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Cytotoxicity of Free *versus* Multilayered Polyelectrolytes

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Abstract

The cytotoxicity of polyelectrolytes commonly employed for layer-by-layer deposition of polyelectrolyte multilayers (PEMUs) was assessed using rat smooth muscle A7r5 and human osteosarcoma U-2 OS cells. Cell growth, viability, and metabolic assays were used to compare the responses of both cell lines to poly(acrylic acid), PAA, and poly(allylamine hydrochloride), PAH, in solution at concentrations up to 10 mM and to varying thicknesses of (PAA/PAH) PEMUs. Cytotoxicity correlated with increasing concentration of solution polyelectrolytes for both cell types and was greater for the positively-charged PAH than for the negatively-charged PAA. While metabolism and proliferation of both cell types was slower on PEMUs than on tissue culture plastic, little evidence for direct toxicity on cells was observed. In fact, evidence for more extensive adhesion and cytoskeletal organization was observed with PAH-terminated PEMUs. Differences in cell activity and viability on different thickness PEMU surfaces resulted primarily from differences in attachment for these adhesion-dependent cell lines.

Keywords

Cell; cytotoxicity; biocompatible; PAH; PAA; metabolism; layer-by-layer

Introduction

Layer-by-layer deposition of polyelectrolytes, with variables such as polymer pairing, layer number, covalent cross-linking, and deposition ionic strength and pH, can produce surfaces with a wide variety of properties suitable for bio-interfaces.^{1–3} Biofunctional applications of PEMUs can be optimized by “tuning” the rigidity, hydrophobicity, temperature sensitivity, charge density, porosity, topology, and chemical composition of multilayers.^{4–8} One possible application for PEMUs would be to improve the bio-interface of implantable materials. The surface composition of implants affects protein adsorption and inflammatory responses as well as cell adhesion, motility, phenotype, and gene expression,^{9–12} as in the case of restenosis after endovascular stent implantation.^{13–15} Inferior cell adhesion to titanium and its alloys used in orthopedic implants frequently results in osseointegration failures that cause loosening of the implants.¹⁶ Controlling the behavior and phenotype of cells *via* the surface properties of their substrate *in vivo* may lead to the improvement of biomedical devices such as coronary stents, orthopedic, spine, and retinal implants.¹²

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Supporting Information Available

Surface topography and thickness of PEMUs obtained by AFM and ellipsometry. A7r5 and U-2 OS cell nuclear area, nuclear perimeter, and cell death on PEMUs. Differential Interference Contrast Images of A7r5 and U-2 OS cell on PEMUs. Live/Dead cell assay of both cell lines and immunofluorescent staining of vinculin, actin, and DNA for A7r5 cells exposed to media containing varying concentration of solution polyelectrolyte. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Extensive work on a variety of PEMUs has shown that cells grow and proliferate on both negative and positive-capped films,^{17–18} although a few multilayers do not support cell attachment and/or growth^{18–20}. Some components of multilayers, particularly polycations, may be cytotoxic^{21–23} and are valued as antibacterial agents.^{5,24–27} Although using such polyelectrolytes for cell substrates would appear to be contraindicated, the kinetic irreversibility of polyelectrolyte complexation in PEMUs yielding stable multilayered films may eliminate cytotoxicity.^{2,28} On the other hand, possible polymer “leaching” from PEMUs over extended use could affect cell viability. The mobility of polyelectrolytes is known to be enhanced by the presence of salt.^{29–32} Additionally, some films are designed to be degraded by cells to release embedded factors⁴ and other films can be made selectively toxic to bacterial cells while supporting the growth of mammalian cells.^{5,11} Prior investigations of solution polyelectrolyte cytotoxicity have shown that polymer molecular weight and charge density, especially positive charge density, are key cytotoxicity parameters, but cytotoxicity of commonly used polyelectrolytes to various cell types remains poorly understood.^{33–34}

The limited number of reports on encapsulating cells with multilayers, compared to growing them on pre-formed PEMUs, illustrates the problems in contacting cells with polycations. For example, Diaspro et al.³⁵, using lower molecular weight PAH, were able to encapsulate a eukaryotic organism (*S. cerevisiae*, yeast cell), while maintaining proliferative and metabolic functions. However, the ruggedness of the *S. cerevisiae* cell wall was probably critical to success in this experiment. More recently, Kadowski et al.³⁶ found severe toxicity of PAH to fibroblasts, but were able to deposit multilayers on these cells only using a low molecular weight isomer of polylysine where the positive charge resides next to the backbone of the polymer.

