

Multifunctional Polyelectrolyte Multilayer Films: Combining Mechanical Resistance, Biodegradability, and Bioactivity

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Cross-linked polyelectrolyte multilayer films (CL PEM) have an increased rigidity and are mechanically more resistant than native (e.g., uncrosslinked) films. However, they are still biodegradable, which make them interesting candidates for biomedical applications. In this study, CL PEM films have been explored for their multifunctional properties as (i) mechanically resistant, (ii) biodegradable, and (iii) bioactive films. Toward this end, we investigated drug loading into CL chitosan/hyaluronan (CHI/HA) and poly(L-lysine)/hyaluronan (PLL/HA) films by simple diffusion of the drugs. Sodium diclofenac and paclitaxel were chosen as model drugs and were successfully loaded into the films. The effect of varying the number of layers in the (CHI/HA) films as well as the cross-linker concentration on diclofenac loading were studied. Diclofenac was released from the film in about 10 h. Paclitaxel was also found to diffuse within CL films. Its activity was maintained after loading in the CL films, and cellular viability could be reduced by about 55% over 3 days. Such a simple approach may be applied to other types of cross-linked films and to other drugs. These results prove that it is possible to design multifunctional multilayer films that combine mechanical resistance, biodegradability, and bioactivity properties into a single PEM architecture.

Introduction

Local drug delivery at a specific implantation site is particularly interesting in many biomedical and tissue engineering applications as it may reduce toxicity and increase the efficiency of the drug. Various approaches include embedding of drugs in biodegradable polymers,^{1,2} chemical grafting of a drug to a natural or polymeric matrix and its subsequent release upon hydrolysis.^{3,4} Recently, a new class of polymeric surface coatings, the polyelectrolyte multilayer films (PEM), has attracted great attention due to its large potential.^{5–9} As PEM films can be coated on any type of implant materials^{10–12} with virtually any shape,^{13,14} they appear as interesting candidates for the localized delivery of bioactive molecules including peptides,¹⁵ drugs,¹⁶ or DNA.^{17,18} Bioactive molecules can be incorporated into such films by different strategies: simple adsorption of the bioactive molecules at a certain step during the film buildup,^{19,15,20–22,16} simple adsorption by dipping the preformed film in a drug-containing solution,^{23,24} which is also called the postdiffusion method or more sophisticated strategies. These more elaborate strategies make use of cyclodextrins either as hydrophobic drugs or plasmid carriers,^{18,25} use a prodrug approach,²⁶ or rely on the preparation of films with polysac-

charides grafted with alkyl chains, which are amphiphilic and allow the formation of hydrophobic nanocavities in the films.²⁷ Bioactive molecules may also be released from degradable PEM or hollow capsules via a diffusion mechanism, via degradation of the film using hydrolytically degradable polycations^{28,29} or enzymatically degradable polyelectrolytes,^{12,30} or via sensitivity to pH or sodium chloride solutions.^{31–34} Layer-by-layer films were also recently used to provide a diffusion barrier to encapsulated glucose oxidase inside alginate microspheres.³⁵ Diffusion of dyes is also the subject of intense study in order to better understand the interaction of small molecules with polymer multilayers.^{36,37}

A usual requirement for the design of bioactive films is that the cell type, which is targeted by the bioactive molecules, has to come in contact and adhere to the film to be in contact with the drug, unless the function of the film would precisely to be nonadhesive or cell resistant.³⁸ Very often, thick and hydrated films such as those that can be designed as drug “reservoirs” are often unfavorable for primary cell adhesion possibly due to their high hydration or high softness.^{38–40} Several strategies have therefore been developed to enhance cell adhesion such as increasing cell stiffness by embedding nanoparticles,⁴¹ chemically cross-linking the films by heating³⁹ or using carbodiimide chemistry,⁴² grafting cell adhesive peptides^{43,44} or sugar molecules,⁴⁵ and more recently capping the film with few layers of synthetic polyelectrolytes.²⁴ All these strategies have consequences on the film chemistry, on its mechanical properties, or on both.⁴⁶

Very interestingly, soft PEM films made of polysaccharides and polypeptides initially exhibiting a poor cell adhesion were found to favor, once cross-linked, cell adhesion and proliferation of various primary cell types.^{44,47} Furthermore, it is possible to

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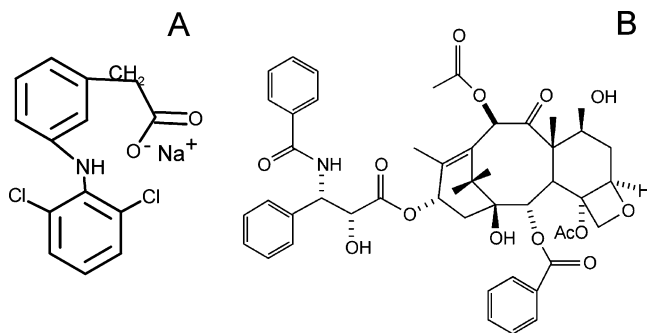


Figure 1. Chemical structures of (A) sodium diclofenac and (B) paclitaxel (Taxol).

take benefit of the biodegradability of films made of natural polyelectrolytes, such as polysaccharides, which can be degraded by various enzymes, and to tune this biodegradability by controlling the concentration of the cross-linking agent.⁴⁸ So far, cross-linked films have always been used without any bioactive molecule inserted in the film bulk. It is known that the cross-linking procedure modifies the relative abundance of chemical groups present in the films,^{42,48} as amide bonds are created upon cross-linking whereas the number of free carboxylic groups is decreased. The charge balance within the films is thus expected to be greatly modified.

