Effect of functionalization of multilayered polyelectrolyte films on motoneuron growth

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Abstract

We studied in vitro cell–substrate interaction of motoneurons with functionalized polyelectrolyte films. Thin polyelectrolyte films were built on glass by alternating polycations, poly(ethylene-imine) PEI, poly(L-lysine) PLL, or poly(allylamine hydrochloride) PAH, and polyanions, poly(sodium-4-styrenesulfonate) PSS or poly(L-glutamic acid) (PGA). These architectures were functionalized with Brain Derived Neurotrophic Factor (BDNF) or Semaphorin 3A (Sema3A). We used Optical Waveguide Lightmode Spectroscopy (OWLS) and Atomic Force Microscopy (AFM) to characterize the architectures. The viability of motoneurons was estimated by the acid phosphatase method, and morphometrical measures were performed to analyse the influence of different architectures on cell morphology. Motoneurons appeared to adhere and spread on all the architectures tested and preferentially on PSS ending films. The viability of motoneurons on polyelectrolyte multilayers was higher compared to polyelectrolyte monolayers. BDNF and Sema3A embedded in the films remained active and thereby create functionalized nanofilms. © 2004 Elsevier Ltd. All rights reserved.

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1. Introduction

In the field of tissue engineering, tissue regeneration and wound healing, a major challenge is the development of cellular microdevices assembling various molecular species into micron-scaled structures. While the selection of the appropriate biomolecules and the development of methods for their incorporation into such complex structures are important, other pertinent issues include the cell viability on the prepared surfaces, and the long-term stability and retention of biological activity of incorporated biomolecules. Surface topography and chemistry have been shown to direct cell–substrate interactions and cellular properties such as cell adhesion, cell–cell reactions and cytoskeletal organiza-

tion. Neurons are highly sensitive to the nature of the substrate whereas other cells such as glial cells more easily adhere on any kind of untreated surfaces. Polypeptides like polylysine or polyornithine have often been used [1,2], together with laminin and fibronectin to optimize neurons cultures [3,4].

Decher [5], developed a technique based on the alternate adsorption of positively and negatively charged polyelectrolytes to build thin films. This approach allows the build-up of films with variable thickness or roughness. The interactions of such multilayered architectures with living cells have been recently investigated [6–12]. Proteins can be embedded in the architecture of thin films [13–18], and keep a secondary structure close to their native form as well as their bioactivity [18,19].

In the present study, we used multilayered polyelectrolyte films built-up by layer-by-layer deposition, and investigated the reaction of motoneurons with these

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nanofilms with respect to the nature of the ending polyelectrolyte type and to the film functionalization either with a growth factor, Brain Derived Neurotrophic Factor (BDNF) [20,21], or a chemorepulsive protein, Semaphorin 3A (Sema3A) [22,23]. The films were characterized by means of Optical Waveguide Lightmode Spectroscopy (OWLS) and Atomic Force Microscopy (AFM). The viability of the motoneurons was estimated by the acid phosphatase method and measurements were completed by morphometrical analyses.

2. Materials and methods

2.1. Polyelectrolyte films preparation

The following polyelectrolytes were purchased from Sigma-Aldrich, Saint Quentin Fallavier, France and have been used for the build-up of multilayer films: poly(ethylene-imine) (PEI; MW 750×10^3), poly(sodium-4-styrenesulfonate) (PSS; MW 70×10^3), poly(allylamine hydrochloride) (PAH; MW 70×10^3), poly(L-glutamic acid) (PGA; MW 54.8×10^3) and the poly(L-lysine) (PLL; MW 23.4×10^3). Solutions of PEI, PSS, and PAH have been prepared to a concentration of 5 mg/ml in 0.15 mol/l NaCl, pH 6.5, while solutions of PGA and PLL were prepared to 1 mg/ml. Multilayer films have been prepared on glass coverslips (VWR International, Strasbourg, France) pretreated with 10 mmol/l SDS followed by 0.12 N HCl for 15 min at 100°C in 24 wells plates (Nunc, VWR) with 3 rinses of pure water after each treatment. 300 µl of each polyelectrolyte solution has been deposited successively on coverslips during 10 min, each deposition being followed by 3 rinses (5 min each) with 0.15 mol/l NaCl. The films might be stored at 4°C during 1 week.