While the literature contains reports of cell proliferation on PEMUs, we have observed consistent differences between cell growth on standard plastic or glass and on PEMUs. The present study had several thrusts. First, we investigated the cytotoxicity of the negatively charged PAA and the positively charged PAH in solution. Then we compared the behavior of the same cells to the same polyelectrolytes immobilized in PEMUs. To differentiate the effect on cell proliferation due to the surface charge, and those due to changes in mechanical properties of the substrate³⁷ we compare “thin” and “thick” PEMUs. The smooth muscle A7r5 cells and human osteosarcoma U-2 OS cells tested represent cell types commonly associated with surfaces of coronary and orthopedic implants. In addition, these cell lines have been used extensively to investigate characteristics of smooth muscle cell and osteoblasts-like cells and there is a rich literature on each cell line. Both of these cell lines are of mammalian origin and highly adhesion-dependent. Further investigation of cells with less adhesion dependence and different membrane compositions (such as yeast and bacteria) would contribute to a broader understanding of polyelectrolyte toxicity.

Materials and Methods

Materials

Poly(ethyleneimine) (PEI; 50% w/v in water; molecular weight = 7.5×10^5 Da), poly(allylamine hydrochloride) (PAH; molecular weight = 7×10^4 Da), and poly(acrylic acid) (PAA; 35% wt% in water; molecular weight = 1.0×10^5 Da) were used as received from Sigma-Aldrich (Saint Louis, Mo, USA). Polyelectrolyte solutions for PEMU buildup were 10 mM with respect to the monomer repeat unit in 25 mM Tris-Cl solutions at pH 7.4. Alamar Blue (Invitrogen, Carlsbad, CA, USA) and a Live/Dead Cell Double Staining Kit (Calbiochem, EMD Chemicals, Gibbstown, NJ, USA) were used according to the suppliers' recommendations.

Solution Polyelectrolytes

1.0 M stock solutions of PAH and PAA, made in 18M3 deionized water (DIH_2O) were used to make 10 mM and 1 mM solutions of PAH and PAA in cell growth media. The 1 mM solutions were serially diluted with culture media to make solutions of 0.1 mM, 0.01 mM, and 0.001 mM PAH and PAA. The pH of all polyelectrolyte solutions in culture media was adjusted to the pH of the normal growth media used for each cell line.

Buildup of Polyelectrolyte Multilayers

Glass cover slips (micro cover glass, 18 mm diameter circle No. 1, VWR) were cleaned in “piranha” solution consisting of 70/30 mixture by volume of concentrated H_2SO_4 and 30% H_2O_2 in water for 20 minutes (*caution*: piranha is a strong oxidizer and should not be stored in closed containers), thoroughly rinsed in DIH_2O , and then dried with a stream of nitrogen. The layer-by-layer build up of polyelectrolyte multilayers was performed either manually in culture dishes (BD Falcon Multiwell Flat-bottom Plates, BD Biosciences, San Jose, CA) or by a robot (StratoSequence V, nanoStrata Inc., Tallahassee, FL, USA). For manual coating, cleaned cover slips were first immersed in 10 mM PEI, rinsed with DIH_2O , and dried with a stream of nitrogen directly in wells of tissue culture plates. Buffered 10 mM polyelectrolyte solutions were added to the culture dishes for a 10 minute coating period before rinsing with DIH_2O for three 1 minute intervals. For robot PEMU production, the glass cover slips were coated by immersion in buffered 10 mM PEI solution for 30 minutes, rinsed in DIH_2O , and dried with a stream of nitrogen. The PEI coated cover slips were mounted on a shaft that rotated at 300 rpm with a fixed dipping time of 10 minutes in buffered polyelectrolyte solutions, followed by rinsing with DIH_2O for three 1 minute intervals. After build up of polyelectrolyte multilayers, cover slips were submerged in 150 mM NaCl, 25 mM Tris-Cl; buffer at pH 7.4 for 24 hours, rinsed with DIH_2O , and dried. Apparent Young's modulus of multilayers $\text{PEI(PAA/PAH)}_1\text{PAA}$, PEI(PAA/PAH)_2 estimated below 50 MPa and $\text{PEI(PAA/PAH)}_{21}\text{PAA}$, PEI(PAA/PAH)_{22} estimated above 200 MPa.³⁸

PEMU Nomenclature

PEMUs are designated as A(B/C)_x (an odd number of layers) or A(B/C)_xB (an even number of layers), in which A is the initial coating of polyelectrolyte (PEI in this investigation), B (PAA) is the first layer, C (PAH) is the second layer in paired polyelectrolyte bilayers, and the subscript (x) is the number of B/C paired bilayers. PEMUs with an odd number of layers are terminated with PAH. Those with an even number of layers are terminated with PAA.