Therefore, an important question that remains so far unanswered is whether the cross-linked films can also be functionalized by insertion of bioactive molecules, which would allow both a control of film mechanical properties and of their bioactivity. In addition, if these films are biodegradable, one could envision combining all these properties within a single multifunctional PEM architecture. In this study, we will show for the first time that two model drugs can be embedded in CL PEM films. The nonsteroidal antiinflammatory drug diclofenac^{49–51} and the anticancer drug paclitaxel (taxol),⁵² which has efficient antiproliferative and antimitotic properties, were studied as model drugs (Figure 1). These drugs have been mostly loaded in gels^{53,54} or in particles⁵⁵ or grafted to polysaccharides⁵³ or polypeptides,⁵⁶ but only a few studies focused on their embedding and bioactivity in films.^{24,26} Two different types of films made either of chitosan (CHI) or poly(L-lysine) (PLL) as polycations and hyaluronan (HA) as polyanion will be investigated. We will demonstrate that it is possible to combine advantages of cross-linked films, e.g., enhanced cellular adhesion, enhanced mechanical resistance, and biodegradability properties with the possibility to render film bioactive.

Experimental Section

Polyelectrolyte Solutions. Film Preparation and Cross-Linking.

HA (sodium hyaluronate, 4×10^5 g/mol) was purchased from Bioiberica (Spain), CHI (5×10^3 g/mol) was purchased from Medipol (Switzerland), and PLL (3×10^4 g/mol) was purchased from Sigma (France). CHI and HA were dissolved at 1 mg/mL in 0.15 M NaCl in water at pH 4.5 for CHI/HA films, and PLL and HA were dissolved at pH 6.5 for PLL/HA films. (CHI/HA)_i and (PLL/HA)_i films were prepared with an automatic dipping machine on 14 mm glass slides as previously described (Dipping Robot DR3, Kirstein and Riegler GmbH, Germany)^{42,12} (VWR Scientific, France), cleaned with 10 mM SDS and 0.1 N HCl, and extensively rinsed. For film cross-linking, we followed the previously published protocol using the water soluble carbodiimide 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and *N*-hydro-sulfosuccinimide (sulfo-NHS) (both purchased from Sigma).⁴² Briefly, EDC and sulfo-NHS were prepared at 70 mg/mL and 22 mg/mL, respectively, in a 0.15 M NaCl solution at pH 4.5. One milliliter of the mixed EDC/sulfo-NHS solution (v/v) at pH 4.5 (for CHI/HA films and

at pH 5 for PLL/HA films) was deposited in the wells containing the film-coated glass slides and left for 18 h at 4 °C. All the experiments were performed at this cross-linker concentration unless otherwise noted.

Atomic Force Microscopy. Atomic force images were obtained in contact mode in air with the Multimode Nanoscope IV from Veeco (Santa Barbara, CA). Cantilevers with a spring constant of 0.03 N/m and with silicon nitride tips were used (Model MLCT-AUHW Park Scientific, Sunnyvale, CA). Deflection and height mode images were scanned simultaneously at a fixed scan rate (between 2 and 4 Hz) with a resolution of 512×512 pixels. The mean roughness of the films was calculated: $R = 1/(N_x N_y) \sum_{i=1}^{N_x} \sum_{j=1}^{N_y} |z_{ij} - z_{\text{mean}}|$ where z_{ij} is the height of a given pixel, z_{mean} is the average height of the pixels, and N_x and N_y are the number of pixels in the x and y directions.

Determination of the Film Mechanical Properties by Force Spectroscopy. The measurements aimed at the determination of the elasticity of the films were performed on (CHI/HA)₂₄ films using a homemade AFM working in the indenter-type mode (IT-AFM).⁵⁷ This number of layer pairs was chosen, as these films can be easily visualized by confocal microscopy and their thickness measured (for input in the curve fitting procedure).⁴⁰ The probe was constituted by a borosilicate sphere of 5 μm in diameter fixed to the cantilever whose spring constant was either 0.06 or 0.38 N m⁻¹ as indicated by the manufacturer (BioForce, Nanosciences Inc., Ames, IA) and confirmed by the thermal fluctuation technique.⁵⁸ All experiments were performed in liquid environment, i.e., the films were immersed in a drop of 0.15M NaCl aqueous solution, and the curves were fitted to extract the Young's modulus E_0 of the film using a modified hertz model that was previously detailed.⁵⁹

Diclofenac Deposition and Measurement of Adsorbed Amount.

Cross-linked (CHI/HA)_i ($i = 12, 24, 48$) films were equilibrated for 1 h in a 0.15 M NaCl solution at pH 7.4. One milliliter of 2 mg/mL diclofenac solution (D899, Sigma, in NaCl 0.15 M, pH = 7.4) was deposited and allowed to adsorb for 2 h. The supernatants were collected and replaced by 1 mL of 0.01 M SDS to extract the diclofenac from the film. After 3 h, the SDS supernatants were collected. Measurements of the sodium diclofenac concentration after diclofenac insertion and after its extraction were performed by UV spectrometry at 276 nm (Beckman Coulter, DU640 Spectrophotometer). The absorbance values were translated to concentration values by comparing to standard solutions of known concentrations that were used to plot the calibration curve (data not shown). This calibration curve followed the Beer–Lambert law. The drug loading was estimated for various numbers of layer pairs i ($i = 12, 24$, and 48).

For the drug release experiments, cross-linked films (CHI/HA)₂₄ charged with diclofenac were put in 1 mL of PBS (Gibco). After 2, 4, 6, 24, 30, and 48 h, 200 μL of the supernatant was collected and replaced by 200 μL of fresh PBS.

Adsorption of Paclitaxel. Paclitaxel, commercially known as taxol, was a generous gift from the Hôpitaux Universitaires de Strasbourg, France. For cell experiments, it was adsorbed overnight at 20 $\mu\text{g/mL}$ on native and cross-linked (PLL/HA)₁₂ films. Films were then rinsed three times in 0.15 M NaCl (pH 6.5). For confocal laser scanning microscopy (CLSM) observations, paclitaxel Oregon Green 488 labeled (paclitaxelGreen 488, Molecular Probes, Eugene, OR) was first dissolved in ethanol/cremophor solution (v/v at 500 $\mu\text{g/mL}$) and then adjusted to 20 $\mu\text{g/mL}$ in a 0.15 M NaCl solution at pH 6.5.

