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Tunable swelling of polyelectrolyte multilayers in cell culture media for modulating NIH-3T3 cells adhesion

Wei Qi,[1,2] Peng Cai,[1,2] Wenjing Yuan,[1,2] Hua Wang[1,2]

[1]Key Laboratory of Life-Organic Analysis, School of Chemistry and Chemical Engineering, Qufu Normal University, 57 Jingxuan West Road, Qufu Shandong 273165, China
[2]Key Laboratory of Pharmaceutical Intermediates and Analysis of Natural Medicine, School of Chemistry and Chemical Engineering, Qufu Normal University, 57 Jingxuan West Road, Qufu Shandong 273165, China

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Abstract: For polyelectrolyte multilayers (PEMs) assembled by the layer-by-layer (LbL) assembly technique, their nanostructure and properties can be governed by many parameters during the building process. Here, it was demonstrated that the swelling of the PEMs containing poly(diallyldimethylammonium chloride) (PDDA) and poly(sodium 4-styrenesulfonate) (PSS) in cell culture media could be tuned with changing supporting salt solutions during the assembly process. Importantly, the influence of the PEMs assembled in different salt solutions on NIH-3T3 cell adhesion was observable. Specifically, the cells could possess a higher affinity for the films assembled in low salt concentration (i.e. 0.15*M* NaCl) or no salt, the poorly swelling films in cell culture media, which was manifested by the large cell spreading area and focal adhesions. In contrast, those were assembled in higher salt concentration, highly swelling films in cell culture media, were less attractive for the fibroblasts. As a result, the cell adhesion behaviors may be manipulated by tailoring the physicochemical properties of the films, which could be performed by changing the assembly conditions such as supporting salt concentration. Such a finding might promise a great potential in designing desired biomaterials for tissue engineering and regenerative medicine. © 2014 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 102A: 4071–4077, 2014.

Key Words: swelling, polyelectrolyte multilayer film, layer-by-layer assembly, supporting salt concentration, cell adhesion

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INTRODUCTION

Recent years witness the wide applications of various biomaterials with unique physical and chemical properties in the biomedical field, such as biosensors, biomimetics, drug delivery, and tissue engineering.[1–8] In particular, the poly electrolyte multilayers (PEMs) assembled by the layer-by-layer (LbL) assembly technique have attracted much interest, so that their nanostructure and properties including compositions, thickness, and surface functional groups can be governed by the experimental conditions during the building processes.[9–16] The LbL technique may constitute a versatile and highly successful surface functionalization method for the PEM deposition.[17–20] Advantageously, the LbL-assembled films can conformally coat substrate materials of any type, size, or shape. Based on these facts, the polyelectrolyte complexes constructed by LbL method are thought to be considerably suitable to address the cell-material interaction issues, which is of great importance in the development of new materials for the large-scale biomedical applications.[21–26]

It is well known that anchorage-dependent cells require good adhesion to a substrate to spread, proliferate and maintain cellular functions.[27,28] Cell adhesion and proliferation behaviors can be largely influenced by the surface physicochemical properties of the substrates, such as the wettability, chemical composition, stiffness, dimensions and topography.[29–31] For example, Rubner and coworkers reported that swelling and hydration behaviors of the LbL-assembled films could be responsible for the multilayer's interaction with living cells.[22] Up to date, however, few detailed studies were carried out purposefully to tune the swelling of the PEMs and further to modulate cell adhesion behavior.

In this work, the swelling of PEMs were purposefully engineered by changing the supporting salt solution during

