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Layer-by-Layer assembled growth factor reservoirs for steering the response of 3T3-cells

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Abstract

Layer-by-Layer (LbL) assemblies of heparin (Hep) and chitosan (Chi) were prepared for use as reservoirs for acidic and basic fibroblast growth factors (aFGFs and bFGFs, respectively). The effects of the architecture and composition of the reservoirs on the viability and proliferation of NIH-3T3 fibroblast cells were studied under starvation conditions. The reservoir stability was monitored by ellipsometry. The aFGF and bFGF loadings were determined using a dissipation-enhanced quartz crystal microbalance (QCM-D). Stability and release assays were performed in a phosphate buffer at physiological conditions. The results demonstrated that the amount of aFGF and bFGF loaded into and released from LbL reservoirs composed of 3 and 6 layer pairs could be controlled. Cell culture assays in low serum culture medium (LSCM) demonstrated that incorporating very small amounts of aFGF and bFGF into the (Hep/Chi)_n multilayers significantly improved the proliferation of the NIH-3T3 fibroblasts. The cells did not proliferate on (Hep/Chi)_n assemblies prepared in the absence of FGF under identical conditions. The LbL reservoirs were highly effective for the long-term storage (up to 9 months) of aFGF and bFGF. This work demonstrates the potential of LbL reservoirs for use as biomaterial coatings.

Keywords

Layer-by-layer assembly; Polyelectrolyte multilayers; Heparin; Chitosan; Acidic fibroblast growth factor (aFGF); Basic fibroblast growth factor (bFGF); Controlled release

1. Introduction

Signaling molecules such as growth factors must be delivered to their target cells in a spatio-temporally controlled fashion to ensure that their specific bioactivity for a given cell type is fully utilized [1]. Although many signaling molecules control the formation of three-dimensional multicellular natural tissues during organogenesis, current tissue engineering approaches often involve the introduction of growth factors via simple scaffold-based delivery systems with very limited control over local concentrations or release kinetics. To engineer complex tissues, three-dimensional patterned architectures in which compartments (reservoirs) containing different signaling molecules are separated by walls (barriers) with adjustable permeability are ideal. The Layer-by-Layer (LbL) assembly method has been used to fabricate different reservoir/barrier architectures containing biomolecules. In this work, two-dimensional, single-component reservoirs for acidic and basic fibroblast growth factors (aFGFs and bFGFs, respectively) were fabricated by LbL assembly, and the effects of the reservoirs on 3T3 cells cultured under starvation conditions were studied.

Layer-by-Layer assembly is a highly versatile method for preparing multi-component coatings on different surfaces [2,3]. Efficient methods for fabricating multilayer films containing biomolecules for biomedical and biomaterial applications have been widely studied [4,5].

Fibroblast growth factors are polypeptide-signaling molecules with a molar mass ranging from 16 to 34 kDa. These molecules, which were discovered by Armelin in 1973, support cell growth and division [6]. FGFs are well known for their role in cell development [7], proliferation, organogenesis (see above), cell differentiation, cell migration [6,8,9], wound healing [10,11], and angiogenesis [12,13]. The release of sufficient amounts of FGFs can induce progenitor cell recruitment without the need for stem cell implantation [14].

Systems with FGFs incorporated into polyelectrolyte multilayers have been reported to mimic physiological conditions and to induce wound healing and tissue regeneration. FGF-containing multilayers have been assembled on flat [14,15–18] and spherical surfaces [19,20] and have also been incorporated into polyelectrolyte multilayers to improve the compatibility of biomaterials. However, FGFs are very labile and can lose their activity in less than one week when stored in solution at 2–8°C. Freeze-thaw cycles can also cause FGF inactivation. In this work, FGFs were incorporated into multilayers prepared by LbL assembly to improve their stability in long-term storage. Specifically, (heparin/chitosan)_n ((Hep/Chi)_n) multilayers with different architectures and FGF contents were used as growth factor reservoirs and delivery systems for cultured cells.

The model proteins employed in this study were acidic FGF (aFGF, isoelectric point pI = 5.6) and basic FGF (bFGF, pI = 9.6). It is known that aFGF can induce mitosis, cell migration, and cell differentiation in most mesodermal cells. It also influences angiogenesis and regulates many other biological responses [13,21]. bFGF belongs to a family of proteins that stimulate fibroblast proliferation [15] and activate angiogenesis, chemotaxis, and periodontal ligament proliferation [22].

Heparin is a polysulfated glycosaminoglycan with a high poly-dispersity and large variations in its saccharide monomer sequence. It has the highest negative charge density of any known biological molecule. The assembly of heparin molecules with FGFs can induce conformational changes in the FGFs, thereby improving their resistance to thermal and enzymatic denaturation; for example, the inactivation of bFGF is reduced at acidic pHs [23]. Chitosan is a naturally derived polycation that has been extensively studied for its numerous positive biological properties [24]. It is positively charged at low pH and can be solubilized in aqueous

media at pH 4.5 or lower. It has been categorized as “generally recognized as safe” (GRAS) by the U.S. Food and Drug Administration.

2. Experimental

2.1. Materials

Poly(ethylene imine) (PEI, $\bar{M}_w = 25.000$ g/mol, Lupasol, BASF), chitosan (low molecular weight, $\bar{M}_w = \sim 35000$ g/mol, Sigma), and heparin sodium salt from porcine intestinal mucosa (Sigma, referred as heparin throughout the text) were used as polyelectrolytes. NaCl suitable for cell culture, mouse aFGF and bFGF, Dulbecco’s modified Eagle’s medium (DMEM), new-born calf serum (NCS), a 0.25% trypsin-EDTA solution, Dulbecco’s phosphate-buffered saline (PBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), penicillin (10000 units/mL), and streptomycin (10 mg/mL) were purchased from Sigma-Aldrich. NIH 3T3 cells (American Type Culture Collection, ATCC®CRL-1658TM) were provided by IGBMC (Institute de Génétique et de Biologie Moléculaire et Cellulaire, Strasbourg, France). Complete culture medium (CCM, DMEM supplemented with 10% NCS, 0.5% penicillin, and 0.5% streptomycin) was employed to subculture the cells, and low serum culture medium (LSCM, DMEM supplemented with 2.5% NCS, 0.5% penicillin, and 0.5% streptomycin) was used in the experiments with the LbL reservoirs. Cell culture plates and flasks (Costar) were used as received. Ultrapure Milli-Q water (specific resistivity ~ 18.2 M Ω ·cm at 25°C) prepared using a water purification system (Milli-Q Gradient, Millipore) was used for dialysis and for preparing aqueous solutions unless otherwise noted.

