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pH-responsiveness of multilayered films and membranes made of polysaccharides

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Abstract

We investigated the pH-dependent properties of multilayered films made of chitosan (CHI) and alginate (ALG) and focused on their post-assembly response to different pH environments using quartz crystal microbalance with dissipation monitoring (QCM-D), swelling studies, zeta potential measurements and dynamic mechanical analysis (DMA). In an acidic environment, the multilayers presented lower dissipation values and, consequently, higher moduli when compared with the values obtained for the pH used during the assembly (5.5). When the multilayers were exposed to alkaline environments the opposite behavior occurred. These results were further corroborated with the ability of this multilayered system to exhibit a reversible swelling-deswelling behavior within the pH range from 3 to 9. The changes of the physicochemical properties of the multilayer system were gradual and different from the ones of individual solubilized polyelectrolytes. This behavior is related to electrostatic interactions between the ionizable groups combined with hydrogen-bonding and hydrophobic interactions. Beyond the pH range of 3-9 the multilayers were stabilized by genipin cross-linking. The multilayered films also became more rigid while preserving the pH-responsiveness conferred by the ionizable moieties of the polyelectrolytes. This work demonstrates the versatility and feasibility of LbL methodology to generate inherently pH stimuli-responsive nanostructured films. Surface functionalization using pH-reponsiveness endows abilities for several biomedical applications such as drug delivery, diagnostics, microfluidics, biosensing or biomimetic implantable membranes.

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Keywords

Layer-by-layer; chitosan; alginate; pH; stimuli-responsive; tissue engineering

Introduction

The layer-by-layer (LbL) technique is a simple, easy, versatile, flexible, handling, inexpensive and powerful tool to create stratified multilayer films with an unprecedented control over the nanometer and micrometer scale. 1, 2, 3 LbL enables the use of a myriad of templates to fabricate ultrathin coatings 4, 5, 6 and to obtain complex structures such as capsules, 7, 8 freestanding membranes, 9, 10, 11 hollow tubular constructs 12 and porous scaffolds 13. This technique relies on the buildup of films based on intermolecular interactions either from electrostatic nature or non-electrostatic, such as hydrogen bonds, *Van der Waals* forces, charge transfer, halogen interactions and covalent bonds. 3, 14

The most commonly used interactions are the electrostatic ones, where LbL relies on the stepwise deposition of oppositely charged polyelectrolytes 1, 14, and the driving force is the charge overcompensation occurring at the top of the film after each new polyelectrolyte deposition.²

Several works reported that the properties of polyelectrolyte multilayers (PEMs) depend on the pH of the polyelectrolytes solutions from which the layers are adsorbed.^{11, 15, 16} However, just a few described the post-assembly effect of pH.^{5, 17, 18, 19, 20} The influence of pH on such systems can be reversible or irreversible, depending on the film composition and on the presence of crosslinks.^{19, 21, 22} The pH-dependent behavior of multilayers award promising features to adjust their mechanical properties, swell-shrink and/or disintegration, permeability and/or to control the fast release of loaded molecules when surrounded by different pH.^{6, 21, 23, 24} So far, techniques such as LbL appear as promising candidates to engineer stimuli responsive systems that endow abilities for drug delivery, diagnostics, microfluidics and biosensors.^{21, 24, 25, 26, 27, 28} Basically, external stimuli include humidity, pH, salts, ultrasound, temperature, light, redox, magnetism, electric field and enzymes.^{20, 29, 30, 31} Among them, pH-sensitive multilayered systems hold a great potential in advanced therapies, namely for controlled drug delivery, due to the diversity of pHs existing in the human body.³² For instance, the pH of gastrointestinal tract ranges from 1 (stomach) to 8 (small intestine).^{32, 33} Moreover, cancer and wound tissues constitute also an acidic environment when compared to healthy tissues.^{32, 34}

In this work, two polysaccharides of marine origin, chitosan (CHI) and alginate (ALG) were used to buildup PEMs. These weak polyelectrolytes were selected in view of their polyionic nature, biocompatibility and also their similar structure to glycosaminoglycans. 12, 35, 36 CHI/ALG multilayers are mainly formed by electrostatic interactions and are sensitive to pH variations of the external environment, especially when it is close to the pK_a values.^{27, 37, 38} The pH-responsive property of CHI/ALG multilayered nanocarriers was already reported in *in vitro* and *in vivo* studies of the release of the anticancer drug doxorubicin.²⁷ The drug release was found to be pH-dependent, but the fundamental mechanism behind such release was not completely elucidated.