HT 29 Cell Culture. Human colonic adenocarcinoma cells HT29 were kindly provided by M. Kedinger (INSERM UMR-S682, Strasbourg, France).⁶⁰ They were routinely cultured in 25 cm² culture flasks in Glutamax I DMEM 25 mM glucose (Invitrogen, France), without sodium pyruvate and supplemented with 10% v/v heat-inactivated fetal calf serum, penicillin (100 U/mL), and streptomycin 0.1 mg/mL (Invitrogen, France). Cultures were incubated at 37 °C in humidified air atmosphere (95%)–CO₂ (5%). Medium was changed every other day. For subcultures, cells were incubated in a mixture of 0.05% trypsin and 0.53 M EDTA and then harvested. The cells were distributed into 24-well plates containing the film-coated glass slides (10⁴/well/ slide)

Table 1. Summary of the Physicochemical and Biodegradability Properties of Native and Cross-Linked (PLL/HA)₂₄ and (CHI/HA)₂₄ Films

parameter	(PLL/HA) films		(CHI/HA) films	
	native	CL	native	CL
film roughness (nm)	1.1 ± 0.4	5.8 ± 0.5	7.3 ± 1 nm	39.6 ± 5.3
E ₀ (kPa)	20 ± 3 ^a	250 ± 30 ^a	15 ± 4	159 ± 40
<i>in vitro</i> enzymatic degradation	yes ^b (in about 10-15 h)	not visible over a 48 h time period ^b	yes, ^c rapid (few hours)	yes, ^c but much slower (only superficial attack is visible)
<i>in vivo</i> biodegradability	NA ^d	NA	yes ^c rapid (few hours)	yes, ^c but slower (several days/weeks)

^a Data taken from ref 40. ^b Data taken from ref 42. ^c From refs 12, 48. ^d NA: nonavailable.

in 1 mL of medium. Viability measurements were performed after 24 h, 48 h, and 72 h in culture. Experiments were performed in triplicate.

Cell Viability. The measurement of viability by the acid phosphatase method makes it possible to quantify the number of living cells with a greater sensitivity than the XTT test.⁶¹ The principle relies on the titration of the enzyme whose activity increases proportionally with the number of viable cells. The culture medium was first replaced with 300 μ L of PBS. The buffer containing 0.1 M sodium acetate (pH 5.5), 0.1% Triton X-100, and 10 mM *p*-nitrophenyl phosphate (pNPP, Sigma) was added in each well. The plates were placed for 3 h at 37 °C in the cell culture incubator. The reaction was stopped by addition of 40 μ L of 1 N sodium hydroxide, and the absorbance of the solution (yellow color) was measured at 405 nm using a spectrophotometer (LabSystems iEMS Reader MF, Gibco). Statistical analysis were performed using Sigma-stat software.

Results and Discussion

Mechanical and Biodegradability Properties of (CHI/HA) and (PLL/HA) Cross-Linked Films. The comparison between both film types in terms of roughness, Young's modulus, and biodegradability properties is presented in Table 1. Mechanical properties of native and cross-linked (PLL/HA) films have already been characterized,⁴⁰ and their biodegradability *in vivo* in contact with hyaluronidase and macrophages have been evaluated.⁶² Cross-linked films are much more rigid than native ones and less susceptible to biodegradation. The CHI/HA films appear to be more susceptible to rapid degradation *in vivo* in contact with various enzymes¹² and to be rapidly degraded by macrophages *in vitro* and *in vivo*.⁴⁸ This more rapid biodegradation may be explained by the fact that CHI is a polysaccharide whereas PLL is a synthetic polypeptide. The mechanical properties of native and cross-linked (CHI/HA) films were measured using the AFM-nano indentation technique (Table 1). They were found to be qualitatively similar to that of (PLL/HA) films: whereas native films are very soft, cross-linked films are about ten times stiffer, which may explain their enhanced resistance *in vivo*.⁴⁸ Consequences of film cross-linking appear thus to lead to common features that are (i) increased roughness, (ii) increased mechanical properties, and (iii) increased resistance to biodegradation.

Incorporation of Diclofenac into Cross-Linked (CHI/HA) Films. A first strategy was tested. It consisted of first adsorbing sodium diclofenac on a native film and then cross-linking the film at pH 5. However, in this case, we noticed that the film became white when the cross-linking solution was added. This may be due to the precipitation of diclofenac in the film. Therefore, a second strategy was investigated. It consisted of first equilibrating the cross-linked film in a 0.15 M NaCl solution at pH 7.4 and then putting the film in contact with diclofenac solution for 2 h. UV spectrometry was used to assess that diclofenac effectively adsorbed in the film by measuring the amount of diclofenac extracted from the films using a surfactant

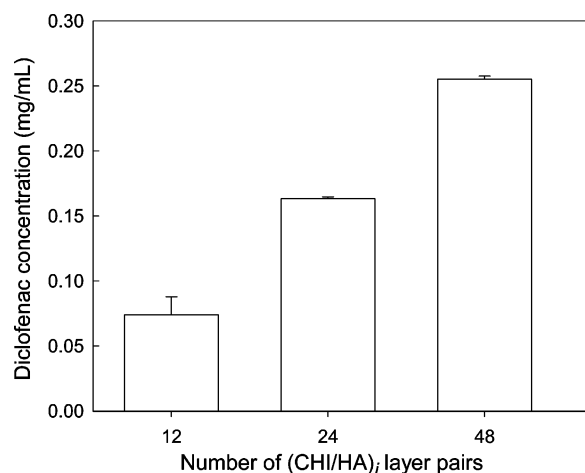


Figure 2. Influence of the number of layer pairs in the films on the diclofenac-loaded concentration in cross-linked (CHI/HA)_i films (with *i* = 12, 24, and 48). The diclofenac solution concentration in contact with the film was fixed at 2 mg/mL. (Error bars are SD of three PEM-coated slides).