Correspondence to: W. Qi; e-mail: qf_qw@163.com or H. Wang; e-mail: huawangqfnu@126.com
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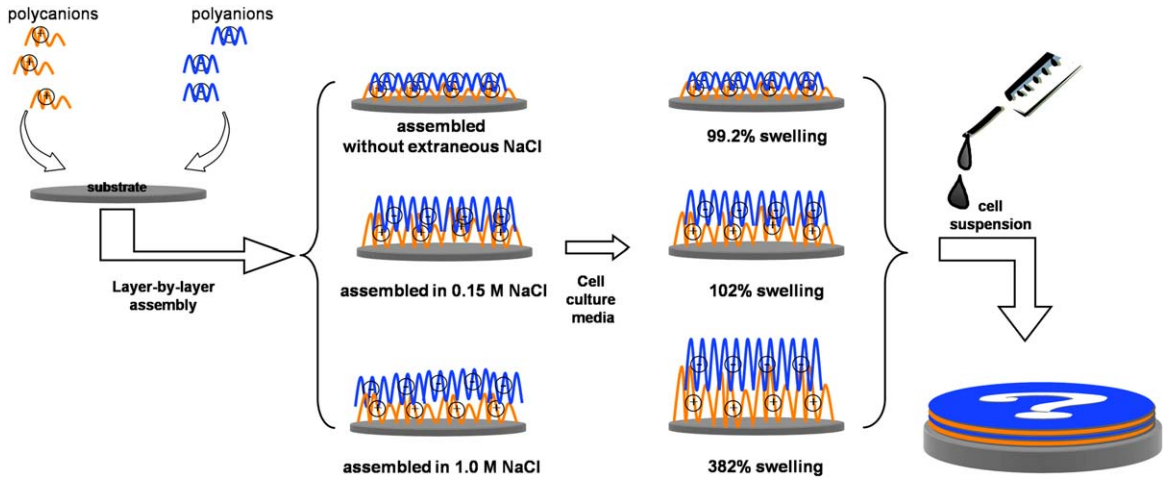

SCHEME 1. Schematic representation of the effect of swelling of the PDDA/PSS multilayer films assembled in different NaCl solutions on NIH-3T3 cell adhesions. PDDA: Poly(diallyldimethylammonium chloride); PSS: poly(sodium 4-styrenesulfonate). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

the assembly process. Herein, the PEMs composed of poly (diallyldimethylammonium chloride) (PDDA) and poly(sodium 4-styrenesulfonate) (PSS) were fabricated by the LbL technique. The physicochemical properties of the PDDA/PSS multilayer films assembled in different ionic strength were studied by quartz crystal microbalance (QCM) and atomic force microscopy (AFM). Subsequently, the swelling of these films in PBS and cell culture media were determined using ellipsometry. Moreover, their effects on the organization of focal adhesions and cytoskeleton in cells were evaluated, as schematically shown in Scheme 1. NIH-3T3 fibroblasts were used as cell models, whose adhesive properties and functions have been well studied previously.[32]

METHODS

Preparation of PDDA/PSS multilayer film via the LbL technique

14 mm-diameter glass coverslips were used as substrates for cell culture and AFM characterization. They were cleaned in piranha solution (7:3 v/v H_2SO_4/H_2O_2) before use, followed by rinsing with water and dried under a smooth stream of N_2. To ensure the successful adsorption, a precursory layer of polyethyleneimine (PEI, Sigma-Aldrich, USA) was deposited on the substrate. PSS (Sigma-Aldrich, USA) and PDDA (Sigma-Aldrich, USA) were then alternately assembled onto the substrate. All the polyelectrolytes were prepared with a final concentration of 2 mg/mL aqueous solutions containing NaCl varying from 0.15*M* to 1.0*M*. The washing step was performed after each deposition with the corresponding salt solution. Here, PEI/PSS/$(PDDA/PSS)_9$ films were built up for characterization and cell culture.

Characterization of the PDDA/PSS multilayer film

For QCM characterization, the quartz crystals (Chenhua, Shanghai, China) were cleaned before use by dipping their surfaces in a solution of 0.01*M* SDS for 10 min followed by an extensive rinse with water.[33] The crystals were excited at their fundamental frequencies (8 MHz). As the typical LbL assembly technique, 0.5 mL of a PDDA solution at 2 mg/mL in different NaCl solutions and 0.5 mL of a PSS solution at 2 mg/mL in different NaCl solutions were injected into the measurement cell alternately (15 min for each). During these periods, the shifts in frequency (ΔF) were continuously recorded.

AFM images were obtained in a contact mode in air with Nanoscope IIIa (Veeco, USA). The thickness and roughness of the films were obtained with the analysis software affiliated to the instrument.