In this work, we performed a systematic study of the mechanism behind the smart responsiveness of (CHI/ALG) PEMs to pH changes of the surrounding environment. Although the production and characterization of such multilayered films has already been reported, to the best of our knowledge, this is the first time that such kind of films were used to study the influence of the postassembly pH on film thickness, swelling, charge density and mechanical properties.

Materials and Methods

Quartz crystal microbalance with dissipation monitoring

The two polyelectrolytes used to process the multilayers were CHI medium molecular weight (M_w 190.000-310.000 Da, ref. 448877, Sigma Aldrich, USA) and low viscosity ALG (538 kDa, $\approx$ 250 cp, ref. 71238, Sigma Aldrich, USA). CHI was purified by a series of filtration and precipitation in water and ethanol steps, adapted from the method described elsewhere.³⁹ The degree of deacetylation is 82.6% as determined by H-NMR.

The buildup of PEMs was followed *in situ* by quartz crystal microbalance with dissipation (QCM-D, Q-Sense, Sweden), using gold coated sensor excited at seventh overtone (35 MHz). The crystals were cleaned in an ultrasound bath at 30 °C using successively acetone, ethanol and isopropanol. Adsorption of the different solutions took place with a constant flow rate of 100 $\mu\text{L}\cdot\text{min}^{-1}$. The polyelectrolyte solutions were freshly prepared at a concentration of 1 $\text{mg}\cdot\text{mL}^{-1}$ and dissolved in a salt containing sodium acetate buffer (0.1 M, pH 5.5) with 0.15 M NaCl. The CHI solution was let adsorbed for 10 min to allow it to reach equilibrium. After rinsing for 10 min with the sodium acetate buffer, the same procedure was followed for ALG deposition. These steps were repeated up to deposit of 5 bilayers. After film buildup, the multilayers were flushed with acidic or alkaline solutions based on sodium acetate with 0.15 M NaCl. The pH was adjusted using appropriate volumes of sodium hydroxide (1M) and acetic acid [2% (v/v)]. The solutions were pumped into the system for 30 min, followed by an injection of sodium acetate buffer/0.15 M NaCl pH 5.5 for another 30 min in order to assess the reversibility of the process. To analyze the influence of pH on film properties of the multilayers two kinds of experiments were performed:

- (i) Cascaded pH experiments were performed by varying the pH in a cyclic way toward more acidic or more alkaline conditions; after that the pH was set back to 5.5.
- (ii) In the individual set of experiments acidic or alkaline solutions were flushed into the multilayers initially at pH 5.5. Afterward, the multilayers were set back to pH 5.5.

In all the experiments the frequency and dissipation changes were monitored in real time. The thickness of the film was estimated using the *Voigt*-based viscoelastic varying the pH in model 40 through the Q-Tools Software (version 3.0.15.553), from Q-Sense. The thickness recovery ratio of multilayers at different pH was further determined as followed (equation 1):

$$R_t (\%) = \frac{Th_f}{Th_i} \times 100 \quad (1)$$

where Th_i and Th_f are the initial and final thicknesses of the multilayers in sodium acetate buffer (pH 5.5) before and after exposure to the media at different pHs.

Production of freestanding membranes

Freestanding membranes were produced using a polypropylene support, as reported before. 11, 12 Polyelectrolyte solutions were alternately deposited on the template to form 100 double layers of CHI and ALG using a home-made dipping robot, especially designed for the automatic fabrication of multilayers. After dried at room temperature, the membranes could be easily detached from the substrate without any defect. Cross-linking of the multilayers was performed with genipin (Wako chemical, USA). A genipin solution (5 mg.mL⁻¹) was prepared by dissolving the adequate amount of lyophilized genipin into dimethyl sulfoxide (DMSO) (Sigma Aldrich, USA)/ sodium acetate buffer [0.1 M acetate buffer, pH 5.5 with 0.15 M NaCl] mixture (1:4, (v/v)]. Afterwards, the membranes were extensively washed into the sodium acetate buffer, and finally with ultrapure water.