(SDS at 0.01 M). By comparing the diclofenac concentration in the supernatant and in the film (after its extraction), it was possible to check that all the drug is either in the film or in the supernatant and that all the drug could be extracted upon addition of the detergent (data not shown).

We then examined the influence of the diclofenac solution concentration on diclofenac loading by varying its concentration over the range 0.2 to 2 mg/mL. A maximum loading was reached for concentrations greater than 1 mg/mL concentration (data not shown). For the highest diclofenac solution concentrations (1 and 2 mg/mL, respectively), the maximum incorporated amount was of the order of 23 to 12%, respectively. In addition, for a given diclofenac concentration (2 mg/mL), drug loading can be modulated by varying the number of layers in the film. We observed that the amount of diclofenac incorporated in the film is directly related to the number of layers in the film, for films containing between 12 and 48 layer pairs (Figure 2). Thus, once some parameters such as the adsorption time²⁷ and the concentration of the drug solution in contact with the film²⁴ have been fixed, it is still possible to modulate the capacity of the drug reservoir by varying the number of layer pairs in the film for a given diclofenac solution concentration. Our results are qualitatively similar to that obtained recently by Rubner et al. for nanoporous films.²³ These authors showed that the total amount of drug released increased as the number of porous layer pairs increased. This indirectly indicates that the adsorbed amounts, although not directly measured in the aforementioned study, were also related to the number of layer pairs. The results suggest that the diclofenac is diffusing through the whole film and that the film behaves as a drug reservoir.

As diclofenac is negatively charged at neutral pH ($pK_a = 4$) and also slightly hydrophobic, it may associate with positive

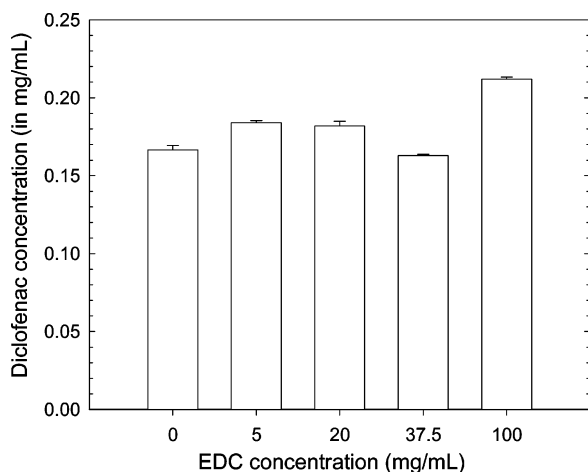


Figure 3. Influence of the cross-linker concentration EDC on the diclofenac-loaded concentration in (CHI/HA)₂₄ films. The initial diclofenac concentration was the same as for Figure 2. (Error bars are SD of three PEM-coated slides).

charges in the film or with hydrophobic domains. We investigated whether the diclofenac adsorbed amount depends on the extent of film cross-linking (Figure 3). We found that the adsorbed amount did not depend on cross-linker concentration, which rather suggests that diclofenac preferentially interacts with hydrophobic film regions as the number of free carboxylic and amine groups is decreased upon cross-linking.⁴² HA three-dimensional structure has been shown to be stabilized by hydrogen bonds and molecular modeling suggested that hydrophobic interactions help to stabilize these structures.⁶³ In a recent work, HA has been shown to interact with graphite, a hydrophobic surface. The authors concluded that HA molecules may interact with graphite through hydrophobic patches along its surface.⁶⁴ Such hydrophobic cavities may also be naturally present in various film types such as poly(acrylic acid)/poly(allyl amine hydrochloride), which have also been found to incorporate hydrophobic drugs such as cytochalasin D and ketoprofen.²³ In addition, recently the number or size of the hydrophobic nanodomains were adjusted by using chemically modified polysaccharides (such as amphiphilic polymers),^{27,65} which were shown to increase the loading capacity of the films.²⁷

Release of Diclofenac. Diclofenac-loaded films were then set in contact with PBS to investigate the release behavior of the films. The release appeared to be in two phases with a first burst effect within about 0.5 h followed by a slower release phase (Figure 4). This kind of two-phase behavior has already been observed for various drug loaded hydrogels or nanoparticles,^{66,67} for which various parameters such as pH and gel of composite nanoparticle formulation were indeed found to influence the release.⁶⁸ For chitosan nanoparticles, the molecular weight of polysaccharide used for preparing the particles was also found to play a role.⁶⁶ In our case, about 90% of the loaded amount is released within about 20 h. This is a relatively high release rate as compared to films containing hydrophobic nanodomains for which 60% of release can be obtained at best in nonphysiologic conditions.²⁷ For other dye-containing films, release was strongly dependent on pH of the release solution and occurred typically in about 15 to 50 min at physiological pH depending on the dye and on film type.^{37,36} In addition, the fraction of dye released never reach 100% and varied from 15% to 40% at most.^{37,36} For (CHI/HA) films containing the drug paclitaxel coupled to HA, half-life of paclitaxel was about 3 h²⁶ whereas nanoporous thin films could release drugs over several days or weeks.²³ It thus seems that release kinetics of

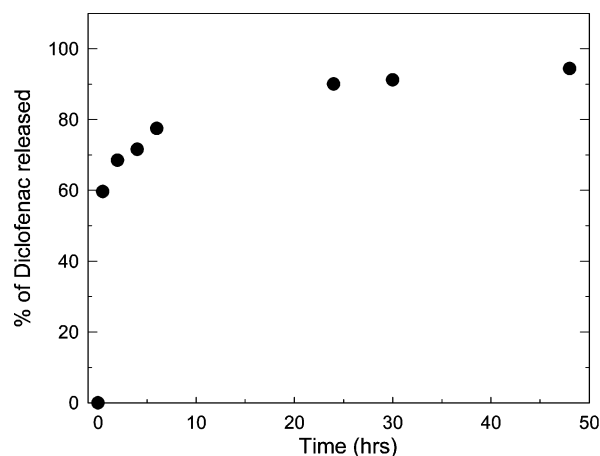


Figure 4. Diclofenac release curve in PBS from a (CHI/HA)₂₄ film. Results are expressed in terms of percentage of the total diclofenac concentration loaded in the film. (Error bars are SD of three measurements but are not visible).

