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Variation of Polyelectrolyte Film Stiffness by Photo-Cross-Linking: A New Way To Control Cell Adhesion

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We report on the preparation of polyelectrolyte films based on biopolymers whose nanomechanical properties can be tuned by photo-cross-linking. Cationic poly(L-lysine) was layer-by-layer assembled with anionic hyaluronan (HA) derivatives modified by photoreactive vinylbenzyl (VB) groups. The study of the multilayer buildup by quartz crystal microbalance with dissipation monitoring showed that the presence of VB groups does not influence significantly the multilayer growth. Then the VB-modified HA incorporated into the films was cross-linked upon UV irradiation. UV spectroscopy measurements showed that the cross-linking rate of the multilayers increases with the amount of VB groups grafted onto HA chains. Force measurements performed by atomic force microscopy with a colloidal probe proved that the rigidity of the cross-linked films increases with the grafting degree of HA chains and consequently the number of cross-links. Cell culture assays performed on non-cross-linked and photo-cross-linked films with myoblast cells demonstrated that cell adhesion and proliferation are considerably improved with increasing film rigidity.

Introduction

Regenerative medicine based on tissue engineering represents a promising therapeutic approach for tissue or organ repair.¹ The challenge is to control the cellular processes on a given substrate to induce the formation of desired functional tissues. Different parameters were identified to play a key role in cellular behaviors. Besides biosignaling molecules, the chemistry and the topography of the material surface^{2–7} and the characteristics of the microenvironment were shown to influence significantly the cellular processes. More particularly, the rigidity of the substrate seems to be of main importance.^{2–11} Indeed, several studies performed on cross-linked 3D polyacrylamide macrogels provided evidence that the adhesion, the spreading, the proliferation, and even the differentiation of cells are conditioned by the substrate stiffness.^{8,12} Such a stiffness dependence has been reported for both cell lines and primary cells.^{13,14}

A challenging development of the tissue engineering field is the design of suitable bioactive thin coatings which can be easily deposited onto the surface of the scaffolds to stimulate the cell response. Among the different approaches explored to prepare such bioactive coatings, the layer-by-layer (LbL) assembly of polyelectrolytes has recently aroused considerable attention.^{15–17} Beside its simplicity and its versatility, this technique, based on the alternate deposition of polycations and polyanions on the solid surface,^{18–20} offers several advantages: it can be used on a broad range of substrates varying by their nature and their geometry, the thickness of the resulting films can be finely tuned at the nanometer scale by adjusting the number of deposited polyelectrolyte pairs, the process requires only the use of aqueous solutions, and the functional properties of the films can be varied by a suitable choice of polyelectrolytes and of post-treatments. The use of the LbL technique was explored for a wide range of potential applications ranging from medicine to microelectronics.^{21–23} Several studies based on synthetic polyelectrolytes have recently described the preparation of LbL films promoting cell adhesion and proliferation.^{24,25} However, the development of films based on biopolymers is more attractive for in vitro and

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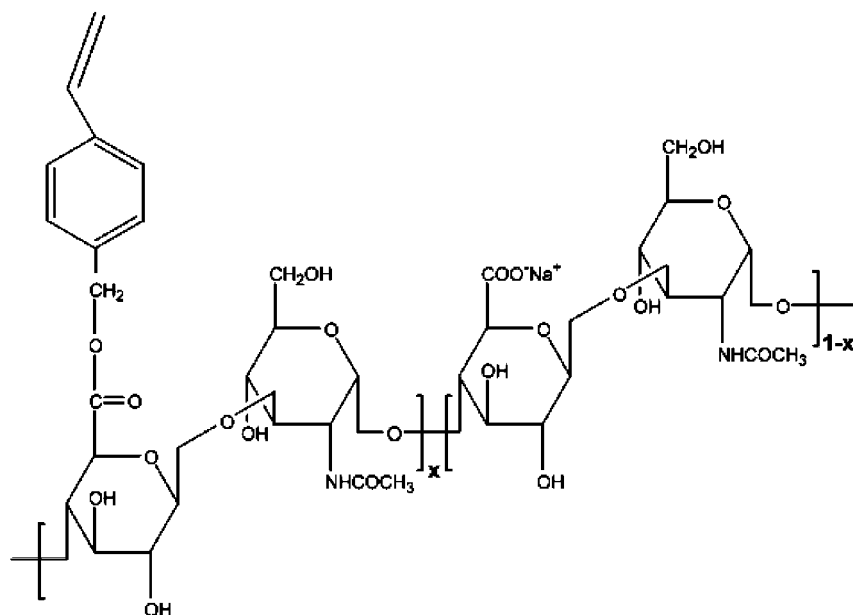
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Scheme 1. Structure of the Anionic HA-*x*VB Derivatives Used in This Study

in vivo applications due to their nontoxicity and their biodegradability.¹⁵ Recently, one of us reported on the fabrication of LbL films based on polysaccharides and/or polypeptides whose rigidity was successfully controlled by cross-linking the carboxylic groups of the polyanion with the amine groups of the polycation.^{14,26} For this, water-soluble 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) combined with *N*-hydrosulfosuccinimide (sulfo-NHS) was added within the films by postdiffusion. It was shown that the elastic modulus of the resulting films increased with increasing EDC concentration.²⁷ Also, cell culture assays performed on these self-assemblies showed that the adhesion, the spreading, and the proliferation of the cells were related to film rigidity. Recently, it was even proved that the stiffness of these films plays a noticeable role in the cell differentiation process.²⁸ However, although the EDC/sulfo-NHS cross-linking strategy proved to be efficient to prepare multilayers of controlled rigidity, it suffers from several drawbacks: the cross-linking reaction is time-consuming since it takes several hours for a micrometer-thick film, the cross-linking cannot be performed in situ in vivo due to the requirement for a thorough rinsing of the films after reaction to eliminate any trace of toxic unreacted chemicals or side products, and the cross-linking process based on the diffusion of EDC and sulfo-NHS molecules within the films precludes any spatial control over the region of cross-linking in the lateral and vertical directions.