Cell Culture and Microscopy

Both the A7r5 rat aortic smooth muscle cells (originally ATCC CRL-1444) and the U-2 OS osteosarcoma cells (originally ATCC HTB-96) were obtained from American Type Culture Collection, but propagated as cell lines in the lab for several years. For the experiments described herein, the A7r5 cells were cultured in high glucose (4500 mg L^{-1} glucose) Dulbecco's modified Eagle medium with L-glutamine (which contains 1.8 mM CaCl_2 and 1 mM MgSO_4 for a total divalent cation concentration of 2.8 mM), pH 7.6 (Sigma-Aldrich) supplemented with 1.5g L^{-1} sodium bicarbonate (recommended for culturing at 5% CO_2 conditions), 10% fetal bovine serum (Standard Hyclone Animal Sera, Thermo Scientific, Waltham, MA), 100 units mL^{-1} penicillin G, 100 $\mu\text{g mL}^{-1}$ streptomycin, 0.25 $\mu\text{g mL}^{-1}$ amphotericin B (Antibiotic-Antimycotic) and 10 $\mu\text{g mL}^{-1}$ gentamicin (Gentamicin Reagent) (both from Invitrogen). The U-2 OS cells were cultured in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham with L-glutamine (which contains 1 mM CaCl_2 , 0.2 mM MgCl_2 , and 0.5 mM MgSO_4 , for a total divalent cation concentration of 1.7 mM) and 15 mM Hepes, pH 7.3 (Sigma-Aldrich) supplemented with 1.2g L^{-1} sodium bicarbonate, 10% Cosmic Calf Serum (Thermo Scientific), 100 units mL^{-1} penicillin G, 100 $\mu\text{g mL}^{-1}$

streptomycin, $0.25 \mu\text{g mL}^{-1}$ amphotericin B and $10 \mu\text{g mL}^{-1}$ gentamicin. Both cell lines were cultured at 37°C with 5% CO_2 (Water Jacket CO_2 incubator Nu-4750, NuAire, Plymouth, MN, USA).

Cells were imaged with a Nikon Ti-E inverted microscope using a Nikon Intensilight C-HGFI and a Cool Snap HQ2 camera from Photometric and Nikon TS100 Tissue Culture and Nikon DS-Ri1 camera.

Alamar Blue Assay

For analysis of solution polyelectrolyte effects on cell metabolism, both A7r5 and U-2 OS cells were plated at 1×10^4 cells per well in BD Falcon 96 multiwell flat-bottom plates (BD Biosciences) containing 100 μL of growth media. The cells were incubated at 37°C with 5% CO_2 for 24 hours. After this initial incubation period, the growth media was removed and replaced with 100 μL of growth media containing polyelectrolytes followed by the addition of 10 μL of Alamar Blue. Both cell lines were incubated in the presence of 0 mM, 0.001 mM, 0.01 mM, 0.1 mM, 1 mM, and 10 mM PAH and PAA for 24 hours. For analysis, 90 μL of cellular growth media from each well was transferred into 96-Well optical-Bottom Plates (Thermo Fisher Scientific, Pittsburg, PA, USA) and reduced Alamar Blue was determined as follows: PEMU effect on cell metabolism was determined with cells grown on PEMU-coated cover slips in BD Falcon 12 multiwell flat-bottom plates. The following surfaces were tested: culture dish (uncoated plastic), piranha cleaned bare cover slip (glass), and cover slips coated with $\text{PEI(PAA/PAH)}_1\text{PAA}$, PEI(PAA/PAH)_2 , $\text{PEI(PAA/PAH)}_{21}\text{PAA}$, PEI(PAA/PAH)_{22} . Both A7r5 and U-2 OS cells were plated at 1.25×10^5 cells per well (note: a well-growth area ratio of $0.32\text{cm}^2/4\text{cm}^2$ for 96-well plates/12-well plates was used to determine the number of cells seeded in a 12 well plate to cover the same amount of growth area as in the 96 well plates). The cells were incubated on the surfaces at 37°C with 5% CO_2 for 24 hours in 1.25mL cell growth media. After the initial incubation period, the growth medium was replaced with fresh 1.25mL cell growth media containing 125 μL of Alamar Blue, and the cells were incubated for another 24 hours before analyzing reduced Alamar Blue.