Surface Zeta Potential Measurements

The analysis of the charged solid/liquid interface was performed with an adjustable Gap Cell in a SurPASS electrokinetic analyzer (Anton Paar, Austria). Basically, using this system an aqueous solution of 1 mM NaCl (electrolyte) flows through the measuring cells with a maximum pressure of 400 mbar. The apparatus include a titration unit allowing automated measurement series with varied solution compositions. However, in this case automatic titration was not possible due to the swelling of the membranes, which requires continuous gap adjustment. The pH of the electrolyte changes from 5.5 through an acidic or alkaline cascade of pH. The reversibility of the process was assessed flushing the two identical surfaces of the membranes with the electrolyte at pH 5.5 after each measurement at acidic or alkaline pH. The resulting potential difference (streaming potential) created between the flat membrane surface mounted in parallel were converted to zeta-potential using a modified Fairbrother–Mastin equation.⁴¹

Water uptake

The water uptake ability of the freestanding membranes was measured by soaking dry films with known weight in sodium acetate buffer (pH 5.5). Briefly, the containers with the freestanding membranes immersed in sodium acetate buffer were placed in a water bath at 37 °C and with agitation (60 rpm). After 30 minutes the excess of solution was removed from the samples using filter papers (Filter Lab, Spain) and the freestanding membranes were weighed with an analytical balance (Denver Instrument, Germany). The water uptake was calculated as followed (equation 2):

$$Water\ uptake\ (\%) = \frac{W_w - W_d}{W_d} \times 100 \quad (2)$$

where W_w and W_d are the weights of swollen and dried freestanding membranes, respectively.

Afterwards, the freestanding membranes were washed and immersed in a sodium acetate solution at different pHs ranging from 2 to 13 for other 30 min and the water uptake was determined, as aforementioned. Finally, to evaluate the reversibility of the process, the freestanding membranes were placed again in a sodium acetate buffer (pH 5.5) and the water uptake determined. The same procedure was repeated for cross-linked membranes.

Determination of the Cross-linking Degree

The cross-linking of PEMs with genipin was evaluated using the trypan blue method which has affinity to free amines 42. The test was performed immersing the non-cross-linked and the cross-linked membranes in trypan blue 0.4% (Invitrogen, USA) diluted 50-fold in sodium acetate buffer [0.15 M NaCl, (pH 5.5)] overnight at 37 °C. The supernatant absorbance was measured at 580 nm in a microplate reader (Sinergy HT, Bio-Tek, USA). A standard curve was prepared by measuring the absorbance for a series of trypan blue solutions at different concentrations. The cross-linking degree was calculated as follows (equation 3):

$$CL\ (\%) = \frac{(NH_3^+ non - Xlinked\ solution) - (NH_3^+ Xlinked\ solution)}{NH_3^+ non - Xlinked\ solution} \quad (3)$$

Mechanical Tests

All viscoelastic measurements were performed using a TRITEC2000B DMA from Triton Technology (UK), equipped with the tensile mode. The measurements were carried out at 37 °C. Freestanding membranes with and without cross-linked were immersed in different media ranging from acidic to alkaline. The pieces of freestanding membranes (length 50 mm, width 5.5 mm) were held between two clamps separated by 10 mm. After measuring the geometry of freestanding membranes, the samples were clamped in the DMA apparatus and immersed in the respective medium bath at 37 °C. The DMA spectra were obtained during a frequency scan between 0.1 and 20 Hz. The experiments were performed using a constant amplitude strain of 30 μ m. Three specimens were tested for each condition.

Statistical analysis

The experiments were carried out in triplicate if not otherwise stated. The results were presented as mean $\pm$ standard deviation (SD). Statistical analysis was performed by Shapiro Wilk normality test using Graph Pad Prism 5.0 for Windows. After this analysis, non-parametric (Kruskal Wallis test) or parametric tests (one way ANOVA followed by Tukey

test) were used depending on whether the samples were from normally distributed populations or not, respectively.

