative ATR–FTIR with AFM nano-indentations made possible a relationship between the Young's modulus and the density of the ionic cross-links to be derived for different types of film containing polysaccharides or polypeptides [100].

9.3. Photo-cross-linking

As an alternative strategy to chemical cross-linking, photo-cross-linking has recently emerged and offers perspectives for the spatial control of film cross-linking. This strategy makes use of photo-sensitive groups that are either inserted within the layers or grafted on to one of the polyelectrolytes (see paragraph 8). Liu et al. [102] thus inserted a positively charged photo-sensitive cross-linker, the p-diazonium diphenyl amine polymer, as interlayers between the negatively charged polysaccharides alginate and heparin. The photo-reaction *in situ* converted the ionic bonds between the neighboring layers into covalent bonds, leading to more stable films than their native counterparts. Similarly, photo-cross-linked (PLL/HA-VB) films were stiffer than uncross-linked ones. Of note, due to the fact that only HA carried the VB groups [80], the maximum stiffness of the films obtained by photo-cross-linking was significantly lower than with chemical cross-linking which allows linkage of both HA and PLL.

10. Conclusions

The LbL films based on polysaccharides have now become a popular tool for developing new functional surface coatings. Applications in the field of food science, biotechnology, paper technologies, or tissue engineering have emerged and grown within the past few years. Fundamental questions on the self-assembly process, and the strength of the molecular interactions within these films, are still largely unresolved, due to the limited number of techniques available. For instance, deuteration of polysaccharides is a technical challenge, which makes their internal dynamics difficult to probe by neutron scattering. Interactions between the counterions, diffusion of polyelectrolytes as well as diffusion of solutes within the films, and film ultrastructure will all be studied in greater depth in the future. Their biomimetic properties, the control over different types of properties (chemical, bioactivity, mechanical) will make it possible to design PEM rationally with specific cellular responses. Polysaccharide-based PEM that combine several stimuli will be developed further in order to recapitulate all the signals received by cells. These films may find future applications as new implant coatings. New types of film in the form of polyelectrolyte microcapsules and free-standing films will open up new avenues for drug delivery and cell targeting by means of polysaccharide-based PEM.

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