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Improved L-929 Cell Growth from Self Assembled PDADMAC/Gelatin Thin Films

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Abstract

This article demonstrates the advantages of using PDADMAC/gelatin over chitosan/gelatin multilayer thin films to improve L-929 mouse fibroblast growth. Although chitosan-based materials are often preferred in cell culture, we put in evidence the problematic low stability and decomposition of chitosan based film under physiological pH. Two kinds of coating were assembled using either chitosan or Poly(diallyldimethylammonium chloride) (PDADMAC) as polycationic polyelectrolyte and gelatin as the anionic counterpart. The non-toxic nature of each coating was confirmed by MTT assay for cell viability and cell proliferation. Using optical microscopy, it was observed that the L-929 fibroblast spread preferentially on the PDADMAC/gelatin surface when compared with the chitosan/gelatin surface. The lack of stability of the chitosan/gelatin films was put in evidence using atomic force microscopy (AFM) by monitoring the changes in thickness for each film when exposed to physiological buffer conditions. While PDADMAC/gelatin films were stable for several hours, chitosan/gelatin films presented a loss of thickness of nearly 40%. This loss of thickness could explain the observed detachment of the cell and poor spreading of the fibroblast cells when using the chitosan/gelatin coating.

Key words : Layer-by-layer, L-929 fibroblast, chitosan, PDADMAC, gelatin.

Introduction

Tissue engineering, as an emerging field in the area of human heath care, has received growing interest in the last few decades. Often seen as an interdisciplinary science, successful research in this field requires an understanding of cellular biology, bioengineering as well as advanced materials science. One of the challenges in the development of tissue engineering application is the need to preserve the cell's ability to grow onto synthetic scaffolds and maintain tissue–specific function which depends critically on factors such as the cell/scaffold and cell/cell interaction.(1) The key processes involved during the in vivo growth of tissue formation and maturation are the cell proliferation, sorting, differentiation, extra-cellular matrix production and organization. In order to address each of these parameters, increasing efforts have been done to develop new coating and improve the biocompatibility of a given surface. In some instance the surface need to be resistant to cellular adhesion when used in the application such as of hemodialysis devices(2) while in some case the opposite behavior is desired to enhance cell attachment and growth for example on scaffolds.

Different kinds of coating techniques have been used such as leaching of glass, sol-gel deposition, chemical vapor deposition (CDV), sputtering, chemical grafting and more recently the layer-by-layer (LBL) method.(3-5) When put in perspective with other deposition strategies, the LBL approach offers several advantages. This simple technique, which has been described in previously published reviews(6-7), relies only on a dipping process and can be applied to surface having very complex morphology. The assembly

occurs mainly in aqueous media and do not requires the use of organic solvents which could be a problem in further cytocompatibility studies. The assembly process based on the adsorption of polyelectrolytes is quick and self-limited which allow for a good control over the surface properties. Finally, biopolymer, oligomer and protein, which are key component of bio-compatibles surface, can be readily used in the assembly due to their electrostatic nature.[8] For the design of scaffolds, various biopolymers can be used mainly as polyelectrolyte complexes or formed into membranes. Chitosan alone, a natural biopolymer derived from chitin, has been extensively studied for its applications in tissue engineering and drug delivery systems.[9] Gelatin, another biopolymer, was found to enhance the activation of macrophages and is known to have high hemostatic effect.[10]

Recently, chitosan has been combined with gelatin into films by varying the ratio of constituents in order to improve the final mechanical properties.[11] The resulting complexes of these two biopolymers showed good cytocompatibility, cell adhesion and proliferation. Further development of the chitosan/gelatin complex led to their preparation into thin films using the LBL technique for the surface modification of titanium surfaces.[12] Although the utilization of chitosan in PEM thin films for cell adhesion present some advantages, the lack of stability of these coatings under physiological pH appears to be a major limitation for their use.[13] The concern for the stability of the bio-coating is of importance especially when one tries to achieve surface modification prior to cell adhesion. In their work, Liu et al.[14] report that a 13 layers film composed of chitosan and Chondroitin sulfate decomposed to less than 70% of its initial thickness within 1 hour when dipped in the phosphate buffer solution. It is the pH dependence of chitosan cationic character which is problematic here. Having a pKa around 6.4 chitosan has an extremely low charge density under physiological buffered conditions (pH 7.4). The resulting loss of electrostatic interaction due to the neutralization of chitosan in the PEM film can lead to decomposition or excessive swelling of the coating has it as been previously reported.[15]