2.2. Protein embedding

The source of Sema3A was human embryonic kidney 293 cells (HEK293 cells) (CRL 1573; American Type Culture Collection, Manassas, VA) stably transfected with an expression vector containing cDNA coding for Flag-His-Sema3A [24], (cell line 602.108), cultured in minimal essential medium containing 5000 U/ml penicillin, 5 mg/ml streptomycin, 200 mmol/l L-glutamine, 10% FCS, and 1 mg/ml G418 (Invitrogen, Cergy-Pontoise, France). Sema3A was purified using an anti-Flag M2 affinity gel (Sigma), and its protein concentration was determined using the Bradford method. For embedding in multilayer films, Sema3A was prepared at 500 µg/ml in 0.15 mol/l NaCl and assembled in films after a PSS layer deposition.

BDNF (Tebu, Le Perray-en-Yvelines, France) was prepared at 1 µg/ml in 0.15 mol/l NaCl and assembled according to the same conditions.

The stability of Sema3A and BDNF in the architectures was determined by western blot analysis using the anti-Flag M2 antibody (Kodak) as described previously [24], or monoclonal anti-BDNF antibody (Sigma) followed by anti-mouse antibody, respectively.

2.3. Optical waveguide lightmode spectroscopy

Optical waveguide lightmode spectroscopy (OWLS) is an optical technique that allows determination of the optical thickness and refractive index of an adlayer deposited on a Si0.8Ti0.2O2 waveguide. The technique has already been theoretically described [25] and experimentally applied to polyelectrolyte multilayers [26]. It allows the determination of the optical thickness and the refractive index of the adsorbed layers.

All experiments were performed on a home-built experimental setup [27] with a He–Ne laser using ASI 2400 waveguides made of Si0.8Ti0.2O2 (Artificial Sensing Instruments, Zrich, Switzerland). Once the cell was mounted, buffer was flushed through the cell at a constant flow rate (10 ml/h) with a syringe-pump.

The build-up of the polyelectrolyte multilayers was performed as follows. After the buffer flow was stopped, 100 µl of one of the polyelectrolyte or protein solution was manually injected in the cell through the injection port. The surface was then maintained in contact with the solution at rest. After 10 min of contact (sufficient to reach a constant optical signal), the polyelectrolyte solution was replaced by a solution of 0.15 mol/l NaCl which was injected in the cell under a constant flow rate of 10 ml/h. The film was then left 10 min in contact with the buffer. This was done alternatively with the polycation and the polyanion solution. These polycation/polyanion adsorption steps were repeated until the multilayer film was built and formed by the desired number of layers. For the evaluation of the adsorbed protein, the homogeneous and isotropic bilayer model was used [27,28]. Within this model, the film is approximated by two adjacent layers each being characterized by its thickness and its refractive index. The refractive index and the thickness of the first layer nearby the substrate, which corresponds to the polyelectrolyte multilayer, were fixed to the values found before protein adsorption, i.e. nA and dA , respectively. The refractive index and thickness of the protein layer (nB , dB) were determined by solving the guided mode equations. Mass data are calculated according to the De Feijter formula (De Feijter et al., 1978): Adsorbed mass = $(dn/dc) - 1 \times (nB - nC) \times dB$ with $(dn/dc) = 0.18 \text{ cm}^3/\text{g}$ and $nC = 1.3343$ which is the refractive index of the buffer.

2.4. Atomic force microscopy

Glass slides (VWR) were used and cleaned with 10 mmol/l SDS/0.1 N HCl and then extensively rinsed

with pure water and dried under nitrogen. After this cleaning procedure, the silica surface appears perfectly flat with a rough mean square (RMS) lower than 0.3 nm. The film was built up in Petri dishes and observed at a given stage of the construction. Each observation at a given step corresponded to a totally new build. For all observations, the samples were kept in 0.15 mol/l NaCl.

Atomic force images were obtained in contact mode with the Nanoscope IIIa from Digital Instruments (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). We always performed several scans over a given surface area. These scans produced reproducible images to exclude sample damage induced by the tip. 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.