Swelling experiments

The in situ swellings of the PEI/PSS/$(PDDA/PSS)_9$ multilayer films assembled in different salt concentrations were obtained using an M2000D spectroscopic ellipsometer (J. A. Woollam, USA).[24] The thickness of the multilayers was determined in air at an incident angle of 75° within a wavelength range of 300–1700 nm. The measurement in PBS or cell culture media was carried out in a liquid cell within a wavelength range of 300–1100 nm. Using a Cauchy model, the thickness was calculated from the ellipsometric parameters, Δ and ψ. Percent swelling is defined as the swollen thickness in buffer/culture media relative to the dry film thickness $\times$ 100%.

Cell culture

NIH-3T3 (mouse embryonic fibroblast cell line) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L D-glucose and supplemented with 10% FBS (fetal bovine serum), 100 units/mL penicillin, and 100 μg/mL streptomycin at 37°C in 5% CO_2 environment. NIH-3T3 cells were seeded on the substrates coated with the PDDA/PSS multilayer films of about 30–50% confluency for the experiments unless otherwise indicated.[2]

Focal adhesion imaging and quantitation

Cells were platted on the different types of PDDA/PSS films and cultured at 37°C in the presence of serum. The film

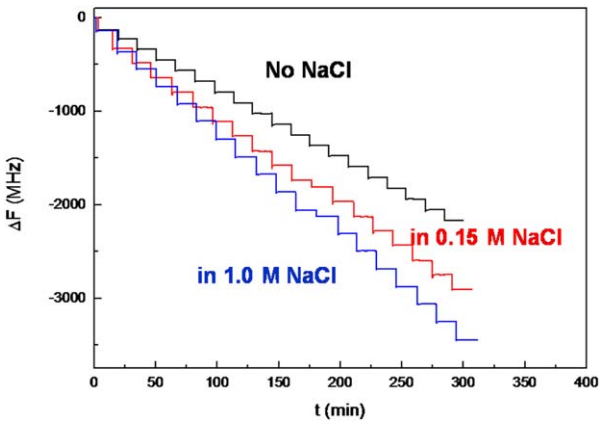

FIGURE 1. Frequency shift (ΔF) measured for the PEI/PSS/(PDDA/PSS)$_9$ multilayer films assembled in different NaCl solutions by QCM as a function of assembly process. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

coated slides were removed from the culture at 12 h or 36 h after plating, washed with PBS, and fixed in 3.7% paraformaldehyde for 4 min. The slides were washed with PBS and then treated with 0.1% Triton X-100 in PBS for 1 min and blocking solution (goat serum) for 30 min.[27] To visualize focal adhesions, cells were treated with anti-vinculin antibody (Sigma, USA) diluted 1:200 in blocking solution for 1 h, followed by incubation with goat anti-mouse DyLight® 594 conjugated antibody (Thermo Fisher Scientific, USA) at the 1:400 dilution in blocking buffer for 1 h. For double labeling, FITC-phalloidin was incubated simultaneously with the second antibody.[2] The slides were washed three times with PBS between each antibody treatment. They were viewed through an Olympus confocal microscope system FV 1000 (Olympus, Japan). Focal adhesion size and spatial distribution were analyzed using ImageJ software (NIH, USA).

Statistical analysis

All experiments were repeated at least three times. Error bars represent standard errors, and statistical analysis was performed using SPSS 13.0 to evaluate the statistical differences ($p < 0.05$) among all samples or between samples and controls, respectively.

RESULTS AND DISCUSSION

Physicochemical properties of the PDDA/PSS multilayer film assembled in different salt concentrations

The buildup of the multilayer films in different salt concentrations was monitored in situ in a step-by-step way by QCM. The resonance frequencies were recorded when a material was deposited from solution. QCM data at 8 MHz was typically represented in Figure 1. One can notes that the data may confirm the significant differences in the film growth, indicating that the growth of PDDA/PSS films in salt solution could depend on the salt concentrations for the buildup.