In the present study, we explore a new approach based on photo-cross-linking to prepare biopolymer-based LbL films of tunable rigidity. In comparison with chemical cross-linking, photo-cross-linking offers several advantages: it is a clean and cheap process which does not require using additional chemicals, it could be performed in various media comprising in vitro and in vivo conditions,²⁹ and the cross-linking reaction can be easily followed by UV spectroscopy. Considering the promising cellular results recently obtained with poly(L-lysine)/hyaluronan (PLL/HA) multilayers cross-linked with EDC/sulfo-NHS,²⁷ we ex-

plored the possibility to prepare similar films by photo-cross-linking. However, a prerequisite condition to prepare photo-cross-linkable LbL films is the presence of photosensitive groups on one of the polyelectrolyte chains used. Advincula and co-workers have recently reported on the preparation and the photo-cross-linking of polyelectrolyte films based on benzophenone-modified poly(acrylic acid) and poly(allylamine hydrochloride).³⁰ Mayes and co-workers have also recently described the preparation of LbL films incorporating a photoreactive poly(acrylic acid) substituted with vinylbenzene (VB) groups.^{31,32} These VB side groups offer the advantage to react easily upon UV irradiation via vinyl moieties to form covalent bounds.³¹ Here, we present an alternative chemistry to synthesize VB-grafted hyaluronans (HA-VB) (Scheme 1). These anionic derivatives were layer-by-layer assembled with cationic PLL to prepare photo-cross-linkable films. The buildup and the photo-cross-linking of these multilayers were systematically investigated as a function of the amount of VB groups grafted onto the HA chains. The rigidity of these polyelectrolyte films was also characterized by atomic force microscopy (AFM) force measurements. Furthermore, these self-assemblies were tested toward cell adhesion.

Experimental Section

Materials. 4-Vinylbenzyl chloride was purchased from Acros (France) and was used without further purification. HA in the sodium hyaluronate form ($M_w \approx 200\,000$) and poly(L-lysine) hydrobromide ($M_w \approx 30\,000$ – $70\,000$) were supplied by Medipol (Switzerland) and Sigma (France), respectively, and were used as received. All the solvents and salts used were analytical reagent grade. Water was Milli-Q grade (resistivity higher than $18.2\text{ M}\Omega\text{ cm}$).

Synthesis of HA-VB Derivatives. VB-modified hyaluronan derivatives were synthesized by grafting VB groups onto carboxylic acid groups of HA chains through ester linkage according to a procedure previously reported.^{33,34} Briefly, an aqueous solution (2%,

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w/v) of HA, Na⁺ salt was transformed into its acidic form (HA, H⁺) by percolating through a cationic-exchange resin (Amberlite IRN-77, H⁺ form) and then neutralized by tetrabutylammonium hydroxide [(C₄H₉)₄N⁺, OH⁻] up to pH 7. After freeze-drying, 0.5 g of the resulting HA salt was dried under vacuum overnight at 40 °C and was dissolved in 30 mL of dried dimethyl sulfoxide (DMSO) at 40 °C. A 0.055 g portion of 4-vinylbenzyl chloride (45 mol % compared to the disaccharide units of HA) was dissolved in 3 mL of dried DMSO. This solution was slowly introduced, and the reaction was continued at 40 °C under magnetic stirring for 48 h. A concentrated aqueous solution of NaCl (10%, w/w) was added to convert the obtained VB-grafted HA into the sodium salt form which was subsequently precipitated in 900 mL of acetone. The precipitate was removed by filtration, washed with 400 mL of acetone, and dried. Then the resulting VB-grafted HA derivative was extensively purified by dialysis against pure Milli-Q water for 6 days and freeze-dried. Different VB-grafted HA derivatives were obtained by varying the amount of VB groups added in the reacting mixture. Coupling was checked by ¹H NMR (Bruker 300 NMR spectrometer), observing the characteristic chemical shifts at 7.4–7.5 and 6.76 ppm for the aromatic (–C₆H₄–) and the vinyl (–CH=) protons, respectively, and by attenuated total reflection (ATR) FTIR (2000 FT-IR spectrometer, Perkin-Elmer), observing the ester absorbance band centered at 1650 cm⁻¹ (results not shown). VB-grafted HA derivatives are denoted HA-*x*VB, with *x* the grafting degree in VB groups defined as the number of VB side groups grafted per 100 disaccharide units. The exact grafting degree *x* in VB groups was determined by UV-vis spectroscopy by measuring the absorbance of a 0.05 g L⁻¹ HA-*x*VB aqueous solution at 252 cm⁻¹ and considering a molar absorptivity ϵ of 18 390 L mol⁻¹ cm⁻¹.³⁵ It has to be noticed that the grafting degree of HA-*x*VB derivatives could not be determined from ¹H NMR measurements due to the poor signal-to-noise ratios of ¹H signals of hyaluronan chains, which result from the high viscosity of the high molar mass polysaccharide solutions.^{36–38}