In the present article, we suggest that a PEM film assembled from a less pH sensitive polyelectrolyte, that is PDADMAC, could lead to a more stable film. The non-pH dependence of PDADMAC provides a fully charged polyelectrolyte in pH ranging from 1 to 14. As a result, thin films from the assembly of PDADMAC/gelatin are expected to be more stable under physiological pH. Although enhanced stability is crucial, the overall cell growth onto the film should remain as good in the case of PDADMAC/gelatin when compared with chitosan/gelatin. In this work, the two polyelectrolyte pairs were compared in terms of cell adhesion and spreading followed by a test of stability under physiological pH. The cells used in our work were L-929 fibroblast mouse cells. The effect of the thin film composition on the in vitro response of the cell has been studied by the MTT assay as well as optical microscopy for the cell morphology. Atomic force microscopy (AFM) was used to investigate the changes in thickness of the PDADMAC/gelatin film versus the chitosan/gelatin pair as a function of the time of exposure to physiological pH.

Experimental

Chemical and Materials

Poly(diallyldimethylammonium chloride) (PDADMAC) and poly(styrene sulfonate, sodium salt) (PSS) were purchased from Aldrich. Chitosan with 84% deacetylation and gelatin type B (from bovine skin), EDTA and MTT solution were obtained from Sigma (St. Louis, Mo, USA). Dulbecco's Modified Eagle's medium (DMEM) for cell culture was obtained from (Hyclone, UT, USA), supplemented with 10% fetal bovine serum (FBS), 100 units / ml penicillin and 100 μg / ml streptomycin.

Layer-by-Layer Deposition

Prior to the layer-by-layer deposition, glass cover slips were cleaned in a mixture of 70% H_2SO_4 (conc.) / 30% H_2O_2 (aq.) ("piranha solution": caution, piranha is a strong oxidizer and should not be stored in a closed container) followed by a 20 min dip in hot H_2O_2 /ammonia/water (1:1:5) and blown-dried with a stream of nitrogen. The concentrations of the PDADMAC, PSS, chitosan and gelatin solutions were 10 mM. Gelatin and chitosan solutions were adjusted to pH 6 with sodium acetate. PDADMAC and PSS solutions were used without any pH adjustment. The sequential adsorption of the polyelectrolyte on glass cover slips was done by manual dipping. The

substrate was immersed in the polycationic solution for 5 min and then rinsed in double distilled water three times. This dipping cycle was completed with the poly-anionic solution over the same time and followed by a similar rinsing step leading to the final deposition of a polyelectrolyte bi-layer. Further deposition of polyelectrolyte layers were accomplished by repeating the same cycle. When we deposited, for example, a number X of bi-layers of the polyelectrolyte A with polyelectrolyte B, the appropriate notation is (A/B)x. According to these conventions, in this research, the followingthin films: (PDADMAC/PSS)4,PDADMAC/PSS)4+(PDADMC /gelatin)8, and (PDADMAC/PSS)4+(chitosan/ gelatin) 8 were used.