All materials, except PEI and chitosan, were used as received. PEI was dialyzed against ultrapure water using a semi-permeable membrane (Carl Roth GmbH, MWCO 14 kDa) to remove short chains and other possible contaminants. After dialysis (pH 7.0), the solution was lyophilized to obtain dry PEI for further use. Chitosan was solubilized in 2% (w/v) acetic acid, precipitated in 1 M NaOH, and dialyzed against Milli-Q water to pH 7.0. Then, the purified chitosan was lyophilized and repurified.

The LbL assembly was performed on either silicon wafers (Si wafers (100); WaferNet, Inc.; product code C-80830) with a native SiO₂ layer or on glass coverslips (15 mm in diameter, 0.13–1.16 mm in thickness, Menzel-Gläser) for the adherent cell cultures. Both substrates were cleaned with ethanol, extensively rinsed with Milli-Q water in an ultrasonic bath, and dried under compressed air. Prior to film deposition, the substrates were treated with O₂ plasma (Plasmaflo Gas Flow Mixer PDC-FMG, Harrick Plasma) to activate the surface (5 min, medium power), and the silicon wafers were characterized by ellipsometry.

2.2. LbL assembly and stability

PEI (0.25 mg/mL) was prepared in Milli-Q water. Heparin and chitosan solutions (0.1 mg/mL) were prepared using 0.15 mol/L NaCl (physiological conditions). Acetic acid (0.2%) was added to the chitosan solution. Co-solutions of aFGF (0.1 or 1 μ g/mL) and heparin and of bFGF (0.1 or 1 μ g/mL) and chitosan were subsequently prepared. The (Hep/Chi)_n reservoirs were assembled by dipping the substrates (silicon wafers or glass slips) alternately into the two polyelectrolyte solutions for 10 min and then rinsing them with Milli-Q water for 1 min 3 times after each immersion. The first layer was PEI, which was used to improve the (Hep/Chi)_n adhesion on the substrates. Using pure polyelectrolyte solutions and the co-solutions, a series of LbL reservoirs containing either aFGF or bFGF were assembled with 3 and 6 layer pairs (3 LP and 6 LP) and stored at 4°C. The FGF reservoirs and the bare (Hep/Chi)_n multilayers are

denoted by their respective codes presented in Table 1. Stability tests were performed by incubating the samples in 0.15 mol/L NaCl for 1, 7, 10, and 14 days at 37°C. The thickness loss was monitored by ellipsometry.

Table 1. Codes for LbL reservoirs used in cell culture assays. Thickness values and swelling by LSCM determined from QCM-D measurements (n.d. means not determined).

Reservoir architecture	Code	Thickness (nm)	Loaded DMEM (ng/cm ²)
PEI(Hep/Chi) ₃ Hep	Hep/Chi-3 LP	2.2	n.d.
PEI(Hep/Chi) ₆ Hep	Hep/Chi-6 LP	4.8	200
PEI(Hep-aFGF0.1/Chi) ₃ Hep-aFGF0.1	aFGF0.1-3 LP	2.7	n.d.
PEI(Hep-aFGF0.1/Chi) ₆ Hep-aFGF0.1	aFGF0.1-6 LP	5.7	361
PEI(Hep-aFGF1/Chi) ₃ Hep-aFGF1	aFGF1-3 LP	n.d.	n.d.
PEI(Hep-aFGF1/Chi) ₆ Hep-aFGF1	aFGF1-6 LP	n.d.	n.d.
PEI(Hep/Chi-bFGF0.1) ₃ Hep	bFGF0.1-3 LP	2.4	n.d.
PEI(Hep/Chi-bFGF0.1) ₆ Hep	bFGF0.1-6 LP	5.0	293
PEI(Hep/Chi-bFGF1) ₃ Hep	bFGF1-3 LP	2.3	n.d.
PEI(Hep/Chi-bFGF1) ₆ Hep	bFGF1-6 LP	5.1	346

2.3. Characterization of the multilayers

The LbL deposition on the silicon wafers was monitored by ellipsometry (PLASMOS SD 2300) using a 632.8 nm laser (He-Ne laser) at an incidence angle of 70°. Due to the small thickness of the LbL reservoirs, their refractive index was assumed to be constant at $n_d = 1.465$, and despite their slightly heterogeneous morphology, they were assumed to be uniform, isotropic films. Although the absolute film thicknesses obtained using these assumptions have small errors, these assumptions allow the relative film thicknesses to be quickly and precisely determined. Ten measurements were performed for each dry sample. Ellipsometry measurements repeated six months after the LbL deposition showed that the thicknesses did not change over time. Each type of LbL assembly described in Table 1 was fabricated at least 10 times. The surface morphologies of the dry samples were examined by atomic force microscopy (AFM, multimode atomic force microscope, Veeco Metrology, USA), and the images were analyzed using the NanoScope Analysis 1.4 software (Bruker Corporation).