As reported previously,[34,35] the added salts (i.e., NaCl) would screen the charges of the polyelectrolytes by the salt ions. As a result, the charge repulsion of the same sign on the polymer chain might be reduced, the repulsion between the polyelectrolytes of the same species could decrease in turn.[13,34] Thus, it is expected that these two effects would make the salt-assembled polyelectrolyte multilayer films thicker, more loop-rich and rougher, which may be confirmed by AFM measurement.

The films assembled in solutions containing 0.15*M*, 0.3*M*, 0.5*M*, 1.0*M* NaCl and without extraneous NaCl were investigated one by one with AFM, shown in Figure 2. The images were analyzed by the software to get thickness and roughness of the films and the results were listed in Table I. As expected, the low thickness (10.4 nm for 20 bilayers) of the films built without extraneous salt was obtained, which is consistent with the reported 5Å/layer.[22] When the assembly was performed at 0.15*M* NaCl, the dried film thickness reached 35.9 nm (Table I), nearly four times of that of the salt-free assembled. Obviously, a significant increase in thickness was achieved. The thickness of PEI/PSS/(PDDA/PSS)$_9$ films, however, appeared a maximal value at an intermediate concentration of NaCl (0.3 or 0.5*M*). And then, a decreased film thickness (22.8 nm) was observed for the films assembled in 1.0*M* NaCl. Such a phenomenon might result from the combinations of polyanions and polycanions which occurred due to the strong charge interaction between them at a higher salt strength.[13,36]

In addition, the salt-free multilayer deposited on a clean glass coverslip was uniformly distributed, exhibiting a low surface roughness, with a root-mean-roughness (Rms) of 1.67 nm. As for the salt-assembled films, Rms increased obviously, and were 7.11 nm, 7.29 nm and 7.36 nm for the films assembled in 0.15*M*, 0.3*M*, and 0.5*M* NaCl, respectively. This might be attributable to the fact that the charge screening of polyelectrolyte by salt ions led to the conformation of polyelectrolyte shifting from the scratched state to the coiled state.[31] Also, it was found that the initial increase in the roughness of the films with increasing salt concentrations could be followed by a decreased roughness (6.05 nm for the film assembled in 1.0*M* NaCl). This implied a salt induced annealing of the films when the salt concentration getting much higher, as suggested by the previous reports.[13,37]

Cell adhesion and organization of focal adhesion on the PDDA/PSS multilayer film assembled in different salt concentrations

Cell adhesion is a critical process and a key determinant of cell viability, proliferation, migration and differentiation. Different substrates were well demonstrated to possess distinct effects on the cell behaviors.[27,38] The cells might communicate with the environments through the cell surface interactions with the substrates, including the formation of focal adhesions via the clustering of integrin receptors.[2,39,40]

Here, cell adhesions and spreading patterns on the multilayers were examined via immunofluorescence technique and were analyzed quantitatively by using the ImageJ software. The cells were stained simultaneously with the FITC-conjugated phalloidin to reveal the actin filament network and