Profilometric section analyses allow, beside RMS, to precisely determine locally at the outer part of the film well or crack depths. They are achieved by cross-sections of the images in the Y orientation, perpendicular to the scanning direction.

2.5. Mouse motoneurons cultures

Motoneurons cultures were performed as described by Martinou et al. [29], with few modifications. The spinal cords of E13 Swiss mouse embryos were dissected and incubated for 15 min at 37°C in 0.25% trypsin solution (Invitrogen). They were added with L-15 medium (Leibovitz medium) (Invitrogen), mechanically dissociated by several passages through the 21 gauge needle of a syringe, and, then centrifuged at $1000 \times g$ during 10 min. The pellet was re-suspended in L-15 medium and centrifuged without acceleration nor brake at 250 g during 10 min at room temperature on a cushion of bovine serum albumin (BSA) 3.5% (w/v) in L-15 medium solution. After the supernatant was discarded, the pellet was re-suspended in L-15 medium and layered over a cushion of Optiprep d: 1.32 g/ml (AbCys, Paris, France), and centrifuged without acceleration nor brake for 15 min at 650 g, at room temperature. The upper phase, containing the purified motoneurons, was collected, re-suspended in L-15 medium and centrifuged at 1000 g, during 10 min at room temperature. The cell pellet was finally suspended in a defined culture medium which consisted of a mixture (v/v) of Dulbecco's modified basal medium of Eagle (DMEM) and Ham F12 (Invitrogen) supplemented with 5 mg/ml insulin, 10 mg/ml human transferrin, 0.1 mM putrescine, 1 pM oestradiol, 20 nM progesterone, 300 ml of a solution of 175 mg/ml sodium selenite and glutamine (all products from Sigma). Cells were seeded with a density of 25×10^3 motoneurons/cm² in 24 wells plates, containing

precoated glass coverslips with different architectures. Cells were kept for 48 h at 37°C in a humidified air (95%) and CO₂ (5%) atmosphere. BDNF was added at a final concentration of 100 ng/ml to the culture medium before seeding or not if already embedded in the film. Identification and purity of motoneurons was assessed by immunolabelling with an anti-choline acetyl transferase (ChAT) antibody. In our experiments, more than 90% of cells were positive by ChAT immunostainings.

2.6. Viability measurement

The measurement of viability by the acid phosphatase method makes it possible to quantify the number of alive cells with a greater sensitivity than the XTT test [30]. It consists in the titration of the enzyme whose activity increases proportionally with the number of viable cells.

In our study, the culture medium was eliminated and each well washed with 300 ml PBS. Then 400 ml of buffer containing 0.1 mol/l sodium acetate (pH 5.5), 0.1% of X-100 Triton and 10 mmol/l of *p*-nitrophenyl phosphate [pNPP] (Sigma) was added in each well. The plates were placed during 2–3 h at 37°C in an incubator with air (95%)–CO₂ (5%) suitably humidified. The reaction was stopped by addition of 40 ml of 1 N sodium hydroxide and the absorbance of the yellow colored solution was measured at 405 nm using a spectrophotometer (LabSystems iEMS Reader MF, Gibco).

The preparations to be observed were fixed during 20 min with formaldehyde 4% in PBS, rinsed with PBS and permeabilized during 30 min by means of a solution containing 0.1% (v/v) X-100 Triton (Sigma) and 3% of BSA. They were then incubated overnight at 4°C with a mouse monoclonal anti-beta tubulin antibody diluted to 1/1000, (Sigma). After 3 rinses of 5 min with PBS, they were incubated during 1 h at room temperature in the presence of a goat anti-mouse antibody labeled with Cy³ (Interbiotech, Montluon, France) diluted to 1/1600 in PBS.

2.7. Morphometrical analysis

Image capture of the neurons was performed with a digital camera (DXM-1200, Nikon). Image visualization and processing were made with the software package Visilog Advanced 5.4 providing all classical image processing tools.

Image analysis procedure: each neuron was extracted automatically by a binary threshold, transforming the gray level image into a binary image. A manual segmentation was used in order to separate the cell body from the neurites. The skeleton of each neurite was determined. A series of four morphometrical parameters was determined for each neuron:

Number of primary neurites: for each cell, the number of neurites coming directly from the cell body was determined.