The amount of material adsorbed (Γ) was monitored using a Q-Sense dissipation-enhanced quartz crystal microbalance (QCM-D, Biolin Scientific). The gold substrate surface was activated by UV plasma (UV/Ozone ProCleaner, BioForce Nanosciences), and the LbL assembly was performed at 22°C using the protocol described for dipping (Section 2.2), except that the samples were rinsed with 0.15 mol/L NaCl to avoid noise in the measurements due to changes

in the solution viscosity. Stability tests were performed by heating 6 LP reservoirs at 37°C for 16 h and then decreasing the temperature to 22°C. The samples were subsequently rinsed to remove weakly bound molecules. The temperature was then increased to 37°C, and LSCM (700 µL) was injected into the reservoirs. The samples were maintained under these conditions for 12 h and subsequently rinsed. Then, the temperature was decreased to 22°C, and the amount of LSCM absorbed was quantified for each system. For uniform, rigid films, the resonance frequency shifts (Δf) can be related to changes in the mass acoustically coupled to the sensor surface (Δm) using the Sauerbrey equation [25],

$$\Delta m = \frac{-(C \times \Delta f)}{n} \quad (1)$$

where n ($n = 1, 3, 5, \dots, 13$) is the overtone number and C is the mass sensitivity constant of the quartz crystal (typically $C = 17.7 \text{ ng}/(\text{cm}^2 \text{ Hz})$ for $f_0 = 5 \text{ MHz}$).

2.4. Release assays

The reservoirs and multilayers (deposited on the glass coverslips) were placed in 24-well plates and incubated with 500 µL of PBS for 1, 7, 10, and 14 days (37°C, 5% CO₂, controlled humidity). The amount of bFGF released from the LbL systems was estimated using a bFGF mouse enzyme-linked immunosorbent assay kit (ELISA kit, Abcam, ab100670) following the manufacturer's protocol. *In vitro* release studies were performed in triplicate.

2.5. Biological investigations

2.5.1. Cell culture assays

NIH 3T3 mouse fibroblasts were incubated with CCM in 75 cm² tissue culture flasks under normal culture conditions (37°C, 5% CO₂, controlled humidity). For *in vitro* experiments with the multilayers, the cells were dispersed in LSCM. The cell suspensions were plated (10⁴ cells/well) on the samples (the LbL deposits on glass coverslips described in Table 1) placed inside the 24-well plates with 1 mL of LSCM and incubated for 1, 7, 10, and 14 days. The LSCM was replenished every 2 days, and MTT assays were performed to evaluate cell proliferation. For the MTT assays, the samples were gently removed from the well plates and placed in new well plates. The cells adhered to the samples were incubated with a 1 mg/mL MTT solution for 3 h. Then, the culture medium was removed, and the formazan crystals were dissolved in 1 mL of isopropanol. The absorbance was measured at 570 nm (Xenius spectrophotometer, SAFAS Monaco), and the cell proliferation was determined using a calibration curve. The cell morphology was examined using a phase contrast microscope (Nikon Eclipse TS 100) equipped with a SPOT Insight camera for image acquisition (SPOT Software, Diagnostics Instruments). The cell growth curves showed that the LSCM prevented overgrowth, enabling evaluation of the NIH 3T3 cell proliferation over longer culture periods. Thus, the LSCM (DMEM supplemented with 2.5% serum instead of 10% serum) was used for cultures of samples with FGFs to limit the nutrition supplied to the cells, thereby allowing cell proliferation to be assessed for 14 days. *In vitro* cell proliferation assays and cell growth curves were performed in triplicate.

2.5.2. Cytotoxicity assays

The cytotoxicity assays were performed following ISO 10933-5:2009 (Biological evaluation of medical devices—Part 5: Tests for *in vitro* cytotoxicity). The NIH/3T3 cells were plated (10^4 cells/well) in 24-well plates and incubated with 500 μ L of CCM. After 24 h and before 100% confluence occurred, the LbL samples on the glass coverslips were gently added to the well plates, which were then incubated for 24 h. The cytotoxicity effects were evaluated by MTT assays and optical microscopy.

2.5.3. Statistical analysis

The data are expressed as the mean \pm standard deviation. Statistical analysis was performed using the two-population Student's *t*-test. The significance level was $p < 0.05$.

3. Results and discussion

3.1. Assembly of the reservoirs

aFGF and bFGF reservoirs were assembled using (Hep/Chi)_n multilayers for *in vitro* release studies. Very small amounts of these biomolecules were used in the LbL assembly process because only nanogram quantities are needed to stimulate biological responses ($ED_{50} < 0.5$ ng/mL). Previous studies showed that depositing a PEI layer first improves the subsequent polyelectrolyte multilayer assembly and makes the adsorption process more homogeneous [26]. Furthermore, PEI promotes adhesion, making it suitable for fabricating multilayers on surfaces with various chemical characteristics. Although PEI is a cytotoxic polycation, its toxicity decreases considerably when it adsorbs to surfaces; therefore, it can be safely used as a “primer” layer for improving the adhesion and homogeneity of LbL films assembled on the PEI layer. Therefore, a “primer” PEI layer was used in this work. The (Hep/Chi)_n multilayers were assembled from their respective polyelectrolyte solutions (0.1 mg/mL) to increase the mass ratio of polyelectrolyte to FGF. The total thickness of the (Hep/Chi)_n multilayers increased linearly to 7.2 ± 0.5 nm at 6 layer pairs as the number of deposited layers increased (Fig. 1a). The thickness profiles of the (Hep/Chi)_n multilayers assembled from 0.1 mg/mL solutions were similar to those of PEI(Hep/Chi)₆Hep prepared from 1 mg/mL solutions, but the final layers prepared from the diluted solutions were slightly thinner (~ 1 nm), which means less homogeneity in surface coverage. The decrease in the polyelectrolyte concentration by a factor of 10 did not affect the (Hep/Chi)_n assembly. Thus, 0.1 mg/mL Hep and Chi solutions were used to prepare co-solutions with aFGF and bFGF, respectively.