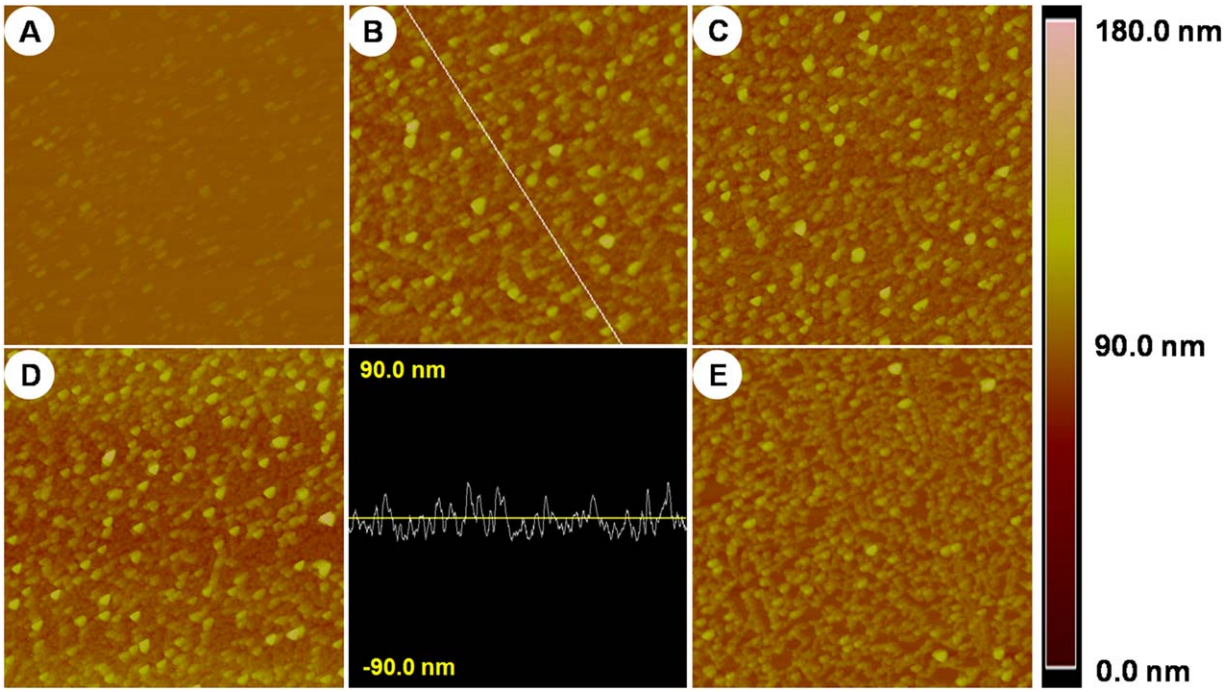

FIGURE 2. AFM height mode images in air obtained for the PEI/PSS/(PDDA/PSS)$_9$ multilayer films assembled A: without extraneous NaCl; B: in 0.15*M* NaCl; C: in 0.3*M* NaCl; D: in 0.5*M* NaCl; E: in 1.0*M* NaCl. The image dimensions are all 3×3 μm^2. The inset is cross-sectional profile for the film assembled in 0.15*M* NaCl marked by the white line in the image B. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

anti-vinculin antibody to detect vinculin at focal adhesions (Figure 3). The cell spreading was quantified by the cell adhesion areas after the cells seeding for 12 h and 36 h, respectively. As shown in Figure 4(a), in 12 h incubation with the films, the cell spreading area on the multilayer assembled in 0.15*M* NaCl solution was the largest showing 1982 ± 67 μm^2 per cell. And for cells on the salt-free assembled film, they had spreading area of 1602 ± 70 μm^2 per cell. When the assembly was performed in 0.3*M*, 0.5*M*, and 1.0*M* NaCl solutions, cell spreading areas decreased to 1065 ± 59, 810 ± 40, and 720 ± 62 μm^2, respectively. As a control, each cell on the glass coverslip presented the spreading area of 828 ± 45 μm^2 averagely. When the culture time was extended to 36 h, the cell spreading area on all the investigated multilayers were improved to 1802 ± 68, 2160 ± 60, 1281 ± 20, 1199 ± 32, 983 ± 21 μm^2 per cell, with increasing the assembly salt concentration from 0 to 1.0*M*, respectively. These results indicated that the cells spread the largest area on the multilayer assembled in 0.15*M* NaCl.

As it was previously reported, the numbers of focal cell adhesions could be related to the affinity of cells for a substrate,[27] implying that stronger cell–substrate interactions could lead to the development of more focal cell adhesion sites. Actually, focal cell adhesions are dynamic complexes of several properties involved in cellular signaling cascades, serving as a connector between the actin filaments and the integrins.[2] Through the engagement of the integrin receptors, cells could adhere to the substrate, spread to assume a flattered morphology, and anchor the cytoskeleton to the substrate.

Herein, the sizes, distribution and numbers of the focal adhesions were analyzed for per cell adherent to the surfaces. All focal adhesions of sizes greater than 0.14 μm^2 were considered mature and therefore counted. As shown in Figure 3, for cells cultured on the multilayer assembled in 0.15*M* NaCl for 36 h, some large vinculin clusters were clearly visible, indicating that many focal adhesion plaques in the cells were assembled and then matured to be more stable structures. Also, it could be seen that the cell actin filaments were distributed throughout the cells with well-organized focal adhesion plaques, resulting in stronger cell adhesion of averaging 256 ± 4 focal adhesions per cell [Fig. 4(b)]. In contrast, for the multilayer assembled in higher investigated concentrations (0.3*M*, 0.5*M*, and 1.0*M* NaCl), some focal adhesion plaques could also be observed, but most of them preferentially localized to the leading edges.