Results

Construction of CHI/ALG multilayers

The assembly of 5 bilayers of CHI/ALG multilayered films and their responsiveness to pH was monitored *in situ* with QCM-D. This technique detects the adsorbed mass of polyelectrolytes onto a gold-coated quartz sensor and the viscoelastic properties of the surface. 43 QCM-D results show the buildup of 5 bilayers and their response to changes in pH in terms of variations on normalized frequency, $f_{7/7}$, and dissipation, D_7 (Figure 1A). As expected, during the buildup of 5 bilayers, the normalized frequency decreases with each polyelectrolyte solution injection, reflecting the increase of mass over the gold sensor.

A pH-dependent response of these multilayered films was also studied within a pH range between 2 and 10, taking as reference the pH 5.5. Figure 1B shows the QCM-D response of the CHI/ALG multilayers to a pH decrease from 4 to 2 (acidic cascade). It can be seen that $f_{7/7}$ decreases when the film is flushed with a polyelectrolyte-free solution at pH 4, and $f_{7/7}$ increases again when the pH returns to 5.5. This was then followed by alternate decreases and increases of pH. It is noticeable that within the pH range 5.5–3 the multilayers respond to pH changes in a similar manner (both $f_{7/7}$ and D_7). Thus, the behavior of the measured D_7 mirrored what is seen with $f_{7/7}$, i.e., increases and decreases in D_7 corresponded to decreases and increases in $f_{7/7}$. These changes are reversible, as shown when the pH increases again to 5.5. In the lowest region of pH (pH 2), D_7 increased when compared with the other acidic pHs. It should be noted that even after lowering the pH until 2 the multilayered films maintained their structural integrity, because an increase of pH back to 5.5 led almost to a full recovery of the initial $f_{7/7}$ and D_7 values. This behavior was further confirmed when the multilayers were subjected to a cascade of acidic solutions with increasing pH (from 2 to 4) (Figure S1).

The behavior of multilayers in a cascade of increasing pH in the alkaline region was also studied. The behavior was exactly the opposite to that found for the acidic conditions: the values of $f_{7/7}$ decreased and the ones of D_7 increased with increasing pH (Figure 1C). However, the kinetic behind the alkaline cascade is different and some hysteresis occurs. An important aspect in these multilayered films is that the reversible behavior is valid but just until pH 9, presenting a reduced relative dissipation difference in this pH range (Figure 1D). The QCM-D responses from CHI/ALG multilayers to acidic and alkaline pH solutions were further analyzed in an individual set of experiments. As expected, the same trend as those observed in cascaded experiments was verified in both alkaline and acidic conditions (Figure 2).

In order to better understand what changes occurred in the film, the wet thickness was estimated using a Voigt-based viscoelastic model. 40 Figure 3 shows the thickness of the multilayers initially at pH 5.5, changing to other pHs (2–10) and then set back to pH 5.5. The thickness of the CHI/ALG multilayer film decreased as the acidic pH changed from 4 to 3 (Figure 3A). At pH 2 a slightly increase of thickness was observed. On the other hand, in

an alkaline environment the wet thickness increased (Figure 3C). The results in acidic and alkaline cascade were further compared with the ones of the individual set of experiments, where the same trend was observed (Figure 3B and Figure 3D). Additionally, as in the direct QCM-D results in Figures 1 and 2, when the films were set back to pH 5.5 after being submitted to a pH range of 2–9 they could recover their wet thickness (Figure 3E).

Surface Zeta Potential Measurements

The zeta potential measurements were performed on CHI/ALG freestanding membranes with ALG as the outermost layer (Figure 4). Freestanding membranes are a versatile and robust kind of multilayer assemblies, allowing the physicochemical characterization of the multilayered films without the influence of the substrate. By simple detachment of the substrate, it is possible to obtain polysaccharides membranes without defect, as previously reported 11, 12, 44. It is nonetheless obvious that, in this case, the thickness of the multilayered films is in the microscale range.

The zeta potential varied between -1.3 ± 0.3 mV and $+7.2 \pm 3.5$ mV when the membranes were exposed to a cascade of acidic solutions (pH 5.5 to 2). When the membranes were exposed to a cascade of alkaline solutions (pH 7 to 10) the zeta potential remained negative and the values ranged between -2.4 ± 0.4 mV and -5.2 ± 0.2 mV. At pH 13 the membranes started to dissolve and the zeta potential presented extreme negative values when compared with the previous ones (-20.8 ± 3.5 mV). After this run it was no longer possible to measure the zeta potential with other pHs due to loss of the structural integrity of the freestanding films. The same procedure was followed with films containing CHI as the outermost layer (Figure S2A and Figure S2B) and the values presented the same trend. Thus, we speculate that the effect of pH influences the properties of the whole film and not only the outermost layer.