To study the effectiveness of embedding FGFs in LbL films, the FGF concentrations on the polyelectrolyte co-solutions used to fabricate the (Hep/Chi)_n reservoirs were only 0.1 and 1 μ g/mL. As expected, the ellipsometry measurements revealed that the film thicknesses were the same whether FGFs were present in the deposition solutions or not. Therefore, the amounts of FGFs embedded in the multilayer films were only estimated qualitatively based on the biological activities observed after seeding the NIH/3T3 fibroblasts. In general, (Hep/Chi)_n reservoirs composed of 3 LPs were approximately 3 nm thick, and those with 6 LPs were approximately 6 nm thick regardless if they contained FGFs or not. It should be noted that these values corresponded to the thicknesses of the (Hep/Chi)_n reservoirs assembled on PEI primer layers. In all cases, the last layer of the 3 and 6 LP samples was either Hep or Hep-aFGF because the cell culture assays revealed that cell proliferation was higher when the top layer was negatively charged. Previous work also revealed that NIH/3T3 fibroblasts are attracted to weakly negative surfaces, although cell membranes have negative surfaces [27].

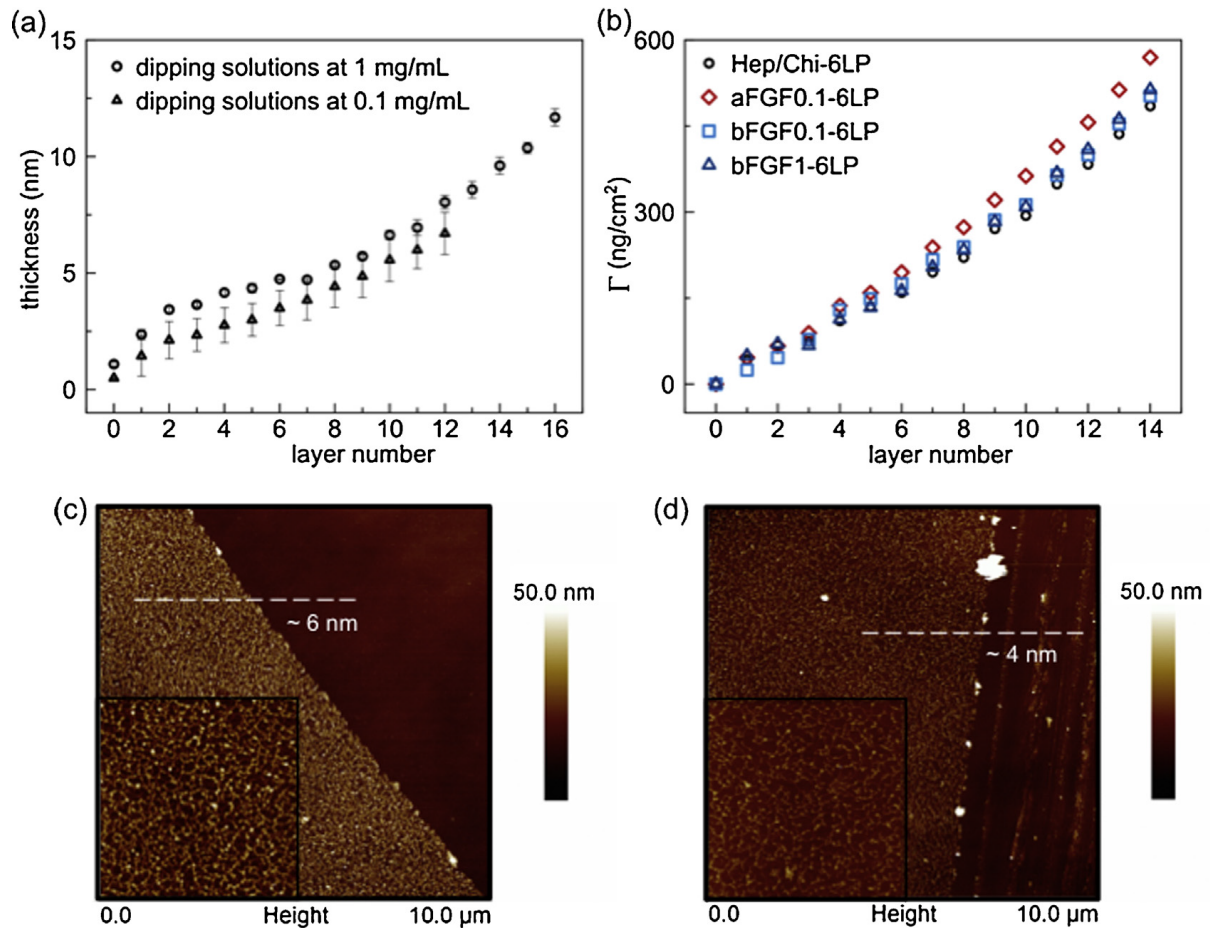


Fig. 1. Thickness increment for $(\text{Hep}/\text{Chi})_n$ multilayers from dipping solutions at 1 or 0.1 mg/mL (a); amount of adsorbed material (Γ) for Hep/Chi-6 LP reservoirs containing aFGF or bFGF assembled from co-solutions with FGF at 0.1 or 1 $\mu\text{g}/\text{mL}$ (b); AFM images and cross section analyses for bFGF reservoirs with 6 LP (c) or 3 LP (d) assembled from Hep 0.1 mg/mL and co-solution Chi 0.1 mg/mL-bFGF 1 $\mu\text{g}/\text{mL}$; insets: AFM images magnification up to $(5 \mu\text{m})^2$ for bFGF reservoirs with 6 LP (c) or 3 LP (d).