Neuritic arborization: from the skeleton of each neurite, the triple points corresponding to arborization knots were found using a hit-or-miss transformation [31]. The neuritic arborization corresponding to the number of arborization knots was then determined by counting all the triple points of each cell.

Neuritic length: a chain coding procedure was applied, transforming the skeleton of each neurite into a succession of segments [31]. The length of each segment was calculated using a least-squares approximation. The neurite length corresponding to the sum of the lengths of the skeleton segments was then automatically determined. For each cell, only the longest neurite was retained for further analyses.

Area of the cell body: from the image of the cell body, the area of each cell body was automatically calculated.

2.8. Statistical analysis

The statistical analysis was performed using SPSS 8.0 statistical package (SAS Institute, Cary, NC). Among the four morphometrical parameters studied, the number of primary neurites and the neuritic arborization were assimilated to discrete variables although the neuritic length and the cell body area were considered as continuous variables. For each investigated architecture, the median and the range of variation (Minimum–Maximum) were determined for the two discrete variables. Summary statistics of the neuritic length and of the cell body area (mean, standard deviation) were also performed. Pairwise between-group comparisons of the four morphometrical parameters were then performed by Mann–Whitney tests [32] to examine differences between the different architectures.

3. Results and discussion

3.1. Multilayered polyelectrolyte films build-up

The build-up of multilayered films arises from electrostatic interactions between alternately deposited polyanions and polycations. In each step, either a polyanion or a polycation layer is added to the film. The number of cycles, the type of polyelectrolytes and the physico-chemical characteristics (salt concentration, pH,...) modulate the thickness and roughness of the multilayered film [33,34].

In our experiments, the solid surface was glass coverslips made of negatively charged silica. We started the build-up with PEI, a highly positively charged polycation. The construction was pursued with the deposition of two pairs of PSS and PAH layers, i.e (PSS/

PAH)₂, which were previously shown to coat homogeneously the surface of the substrate [26]. We ended the film by adding at least two pairs of PGA and PLL layers (PGA/PLL)₂. The build up of the architectures was followed step-by-step using Optical Waveguide Lightmode Spectrometry (Fig. 1a). Typically, a PEI/(PSS/PAH)₂/(PGA/PLL)₄ structure had a thickness of around 45 nm.

A total coating of the glass coverslip surface was obtained (Fig. 1b). The adhesion of the multilayers and the integrity of the architectures was tested by scratching the surface of the film. Only hard material such as metal, was able to completely remove the film. No peeling could be achieved showing the strong adhesion of the film to the substrate.

This observation is in agreement with other data from the literature [35] indicating that such films adhere on many different types of support such as glass [36], metals [37,38], non charged polymers [39,40] and on silicone [41].

Fig. 1. Characterization of a polyelectrolyte multilayer film. (a) Evolution of the thickness of a multilayer film followed by Optical Waveguide Lightmode Spectrometry. Each polyelectrolyte deposition increases the overall thickness of the structure to reach 45 nm after the adsorption of 6 layer pairs. (b) Top view of a scratched multilayer film with a final layer of PLL^{FITC}. Scratching was performed to test the integrity of the structure. Bar: 10 µm.

3.2. Neuronal cultures on mono- or multilayers

To compare neuronal viability on different architectures, we performed acid phosphatase activity tests. The tests were achieved on cells grown either on PLL (taken as the 100% reference) or PAH monolayers or on films constituted by 5 pairs of layers either ending by PLL or PAH. As already indicated, the use of cationic monolayers was inherent to the negative charge of the glass surface. Compared to the cell viability on the monolayers, viability was increased on multilayered films (Fig. 2 a, b). This increase was more than 95% for PLL (PLL monolayer $100\% \pm 0.00218$; PEI/(PSS/PAH)₂(PGA/PLL)₃ multilayers $196\% \pm 0.00272$) and by more than 190% on multilayers ending by PAH (PAH monolayer $100\% \pm 0.00057$; PEI/(PGA/PLL)₂(PSS/PAH)₃ multilayers $291\% \pm 0.0037$).