L-929 Cell Culture

L-929 mouse fibroblasts were cultured in DMEM, (Hyclone, UT) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. The cells were incubated at 37°C in humidified atmosphere containing 5% CO2. When the cells reached 80% confluence, they were serially subcultured. Polyelectrolyte coated glass slips used in cytotoxicity test and proliferation assay were sterilized using 70% ethyl alcohol for 5 min in the 24-well polystyrene culture plates (Nunc, Rockford, IL), rinsed twice for 5 minutes with sterilized water, and air dried. One milliliter of culture medium was added to each culture well to equilibrate the samples for 20 min before cell seeding. Then, cells were seeded on blank wells (as controls) and wells containing polyelectrolyte coated glass slips at an initial density of 5x104cells per well. The experiments were conducted in triplicate. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO2. Cytotoxicity test was performed at 24 hours and proliferation assay was conducted between 8-24-48 hours. Both analyses were determined for the number of viable cells by MTT assay.[14] All the experiments were repeated 3 times. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay was based on the reduction of the yellow tetrazolium salt to purple formazan crystals by dehydrogenase enzymes secreted from the mitochondria of metabolically active cells. The amount of purple formazan crystals was proportional to the number of viable cells. First, the culture medium was aspirated and replaced with 0.5 mg/ml MTT solution. After that, the culture was incubated for 30 min at 37°C. The solution was then aspirated, and 900 μl of DMSO containing 125 μl of glycine buffer (0.1M glycine, 0.1 M NaCl, pH 10) added to dissolve the formazan crystals. Finally, after 10 min of rotary agitation, the absorbance of the DMSO solution at 570 nm was measured using a Thermospectronic Genesis10 UV-vis spectrophotometer (Rochester, NY).

Morphological Study

Cells were seeded on polyelectrolyte coated glasses and controls as described above. The cultured media were removed at the designated times at 8, 24 and 48 hours after seeding. Cells were washed twice with a sterile phosphate buffer saline (PBS) solution to eliminate free cells. Cell morphological imaging was performed using a phase-contrast microscope-attached Olympus 5050 digital camera.

Film Stability Test

To simulate the pH changes occurring during cell growth on the substrate, the coated glass slides were immersed in the same solution used in cell test. The samples were supplemented with DMEM for 4, 8, 24, and 48 hrs. Initially, the pH of the cultured medium was 7.4. The thicknesses of the PDADMAC/gelatin and chitosan/gelatin films were evaluated by atomic force microscopy (AFM). The AFM from digital Instruments Nanoscope IIIa (USA) was used in tapping mode. All samples were measured in air after variable exposure to the cell growth medium. The topographic images were recorded with a standard silicon tip on a cantilever with a resonant frequency of 290 kHz. Prior to the AFM measurement a scratch was made on the film and the AFM image was taken at the step edge in order to obtain the exact thin film thickness.

Results and Discussion

Our goal in this article is to compare two pairs of polyelectrolytes (chitosan/gelatin) and (PDADMAC/gelatin) on cell viability, proliferation, spreading of cells and film stability under physiological pH. The objective is to develop more stable coatings, which would be used under physiological pH and with a good cytocompatibility and favor cell spreading. While gelatin was used as polyanion in both thin films, either chitosan or PDADMAC was used as polycation. Beside their different chemical structures, one of the major

differences between chitosan and PDADMAC is their degree of ionization as a function of the pH. Chitosan has a pKa of 6.4 and therefore presents a lower charge density at pH 7.4. It is precisely this low charge density which might impair its low stability to the film assembled from chitosan and gelatin. As a reference in the cell adhesion, we chose to use a 4 bi-layers thin film of PSS/PDADMAC since it can form a strong non-pH dependant coating on the substrate. The performance of the PDADMAC/gelatin and chitosan/gelatin was compared to that of the reference PSS/PDADMAC coating.