The assembly of the bare $(\text{Hep}/\text{Chi})_n$ multilayers or FGF reservoirs from their respective solutions or co-solutions was also monitored by QCM-D. The amount of material adsorbed (Γ) for the bare $(\text{Hep}/\text{Chi})_n$ multilayers and for the aFGF or bFGF reservoirs is shown in Fig. 1b. The increase in Γ with increasing number of layers deposited indicated that both $(\text{Hep}/\text{Chi})_n$ multilayers and FGFs reservoirs were assembled. The Γ values of the FGF reservoirs were slightly higher than those of the bare $(\text{Hep}/\text{Chi})_n$ multilayers, possibly due to changes in the hydrogel network. The thicknesses were estimated from the QCM-D mass measurements assuming that the reservoir density was $1 \text{ g}/\text{cm}^3$ (Table 1). The estimated values are consistent with the ellipsometry data and confirmed that the structure of the (Hep/Chi) networks did not change upon addition of the FGFs. In fact, the deposited reservoirs exhibited viscoelastic hydrogel behavior and might have rearranged to accommodate the FGFs for their storage. Although the aFGF and bFGF concentrations in the dipping co-solutions were the same (0.1 $\mu\text{g}/\text{mL}$), more material was embedded in the aFGF reservoirs than in the bFGF reservoirs, probably due to favorable interactions between Hep and aFGF in their dipping co-solution. It is well known that both aFGF and bFGF have binding sites for heparin residues [23]. However, binding interactions between chitosan and bFGF might not occur in their co-solution. Another interesting process is the swelling of the reservoirs by LSCM (Table 1, Fig. A1 in Appendix A). The bare $(\text{Hep}/\text{Chi})_n$ multilayers absorbed approximately 200 ng/cm^2 LSCM,

whereas the FGF reservoirs absorbed at least 50% more of the loaded culture medium. The higher uptake of the LSCM components by the FGF reservoirs might contribute to the enhanced cell proliferation because they are inherently bioactive.

AFM measurements showed that the bFGF1-6 LP reservoirs (Fig. 1c) were slightly rougher than the bFGF1-3 LP reservoirs (Fig. 1d); the roughness mean square (r.m.s) value of the 6 LP reservoirs (r.m.s. 7.3 nm) was twice as high as that of the 3 LP reservoirs (r.m.s. 3.5 nm). Cross-sectional analysis results obtained from trenches cut in the reservoirs are consistent with the thicknesses determined by ellipsometry. Specifically, the thicknesses of the 6LP (inset Fig. 1c) and 3 LP (inset Fig. 1d) reservoirs were 6 and 4 nm, respectively. Based on all the data from the different characterization methods it was concluded that, macroscopically, the (Hep/Chi)_n reservoirs were quasi-continuous films with a submicron structure similar to that of a fibrillar network. The network structure became denser and thicker as the number of deposited layer pairs increased. Thus, the reservoirs can be considered to be thin Hep/Chi complex deposits embedded with FGFs prepared by LbL assembly.

3.2. Reservoir stability and release

The stability of the FGFs reservoirs over 14 days was studied under physiological conditions (0.15 mol/L NaCl, pH 7.4) at 37°C to mimic cell culture environments. Ellipsometry measurements showed that the bare (Hep/Chi)_n multilayers lost approximately 30% of their thickness after 14 days of incubation regardless of the number of LP (Fig. 2). Compared to the bare (Hep/Chi)_n multilayers, the aFGF and bFGF reservoirs exhibited interesting stability characteristics. Although the 3 LP reservoirs (Figs. 2a) were less stable than bare (Hep/Chi)_n multilayers, losing approximately 60% of their thickness, the 6 LP reservoirs (Figs. 2b) were more stable (thickness loss of approximately 25%), even after 14 days. The thicknesses of both bare (Hep/Chi)_n multilayers and FGF reservoirs decreased during the first 7 days of incubation and then plateaued after 10 days. It should be noted that the FGF concentration of the dipping co-solution (0.1 or 1 µg/mL) did not appear to influence the stability or thickness of the reservoirs.

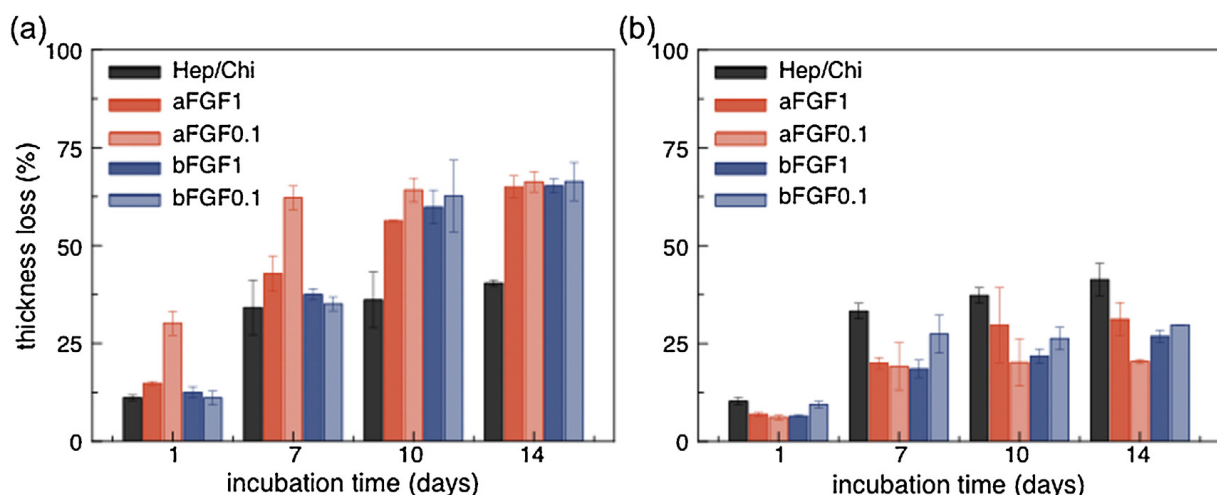


Fig. 2. Stability for FGF reservoirs with 3LP (a) and 6 LP (b) during 14 days compared to respective bare multilayers (Hep/Chi-3 LP and Hep/Chi-6 LP).