To compare a surface coated by a PLL monolayer or by a PEI/(PSS/PAH)₂(PGA/PLL)₃ multilayer film, we used Atomic Force Microscopy. This technique allows to map the surface layer in a liquid environment, without any dehydration step. In the case of PLL monolayer adsorption, we found that the polyelectrolyte layer directly deposited on the top of the glass coverslip was extremely sparse (Fig. 2c) and that large surface domains remained free of any polycation deposition. A section analysis of the surface demonstrated that the polyelectrolyte deposits did not exceed 1 nm in height with an extremely small mean roughness (RMS: 0.27 nm), very similar to the RMS of the bare glass surface (always under 0.3 nm). On the contrary, the surface of PEI/(PSS/PAH)₂(PGA/PLL)₃ multilayer architectures appeared fully coated with polyelectrolytes (Fig. 2d), with a maximum measured roughness of

Fig. 2. Motoneurons viability on polyelectrolyte monolayers and multilayered films: Motoneurons viability was tested by measurement of the acid phosphatase activity on a PLL monolayer or a PEI/(PSS/PAH)₂(PGA/PLL)₃ polyelectrolyte multilayer (a) and on a PAH monolayer or a PEI/(PGA/PLL)₂(PSS/PAH)₃ multilayer (b). In both cases the normalized neuronal viability was increased on the multilayer. (c, d) Surface analysis by Atomic Force Microscopy of the roughness of a PLL monolayer surface (c) and a PEI/(PSS/PAH)₂(PGA/PLL)₃ multilayer (d). Each image represents a square of 4 by 4 μm² of the surface of the sample. In both experiments, a section analysis was performed in the middle of the image to calculate the mean roughness of the surface.

8.59 nm and a mean of 2.86 nm (RMS) for a film thickness of about 40 nm. Similar results were found with PAH. We were only able to investigate monolayers of polycations due to the negative charge of the glass surface.

These data suggest that one of the possible origins of the better neuron viability on polyelectrolyte multilayered films is relative to the perfect covering of the substrate by a multilayered film in comparison to a simple monolayer.

3.3. Neurons development on polyelectrolytes multilayers

To investigate the influence of the ending polyelectrolyte layer on neuronal growth, we ended the architectures by two different polycations or by two different polyanions, respectively: either PLL, i.e. [PEI/(PSS/PAH)₂/(PGA/PLL)₂/PGA/PLL], or PAH, i.e. [PEI/(PGA/PLL)₂/(PSS/PAH)₂/PSS/PAH]; either PGA i.e. [PEI/(PSS/PAH)₂/(PGA/PLL)₂/PGA], or PSS, i.e. [PEI/(PGA/PLL)₂/(PSS/PAH)₂/PSS].

After 2 days of in vitro culture, the neurons were fixed and labeled with beta-tubulin to stain all neuritic extensions (Fig. 3) and to measure all the morphological parameters.

The data concerning the four morphometrical considered parameters are listed in Table 1, whereas the pairwise comparisons between the groups for the different architectures are gathered in Table 2. Sig-

nificant differences in neurons development are revealed by these comparisons. The films ending with PSS present a better neuronal growth than any other multilayered films. This result is demonstrated by the relative importance of the number of primary neurites as compared to the other multilayered films, by the significant increase of neuritic arborization and by neuritic length (Tables 1 and 2). No significant differences were, however, observed on the cell body areas for the different polyelectrolyte multilayered films (Tables 1 and 2) suggesting that the overall growth condition for the neurons was satisfactory.

The effect of PSS on the morphology of the neurons is probably not simply linked to the surface charge of the film, since no comparable behavior was observed with an other anionic polyelectrolyte such as PGA. This specific effect could have different origins. (1) First it can be linked to the presence in the polymer structure of a phenyl group which increases the hydrophobic character of the outer layer. (2) It was also earlier observed that PSS/PAH architectures are deposited as more dense structures compared to PGA/PLL ones; such film rigidification favors generally cellular adhesion [42,43].