Reduced Alamar Blue in aliquots of the growth media was determined using a Molecular Devices SpectraMax MicroPlate Reader with the following parameters: fluorescence analysis, bottom well reading, excitation at 540nm (Alamar Blue excitation range 540nm–570nm), emission at 600nm (Alamar Blue emission range 580nm–610nm), automatic cutoff, sensitivity at six readings per well reporting average relative fluorescence units (RFUs). Two trials were conducted for each PEMU and polyelectrolyte concentration. Data was analyzed using SoftMax Pro software. The ratio of reduced Alamar Blue in each of the experimental samples compared to controls (culture media without polyelectrolytes and tissue culture dish plastic) was plotted. Statistically significant differences from control samples were determined using the student T-test ($p < 0.05$).

Cell Counting for Growth Curves

Both A7r5 and U-2 OS cells were plated at 0.5×10^4 cells per well into BD Falcon 12 multiwell flat-bottom plate either directly onto the plastic or onto PEMU-coated cover slips. The cells were incubated in 2 mL of growth media at 37°C with 5% CO_2 for 24 hours. After the initial incubation period, the normal growth media in the wells was removed and replaced with 2 mL of growth media containing solution concentrations of up to 10 mM PAH and PAA as described above. The cells were incubated in the presence of these polyelectrolyte concentrations for up to 12 days. On days 1, 6, 8, and 12 of exposure to the solution polyelectrolytes and days 1, 3, 7, and 10 of exposure on PEMUs, the cells were harvested by adding 0.5mL of TrypLE (Invitrogen) at room temperature for 5 minutes and

using a cell scraper (Corning, NY) to detach all the cells from substrate. The cells were counted using a Phase Hemocytometer (Hausser Scientific, Horsham, PA) and a Nikon TS100 Tissue Culture Phase Contrast Microscope. The total number of cells counted for each experimental condition was plotted against the number of days the cells were incubated under those conditions. Three trials were conducted for each PEMU and polyelectrolyte concentration.

Live/Dead Cell Double Staining

For analysis of solution polyelectrolyte and PEMU effects on cell proliferation/survival, both A7r5 and U-2 OS cells were plated at 0.5×10^4 cells per well into BD Falcon 12 multiwell flat-bottom plate and incubated for 4 days either on clean glass cover slips immersed in 2 mL of growth media containing concentrations of PAH and PAA polyelectrolytes up to 10 mM or PEMU-coated cover slips. On the fourth day of incubation, the Live/Dead Double Staining Kit was used according to the supplier's recommendation (EMD Chemicals, Calbiochem, Gibbstown, NJ, USA). The kit uses a cell-permeable green fluorescent Cyto-dye (maximum excitation 488 nm; maximum emission 518 nm) to stain live cells and an impermeable red fluorescent dye propidium iodide, (maximum excitation 488 nm; maximum emission 615 nm) to stain dead cells. The cells were imaged using FITC and Texas Red filters. Nuclei area, perimeter, and cell counts for percentage of dead cells were calculated with ImageJ software.

Results and Discussion

Effect of Polyelectrolytes on Cell Metabolism

The effects of solution PAA and PAH at concentrations ranging from 0.001–10 mM in cellular growth media on the metabolism of A7r5 and U-2 OS cells were compared to the effects of the same polymers immobilized in polyelectrolyte multilayers. Alamar Blue, a sensitive oxidation/reduction fluorescent and colorimetric indicator^{39–41}, was used to measure cellular metabolism. To evaluate the effects of the solution polyelectrolytes, both cell types were allowed to attach to tissue culture plastic wells for 24 hours. The cells were incubated for an additional 24 hours in fresh growth media containing the various concentrations of PAA or PAH and Alamar Blue. The two cell types were also cultured for 24 hours in growth media containing Alamar Blue on PEMUs of 1, 2, 21, and 22 bilayers with a PAH or an additional PAA terminal layer. All assays were done in duplicates. After the 24 hour incubation period, the Alamar Blue reduction in the media for each sample was determined. The ratio of the Alamar Blue reduction mean values for each experimental condition compared to that of the control samples was determined for each cell line.