For practical reasons, the observed thickness changes were used to describe the stability of the reservoirs. Unfortunately, it is impossible to precisely distinguish between swelling/deswelling, polyelectrolyte desorption and morphological changes in

inhomogeneous thin films. However, further QCM-D investigations (Appendix A, Fig. A1) revealed a small change in the resonance frequency when the temperature was increased to 37°C and then decreased back to 22°C, suggesting that the increase in the thermal energy produces a mass loss, which disturbs the hydrogel network (Appendix A, Fig. A1, events 2 and 3). Further experiments performed at 37°C with 0.15 mol/L NaCl for 16 h indicated that reservoirs were quite stable and that adsorbed components did not desorb within the first day. These QCM-D data suggested that the thickness losses observed during the first day of release were mostly due to structural rearrangements combined with a small mass loss due to the temperature changes.

The cumulative release profiles for the bFGF reservoirs presented in Fig. 3 showed that the 3 LP reservoirs released more bFGF than the 6 LP reservoirs. Furthermore, the release rates are consistent with the stability of the layers as monitored by ellipsometry. In general, a small release was observed initially, and a burst release occurred after 7 days for reservoirs prepared with a small bFGF concentration. Under the same conditions, the burst release occurred after 10 days for reservoirs prepared with a higher bFGF concentration. The reservoirs assembled from 0.1 µg/mL bFGF co-solutions released more bFGF than those fabricated from 1 µg/mL bFGF co-solutions. No release was observed for the bare (Hep/Chi)_n multilayers, thus demonstrating the robustness of our fabrication process.

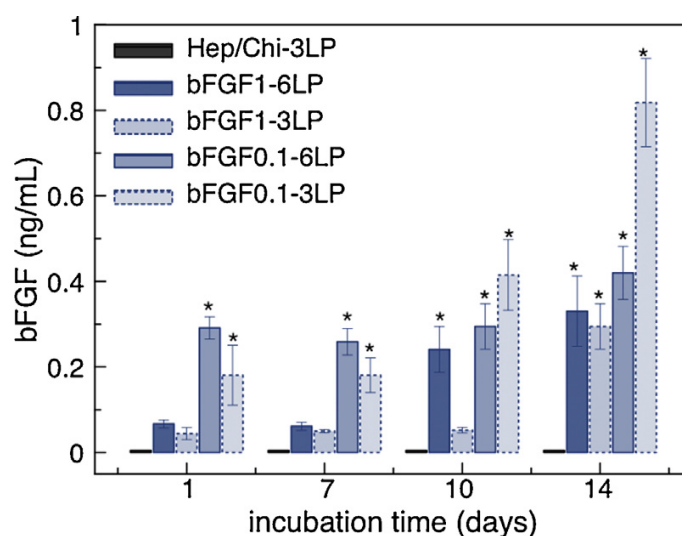


Fig. 3. Release profiles for bFGF reservoirs assembled with 3LP or 6 LP and from co-solutions with bFGF at 0.1 or 1 µg/mL.*Statistics analyses for t-test ($p < 0.01$) compared to bare Hep/Chi-3 LP multilayers.

3.3. Cell proliferation for the aFGF and bFGF reservoirs

The cell culture conditions were initially adapted because the complete culture medium (10% NCS in DMEM) produced overgrowth in a short period of time (7 days), preventing appropriate evaluation of the effects of the FGFs on cell proliferation. Based on the cell growth curves, new cell culture conditions were determined for longer incubation times (14 days). Specifically, a culture medium supplemented with a smaller amount of serum (2.5% NCS, results not shown), which slowed cell proliferation, was employed. This strategy is commonly used to study cell adhesion and proliferation to evaluate biological responses to FGFs coated on surfaces or embedded in biomaterials. In previous research, a culture medium supplemented with 0.5 or 1% serum was employed to evaluate the effects of FGF-2 (bFGF) inserted into polyelectrolyte multilayers [28,29]. The cell culture assays performed with both

aFGF and bFGF reservoirs revealed interesting properties of the different assembled architectures. Over 14 days, the amount of cells plated on the bare (Hep/Chi)_n multilayers did not change for the 6-LP multi-layers and increased only slightly for the 3-LP multilayers (Fig. 4). These results indicated that although the cells adhered to the bare (Hep/Chi)_n multilayers and survived for 14 days, no significant proliferation occurred. The addition of aFGF and bFGF to the multilayer assemblies enhanced cell proliferation for all the systems listed in Table 1. Intriguingly, cell proliferation was higher when the 3 LP reservoirs (Fig. 4a) were used than when the 6 LP reservoirs (Fig. 4b) were employed.