3.4. Insertion and stability of proteins into the polyelectrolyte multilayer films

The buildup of functionalized multilayered films (typical architecture: PEI-(PGA-PLL)₂-PSS-PAH-PSS-BDNF-PSS or PEI-(PGA-PLL)₂-PSS-PAH-PSS-Sema3A-PSS) including protein uptake and further polyelectrolyte adsorption was followed in situ by OWLS (Fig. 4a and b). One observes that the protein adsorption does not modify the further build-up process of the following layers. Calculations based on the effective refractive indexes led to adsorbed protein amounts of about 96.4 ng/cm² for BDNF and 25.4 ng/cm² for Sema3A.

We evaluated the stability of the embedded proteins by immunodetection of the proteins with anti-BDNF antibody or anti-Flag antibody recognizing recombinant Flag-tagged Sema3A. The proteins could be detected only in the control solutions and in the multilayers (data not shown). After 2 days of culture, the detection of the proteins by western-blot showed that BDNF and Sema3A were still present in the films. Protein could neither be detected in the rinsing solution (0.15 mol/l NaCl) nor in the culture medium suggesting an absence of protein release during cellular growth.

3.5. Activity of the proteins immobilized in the multilayered films

After BDNF or Sema3A embedding in the multilayers, we cultivated motoneurons at the surface of the

Fig. 3. Morphology of cultured motoneurons on multilayered films architectures. We tested substrates made of polyelectrolyte multilayers ending with two different polycations, PLL (panel a, full architecture: PEI/(PSS/PAH)₂/(PGA/PLL)₂/PGA/PLL) or PAH (panel b, PEI/(PGA/PLL)₂/(PSS/PAH)₂/PSS/PAH); or with two different polyanions, PGA (panel c, PEI/(PSS/PAH)₂/(PGA/PLL)₂/PGA) or PSS (panel d, PEI/(PGA/PLL)₂/(PSS/PAH)₂/PSS). The neurons were fixed, immunostained with a mouse anti-beta-tubulin antibody and revealed with a Cy³-coupled goat anti-mouse antibody. Bar: 25 μm.

Table 1

Morphometrical parameters of the neurons for the different architectures: PLL [PEI/(PSS/PAH)₂/(PGA/PLL)₂/PGA/PLL], PAH [PEI/(PGA/PLL)₂/(PSS/PAH)₂/PSS/PAH], PGA [PEI/(PSS/PAH)₂/(PGA/PLL)₂/PGA] or PSS [PEI/(PGA/PLL)₂/(PSS/PAH)₂/PSS]. Summary statistics

Multilayer films endings	Number of neurons	Number of primary neurites		Neuritic arborization		Neuritic length (μm)		Cell body area (μm ²)	
		Median	Range (Min–Max)	Median	range (Min–Max)	Mean	S.D.	Mean	S.D.
PSS	30	4	3–6	6	4–14	48.9	21.3	144.4	25.3
PGA	34	2	1–4	2	0–11	38.6	21.1	163	41.4
PLL	18	2	1–4	2	0–5	36.8	19.5	156.6	38.6
PAH	29	4	2–7	4	0–13	32.2	12.5	142	37.8

Min: Minimum; Max: maximum; S.D.: Standard deviation.

Table 2

Pairwise comparisons between the groups for the morphometrical parameters according to the different polyelectrolyte layers studied

Morphometrical parameters	PSS vs. PGA	PSS vs. PLL	PSS vs. PAH	PGA vs. PLL	PGA vs. PAH	PLL vs. PAH
Number of primary neurites	$P < 0.0001$	$P < 0.0001$	n.s.	n.s.	$P < 0.0001$	$P < 0.0001$
Neurite arborization	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	n.s.	$P < 0.001$	$P < 0.0001$
Neuritic length	$P < 0.02$	$P < 0.02$	$P < 0.001$	n.s.	n.s.	n.s.
Cell body area	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

n.s. indicates a P value greater than 0.05.

Results of the Man–Whitney tests.