Preparation of PLL/HA-*x*VB Films and Photo-Cross-Linking Procedure. PLL and HA-*x*VB were dissolved in 0.15 M NaCl with a concentration of 0.5 and 1 g L⁻¹, respectively. The pH of the solutions was set at pH 7.4 by adding 0.1 M NaOH. HA-*x*VB solutions were stored in the dark to avoid any photo-cross-linking of polyelectrolyte chains. All solutions were filtered through an 8 μ m Millipore membrane before use. The substrates used for multilayer growth were SUPRASIL-type fused silica plates (Hellma, France) or 14 mm microscope cover glasses. They were cleaned by treatment in a hot piranha solution (H₂O₂ (35%)/H₂SO₄ (98%), 1:1, v/v) for 20 min (caution: piranha solution is extremely corrosive) and then thoroughly washed with pure Milli-Q water. The films were fabricated manually or with an automatic dipping machine (Dipping Robot DR3, Kirstein and Riegler GmbH, Germany) by alternately dipping the substrate in aqueous solutions of PLL and HA-*x*VB for 10 min each. Between each deposition step, the substrate was thoroughly rinsed in 0.15 M NaCl solution buffered at pH 7.4 to remove the excess polyelectrolyte. For the cell culture assays, the film-coated glass slides were introduced into 24-well plates and were stored at 4 °C. A film obtained by dipping the substrate *N* times into the solution of PLL and *N* times into the solution of HA-*x*VB is named (PLL/HA-*x*VB)_{*N*}. Multilayers were cross-linked directly in buffer solution by exposure at a distance of 5 cm to a model VL-215.LC (Vilber Lourmat) short-wave ultraviolet lamp (30 W) transmitting at 254 nm. The incoming UV intensity measured in these conditions with a VLX-3W radiometer was 1.9 mW cm⁻².

Film Characterization. Quartz Crystal Microbalance (QCM). A QCM D-300 (Q-Sense, Sweden) system employing SiO₂-coated

quartz crystal resonators with a nominal frequency of about 5 MHz was used to monitor in situ the growth of multilayers. Before use, the silica crystals were cleaned with a plasma UV-ozone cleaner. PLL/HA-*x*VB multilayers were built in situ at 21 °C by successive injections of 0.5 mL of PLL and then HA-*x*VB in the flow cell. Between each adsorption step, the flow cell was thoroughly rinsed by injecting 2 \times 5 mL of 0.15 M NaCl (pH 7.4) for 5 min. After each injection, the QCM signal was recorded until a steady state was reached. The frequency shift (Δf_n) due to polyelectrolyte adsorption was recorded at three different overtones *n* (15, 25, and 35 MHz).

UV-Vis Spectroscopy. The photo-cross-linking of the films was monitored by UV-vis spectroscopy. Fused silica slides coated by PLL/HA-*x*VB films were rapidly blown dry with a stream of pure air and mounted in a Kontron UVIKON860 spectrometer sample holder. Then the spectra were collected using an uncoated fused silica slide as a reference. To ensure the accurate quantification of the cross-linking, the relative absorbance (*A*_{rel}) of the sample was defined as

$$A_{\text{rel}} = \frac{A_{252}(t) - A_{400}(t)}{A_{252}(0) - A_{400}(0)} \quad (1)$$

with *A*₂₅₂(0) and *A*₂₅₂(*t*) the absorbances corresponding to the maximal absorbance of the VB groups measured initially and after an UV exposure time of *t* (min), respectively, and *A*₄₀₀(0) and *A*₄₀₀(*t*) the absorbance measured in the baseline region (400 nm) initially and after an UV exposure time of *t*, respectively.

Atomic Force Microscopy. All AFM measurements were carried out in a liquid (Hepes–NaCl buffer containing 0.15 M NaCl and 20 mM Hepes at pH 7.4) using a Nanoscope V atomic force microscope (Veeco, California), and the spring constants of all cantilevers were determined individually using the thermal noise method.³⁹

Imaging. All AFM images were obtained in contact mode in a liquid using pyramidal silicone nitride cantilevers (MLCT-Microlever Probes, Veeco Instruments, Germany) with force constants around 60 mN m⁻¹. Substrate topographies were imaged with 512 \times 512 pixels² at line rates of 1 Hz. For surface roughness analysis, 5 \times 5 μ m² AFM images were obtained, and the root mean squared deviation *R*_{rms} from the principal *x*–*y* plane was calculated according to

$$R_{\text{rms}} = \sqrt{\frac{1}{N_x N_y} \sum_{i=1}^{N_x} \sum_{j=1}^{N_y} (z_{ij} - z_{\text{mean}})^2} \quad (2)$$

where *z*_{*ij*} is the height of a given pixel, *z*_{mean} is the average height of the pixels, and *N*_{*x*} = *N*_{*y*} = 512 are the number of pixels in the *x* and *y* directions.

Force Measurements. Force-indentation profiles were recorded in a liquid using borosilicate sphere-tipped cantilevers of 2.5 μ m radius (Bioforce Nanoscience, Iowa) having a spring constant of 60 mN m⁻¹. Young moduli *E* were extracted from the above profiles by using the finite thickness corrected Hertz sphere model.⁴⁰ Because of their high water content, we assumed incompressibility of PLL/HA-*x*VB films (Poisson's ratio ν = 0.5), and the expression of the indentation force is thus given by

$$F = \frac{16E}{9} R^{1/2} \delta^{3/2} [1 + 1.133\chi + 1.283\chi^2 + 0.769\chi^3 + 0.0945\chi^4] \quad (3)$$

where $\chi = (R\delta)^{1/2}/h$, *R* is the indenter radius, *h* is the sample thickness, and δ is the indentation. The indentation δ is obtained by subtracting the deflection *d* to the movement of the piezoelectric ceramic ($\Delta z = z - z_0$) in the *z* direction, where *z*₀ is the contact point. The contact point *z*₀ was determined following the method proposed by Crick

(35) The concentration of VB groups was determined from Beer–Lambert's law according to $A = \epsilon l C$, with *A* the absorbance measured at 252 cm⁻¹, ϵ the molar absorptivity determined from standard dimethyl sulfoxide solutions of 4-vinylbenzyl chloride (ϵ = 18 390 L mol⁻¹ cm⁻¹), *l* the path length, and *C* the concentration of VB groups.