Solution PAA and PAH elicited different responses on cell metabolism (Figure 1). At 0.1 mM, neither solution PAA nor PAH was toxic to either cell type (Figure 1, A). PAH above 0.1 mM caused metabolic activity in both cell lines to fall below 50% of the control cells. In contrast, concentrations of PAA above 0.1 mM caused no change in A7r5 cells and U-2 OS, except at 10 mM PAA concentrations where U-2 OS cells demonstrate 40% increase. This PAA-induced increase in metabolism may indicate that the higher PAA concentrations, while not lethal, causes cell stress that increases metabolism.

When grown on the thin PAA-terminated PEMU, both A7r5 and U-2 OS cells exhibited little metabolic difference from control cells grown on tissue culture plastic, but growth on the thin PAH-terminated surface decreased the metabolic activity of the U-2 OS cells by approximately 30%. On the thicker PEMUs, both cell lines exhibited decreased metabolic activity, but the differences between the terminating polyelectrolytes were less pronounced, with only a 5% metabolic difference between cells grown on the thicker PAA- and PAH-

terminated PEMUs. The improved biocompatibility of polycations when complexed with polyanions is clearly evident by comparing the results for 1 and 10 mM PAH in Figure 1B with those for the PEMUs.

Phase contrast imaging of cells exposed to dissolved polyelectrolytes (Figure 2) revealed trends similar to those from the Alamar Blue assay. Morphology changes attributable to toxicity of solution PAH at high concentrations (1 mM and 10 mM) for both A7r5 and U-2 OS are clearly observed within the first 30 minutes following addition of media containing the polyelectrolytes. Figure 2 illustrates the destruction of cellular membrane of U-2 OS cells in the presence of 1 mM and 10 mM PAH. Following PAH extraction of the cell membrane and cell contents into floating globules, the globules become deposited across the culture dish. In contrast, solution PAA at similar concentration did not produce comparable destruction of cell membranes. A control with PAH and cell media only (2E) showed globule deposition was not attributed to cell media or its interaction with PAH.

Long Term Exposure to Solution PAA/PAH Polyelectrolytes or PEMUs Effects on Cell Proliferation

To assess effects of PAA and PAH on A7r5 and U-2 OS cell proliferation, growth plots were constructed for cells exposed to different concentrations of both polyelectrolytes (Figure 3) and to thin and thick PAA- or PAH-terminated PEMUs (Figure 4). Compared to control cultures with no added polyelectrolyte, exposure to solution PAH and PAA caused a general reduction in cell proliferation over 12 days (Figure 3). Each tenfold increase in solution PAH concentration above 0.1mM caused approximately a 50% decrease in cell count for both cell types. Solution PAH at 1 mM and 10 mM killed all the seeded cells within 24 hours. In contrast, both cell types tolerate solution PAA much better. Exposure of U-2 OS cells to PAA at concentrations up to 1 mM result in no significant cell count difference compared to controls even after 12 days. At 10 mM PAA concentrations, both U-2 OS and A7r5 cell counts decreased after 12 days of exposure. However, unlike exposure to 10 mM PAH, both cell types maintained approximately 40% cell counts compared to control conditions. Although necrotic membrane destabilization occurs at PAH concentrations greater than 0.1 mM PAH in normal growth media (Figure 2), cell necrosis was not found at lower PAH concentrations, in which cells maintained normal metabolic activity (Figure 1) and proliferation (Figure 3). Using lower concentrations as well as lower molecular weight forms of PAH were key to early attempts to encapsulate cells with PEMUs.^{21,23,35,42} Similarly, Kadowaki et al. cultured fibroblast cells on films made with cytotoxic polycations (including PAH) with no adverse effects on cell proliferation or morphology.³⁶ In contrast, polyelectrolyte films deposited on top of the cells decreased proliferation and reduced cell spreading.³⁶