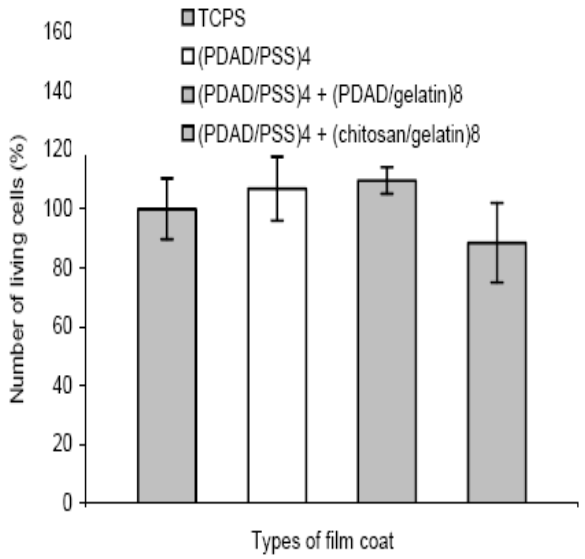

Figure 1. Relative percentage of cell number of L929 mouse fibroblasts on different polyelectrolyte film coatings at 24 hr. Error bars represent means ± SD for n = 3

Cytotoxicity and Cell Proliferation

Cytotoxicity test of the three coatings, i.e. (PDADMAC/PSS)4, (PDADMAC/PSS)4+ (PDADMAC/Gelatin)8 and (PDADMAC/PSS)4+ (chitosan/gelatin)8 were conducted using L-929 mouse fibroblasts. Figure 1 illustrates the percentages of the living cells normalized to that of the control. After 24 hour-culture, no significant difference of the percentages of the living cells could be seen for all types of films when compared with the control. The results demonstrated that none of the films were cytotoxic. Proliferation ability of the L-929 fibroblast growth on various film coats was examined. Phase-contrast microscope imaging revealed that all of the seeded cells attached to the culture plate after 8 hours. The result analyzed by MTT assay, as shown in Figure 2, confirmed that the relative percentage of the living cells after seeding on the films of (PDADMAC/PSS)4, (PDADMAC/PSS)4+ (PDADMAC/Gelatin)8 and (PDADMAC/PSS)4+ (chitosan/gelatin)8 were comparable at 8 hours, and again demonstrated no significant change of the cell number during the first 24 hours. By 48 hours, cell number significantly increased on the coating of (PDADMAC/PSS)4 and (PDADMAC/PSS)4+ (PDADMAC/Gel)8 with slightly lower cell number on (PDADMAC/PSS)4+(chitosan/gelatin)8 compared to those at 24 hours. These results indicated that L-929 cells could grow and proliferate on all types of film coat even with slightly different ability.

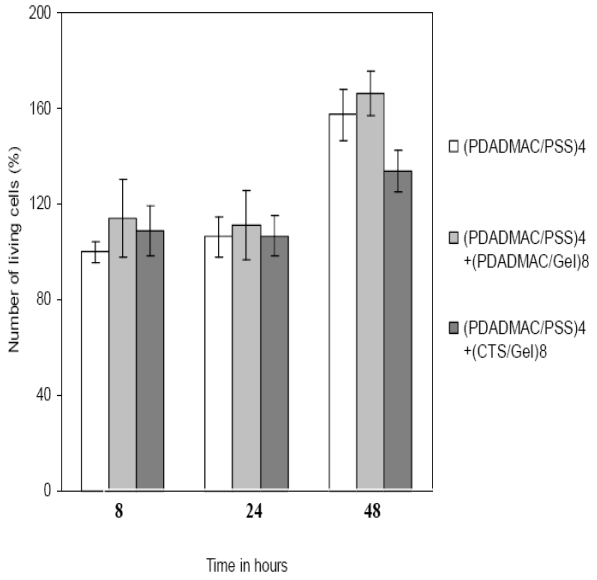

Figure 2. Proliferation of L-929 mouse fibroblasts on different polyelectrolyte film coat at 8, 24 and 48 hours. Error bars represent means ± SD for n = 3