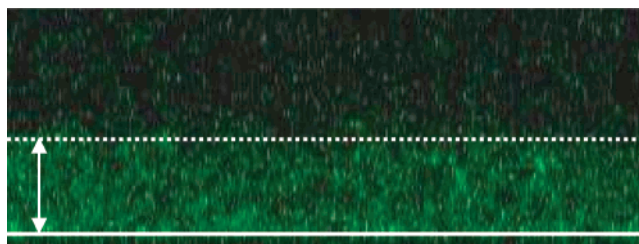


Figure 5. Confocal laser scanning (CLSM) image of a CL (PLL/HA)₂₄ film loaded with fluorescent paclitaxel (paclitaxelGreen 488) at 20 µg/mL. The white line corresponds to the glass slide and the white arrow to the film thickness (approximately 4 µm).

PEM films strongly depend on film architecture and on dye/film or drug/film interactions. The exact mechanisms that lead to entrapment and subsequent release of dyes and drugs are not very well understood. In our case, as cross-linked films are stable in PBS, we hypothesize that the release is due to a passive diffusion out of the film, as the drug is not covalently bound to the film and as it may be entrapped in hydrophobic domains.

Incorporation of Paclitaxel in (PLL/HA) Films and Cell Activity. To further validate the concept of drug loading into CL films, another type of drug, paclitaxel, was loaded in another film architecture composed of cross-linked (PLL/HA) films. Paclitaxel was recently coupled to hyaluronan (HA-Pac) and was found to remain active on macrophages when inserted in (chitosan/HA-Pac) films.²⁶ It was also loaded in native (PLL/HA) films capped with (PSS/PAH) synthetic layers that were needed for improving cell adhesion.²⁴ In the present study, we attempted to maintain a constant paclitaxel concentration for loading and did not investigate the influence of its concentration. We first verified the incorporation of paclitaxel in the film by observing CL (PLL/HA)₂₄ loaded with fluorescently labeled paclitaxel by CLSM (Figure 5). The visualization of a green band indicates that paclitaxel is diffusing throughout the CL film similarly to native (PLL/HA) films.²⁴ The low fluorescence intensity is due to the low fluorescent paclitaxel concentration. This diffusion is analogous to what occurs for diclofenac in (CHI/HA) films.

Human colonic adenocarcinoma (HT29) cell activity was then evaluated on CL films that contained paclitaxel. First, we verified that initial HT29 cell activity was significantly increased on CL films as compared to native ones (Figure 6). Good cell adhesion is effectively an important prerequisite for testing the subsequent action of paclitaxel on cells. Only a few HT29 cells

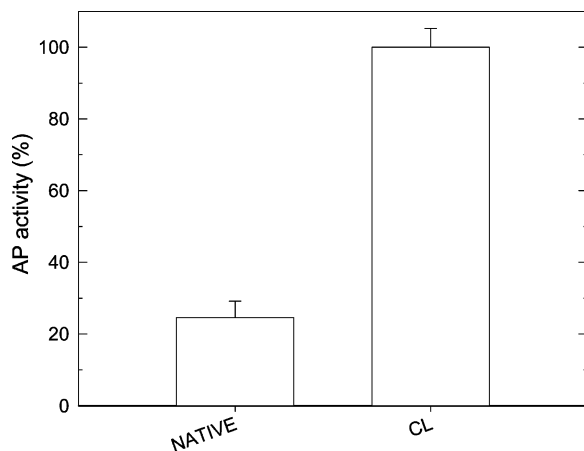


Figure 6. Acid phosphatase (AP) activity for HT29 cells cultured on native or cross-linked (PLL/HA)₁₂ films after 24 h. The error bars represent the standard deviation. The value of 100% has been arbitrarily attributed to CL films (** $p < 0.001$).

adhered and remained viable on native films, which is similar to results obtained for other cell types such as chondrosarcomas, primary chondrocytes, and primary motoneurons.^{42,62} Even if the high thickness and gel-like character of native (PLL/HA) films make them a good reservoir for drugs, such films are clearly not appropriate when cells have first to come in contact and adhere onto films before being able to “sense” bioactive molecules.

We first investigated paclitaxel activity when paclitaxel was loaded in (PLL/HA) films prior to film cross-linking. However, in this case, no paclitaxel activity was observed (data not shown), which is probably due to a loss of activity of paclitaxel upon cross-linking during the formation of amide bonds. As a consequence, paclitaxel was loaded into CL films after cross-linking. Cell viability was followed over a 3 day period, and the number of viable cells was counted everyday (Figure 7). Figure 7A and 7B shows the same data but in a different manner. In Figure 7A, cell viability was set at 100% for the control CL films at 24 h. Cell viability is clearly increasing on the CL films, similarly to what was already found for chondrosarcoma cells and primary chondrocytes on such CL (PLL/HA) films.^{47,62} In comparison, cell viability was steadily decreasing on the paclitaxel-loaded CL films. In Figure 7B, CL films without drug were taken as a reference (cell viability being set at 100% for comparison) for each time period in culture. After 24 h in culture, there was already a significant difference between paclitaxel-loaded CL films and controls. The differences were even increasing over the 3 day culture period. Paclitaxel had a dramatic effect on HT29 viability over this period, as less than 46% of the initial cells remained viable. When the cell number on drug-loaded films is compared to the cell number on control CL films, this percentage decreases to 10% over the 72 h time period. The differences between both film types were significantly different at each time period. The activity of the drug incorporated into CL films was thus fully conserved. The mechanism of paclitaxel action requires its internalization by the cells. Such internalization has indeed recently been shown on adherent cells anchoring to films capped with PSS/PH layers that contained fluorescently labeled paclitaxel loaded at high concentration (100 $\mu\text{g/mL}$).⁶⁹ It is however difficult to know whether the molecules are diffusing toward the cell membrane through the film or whether the cells would be able, via membrane protrusions (pseudopodia or filopodia), to detect the presence of the molecules in the film. In any case, if paclitaxel were diffusing out of the films, its diffusion would probably