When incorporated into PEMUs, PAH and PAA have significantly less effect on proliferation of both cell lines, despite existing in locally high concentrations within the microenvironment of cell contact (Figure 4). Although both cell types proliferated on the PEMUs over 10 days, there were fewer A7r5 cells on both the thin and thick PEMUs than on the tissue culture plastic and glass cover slips by day 3. Exposure to the thick PEMUs caused the most significant differences in the total cell counts over the course of ten days. Although the PAH-terminated PEMUs yielded lower cell counts regardless of thickness, the difference in total cell count between the PAA- and PAH-terminated PEMU was less on the thick PEMUs. The characteristics influencing cell morphology that change most with increasing PEMU thickness are swelling and stiffness. Mendelsohn et al. demonstrated that PAH/PAA PEMUs at 20 layers maintained overall cytophilic or cytophobic characteristics independent of the last polyelectrolyte deposited, because PEMU swelling influenced cells adhesion more.⁴³ Similarly, Mehrotra et al. utilizing PDADMAC/PSS films correlated changes in cell adhesion with changes in a films stiffness as film thickness increases.⁴⁴ Both

of these investigations highlight surface modulus as a predominant variable influencing cell adhesion. Analysis of surface topography (Supporting Information) for the PEMUs we used showed roughness to be negligible regardless of film thickness. In addition, divalent cations can affect polyelectrolyte activity, but the difference between the 2.8 mM total divalent cation concentration in the A7r5 culture medium and the 1.7 mM total concentration in the U-2 OS cell culture medium was judged insignificant for these studies and not tested for effect on cell adhesion to PEMUs.

PEMU effect on cell survival

To determine whether exposure to PEMUs has a direct cytotoxic effect on the cells, cell death for both cell types on PAA/PAH PEMUs and varying concentrations of solution polyelectrolytes (Supporting Information) was assessed with a dual fluorescence dye staining kit that stains live cells with a membrane permeable green fluorescent Cyto-dye and dead cells also with a membrane impermeable propidium iodide red fluorescent dye. Uptake of both dyes into cells with compromised plasma membranes stains nuclei orange. After four days in culture, few dead cells were found on any of the PEMUs tested, but the thin PEI(PAA/PAH)₁PAA and PEI(PAA/PAH)₂ PEMUs had a lower total cell death percentage than the thicker PEI(PAA/PAH)₂₁PAA and PEI(PAA/PAH)₂₂ PEMUs. The thick PAH-terminated PEMU had the highest percentage of dead cells (Figure 5).

Survival of the majority of cells even on the thick PAH-terminated PEMU indicates that incorporation of PAA and PAH into a PEMU neutralizes PAH cytotoxicity through the formation of stable polyvalent polycation-polyanion interactions that inhibit the membrane destructive forces illustrated in Figure 2. Stable incorporation in a PEMU also likely prevents possible internalization of toxic amounts of polyelectrolyte by mechanisms such as endocytosis, which is the mechanism by which cationic liposomes facilitate the uptake of DNA into cells for transfection.⁴⁵

The metabolic activities, cell growth curves and cell survival rates point toward a consistent picture for the impact of polyelectrolytes on the two cell types: in solution, the toxicity of PAH is clearly seen by the lack of metabolic output (Figure 1) and cell growth (Figure 3). The cause of this toxicity is clearly revealed in the micrographs in Figure 2, which show wholesale mobilization and then redeposition of membrane components. The polyanion has less effect on cell metabolism and growth. In fact, Figure 1 indicates accelerated metabolism, which may be a result of cell stress, yet the growth curves in Figure 3 show few differences when compared to the control except for the very highest concentration of PAA (10 mM).

When incorporated in multilayers, the toxic effects of PAH are clearly attenuated, but the metabolic rate and growth curves are somewhat lower on PEMUs than on the control uncoated substrates. The concept of “toxicity” must be used with caution, however, as there is little evidence of cell damage on PEMUs (Figure 5). The two cell lines investigated here depend on adhesion for growth, survival and proliferation. Thus, factors that directly or indirectly influence adhesion will also control growth and anoikis - apoptosis caused by lack of adhesion. Adhesion is a prerequisite in preventing apoptosis and maintaining biological functions such as growth, motility, and survival.⁴⁶ It has been well established that PEMU type controls adhesion^{17–18} and morphology (including phenotype)^{12,17,20} and ultimately proliferation^{18–19,21}. An important component of this control is the mechanical compliance of the surface, with stiffer substrates favoring adhesion.^{47–50} Because the apparent modulus of the film is coupled to the modulus of the substrate,^{38,50} thinner films have greater apparent modulus than thicker ones. This would explain the decrease in metabolic activity (Figure 1) and the lower growth rates (Figure 4) of the cells on the thicker compared to the cells on the thinner PEMUs. Cells are able to detect the thickness and stiffness of compliant