Cell Adhesion and Spreading

Under microscopic observation and MTT analysis at 8 hours we found that all L-929 cells attached on all the coatings as well as the control surface. Most of the cells which attached on the polystyrene control started spreading as it can be seen by morphological change from round to polygonal or spindle shapes, while cells on all polyelectrolyte film coats remained round. At 24 hours, all cells on the control surface were fully spread by extending the cytoplasmic process, having a short spindle shape, which is the expected morphology for L-929. The coverage was found to extend throughout the culture surface. It can also

be seen that some cells on the (PDADMAC/PSS)4 + (PDADMAC/Gelatin)8 started to spread and present a short spindle shape. However, cell morphology on (PDADMAC/PSS)4+(PDADMAC/Gelatin)8 was comparable to those on the control surface at 48 hours. This indicated that this coating could support cell adhesion and spreading as well as the polystyrene culture plate. On the other hand, cells on (PDADMAC/PSS)4+ (chitosan/Gelatin)8 firmly attached and kept a round shape without any cytoplasmic extension even until 48 hours (Figure 3A) or longer culture period (Figure 3B). However, at 24 hours, the majority of the cells on (PDADMAC/PSS)4 were still in an early stage of spreading as a round shape with multiple small cytoplasmic projections and by 48 hours, most of the population turned to have a short spindle shape (Figure 3A). These results suggested that surface coating with (PDADMAC/PSS)4+ (PDADMAC/gelatin)8 was more compatible to the cells than (PDADMAC/PSS)4+ (chitosan/gelatin)8 in the aspects of supporting cell attachment and spreading. The good spreading of the cells on the PDADMAC/gelatin coating is probably due to the presence of gelatin which contains RDG sequence and therefore induces cell attachment and spreading. The hydrophobic nature of gelatin also favors adsorption of protein produced by the cell itself. Interestingly, when the number of the seeded cells was increased or left in culture for a longer period, a number of cells on (PDADMAC/PSS)4 + (chitosan/gelatin)8 formed clumps that grew larger and started to detach from the culture plate, while the other cells remained in monolayer firmly attached to the cultured surface (Figure 3B). The possible cause of clump formation could be that the adhesion among L-929 fibroblast themselves was higher than that cells used for adhering to the coating material, (PDADMAC/PSS)4+ (chitosan/ gelatin)8, in conjunction with the contractile nature of interconnected fibroblasts which are generated by contraction of actin microfilament bundles inside the cells. Previous studies demonstrated that a single fibroblast is capable of a generating force in the immediate surrounding matrix in vitro.[15-17] Therefore, if the contractile force between interconnected network of cells was stronger than the adhesion force between fibroblasts and (chitosan/gelatin)8, cells would roll up, form cell clump and detach eventually. These results demonstrated that the L-929 responded to (PDADMAC/PSS)4+(PDADMAC/gelatin)8 better than (PDADMAC/PSS)4+(chitosan/gelatin)8 by exhibiting normal cell proliferation and spreading in the condition of high cell density (Figure 4B). The poor adhesion on the surface is probably also due to the swelling and decomposition of the chitosan based coating. The next paragraph will give more evidence on the chitosan/gelatin film decomposition.