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Table 1. Characteristics of HA-*x*VB Derivatives Used in This Study

derivative	concn of 4-vinylbenzyl chloride added to the reaction (mol %)	effective grafting degree x^a (%)
HA-14VB	15	14
HA-18VB	24	18
HA-29VB	35	29
HA-37VB	45	37

^a Value determined from UV spectroscopy measurements.

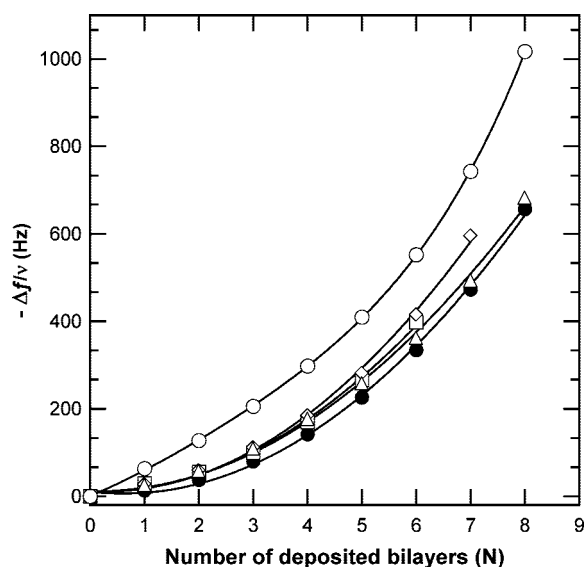


Figure 1. QCM frequency shift (15 MHz harmonic) vs number of deposited layer pairs for the (PLL/HA)_{*N*} films (filled circles) and for the HA-*x*VB derivatives having different grafting rates: 14% (squares), 18% (tilted squares), 29% (triangles), 37% (empty circles). The lines are drawn as guides for the eye.

and Yin.⁴¹ To avoid large strain effects, we analyzed force-indentation curves for micrometer-thick films in the 0–50 nm indentation domain. For each tested sample, two measurements were realized at five different positions on each sample and for two different slides. Young moduli were calculated by least-squares fitting the obtained force-indentation curves.

Cell Culture Assays. C2C12 cells (<20 passages, kindly provided by Cécile Gauthier-Rouvière, CRBM, Montpellier, France) were maintained in polystyrene flasks in a 37 °C, 5% CO₂ incubator and cultured in 1:1 Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 medium (growth medium, GM; Gibco, Invitrogen, Cergy-Pontoise, France) supplemented with 10% fetal bovine serum (FBS; PAA Laboratories, Les Mureaux, France) containing 100 U mL⁻¹ penicillin G and 100 μg mL⁻¹ streptomycin (Gibco, Invitrogen, Cergy-Pontoise, France). Cells were subcultured prior to reaching 60–70% confluence (approximately every 2 days). For the cell experiments, the films were deposited on 12 mm diameter glass slides and introduced into 24-well plates. C2C12 cells were seeded on non-cross-linked and photo-cross-linked (upon UV irradiation for 50 min) films at 5×10^4 cells cm⁻² in GM and were observed in phase contrast microscopy using a 10× objective under a Zeiss Axiovert 200 (Zeiss, Germany) inverted microscope. Images were obtained using a CoolSNAP EZ CCD camera and acquired with Metavue software (both from Roper Scientific, Evry, France).

Cell Counting Assays. For the cell counting tests, films were directly fabricated into 96-well plates. Briefly, 50 μL of PLL solution was introduced into each well and allowed to adsorb for 8 min. The wells were then washed twice with the rinsing solution. Then 50 μL of HA-*x*VB solution was introduced into each well and allowed to