substrates via adhesion-induced deformations⁵¹ and as demonstrated by Mehrotra et al. changes in cellular adhesion on PDADMAC/PSS films correlated to changes in film stiffness and thickness.⁴⁴ Cells on thin PEMUs will “feel” a stiffer substratum possibly because of reduced traction-induced displacements caused by supportive mechanical contributions of the stiffer substrate underneath the film.⁵¹ Adjustments to focal adhesion size and morphology are in response to changes in the effective stiffness to maintain a homeostatic energy level equivalent to the work done by cells to the films.⁴⁴ Increasing PAH/PAA PEMUs thickness decreases compliance³⁸ and for both cell types cultured on these films (greater than 20 layers) swelling also influenced cell adhesion.⁴³ Inspection of the morphology of cells also reveals differences in adhesion behavior. For example, cells on the thicker PEMUs (Figure 5) were more clustered, indicating poorer adhesion and more self-association. By day 10, cells on thicker PEMUs exhibited the same degree of slower growth regardless of the top layer (Figure 4). Another indicator of differential adhesion is the apparent differences in nucleus size between the cells on the PEMUs and those on the control plastic and glass surfaces (see Supporting Information). The apparently smaller diameters of the nuclei in the images of the cells on the PEMUs, especially the thicker PEMUs, indicate that the nuclei are less compressed than in the better spread cells on the control surfaces. Greater cell death percentages for both cell types were observed for cells on thicker PEMUs (see Supporting Information), however, unlike the necrotic cell death observed with increasing concentrations of solution PAH (Figure 2) dead cells were determined to have undergone anoikis, an apoptotic cells death resulting from a reduction of cellular adhesion. Cells demonstrated typical morphological characteristics of anoikis (see Supporting Information) - fragmented nuclei, rounded cell bodies, dendritic extensions and blebbing of the plasma membrane (indicating poor adhesion).^{52–54}

More subtle morphological differences in surface charge were seen for thinner PEMUs. For example, PAH appeared to be favored over PAA for adhesion. Despite the significant solution cytotoxicity of PAH, cell types cultured on the PAH-terminated PEI(PAA/PAH)₂ (Figure 6, A3 and B3) had prominent vinculin-containing focal adhesions in lamellipodia and robust tensin-containing fibrillar adhesions that terminated in the focal adhesions, especially in the A7r5 cells. In both cell lines cultured on the PAA-terminated PEMUs (Figure 6, A2 and B2), tensin was less extensively organized and the fibrillar adhesions were confined to the center of the cell and most did not extend to the vinculin-containing focal adhesions. Interestingly, the vinculin and tensin localization of the A7r5 cells grown on PAA-terminated PEMU more closely resemble those characteristics of cells on uncoated glass cover slips, whereas the characteristics of the U-2 OS cells PAH-terminates PEMU more closely resembled those of the U-2 OS cells on glass. In all cases tested, the PEI initial layer had no detectable negative effect on cell growth. In contrast to cell adhesive morphology on thicker PEMUs (see Supporting Information), cells on thin films exhibited morphologies and behavior consistent with a stiffer environment due to finite substratum thickness.⁵¹ Importantly, these morphological differences were not observed in either cell type grown in media containing ‘non-necrotic’ solution polyelectrolyte concentrations (Supporting Information).

Conclusion

Analysis of polyelectrolyte effects on A7r5 and U-2 OS cell metabolism, proliferation, and survival demonstrated that solution positively charged PAH was much more toxic to both cell types than was solution negatively charged PAA. Even at the lowest concentration (0.001 mM), solution PAH significantly decreased proliferation of both cell types. Necrotic cell disruption was clearly observed with increasing concentration of solution PAH. In contrast, PAA was well tolerated at concentrations up to 1 mM, a concentration at which PAA actually caused stimulation of metabolism in both cell types. Incorporation of the PAA

and PAH polyelectrolytes into PEMUs neutralizes the cytotoxic/cytostimulatory effects through the formation of stable polyvalent polycation-polyanion interactions, which effectively remove the availability of these components for membrane interactions or cell uptake. Slower cell growth on PEMUs, more apparent for the A7r5 line, is attributed to adhesion and morphological changes due to the substrate surface charge and/or the apparent modulus of the multilayer and not to direct toxic effects of the PEMUs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Alamar Blue reduction ratios for cells in solution PAA and PAH polyelectrolyte and on PEMUs