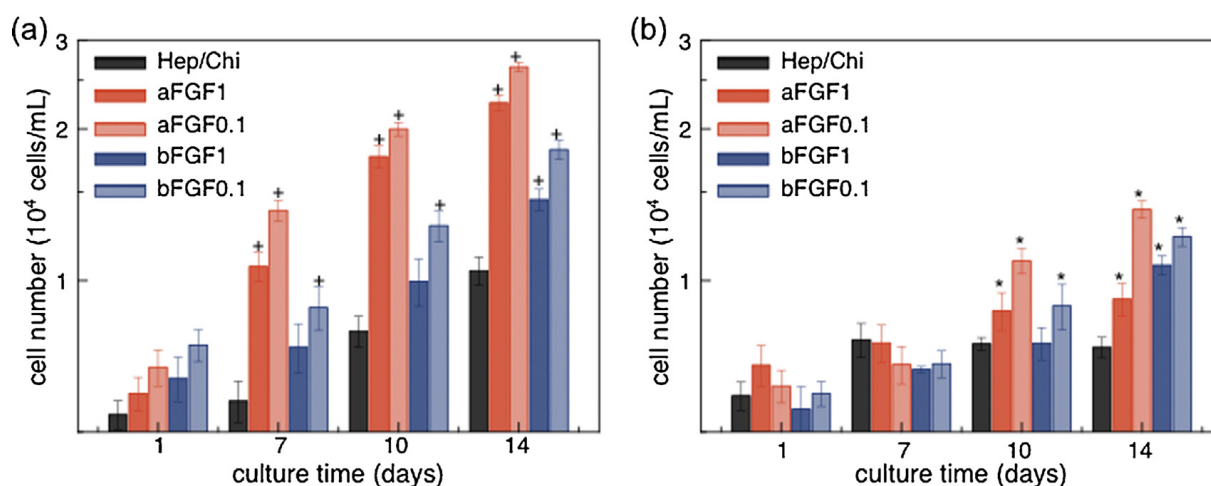


Fig. 4. Proliferation of NIH/3T3 fibroblasts (under starving culture conditions) on FGF reservoirs with 3LP (a) or 6LP (b). Statistics analyses for *t*-test ($p < 0.05$) compared to bare Hep/Chi-3 LP (+) or Hep/Chi-6 LP (*) multilayers.

Enhanced cell proliferation was clearly observed after 7 days for the 3 LP reservoirs, whereas cell proliferation did not improve until 10 days of incubation for the 6 LP reservoirs. For the 3 LP FGF reservoirs, cell concentrations of approximately 2.5 and 1.5 × 10⁴ cells/mL were obtained for aFGF and bFGF, respectively, whereas a cell concentration of less than 1.5 × 10⁴ cells/mL was obtained with the 6 LP FGF reservoirs. These results can be explained by the stability tests: because the 3 LP reservoirs were less stable than the 6 LP reservoirs, they probably released higher amounts of FGFs. Furthermore, roughness results from AFM measurements indicated that 6 LP reservoirs (r.m.s. 7.3 nm) were twice as rough as the 3 LP reservoirs (r.m.s. 3.5 nm). During the first day of proliferation, the cell adhesion to all the surfaces was the same within the experimental error. Therefore, the rougher 6 LP reservoir surfaces might have led to a decrease in cell proliferation. From Fig. 4, it can be inferred that the NIH/3T3 fibroblasts exhibited higher cell proliferation on the FGF reservoirs assembled from the 0.1 μg/mL FGF co-solutions than on the FGF reservoirs fabricated using the 1 μg/mL FGF co-solutions. The release profiles (Fig. 3) showed that the 6 LP reservoirs released less bFGF, which is consistent with these results. Higher cell proliferation was observed for the 3 LP aFGF reservoirs than for the bFGF reservoirs. These findings can be explained by the fact that the last layer in the aFGF reservoirs was composed of a Hep-aFGF mixture, whereas that in the bFGF reservoirs consisted of only heparin (Table 1). Furthermore, because heparin binds to FGFs and improves their stability [23], the amount of FGF loaded in the reservoirs was likely higher in the aFGF reservoirs due to favorable intermolecular interactions in the dipping co-solution. This explanation is consistent with the QCM-D results.

Morphological analyses of the NIH/3T3 fibroblasts during the cell proliferation assays (Appendix A, Supplementary data, Figs.A3 and A4) are consistent with the MTT assay results. Optical microscopy (OM) images clearly showed that the cells attached to the bare (Hep/Chi)_n multilayers, but they did not proliferate. The cells maintained their round shape and survived up to 14 days of incubation (Fig A3: a–d). The addition of aFGF (Fig A3: e–p) or bFGF (Fig A4) to the reservoirs promoted cell adhesion and proliferation as shown by the spreading of the NIH/3T3 fibroblasts and change in their morphology to spindle shape within 7 days. The cell proliferation was higher on the 3-LP reservoirs than on the 6-LP reservoirs regardless of the character of the FGF molecules (*i.e.*, acidic or basic). After 10 days, a cobblestone-like morphology was observed for cells seeded on 3 LP aFGF reservoirs (Fig A3: k–p). For the bFGF reservoirs, the cell proliferation on the 3 LP reservoirs was also higher than that on the 6 LP reservoirs; however, the cells did not reach 100% confluence and appeared spindle-like (Fig A4: g, h, o, p). These results indicated that the NIH/3T3 fibroblasts achieved a higher confluence on the aFGF reservoirs and are consistent with the cell proliferation results obtained from the MTT assays. OM images of the proliferation assays with cells seeded on the aFGF reservoirs revealed that the cells preferred the reservoirs with a Hep-aFGF top layer (FigA3: m–p) over those with a Chi top layer (Fig A3: i–l), mainly due to differences in cell adhesion after 1 day (Fig A3: i, m).

For the cytotoxicity studies, the cells were cultured for 1 day. Then, the reservoirs and the bare (Hep/Chi)_n multilayer samples, which were previously adsorbed on glass coverslips, were gently placed on the spread cells sheet and were reincubated. After 1 day, the reduction in cell viability was determined via MTT assay, and the results are presented in Fig. 5. For comparison, glass slides used as controls displayed 82% of cell viability (results not shown). According to the standard method employed, a decrease of more than 30% in the cell viability is considered to be due to a cytotoxic effect. Except for the 6 LP bFGF reservoirs, all the reservoirs exhibited a lower cytotoxic effect than the bare (Hep/Chi)_n multilayers. The higher cytotoxicity of the 6 LP bFGF reservoirs can explain the lower NIH/3T3 cell proliferation rates observed when the fibroblasts were seeded on them.