Fig. 4. BDNF and Sema3A embedding into polyelectrolyte multilayer films. Deposition and embedding of the BDNF (a) and Sema3A (b) during the buildup of multilayer films as detected by Optical Waveguide Lightmode Spectrometry. The amount of BDNF absorbed is $0.0964 \mu\text{g}/\text{cm}^2$ and that of Sema3A $0.0254 \mu\text{g}/\text{cm}^2$.

functionalized films PEI/(PGA/PLL)₂/PSS/PAH/PSS/BDNF/PSS or PEI/(PGA/PLL)₂/PSS/PAH/PSS-Sema3A/PSS (Fig. 5a and b). The functionalization always affected clearly either survival or neurons growth. Neurons that grew on BDNF functionalized films had a 84% increased survival rate (Control: $100\% \pm 7.1$; BDNF: $184\% \pm 5.8$; $P < 0.001$) while a 25% decrease was observed in the case of Sema3A functionalized films (Control: $100\% \pm 5.5$; Sema3A: $74.8\% \pm 2.8$; $P < 0.05$).

In terms of morphological effects, there are no statistically significant differences between neurons grown on PEI/(PGA/PLL)₂/PSS/PAH/PSS/BDNF/PSS and neurons grown on PEI/(PGA/PLL)₂/PSS/PAH)₂/PSS multilayers with BDNF added to the culture medium, on the cell body area, the number of primary

neurites or on the neuritic length (Table 3), while a difference could be found on the arborization of the neurons ($P < 0.02$). These observations clearly show that motoneurons were able to interact with BDNF embedded in the polyelectrolyte multilayers.

The Sema3A embedded in the multilayers (Table 3) reduced the neuritic length, the neuritic arborization and the number of primary neurites ($P < 0.001$) without modifying the area of the cell body, indicating again that cells could interact with the embedded proteins.

The obtained results on neuronal viability are consistent with the known role of BDNF in the survival of motoneurons in culture [44]. Culture medium taken from BDNF loaded multilayered films was used to culture motoneurons. No neurons survived after 48 h

Fig. 5. BDNF and Sema3A activity while inserted in polyelectrolyte multilayer films. Fluorescence micrographs of motoneurons cultivated on multilayer films functionalized by BDNF (a) and Sema3A (b). Both proteins modify the growth of the neurons either by increasing it (BDNF) or by reducing the neurite length (Sema3A). (c, d) Motoneurons viability tested by acid phosphatase activity measurement on multilayer films containing BDNF (c) or Sema3A (d). The normalized neuronal viability was increased on the multilayer containing BDNF and decreased with Sema3A.

Table 3

Summary statistics and comparisons of motoneuron growth on multilayered films ending by PSS with embedded BDNF or Sema 3A

Morphometrical parameters	Control: PSS ending multilayer (BDNF in culture medium)		BDNF embedded in PSS ending film		Sema3A embedded in PSS ending film	
	Summary statistics		Summary statistics		Summary statistics	
Number of primary neurites	Median: 4	Range: 3–6	Median: 4 n.s.	Range: 3–6	Median: 3 $P < 0.001$	Range: 1–5
Neuritic arborization	Median: 6	Range: 4–14	Median: 7 $P < 0.02$	Range: 5–14	Median: 2 $P < 0.001$	Range: 1–3
Neuritic length (µm)	Mean: 48.9	S.D.: 21.3	Mean: 48.8 n.s.	S.D.: 17.8	Mean: 17.2 $P < 0.001$	S.D.: 5.8
Cell body area (µm ²)	Mean: 144.4	S.D.: 25.3	Mean: 142.3 n.s.	S.D.: 22.6	Mean: 122.7 n.s.	S.D.: 34.5

Range indicates minimum and maximum values; S.D.: Standard deviation; n.s. indicates a P value larger than 0.05.

demonstrating that no BDNF significant amount was released from the multilayered films. This experiment confirmed that BDNF was embedded in a stable manner in the polyelectrolyte architectures.

4. Conclusion

Our data indicate that the response of motoneurons cultured on polyelectrolyte films can be tuned by protein embedding and are strictly influenced by the ending polyelectrolyte. The immobilization of proteins directly into the growing substrate where they remain still functional and available, even embedded under two

layers of polyelectrolytes, is particularly advantageous since it allows the direct presentation of growth factors in the injury environment. In comparison to conventional coating methods, the polyelectrolyte multilayer films is a very easy-to-use technique and could lead to a new family of substrates aimed to repair nervous system injuries.

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