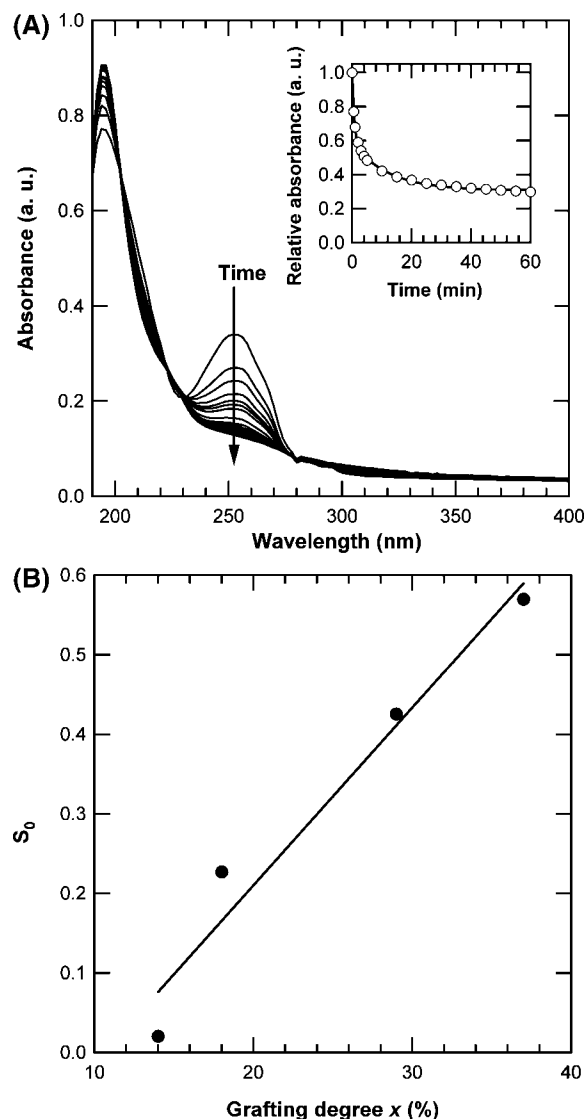


Figure 2. (A) UV absorbance spectrum measured for a (PLL/HA-37VB)₁₅ film exposed for different times upon UV irradiation (the absorbance peak measured at 252 nm decreases as a function of the time). The inset represents the variation of the relative absorbance *A*_{rel} (see the Experimental Section) vs UV exposure time. The line is a double-exponential fit to the data. (B) Variation of the initial slope *S*₀ extracted from the exponential fit of *A*_{rel} vs exposure time, represented as a function of the grafting degree *x* of HA-*x*VB derivatives incorporated into the LbL films.

adsorb for 8 min, and the wells were subsequently rinsed. The process was repeated until 12 layer pairs were deposited. Then the films were either UV cross-linked or not, prior to cell seeding at 7500 cells/cm² in growth medium. Cell numbers were assessed after 4 h in culture using a cell counting kit (CyQUANT, Molecular Probes, Invitrogen, Cergy-Pontoise, France). In brief, the cells were washed three times with phosphate-buffered saline (PBS) and frozen at -80 °C overnight. After the cells were thawed at room temperature (rt), a mixture of the cyQUANT GR dye and cell-lysis buffer was introduced and fluorescence of the plates was directly measured using a fluorescence microplate reader (Twinkle LB 970, Berthold, Germany) with the excitation filter set at 485 ± 7 nm and emission filter at 535 ± 12 nm. Experiments were performed in duplicate with three independent wells per experimental condition.

Results and Discussion

To prepare PLL/HA-*x*VB multilayered films of various cross-linking rates, four different HA-*x*VB derivatives with *x* increasing

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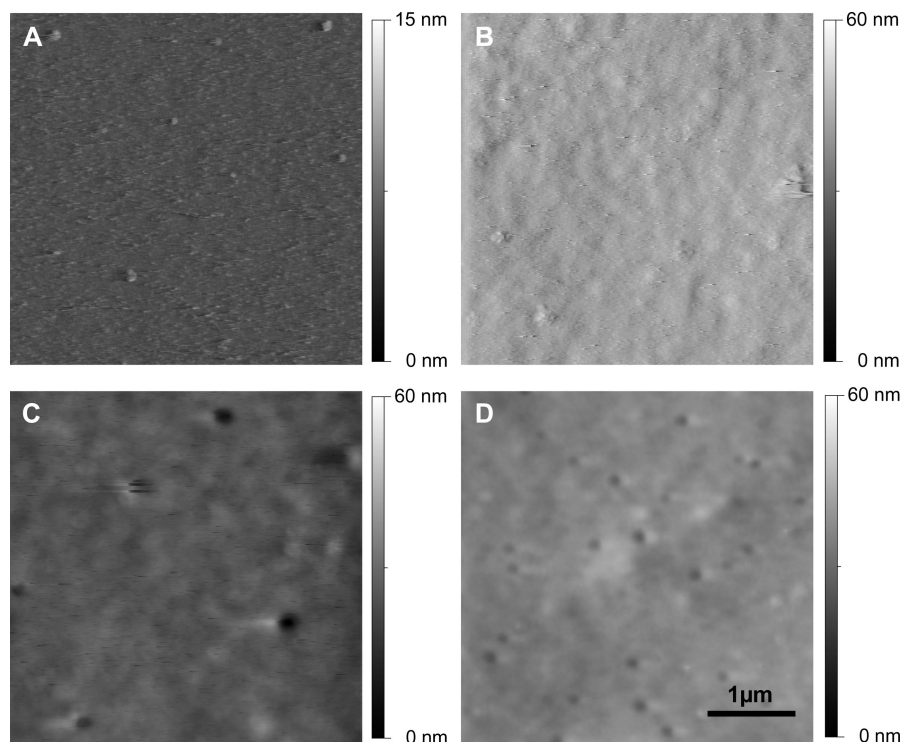


Figure 3. Topographic AFM images ($5 \times 5 \mu\text{m}^2$) of photo-cross-linked $(\text{PLL}/\text{HA}-x\text{VB})_{24}$ films with the grafting degree x equal to 14% (A), 18% (B), 29% (C), and 37% (D).