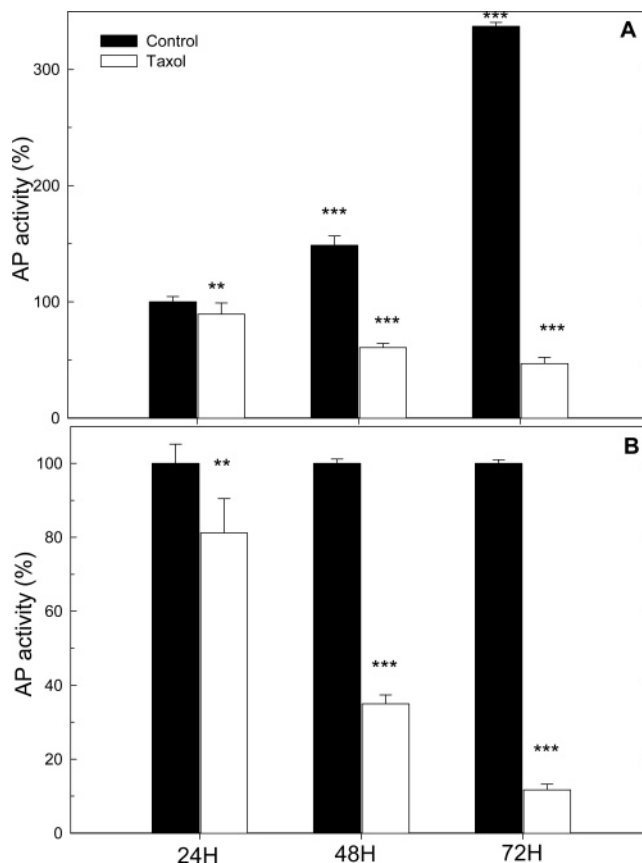


Figure 7. Acid phosphatase (AP) activity for HT29 cells cultured on cross-linked (PLL/HA)₁₂ films loaded or not with paclitaxel, after different time periods of 24, 48, and 72 h in culture. The error bars represent the standard deviation. Two different representations are shown: (A) The value of 100% has been arbitrarily attributed to CL films at 24 h (** $p < 0.01$; *** $p < 0.001$ versus control at 24 h for control films or versus paclitaxel at 24 h for paclitaxel-loaded films). (B) The value of 100% has been arbitrarily 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 24, 48, and 72 h, respectively).

occur within hours or even days, as the effect of paclitaxel was still visible 3 days after cell plating even if the medium was changed every 2 days.

The optimal duration of release *in vivo* would strongly depend on the type of biomedical application. For instance, in vascular therapy, it has been suggested that a sustained release of the drug for at least three weeks after stent deployment is required to present the cascade of biological events that leads to restenosis.⁷⁰ In the case of solid tumors however (like metastatic breast tumors), the effect of drugs should be observed after only a few days, as 1 day is the time by which the tumors under normal conditions have almost doubled in volume.⁷¹

Conclusion

We have developed multifunctional polyelectrolyte multilayer films that are (i) mechanically resistant, (ii) biodegradable, and (iii) bioactive. The films made of polypeptides and polysaccharides are mechanically resistant, as they are cross-linked and are also cell adhesive. Two types of film architectures (PLL/HA) and (CHI/HA) were chosen, as they were already well characterized. They are biodegradable, as they can be degraded *in vitro* and *in vivo*. In addition, they are bioactive and can

function as drug carriers. To render the film bioactive, the model drugs sodium diclofenac and paclitaxel were loaded after film cross-linking using the simple postdiffusion method. The amount of drug could be controlled by changing the number of layers in the film. Paclitaxel was found to remain active when loaded in CL (PLL/HA) films leading to a dramatic decrease in cell viability over a 3 day period. Overall, these results prove that it is possible to prepare multifunctional films with defined mechanical properties, biodegradability properties, and bioactivity. These films will now be employed to investigate the respective roles of film bioactivity versus film mechanical properties on cell adhesion and differentiation.