The zeta potential measurements revealed that the films at pH 5.5 were close to neutrality. The zeta potential values remained negative at pH 4 and an increase of pH back to 5.5 led to an increase in the charge density. The values of zeta potential only became positive below the pKa of glucuronic and manuronic acid ALG units, i.e. below pH 3.4. Likewise, when the films were exposed to a higher pH than the pKa of CHI, an increase in zeta potential was noted. All together, these results confirmed the pH-responsiveness observed in QCM-D because a partial recovery of the multilayer zeta potential was observed when they were brought back to the initial assembly pH (pH 5.5). Additionally, behavior of the films at the extreme pHs of 2 and 10 was confirmed, with a drastic change in zeta potential at these pHs.

Swelling ability

The freestanding multilayered films are composed by polyelectrolytes with abundant hydrophilic groups, such as hydroxyl, amine, and carboxyl groups, which can promote water uptake.⁴⁵

In this work, the swelling ability of freestanding membranes was evaluated in solutions at different pH ranging from 2 to 13 for membranes that were either non-cross-linked or cross-linked (Figure 5). The freestanding membranes were cross-linked with genipin to improve their stability and to retain their properties over the whole pH range. The cross-linked

membranes present a cross-linking degree of 64.2% determined by trypan blue assays, as previously reported. 12

The water uptake values for native freestanding membranes decreased in the pH range 4–3 with values of $62.5\% \pm 6.2$ and $59.2\% \pm 4.2$ for pH 4 and 3, respectively (Figure 5A). It would be expected that the films started to swell below pH 3.4. However, a slightly increase of water uptake was just observed at pH 2 ($78.1\% \pm 22.9$) relatively to the other acidic pHs (3 and 4). In an acidic pH range, the freestanding membranes present a reversible behavior, because their swelling values return back to their initial swelling values when they are immersed again in a medium at the initial assembly pH.

When the freestanding membranes without cross-linking are immersed in an alkaline environment (pH > 7) the multilayers maintained their structural integrity, especially up to pH 9. The water uptake values increase substantially, ranging from $207.1\% \pm 69.5$ (pH 7) to $391.9\% \pm 27.1$ (pH 13) (Figure 5B). However, when they are brought to the pH of the assembly, the multilayers presented an almost reversible behavior. However, this trend was not verified at pH 10 and especially at pH 13, meaning that at these conditions the film disassembled.

The results were further compared with the ones in cross-linked membranes when immersed in acidic and alkaline mediums (Figure 5C and 5D). In all the conditions the water uptake ability of the cross-linked membranes is lower than of the non-cross-linked membranes. However, the water uptake ability at cross-linked membranes presents the same trend of non-cross-linked membranes, i.e., the water uptake is lower in acidic environments than in the alkaline ones.

Altogether, the results revealed that the native multilayered films are stable in a range of pH between 3 and 9 presenting a swelling/deswelling reversibility, which is an important aspect for the maintenance of their functionality in the physiological relevant pH range. The cross-linking mechanism is an effective method that increases the pH range where film stable remain stable, while preserving the pH responsive properties.

Mechanical properties

In this work, DMA experiments were performed in solutions at different pHs. The motivation for this experiment is that if the pH of the medium affects the dissipation, thickness, zeta potential and water uptake of multilayered films, their mechanical properties should also be influenced.

DMA experiments were used to monitor the change in the viscoelastic properties of the samples immersed in acetate solution / 0.15 M NaCl at pH 3, 5.5, 7.4 and 13. The values of storage modulus (E') and loss factor ($\tan \delta$) are shown as a function of frequency (Figure 6). The $\tan \delta$ is the ratio between the energy loss by viscous mechanisms and the energy stored in the elastic component, providing information about the damping properties of the material. 46 Freestanding membranes immersed in a medium at pH 5.5 and 3 presented a similar E' and $\tan \delta$ variation with time (Figure 6A and Figure 6C). However, E' was slightly higher at pH 3 than at 5.5, which corroborated the QCM-D and swelling results. On

the other hand, the films immersed at pH 7.4 presented a strong decrease in E' as compared with the more acidic conditions, accompanied by an increase on $\tan \delta$ at high frequencies. The decrease in $\tan \delta$ indicated that the membranes acquired more elastic properties and should be also related with the release of water molecules from the multilayers.