TABLE I. Thickness and Roughness of the PEI/PSS/(PDDA/PSS)$_9$ Multilayers Assembled in Different NaCl Solutions as Measured by AFM Technique

PEI/PSS/(PDDA/PSS)$_9$ Multilayer Films	Film Thickness (nm)	Film Roughness Rms (nm)
Salt-free assembled	10.4	1.67
Assembled in 0.15*M* NaCl	35.9	7.11
Assembled in 0.3*M* NaCl	34.6	7.29
Assembled in 0.5*M* NaCl	36.7	7.36
Assembled in 1.0*M* NaCl	22.8	6.05

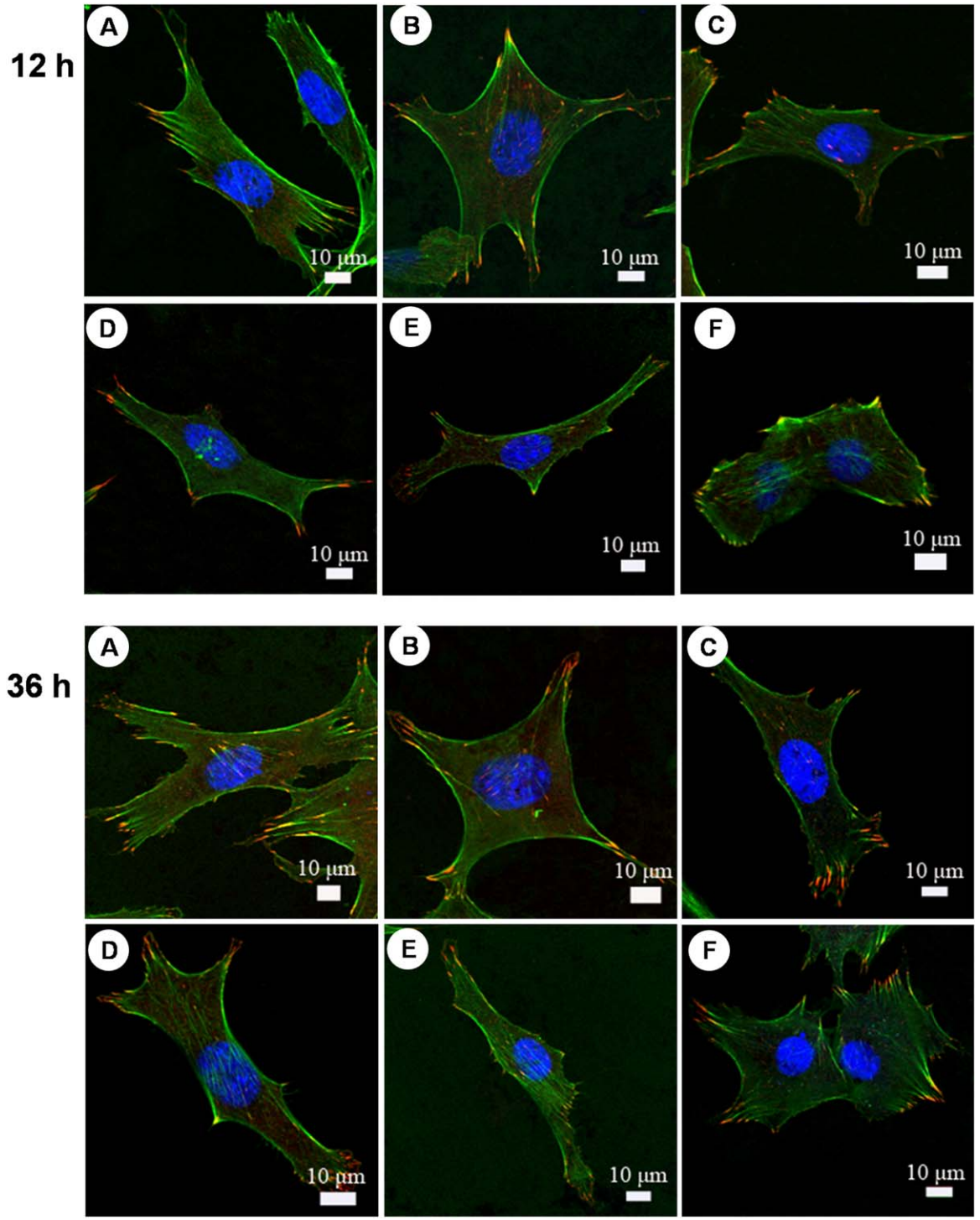

FIGURE 3. Fibroblast morphology and cell adhesions on the PEI/PSS/$(PDDA/PSS)_9$ multilayer films assembled in different NaCl solutions after being plated for 12 h and 36 h, respectively. A: without extraneous NaCl; B: 0.15*M* NaCl; C: 0.3*M* NaCl; D: 0.5*M* NaCl; E: 1.0*M* NaCl, and on bare glass coverslip (F). For NIH-3T3 cells, actin and vinculin were stained with FITC-phalloidin (green) and a monoclonal anti-vinculin antibody (red), respectively. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Of note, the cells cultured on these multilayers for 36 h could achieve 201 ± 4, 177 ± 6, 155 ± 8 focal adhesions per cell, respectively.