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References and Notes

- Seal, B. L.; Otero, T. C.; Panitch, A. *Mater. Sci. Eng. C* **2001**, *34*, 147–230.
- Whittlesey, K. J.; Shea, L. D. *Exp. Neurol.* **2004**, *190*, 1–16.
- Luo, Y.; Prestwich, G. D. *Curr. Cancer Drug Targets* **2002**, *2*, 209–226.
- Prestwich, G. D.; Marecak, D. M.; Marecek, J. F.; Vercruysse, K. P.; Ziebell, M. R. *J. Controlled Release* **1998**, *53*, 93–103.
- Decher, G. *Science* **1997**, *277*, 1232–1237.
- Hiller, J.; Mendelsohn, J. D.; Rubner, M. F. *Nat. Mater.* **2002**, *1*, 59–63.
- Tang, Z.; Kotov, N. A.; Magonov, S.; Ozturk, B. *Nat. Mater.* **2003**, *2*, 413–418.
- Decher, G.; Hong, J. D.; Schmitt, J. *Thin Solid Films* **1992**, *210–211*, 831–835.
- Lvov, L.; Haas, H.; Decher, G.; Moehwald, H.; Mikhailov, A.; Mchedlishvili, B.; Morgunova, E.; Vainshtein, B. *Langmuir* **1994**, *10*, 4232–4236.
- Zhu, Y.; Gao, C.; He, T.; Liu, X.; Shen, J. *Biomacromolecules* **2003**, *4*, 446–452.
- Tan, Q.; Ji, J.; Barbosa, M. A.; Fonseca, C.; Shen, J. *Biomaterials* **2003**, *24*, 4699–4705.
- Etienne, O.; Schneider, A.; Taddei, C.; Richert, L.; Schaaf, P.; Voegel, J.-C.; Egles, C.; Picart, C. *Biomacromolecules* **2005**, *6*, 726–733.
- Schultz, P.; Vautier, D.; Richert, L.; Jessel, N.; Haikel, Y.; Schaaf, P.; Voegel, J. C.; Ogier, J.; Debry, C. *Biomaterials* **2005**, *26*, 2621–2630.
- Gao, C.; Leporatti, S.; Moya, S.; Donath, E.; Mohwald, H. *Langmuir* **2001**, *17*, 3491–3495.
- Etienne, O.; Picart, C.; Taddei, C.; Haikel, Y.; Dimarcq, J.-L.; Schaaf, P.; Voegel, J.-C.; Ogier, J. A.; Egles, C. *Antimicrob. Agents Chem.* **2004**, *48*, 3662–3669.
- Thierry, B.; Winnik, F. M.; Merhi, Y.; Silver, J.; Tabrizian, M. *Biomacromolecules* **2003**, *4*, 1564–1571.
- Jewell, C. M.; Zhang, J.; Fredin, N. J.; Lynn, D. M. *J. Controlled Release* **2005**, *106*, 214–223.
- Jessel, N.; Oulad-Abdelghani, M.; Meyer, F.; Laval, P.; Haikel, Y.; Schaaf, P.; Voegel, J. C. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 8618–8621.
- Ai, H.; Jones, S. A.; Lvov, Y. M. *Cell Biochem. Biophys.* **2003**, *39*, 23–43.
- Etienne, O.; Gasnier, C.; Taddei, C.; Voegel, J. C.; Aunis, D.; Schaaf, P.; Metz-Boutigue, M. H.; Bolcato-Bellemin, A. L.; Egles, C. *Biomaterials* **2005**, *26*, 6704–6712.
- van den Beucken, J. J.; Walboomers, X. F.; Boerman, O. C.; Vos, M. R.; Sommerdijk, N. A.; Hayakawa, T.; Fukushima, T.; Okahata, Y.; Nolte, R. J.; Jansen, J. A. *J. Controlled Release* **2006**, *113*, 63–72.
- Vodouhe, C.; Schmittbuhl, M.; Boulmedais, F.; Bagnard, D.; Vautier, D.; Schaaf, P.; Egles, C.; Voegel, J. C.; Ogier, J. *Biomaterials* **2005**, *26*, 545–554.
- Berg, M. C.; Zhai, L.; Cohen, R. E.; Rubner, M. F. *Biomacromolecules* **2006**, *7*, 357–364.
- Vodouhe, C.; Guen, E. L.; Garza, J. M.; Francius, G.; Dejugnat, C.; Ogier, J.; Schaaf, P.; Voegel, J. C.; Laval, P. *Biomaterials* **2006**, *27*, 4149–4156.
- Jessel, N.; Schwinté, P.; Falvey, P.; Darcy, R.; Haikel, Y.; Schaaf, P.; Voegel, J.-C.; Ogier, J. *Adv. Funct. Mater.* **2004**, *14*, 174–182.
- Thierry, B.; Kujawa, P.; Tkaczyk, C.; Winnik, F. M.; Bilodeau, L.; Tabrizian, M. *J. Am. Chem. Soc.* **2005**, *127*, 1626–1627.
- Guyomard, A.; Nysten, B.; Muller, G.; Glinel, K. *Langmuir* **2006**, *22*, 2281–2287.
- Vazquez, E.; Dewitt, D. M.; Hammond, P. T.; Lynn, D. M. *J. Am. Chem. Soc.* **2002**, *124*, 13992–13993.
- Wood, K. C.; Boedicker, J. Q.; Lynn, D. M.; Hammond, P. T. *Langmuir* **2005**, *21*, 1603–1609.
- Serizawa, T.; Yamaguchi, M.; Akashi, M. *Angew. Chem. Int. Ed.* **2003**, *42*, 1115–1118.
- Schüler, C.; Caruso, F. *Biomacromolecules* **2001**, *2*, 921–926.
- Antipov, A. A.; Sukhorukov, G. B. *Adv. Colloid Interface Sci.* **2004**, *111*, 49–61.
- Balabushevich, N. G.; Tiourina, O. P.; Volodkin, D. V.; Larionova, N. I.; Sukhorukov, G. B. *Biomacromolecules* **2003**, *4*, 1191–1197.
- Sukhishvili, S.; Granick, S. *Macromolecules* **2002**, *35*, 301–310.
- Srivastava, R.; Brown, J. Q.; Zhu, H.; McShane, M. J. *Macromol. Biosci.* **2005**, *5*, 717–727.
- Burke, S. E.; Barrett, C. J. *Macromolecules* **2004**, *37*, 5375–5384.
- Kharlampieva, E.; Sukhishvili, S. A. *Langmuir* **2004**, *20*, 9677–9685.
- Mendelsohn, J. D.; Yang, S. Y.; Hiller, J.; Hochbaum, A. I.; Rubner, M. F. *Biomacromolecules* **2003**, *4*, 96–106.
- Yang, S. Y.; Mendelsohn, J. D.; Rubner, M. F. *Biomacromolecules* **2003**, *4*, 987–994.
- Richert, L.; Engler, A. J.; Discher, D. E.; Picart, C. *Biomacromolecules* **2004**, *5*, 1908–1916.
- Koktysh, D. S.; Liang, X.; Yun, B. G.; Pastoriza-Santos, I.; Matts, R. L.; Giersig, M.; Serra-Rodríguez, C.; Liz-Marzán, L. M.; Kotov, N. A. *Adv. Funct. Mater.* **2002**, *12*, 255–265.
- Richert, L.; Boulmedais, F.; Laval, P.; Mutterer, J.; Ferreux, E.; Decher, G.; Schaaf, P.; Voegel, J.-C.; Picart, C. *Biomacromolecules* **2004**, *5*, 284–294.
- Berg, M. C.; Yang, S. Y.; Hammond, P. T.; Rubner, M. F. *Langmuir* **2004**, *20*, 1362–1368.
- Picart, C.; Elkaim, R.; Richert, L.; Audoin, F.; Da, Silva, Cardoso, M.; Schaaf, P.; Voegel, J.-C.; Frisch, B. *Adv. Funct. Mater.* **2005**, *15*, 83–94.
- Schneider, A.; Bolcato-Bellemin, A.-L.; Francius, G.; Jedrzejewska, J.; Schaaf, P.; Voegel, J.-C.; Frisch, B.; Picart, C. *Biomacromolecules* **2006**, *10*, 2882–2889.
- Thompson, M. T.; Berg, M. C.; Tobias, I. S.; Lichter, J. A.; Rubner, M. F.; Van, Vliet, K. J. *Biomacromolecules* **2006**, *7*, 1990–1995.
- Schneider, A.; Francius, G.; Obeid, R.; Schwinté, P.; Frisch, B.; Schaaf, P.; Voegel, J.-C.; Senger, B.; Picart, C. *Langmuir* **2006**, *22*, 1193–1200.
- Picart, C.; Schneider, A.; Etienne, O.; Mutterer, J.; Egles, C.; Jessel, N.; Voegel, J.-C. *Adv. Funct. Mater.* **2005**, *15*, 1771–1780.
- Weindl, G.; Schaller, M.; Schafer-Korting, M.; Korting, H. C. *Skin Pharmacol. Physiol.* **2004**, *17*, 207–213.
- Mulhall, K. J.; Curtin, W. A.; Given, H. F. *Orthopedics* **2003**, *26*, 1219–1223.
- Marsolais, D.; Cote, C. H.; Frenette, J. *Lab. Invest.* **2003**, *83*, 991–999.
- Schiff, P.; Fant, J.; Horwith, S. *Nature* **1979**, *277*, 665–667.
- Luo, Y.; Ziebell, M. R.; Prestwich, G. D. *Biomacromolecules* **2000**, *1*, 208–218.
- Ruan, G.; Feng, S. S. *Biomaterials* **2003**, *24*, 5037–5044.
- Sipahigil, O.; Gursoy, A.; Cakalagaoglu, F.; Okar, I. *Int. J. Pharm.* **2006**, *311*, 130–138.
- Milas, L.; Mason, K. A.; Hunter, N.; Li, C.; Wallace, S. *Int. J. Radiat. Oncol. Biol. Phys.* **2003**, *55*, 707–712.
- Hemmerle, J.; Altmann, S. M.; Maaloum, M.; Horber, J. K.; Heinrich, L.; Voegel, J. C.; Schaaf, P. *Proc. Natl. Acad. Sci.* **1999**, *96*, 6705–6710.
- Lévy, R.; Maaloum, M. *Nanotechnology* **2002**, *13*, 33–37.
- Francius, G.; Hemmerle, J.; Ohayon, J.; Schaaf, P.; Voegel, J.-C.; Picart, C.; Senger, B. *Microsc. Res. Tech.* **2006**, *69*, 84–92.

