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

Alliny F. Naves[a,b,*,1], Marvin Motay[a,b], Rémi Mérindol[a,b], Christiane P. Davi[a,c], Olivier Felix[a], Luiz H. Catalani[d], Gero Decher[a,b,e,**]

[a] C.N.R.S., Institut Charles Sadron, 23 rue du Loess, F-67034 Strasbourg, France
[b] Université de Strasbourg, Faculté de Chimie, 1 rue Blaise Pascal, F-67008 Strasbourg, France
[c] Centro de Ciências Naturais e Humanas CCNH, Universidade Federal do ABC, Rua Santa Adélia, 166, 09210-170, Santo André, Brazil
[d] Departamento de Química Fundamental, Instituto de Química, Universidade de São Paulo, Av. Prof. Lineu Prestes, 748, CP 26077, 05513-970 São Paulo, Brazil
[e] International Center for Frontier Research in Chemistry, 8 allée Gaspard Monge, F-67083 Strasbourg, France

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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.

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,

* Corresponding author at: C.N.R.S., Institut Charles Sadron, 23 rue du Loess, F-67034 Strasbourg, France. Fax: +55 11 38 15 55 79.
** Corresponding author at: Université de Strasbourg, Faculté de Chimie, 1 rue Blaise Pascal, F-67008 Strasbourg, France. Fax: +33 03 88 41 40 99.
E-mail addresses: alliny@iq.usp.br (A.F. Naves), decher@ics-cnrs.unistra.fr (G. Decher).
[1] Present address: Departamento de Química Fundamental, Instituto de Química, Universidade de São Paulo, Av. Prof. Lineu Prestes, 748, CP 26077, 05513-970 São Paulo, Brazil.

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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 polydispersity 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, $\bar{M}_w$ = ~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), newborn 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-1658™) were provided by IGBMC (Institute de la Génétique et de la 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 MΩ 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.

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

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 SiO_2 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 O_2 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 μg/mL) and heparin and of bFGF (0.1 or 1 μg/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.