Taken cell spreading area and organization of focal adhesions together, it is concluded that the cells could possess higher affinity for the multilayer assembled in 0.15*M* NaCl than for others assembled in higher salt concentrations. Despite the dry multilayers assembled in 0.15*M* NaCl, 0.3*M* NaCl, and 0.5*M* NaCl having nearly similar roughness and thickness, the cell area and the focal adhesion points per

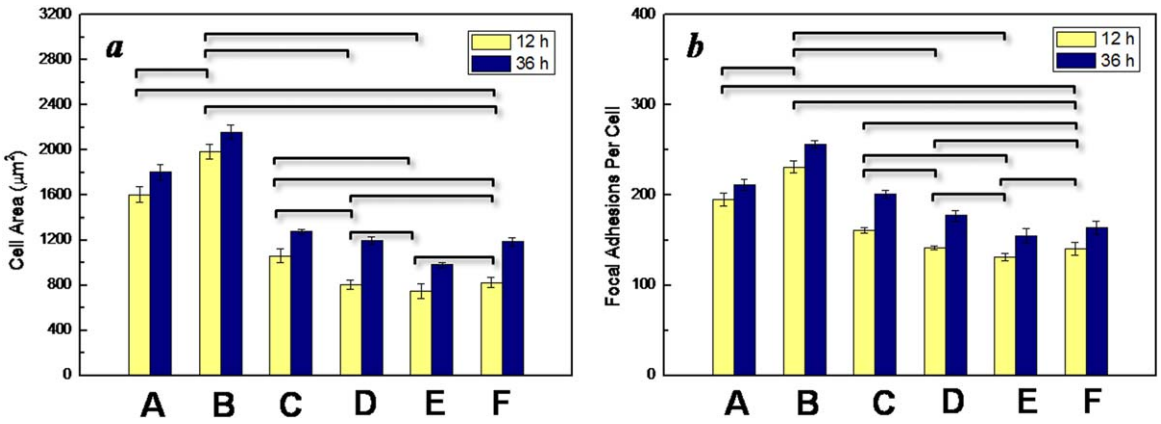

FIGURE 4. Quantitative analysis of cell adherence to the PEI/PSS/(PDDA/PSS)$_9$ multilayer films assembled in different NaCl solutions with respect to (a) cell spreading area per cell; (b) advantage focal adhesion number per cell. A: without extraneous NaCl; B: 0.15*M* NaCl; C: 0.3*M* NaCl; D: 0.5*M* NaCl; E: 1.0*M* NaCl, and bare glass coverslip (F). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

cell were significantly different. Thus, here, it might deduce that the roughness and thickness of the dry films were not the dominant factors for the cell adhesions.