- (60) Simon-Assmann, P.; Leberquier, C.; Molto, N.; Uezato, T.; Bouziges, F.; Kedinger, M. *J. Cell Sci* **1994**, *107* (Pt 3), 577–587.
- (61) Ueda, Y.; Walsh, E.; Nakanishi, H.; Yoshida, K. *Neurosci. Lett.* **1994**, *165*, 203–207.
- (62) Richert, L.; Schneider, A.; Vautier, D.; Jessel, N.; Payan, E.; Schaaf, P.; Voegel, J.-C.; Picart, C. *Cell Biochem. Biophys.* **2006**, *44*, 273–276.
- (63) Scott, J. E.; Heatley, F. *Biomacromolecules* **2002**, *3*, 547–553.
- (64) Spagnoli, C.; Korniaikov, A.; Ulman, A.; Balazs, E. A.; Lyubchenko, Y. L.; Cowman, M. K. *Carbohydr. Res.* **2005**, *340*, 929–941.
- (65) Rinaudo, M.; Auzely, R.; Vallin, C.; Mullagaliev, I. *Biomacromolecules* **2005**, *6*, 2396–2407.
- (66) Fernandez-Urrusuno, R.; Calvo, P.; Remunan-Lopez, C.; Vila-Jato, J. L.; Alonso, M. *J. Pharm. Res.* **1999**, *16*, 1576–1581.
- (67) Boonsongrit, Y.; Mitrevej, A.; Mueller, B. W. *Eur. J. Pharm. Biopharm.* **2006**, *62*, 267–274.
- (68) Fitzpatrick, D.; Corish, J. *Int. J. Pharm.* **2005**, *301*, 226–236.
- (69) Vodouhè, C.; Guen, E. L.; Garza, J. M.; Francius, G.; Dejumat, C.; Ogier, J.; Schaaf, P.; Voegel, J. C.; Lavalle, P. *Biomaterials* **2006**, *27*, 4149–4156.
- (70) Kamath, K. R.; Barry, J. J.; Miller, K. M. *Adv. Drug. Delivery Rev.* **2006**, *58*, 412–436.
- (71) Ahmed, F.; Pakunlu, F. I.; Brannan, A.; Bates, F.; Minko, T.; Discher, D. E. *J. Controlled Release*, **2006**, in press.

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