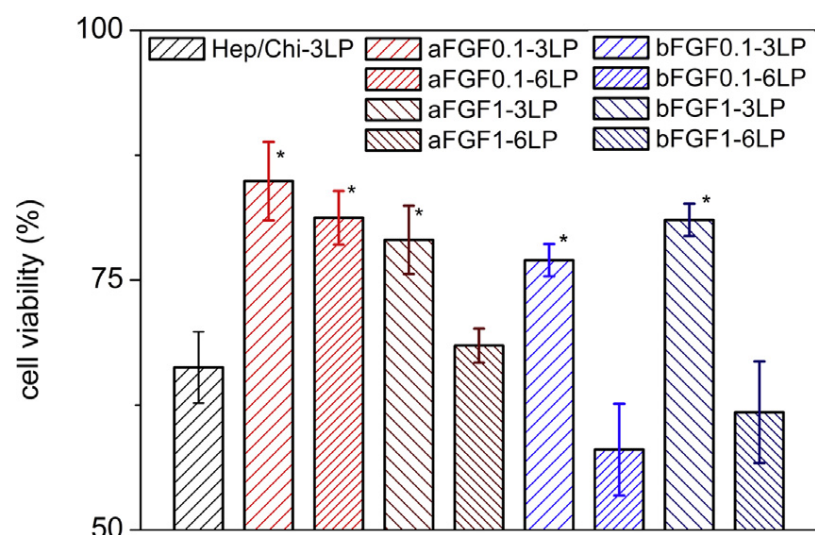


Fig. 5. Cytotoxicity studies for Hep/Chi multilayers containing aFGF or bFGF using NIH/3T3 fibroblasts under normal culture conditions. *Statistical analysis for t-test($p < 0.05$) compared to bare Hep/Chi-3 LP multilayers.

3.4. Long-term activity of aFGF and bFGF in the reservoirs

Cell proliferation assays for long-term evaluation of FGF reservoirs were performed under LSCM conditions. In most of the experiments, the co-solutions were prepared the day before assembling the FGF reservoirs and stored at 4°C. To study the stability of the aFGF and bFGF bioactivities in the co-solutions, the co-solutions were stored at 4°C for 30 days before assembling the multilayer systems. The enhanced cell proliferation observed on the samples assembled 30 days after the Hep-aFGF and Chi-bFGF co-solutions were prepared (Appendix A, Fig. A2) showed that the FGF activity was maintained in these solutions. The same trends were observed for both the reservoirs prepared from stored co-solutions and those prepared from freshly made co-solutions.

In another experiment, the long-term activity of the FGFs in the reservoirs was evaluated using LbL samples stored at 4°C. The results indicated that the (Hep/Chi)_n multilayers prevented the denaturation of both aFGF and bFGF over long periods of time. After 14 days of incubation, the cell proliferation on FGF reservoirs that were stored for 1, 2, and 9 months was higher than that on the bare (Hep/Chi)_n multilayers. In fact, no increase in cell proliferation was observed for the bare multilayers regardless the storage time (Fig. 6, results for 2 and 9 months not shown). Previous studies reported the use of (heparin/PEI)_n multilayers to store and preserve aFGF for 3 months at -20°C [13]. Other work described using electrospun chitosan fiber networks decorated with heparin-containing polyelectrolyte nanoparticles to store and release bFGF, which remained bioactive for 30 days [30]. In this work, the FGF activity was maintained for up to 9 months when stored in the FGF reservoirs assembled from (Hep/Chi)_n multilayers at 4°C. Therefore, these reservoirs could easily be used to coat biomaterials that require more effective wound healing processes. Very small amounts of FGF were used, and the deposition process was reproducible and easy to perform on surfaces with different chemical natures (hydrophilic or hydrophobic).

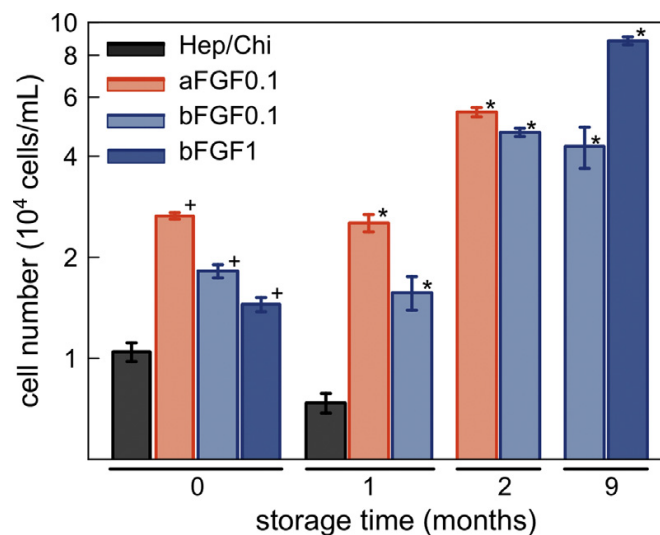


Fig. 6. Proliferation of NIH/3T3 fibroblasts (under starving culture conditions) on freshly prepared or stored aFGF or bFGF reservoirs (3 LP) to verify long-term activity. Statistical analysis for t-test ($p < 0.05$) compared to bare Hep/Chi-3 LP multilayers freshly prepared (+) or stored (*).

4. Conclusions

(Hep/Chi)_n multilayers were used to store aFGF and bFGF. The adsorption of the polyelectrolyte multilayers and FGF reservoirs was confirmed by ellipsometry and QCM-D measurements. The preparation of aFGF and bFGF reservoirs using polyelectrolyte-FGF co-

solutions with very low FGF concentrations is an attractive method for coating surfaces. Both the biomolecule load and release could be easily controlled by changing the FGF concentration or the number of layer pairs. NIH/3T3 fibroblasts were cultured on the aFGF and bFGF reservoirs in a low serum medium. Enhanced cell proliferation was only observed when the cells were seeded on the FGF reservoirs and not when they were seeded on the bare (Hep/Chi)_n multilayers. These reservoirs could also be used to store aFGF and bFGF at 4°C for long periods of time without loss of their bioactivity. Additionally, the reservoirs can be assembled on any surface to fabricate a coating that can deliver FGFs, thus making them very attractive for biomedical applications.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.colsurfb.2015.11.019>.

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