The results were further compared with the ones performed with cross-linked membranes when immersed in the same medium (Figure 6B and Figure 6D). E' was higher for the cross-linked membranes in all conditions. Again, we found that E' was higher at lower pH. Moreover, it was possible to analyze the cross-linked films immersed at pH 13, which was not possible for the non-cross-linked ones. Regarding $\tan \delta$, the decreased values suggested the release of water molecules upon cross-linking. Thus, the genipin cross-linking increased the freestanding membrane stability, while preserving their pH-responsiveness (Figure S3).

Discussion

In this work we studied the postassembly response of CHI-ALG multilayers to pH variations. We found that in acidic media the multilayers presented lower dissipation and higher frequency values. The decrease in D_7 revealed that the multilayers became more rigid, which is associated with the diffusion of water molecules from the multilayers, reflected by a decrease in $f_{1/7}$. The water molecules have a plasticizing effect in such kind of polysaccharides, increasing their molecular mobility and decreasing their stiffness.⁴⁶ Multilayers composed by strong polyelectrolytes bear completely ionized groups over a broad pH range.⁴⁷ On the other hand, multilayers based on weak polyelectrolytes (pKa values between 3 and 10) present sensitivity to pH, which affect their swelling ability.³¹ One example is the carboxyl and amine groups present in ALG and CHI multilayers, respectively, which exhibit a change in the ionization state (charge density) as a function of pH, which led to higher water uptake and lower E' values.³⁷ However, these changes are reversible, as shown when the pH increased again to 5.5 (pH set as reference). This behavior can be explained by the strengthening of the electrostatic interactions between CHI and ALG multilayers when the pH varies from 5.5 to 3. In this pH range the charge of CHI increases but the one from ALG does not significantly decrease. One can assume that a combination of the aforementioned factors (ionization of carboxylic and amine groups) caused the film to shrink (decrease in wet thickness) and, consequently, led to lower water uptake. A similar behavior was already reported for gelatin-poly (galacturonic acid).¹⁷ In the lowest region of pH (pH 2), the D_7 and the wet thickness increased when compared with the other acidic pHs. Below the pKa of ALG (pKa ALG $\approx$ 3.4 or 3.65 for glucuronic and manuronic units, respectively)⁴⁸, the carboxyl groups became protonated and, consequently, a decreased in the ion pairing occurred. However, it should be pointed out that the dissociation behavior of the polyelectrolytes (pKa) could be shifted by 1 or 2 pH units to the alkaline or acid region due to hydrophobic effects when it is incorporated in PEMs.²⁰ This shift in the pKa clarifies the seemingly contradictory effects at pH 3. Thus, at pH lower than 3 the decrease in ion pairing may result in some uncompensated positive charges of amino groups (positive zeta potential values) and in an increase of the electrostatic repulsion between the chains, although the positive charges could still be compensated to a certain extent by the chloride and acetate counteranions of the external medium. The increase of the osmotic pressure, due to the counterions that penetrate into the film to compensate the

excess of charges, causes diffusion of water and the swelling of the system, as reflected by the water uptake results.¹⁸ Additionally, depending on the hydration shell of the anions present in the external environment different degrees of swelling can be achieved i.e. larger hydration shell should lead to the higher water uptake.⁴⁹ It should be noted that even after lowering the pH until 2 the multilayered films maintained their structural integrity, because an increase of pH back to 5.5 led almost to a full recovery of the initial $f_{7/7}$, D_7 , wet thickness, zeta potential and water uptake values. We hypothesize that hydrogen bonding, which becomes effective after protonation of the carboxylic groups at pH 2, should play an important role on the film stability at this pH range. In fact, the polymers used in the present study incorporate moieties that, can act as hydrogen bonding donors and acceptors. (e.g. carboxylic, amino and hydroxyl groups). This type of non-electrostatic interactions has been used to assemble distinct LbL films composed of uncharged polymers and it has been shown to be strong enough to maintain their integrity.^{18, 50} Probably, chain entanglements and hydrophobic interactions also contribute to the film stability.