from 14% to 37% were synthesized (Table 1). These derivatives were successfully characterized by ATR-FTIR, ^1H NMR, and UV-vis spectroscopy (results not shown). An HA- $x\text{VB}$ derivative synthesized by adding 50 mol % 4-vinylbenzyl chloride during the reaction was also synthesized. However, this sample was not used in this study since it was not water soluble. To investigate the influence of the presence of VB groups grafted onto HA chains on the multilayer buildup, the growth of PLL/HA- $x\text{VB}$ multilayers was systematically monitored in situ by QCM. Figure 1 displays the frequency shift versus the number of deposited pair layers for the PLL/HA- $x\text{VB}$ films incorporating HA- $x\text{VB}$ derivatives of increasing x . The growth measured for the native PLL/HA film was added for comparison. Whatever the HA- $x\text{VB}$ derivative used, the frequency increment increases in a nonlinear fashion with increasing number of deposited pairs. This behavior, reported previously for native PLL/HA films, was explained by an “in” and “out” diffusion of the PLL chains through the self-assembled film during the buildup process.⁴² The inspection of the data reveals also that the presence of VB groups does not affect significantly the multilayer growth, compared to PLL/HA native films, for x lower than 37%. From the measurements performed on native PLL/HA films, the thickness of the $(\text{PLL}/\text{HA}-x\text{VB})_8$ films can be estimated to be about 450 nm for x lower than 37%.⁴³ However, for a higher grafting degree x , larger frequency shifts Δf were observed after each deposited layer pair, which reflects a higher mass of polyelectrolyte deposited and consequently the formation of thicker films. A similar behavior was previously observed for LbL films incorporating hydrophobically modified anionic polysaccharides and was attributed to hydrophobic interactions between amphiphilic polyelectrolyte chains.³³ Considering the hydrophobicity of VB groups, the presence of HA- $x\text{VB}$ aggregates during the multilayer buildup can be hypothesized as

well to explain the higher thickness of the films based on the higher modified HA-37VB derivative. The presence of photo-reactive VB side groups on the HA chains offers the possibility to cross-link the PLL/HA- $x\text{VB}$ films upon exposure to UV irradiation. It has to be noticed that the photo-cross-linking reaction in this case involves only the VB-modified HA chains incorporated within the films. This contrasts with the EDC/sulfo-NHS cross-linking strategy developed previously for PLL/HA native films, which is based on the formation of amide linkages between carboxylic and amine groups of HA and PLL, respectively.¹⁴ The photo-cross-linking of 15-bilayer PLL/HA- $x\text{VB}$ films was systematically investigated by UV-vis spectroscopy. Figure 2A shows UV-vis spectra of a $(\text{PLL}/\text{HA}-37\text{VB})_{15}$ film deposited on a fused silica slide and exposed to UV light for different times. A significant peak observed at 252 nm is attributable to the VB side groups. Upon UV irradiation, this peak was seen to decrease with increasing exposure time due to the reaction of the vinyl groups. The relative absorbance (see the Experimental Section) measured at 252 nm and related to the percentage of remaining unreacted vinyl groups was traced versus the irradiation time (inset, Figure 2A) to evaluate the cross-linking kinetics. After only 20 min of exposure, the peak present at 252 nm strongly decreased and was no longer visible so that the relative absorbance reached a plateau, which indicates the complete conversion of the vinyl groups. Similar experiments performed on $(\text{PLL}/\text{HA}-37\text{VB})_N$ films with N varying from 8 to 15 revealed that the time required to achieve the entire conversion of the vinyl groups does not depend on the film thickness. Indeed, the tested multilayers were thin enough to ensure the transmission of the UV light throughout the whole film and consequently the total conversion of the cross-linking vinyl groups. To evaluate more accurately the cross-linking kinetics, the decrease of the relative absorbance A_{rel} measured at 252 nm as a function of the exposure time was fitted by a double-exponential function, as shown in the inset of Figure 2A. A double-exponential function had to be taken to represent the

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data, suggesting that the cross-linking occurred through at least two mechanisms probably involving different diffusion rates. The slope value at the origin (S_0), extracted from this equation when the exposure time tends to zero, is directly related to the cross-linking kinetics and can thus be used to compare the initial rate of the cross-linking for the different tested films. The variation of S_0 with the grafting degree x displayed in Figure 2 shows a linear increase of S_0 with x . Thus, the higher the content of VB groups in the film, the higher the initial rate of the cross-linking reaction. When the concentration of VB groups increases in the film, the probability of a VB group to react with a second adjacent one increases so that the kinetics of the cross-linking reaction is improved. Crucially, the photo-cross-linking via VB side groups is observed to be a faster process than the chemical cross-linking based on the EDC/sulfo-NHS strategy. Indeed, a complete cross-linking of the films was achieved after a UV exposure of 20–30 min only, independently of the film thickness, whereas more than about 8 h is usually required for the EDC/sulfo-NHS approach.¹⁴

The morphology of the photo-cross-linked 24-bilayer films exposed for 50 min to UV irradiation was evaluated by performing in situ AFM imaging measurements in contact mode. The inspection of the topographic images (Figure 3) recorded for the multilayers incorporating HA- x VB with x increasing reveals the presence of continuous films. The root-mean-squared deviation R_{rms} computed from the AFM images varies from 0.3–9 nm for x increasing from 14% to 37%. These values are in the same range as those measured previously for EDC/sulfo-NHS cross-linked PLL/HA films, which provides the evidence that the presence of VB groups does not perturb significantly the morphology of the films.²⁷

The mechanical properties of the cross-linked films were investigated by AFM by applying a deformation with a sphere-tipped cantilever used as a microindenter. This technique was previously proved to be an accurate and powerful method to probe the nanomechanical properties of thin films such as LbL assemblies.^{44–49} Figure 4A displays typical force–indentation profiles measured for (PLL/HA- x VB)₂₄ films with x increasing from 14% to 37%. Considering the results previously reported for PLL/HA native films, the thickness of these films is estimated to be about 4 μm .⁵⁰ The inspection of the force–indentation curves reveals that the stiffness of the multilayers increases with x . The Hertz sphere model was used to analyze the force measurement data and to estimate the average Young moduli E of the cross-linked films. Figure 4B displays the variation of the measured average elastic modulus E versus the grafting degree x of the HA- x VB derivatives used for the film fabrication. Interestingly, the elastic modulus increases linearly with x . This behavior is related to the increase of the cross-linking rate with x increasing: The rigidity modulus of a network is inversely proportional to the average length of the segments between two cross-links,⁵¹ hence proportional to the number of VB groups grafted onto HA chains. Therefore, the stiffness of the film can