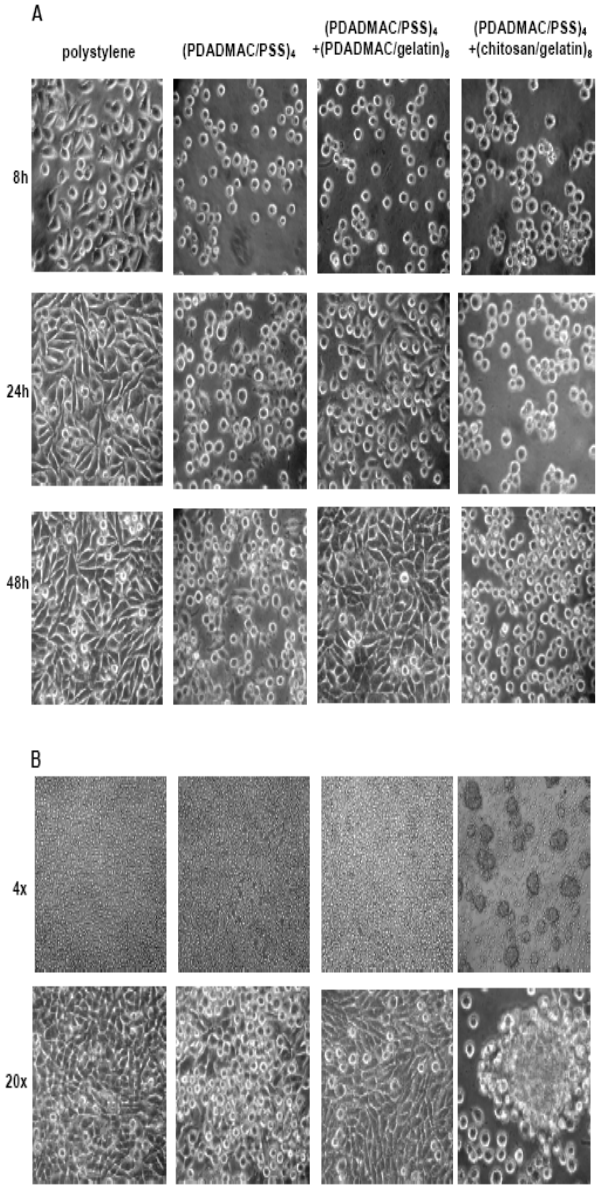

Figure 3. Morphology and distribution of L-929 cells on different polyelectrolyte film coats and polystyrene culture plates at 8 , 24 and 48 hours by using phase-contrast microscope.

Film Stability

As our objective is to use these coatings to improve cell growth, the stability of the film in the

culture medium was investigated. The prepared films were dipped in similar buffered solution as the one used in cell culture for 4 hrs, 8 hrs, 24 hrs and 48 hrs and their thickness was measured using AFM. Figure 4 shows the changes in the thickness of films after dipping in medium solution pH 7.4. The PDADMAC/Gelatin film displayed a good stability with a thickness remaining nearly constant with the increasing dipping time. On the other hand, the chitosan/gelatin film quickly decomposed and its thickness was reduced to 55% after 24-hour exposure to the buffer solution. It is believed that, as stated earlier, the pH change induces deprotonation of the amino groups from NH3+ to NH2 of the chitosan leading to a loss of electrostatic interaction between the two polyelectrolytes and consequently the decomposition of the chitosan/gelatin film. The instability of (PDADMAC/PSS)4+ (chitosan/gelatin)8 film is probably the cause of the poor cell adhesion and spreading of the L-929 cells on the coated substrates.

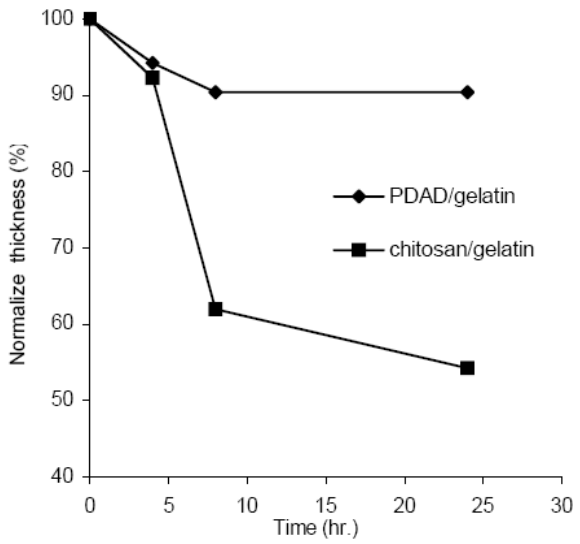

Figure 4. Percent change film thickness of PDADMAC/ Gelatin and chitosan/Gelatin as a function of the dipping time in the buffer solution.

Conclusion

We have shown that multilayer thin films, assembled from PDADMAC and Gelatin, provide a more stable film for the culture of L-929 fibroblast cells than the chitosan/gelatin pair. While both films show good cytocompatibility, thickness measurement with an atomic force microscope showed that the chitosan based film can decompose under physiological pH due to the loss of electrostatic interaction leading to cell detachment and poor spreading. We believe that this layer-by-layer coating from PDADMAC and Gelatin could be of great interest in the development of scaffold for tissue engineering applications.

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