The behavior of multilayers in a cascade of increasing pH in the alkaline region was also studied. At increasing pH and above the pK_a of CHI (pK_a_{CHI} $\approx$ 6.5)³³, the amine groups should be progressively uncharged (NH₂) and the carboxylic groups are ionized (higher negative zeta potential values). However, at four pH units above the pK_a of ALG, all the carboxyl groups should be charged, which explain the non-significant changes occurring between pH 8 and 9. Thus, in this alkaline pH range, the electrostatic repulsion between the negative charged groups led to higher wet thicknesses and water uptake and lower E . However, as mentioned above some charge compensation would occur due to the counterions of the external medium that penetrate within the film. Therefore, because the multilayers maintained their structural integrity, we can attribute this behavior to other non-electrostatic interactions aforementioned i.e. hydrogen bonding, hydrophobic interactions and chain entanglements within the polymeric chains of the multilayers. For pH higher than 9 the reversible behavior of these multilayers was no longer valid. At pH 10 the electrostatic repulsions between the negative charges of ALG increased (drastic increase of zeta potential) and the osmotic pressure within the films (due to the diffusion of counterions) attained such a high value that it was no longer counterbalanced by hydrogen bonding, hydrophobic interactions and chain entanglements. This could explain why, at pH higher than 10, the structural integrity, was not maintained, resulting in a partial dissolution of the film and, thus, irreversible changes. Such a critical pH was already reported for other PEMs.^{5, 19, 24, 51} Weaker electrostatic interactions result in lower critical pH values at which film disintegration occurs.⁵ The partial or full disassembly of LbL films has been reported to be not only directly dependent on ion pairing, but also in a large extent, to non-electrostatic interactions, as previous mentioned. Increased osmotic pressure causes diffusion of water into the film and results in swelling.¹⁸ However, when the osmotic pressure built within the film is no longer counterbalanced complete dissociation of the film may occur.^{18, 50, 51}

Scheme 1 represents schematically the rearrangement between the polyelectrolyte chains, as well as their stability, across all the pH range, supporting the results that have been discussed in this work.

To improve the stability and to retain the properties of the freestanding membranes over the whole pH range genipin was used as crosslinker. Genipin has the ability to cross-link the amine groups present on CHI with the ester groups of genipin, leading to the formation of amide groups.⁵² When CHIT/ALG films are cross-linked with genipin, because ALG does not contain primary amines, genipin will give rise to semi-interpenetrating polymer networks with free ALG chains entrapped inside cross-linked CHIT multilayers, as previously reported in other multilayered systems.⁵³ However, the multilayers were found to be stable upon cross-linking and ALG is not completely released from the films.⁵⁴ The cross-linking procedure will prevent the dissolution of PEMs and in the meantime the films become rigid.²⁶ In all the pH range tested the cross-linked membranes presented lower water uptake when compared with the non-cross-linked ones. The chemical cross-linking with genipin led to a higher reduction on water uptake due to the smaller free volume and intermolecular space between the polyelectrolyte chains, which limit the molecular mobility of the polymer chains at the nanoscale level. The same trend was observed in the mechanical properties where higher modulus was observed for all the pH media. E' was higher for the cross-linked freestanding membranes at lower pHs, but the water uptake was lower, indicating that only a fraction of amino groups was involved in the cross-linking. In fact, at low pH, the films are both covalently crosslinked and stabilized by electrostatic interactions and hydrogen bonds.

Given the responsiveness of CHI and ALG to pH, one expects that PEMs made of these polysaccharides also respond to pH changes in the same fashion. However, when we compared our results with potentiometric titration curves of these raw materials more gradual changes are observed in PEMs, because the alterations in the physicochemical characteristics are less abrupt. CHI/ALG multilayers present a multimode of interactions within the film when compared with the ones occurring with the raw materials in solution.^{31, 55, 56} A similar behavior has been found in other studies where the responsiveness of polyelectrolytes brushes and multilayers were compared with polyelectrolyte monolayers and a different trend was also observed.⁵¹ Altogether, the results revealed that the multilayered films without cross-linking are stable in a range of pH between 3 and 9 presenting a swelling/deswelling reversibility, which is imperative to maintain their structural integrity as well as their functionality in the physiological relevant pH range. The cross-linking mechanism is an effective method that increases the pH range where film stable remain stable, while preserving the pH responsive properties. This reversible swelling-deswelling in multilayers is often accompanied by the competition between repulsive electrostatic interactions of polyelectrolyte chains with attractive electrostatic or hydrophobic/hydrogen bonding interactions between the polyelectrolytes and/or the substrate.