Swelling of the PDDA/PSS multilayer film assembled in different salt concentrations

The anchorage-dependent cells must adhere to a substrate with enough strength before any other cellular events. On very soft surfaces such as highly hydrated and swollen ones, the substrates cannot provide strong enough adhesion force for the cells to anchor and spread. By contrast, a rigid substrate can provide the enough adhesion force.[22,24,41]

Accordingly, swelling experiments were performed in phosphate buffer solutions (PBS, pH = 7.4) and cell culture media, which were probed by using the ellipsometry. As shown in Figure 5, the film assembled in 0.15*M* NaCl displayed the smallest welling ratio in cell culture media with $102 \pm 9.9\%$ among all the salt-assembled films. Its swelling and hydration was almost the same as that of the salt-free assembled film ($99.2 \pm 7\%$). While the multilayer assembled in 1.0*M* NaCl swelled $382 \pm 19\%$ of its original thickness in cell culture media. The nearly same results were obtained in PBS. This swelling behavior may be driven by the osmotic pressure.[35,42] Actually, the change in ionic strength led to a difference in the chemical potential of the ions inside and outside the multilayer, which produced a swelling process on the multilayer. For the multilayer constructed at high salt concentrations, the difference in chemical potentials was bigger and a more obvious swelling process was observed. This implied that the film assembled in 0.15*M* NaCl might possess the strongest stiffness in the culture media, and that one assembled in 1.0*M* NaCl was the most swellable.

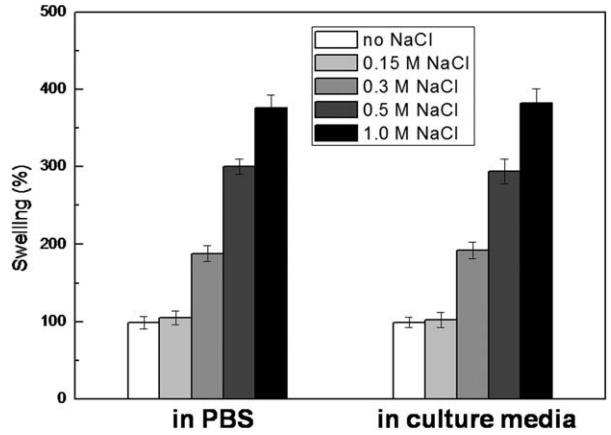

FIGURE 5. Percent swelling of the PEI/PSS/(PDDA/PSS)$_9$ multilayer films assembled in different NaCl solutions in PBS (pH = 7.4) and cell culture media as measured by ellipsometry.

According to the cell response results that the cells had a higher affinity for the multilayer assembled in 0.15*M* NaCl than for others assembled in higher salt concentration, the smallest swelling behavior of the multilayers (assembled in 0.15*M* NaCl) played a major role in the best cell adhesions. Maybe changes in thickness, or in roughness or in other parameters derived from the swelling of films were actually responsible for the manipulation of cell adhesion behavior. Certainly, the definite relationship between swelling of PEMs and cell adhesion behavior needs more evidences.

CONCLUSIONS

In this work, the swelling of PDDA/PSS multilayers was tuned purposefully to study its effect on cell adhesion behavior. It was found that cells could conduct the well-spreading behaviors on the lowly swelling films, with the larger cell spreading areas and focal adhesions. In contrast, cells on the highly swelling films showed a reduced spreading area and a decreased number of focal adhesions. Particularly, for the multilayer films fabricated with LbL technique, their swelling could be easily tuned by the multilayer assembly conditions involved. Here, the swelling could be tuned by the salt concentrations of the polyelectrolyte solutions used to deposit films. At high ionic strength, i.e., 1.0*M* NaCl solution, the assembled films might be swelled

by nearly 400% of its original thickness on the average. Moreover, that assembled in 0.15*M* NaCl nearly did not change their thickness after being incubated in the culture media or PBS. Therefore, this study provided an efficient strategy by which to fabricate the desired and engineered cell-interacting materials by controlling the multilayer processing conditions, indicating the potential applications in regenerative medicine and tissue engineering.

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