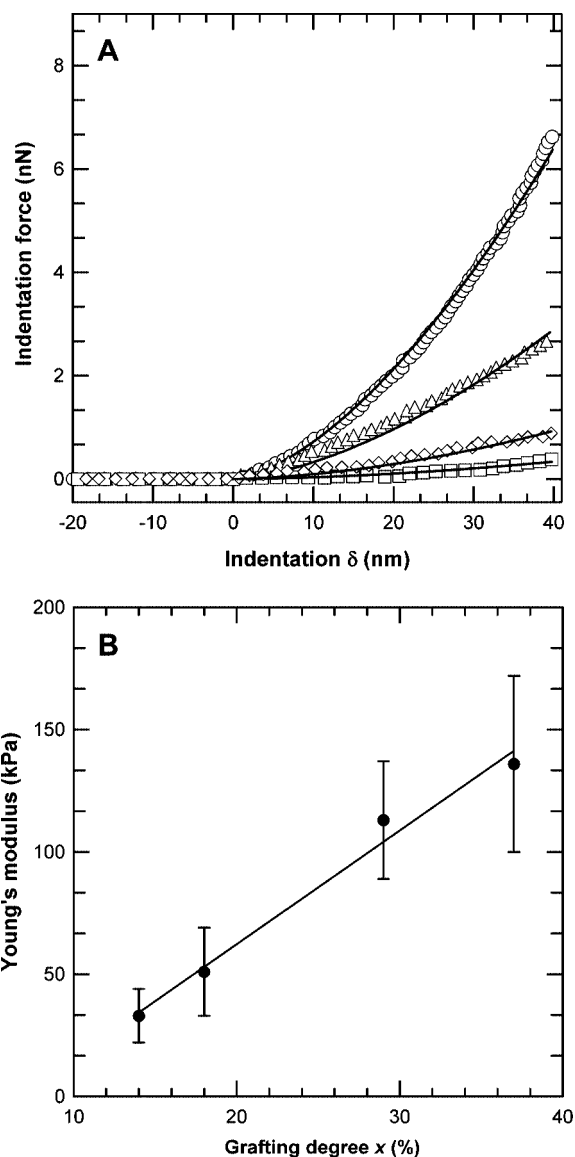


Figure 4. (A) Comparison of indentation curves represented by the force vs deformation and measured by AFM for (PLL/HA- x VB)₂₄ films with x equal to 14% (squares), 18% (tilted squares), 29% (triangles), and 37% (circles). The lines are fits to eq 3 (see the Experimental Section). (B) Variation of Young modulus extracted from force–indentation profiles of (PLL/HA- x VB)₂₄ films vs the grafting degree x .

be accurately adjusted simply by varying the grafting degree x of VB-modified HA chains incorporated into the films. However, in contrast with the strategy based on EDC/sulfo-NHS chemical cross-linking, for which the maximum Young modulus reached is about 380 kPa,²⁷ the photo-cross-linking does not allow preparation of highly cross-linked films (i.e., characterized by a high Young modulus). This might be due to the fact that, in the present case, only HA–VB chains can react in the cross-linking reaction whereas both PLL and HA take part in the EDC/sulfo-NHS chemical cross-linking.¹⁴ The comparison between the elastic moduli of PLL/HA films cross-linked by EDC/sulfo-NHS and photo-cross-linked PLL/HA–37VB films allows us to conclude that the stiffness of the PLL/HA–37VB film is quite similar to that of a PLL/HA film cross-linked with 10–30 mg mL^{−1} EDC.²⁷ This indicates that the stiffest photo-cross-linked films are only moderately rigid. In this EDC concentration range, cells began to adhere and spread on the films. Of note, in the case of the chemical cross-linking, a plateau in the Young modulus was reached for EDC concentrations greater than 70 mg mL^{−1}.²⁷