Conclusions

The pH-responsiveness of multilayered films incorporating weak acidic and basic polyelectrolytes was investigated in detail. Their thickness, swelling-deswelling zeta potential and mechanical properties could be tuned by simple postassembly adjustment of the external pH. The results suggested that any shift in the outer pH from acidic to alkaline relatively to the one used during the buildup (pH 5.5) altered most of the properties of the

CHI/ALG multilayers. The multilayers were stable in the pH range from 3 to 9, undergoing reversible swelling with pH 5.5. At pH 2, the carboxylic groups were uncharged and more amino groups were uncompensated, resulting in an unstable behavior of the multilayer. An opposite behavior occurred at pH 10 and 13. In this pH range the films lose their reversible behavior, and a partial or full disassembly of the film would occur, meaning that the critical pH was reached. Whereas the electrostatic interactions were not sufficient to stabilize the structure, cross-linking the films appeared as an effective method to render it more stable and rigid while preserving the pH responsiveness. Altogether, the results showed that CHI/ALG multilayers even without cross-linking were able to maintain the structural integrity in the physiological range, which is crucial for many applications. This kind of multilayers may represent an interesting alternative to the traditional methods in materials science and biotechnological /pharmaceutical applications for use in drug release upon controlled disassembly. It is also known that multilayers with a high swelling degree present resistance to cell adhesion and spreading whereas the decrease of swelling promotes cell adhesion and spreading 6, 18, 19, 30. Thus, the reversible swelling-shrinking behavior of the films controlled by pH, could also be used for controlled drug release and even for controlling cell response.

Supporting Information

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

QCM-D responses in normalized frequency (f_{17}) and dissipation changes (D_7) during the buildup mechanism (A) and to modifications in pH post-assembly as a function of the time: Acidic cascade (B) alkaline cascade (C). Relative dissipation difference between the postassembly and assembly pH (pH 5.5) (D). Significant differences were found for (***) $p < 0.001$, (**) $p < 0.01$ and (*) $p < 0.05$ (Mean +SD of three independent experiences).

Figure 2.

Individual QCM-D responses in normalized frequency (Δf_{norm}) and dissipation changes (ΔD_7) to modifications in pH postassembly as a function of the time: Acidic pHs (A, B), alkaline pHs (C, D). Relative dissipation difference between the postassembly and assembly pH (5.5) (E). Significant differences were found for (***) $p < 0.001$ (Mean +SD of three independent experiences).

Figure 3.

Normalized thickness of CHI/ALG film as a function of the external pHs, calculated according with a Voigt Model for acidic (A) and (C) alkaline cascades as well as for individual set of experiments (B, D). Significant differences were found for (***) $p < 0.001$, (**) $p < 0.01$ and (*) $p < 0.05$. Thickness recovery (R_t) of CHI/ALG multilayers in individual and cascade experiments after exposure to different pHs (E) (Mean +SD of three independent experiences).

Figure 4. Evolution of the zeta potential (mV) on CHI/ALG films as a function of the pH: acidic (A) and alkaline (B). Significant differences were found for (***) $p < 0.001$, (**) $p < 0.01$ and (*) $p < 0.05$.

Figure 5. pH-dependent swelling behavior of CHI/ALG films in acidic and alkaline environment for membranes without (A,B) and with cross-linking (C,D). Statistical analysis was performed, and data was considered statistically different for p values < 0.05 . (#) denotes significant differences when compared to pHs different than 5.5. (*) denotes significant differences relative to pH 5.5 before and after the flushing of multilayered film with acidic or alkaline pHs (Mean $\pm$ SD of three independent experiences).

Figure 6. Frequency dependency of storage modulus (E') and the loss factor ($\tan \delta$) of (CHI/ALG)₁₀₀ membranes without (A, C) and with cross-linking (B, D) at 37°C while immersed in solutions at different pHs.

Scheme 1.

Schematic illustration shows the effect of pH on the molecular mobility of multilayered films as well as on the charges and interaction behind multilayered films.