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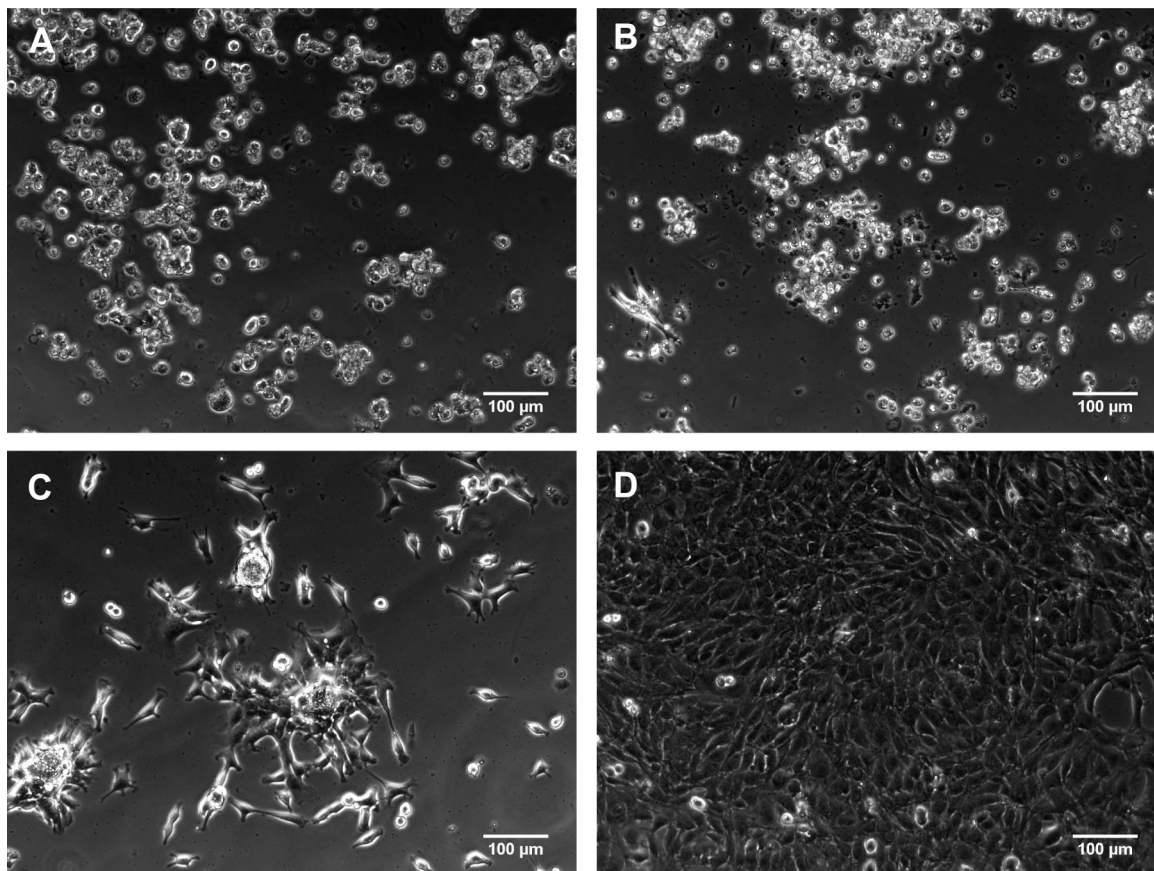


Figure 5. Morphology of C2C12 cells adhered on non-cross-linked (A, B) and photo-cross-linked (C, D) (PLL/HA-37VB)₁₂ films after a culture time of 15 h (left) and 72 h (right).

To evaluate the influence of the photo-cross-linking of the multilayers on the adhesion and proliferation of the cells, cell culture assays were thus performed only for the films built with the most grafted HA-37VB, either exposed for 50 min or not to UV irradiation. Mouse myoblasts (C2C12 cells), which are of interest to study the differentiation of skeletal muscle cells toward myotubes, were selected for these assays, as they were previously used for testing adhesion, proliferation, and differentiation on the chemically cross-linked films.²⁸ Figure 5 displays the morphology of C2C12 cells adhered on non-cross-linked (Figure 5A,B) and photo-cross-linked (Figure 5C,D) (PLL/HA-37VB)₁₂ films after a culture time of 15 h (Figure 5, left) and 72 h (Figure 5, right). The adhesion of C2C12 cells after 15 h of culture was observed for both non-cross-linked and photo-cross-linked films. However, a drastic variation of the cell morphology was observed between both films tested: The cells adhered onto the non-cross-linked film adopted a rounded shape, whereas the cells adhered onto photo-cross-linked films spread. Moreover, in contrast with the non-cross-linked multilayer, an extensive proliferation of the cells was observed on the photo-cross-linked film after 72 h of culture. Early adhesion of the cells at 4 h was also quantified by a fluorescence-based assay. The cell number was significantly higher on UV cross-linked films as compared to non-cross-linked ones (Table 2). No effect was observed for PLL/HA films that did not contain photo-cross-linkable groups (data not shown). The cross-linking reaction between vinyl groups does not modify significantly the surface chemistry of the PLL/HA-*x*VB multilayers. As a consequence, the larger spreading and proliferation observed for C2C12 cells adhered on photo-cross-linked films can be attributed to the higher rigidity of these multilayers. These results confirm that the cross-linking of LbL films promotes cell growth as evidenced previously

Table 2. Quantification of the Number of Viable Cells Adhered onto the Films after a Culture Time of 4 h

condition	no. of cells adhered on the (PLL/HA-0.37VB) ₁₂ film (cell number cm ⁻²)	no. of cells adhered on tissue culture polystyrene ^a (cell number cm ⁻²)
without UV exposure	883 ± 220	5669 ± 316
with UV exposure	3358 ± 316	6701 ± 551

^a Cell countings measured on plastic well plates exposed or not to UV irradiation were added for comparison.

for PLL/HA films.²⁷ Therefore, photo-cross-linking is an efficient and elegant process which can be used as an alternative to chemical cross-linking to increase the rigidity of the LbL films and to promote the adhesion and the proliferation of cells.

Conclusion

Photosensitive HA-*x*VB derivatives were successfully synthesized and incorporated into biopolymer-based LbL films. These multilayers were shown to cross-link quickly and easily upon exposure to UV irradiation. The cross-linking rate of the films was seen to depend on the grafting degree *x* of the HA-*x*VB derivatives. Moreover, nanomechanical measurements demonstrated that the rigidity of the cross-linked films can be modulated over several orders of magnitude by simply varying the grafting degree *x*. Cell adhesion assays performed on these multilayers confirm the emerging idea that the cellular behavior is strongly influenced by the stiffness of the substrate, independently from the chemistry of the surface. The fabrication of photo-cross-linkable biopolymer-based LbL films of tunable rigidity is of great interest for the development of bioactive coatings for tissue

engineering. The experimental conditions described here should be adapted for such applications. The photo-cross-linking process can be performed easily and rapidly. Moreover, photo-cross-linking should offer a spatial control which is of potential interest to design microstructured coatings able to direct the cell proliferation at the micrometer scale.

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