The metabolism of A7r5 (triangles) and U-2 OS (squares) cells grown for 24 hours on tissue culture plastic in the presence of solution polyelectrolytes PAA or PAH (A), or on PEMUs (B), was measured using an Alamar Blue reduction assay. The ratio of the mean Alamar Blue reduction value for each experimental condition to untreated controls are plotted for solution PAA and PAH concentrations between 0.001 mM and 10 mM (A) and for thin and thick PEMU layers terminated with PAA or PAH (B). Mean Alamar Blue reduction values are calculated from two experimental trials consisting of two independent samples for each condition. Statistically significant differences from control samples determined using the student T-test ($p < 0.05$) are marked with an asterisk (*).

Figure 2. Phase Contrast Images of U-2 OS cells in solution PAH and PAA

U-2 OS cells after 24 hour culture were treated for 30 min with normal growth media (A), media containing 10 mM PAA (B), 1 mM PAH (C), and 10 mM PAH (D). Scale Bar, 100 μ m (A–D). Media containing 10mM PAH after 24 hour incubation on culture dish plastic in the absence of cells (E) scale Bar, 50 μ m. The boxed areas in the lower row of images are enlarged in the upper row of images.

Figure 3. Growth of A7r5 and U-2 OS cells in media containing various solution PAH and PAA polyelectrolyte concentrations

Total cell counts of A7r5 (A) and U-2 OS (B) cells on days 1, 6, 8, and 12 of growth in the presence no added polyelectrolyte (a, gray) or solution PAA (b–f, red) and PAH (g–k, blue) at concentrations of 0.001 mM (b and g), 0.01 mM (c and h), 0.1 mM (d and i), 1 mM (e and j), and 10 mM (f and k). Both cell lines were seeded at 5.0×10^3 cells per well on day 0 (◆). Inset (B) day 1 plotted with different cell count scale. Star marked cell count columns are significantly different from those of control cells grown in the absence of added polyelectrolyte (P-value < 0.05).

Figure 4. Growth of A7r5 and U-2 OS cells on thin and thick PEMUs terminated with PAA and PAH

Total cell counts on days 1, 3, 7, and 10 after seeding of A7r5 (A) and U-2 OS (B) cells on: (a, white) culture dish plastic, (b, gray) glass cover slip, (c, red) PEI(PAA/PAH)₁PAA, (d, blue) PEI(PAA/PAH)₂, (e, red) PEI(PAA/PAH)₂₁PAA, and (f, blue) PEI(PAA/PAH)₂₂ PEMUs on glass cover slips are plotted. Both cell lines were seeded at 5×10^3 cells per well on day 0 (◆). Inset (B) day 1 plotted with different cell count scale. Star marked cell count columns are significantly different from those of control cells grown on uncoated culture dish plastic (p-value < 0.05).

Figure 5. Live/Dead double staining assay of A7r5 and U-2 OS cells on thin and thick PEMU surfaces terminated with PAA and PAH

A7r5 (PA,GA, A1–A4) and U-2 OS (PB, GB, B1–B4) cells grown for four days on plastic culture dish (PA, PB), glass cover slips (GA, GB), PEI(PAA/PAH)₁PAA (A1,B1), PEI(PAA/PAH)₂ (A2,B2), PEI(PAA/PAH)₂₁PAA (A3,B3), and PEI(PAA/PAH)₂₂ (A4, B4) were stained using the Calbiochem Live/Dead Double Staining Kit. Cyto-dye (green) and propidium iodide (red) staining of dead cells yielded orange-red nuclei. Scale Bar, 100 μ m.

Figure 6. A7r5 and U-2 OS cells on PAA/PAH PEMU surfaces with PEI as the initial layer A7r5 (A1–A3) and U-2 OS (B1–B3) cells cultured on uncoated glass cover slips (A1, B1), PEI(PAA/PAH)₁PAA (A2, B2), and PEI(PAA/PAH)₂ (A3, B3) surfaces for 48 hours, were stained for vinculin (red), tensin (green) and DNA (blue). Three panels under each of the major images (A1–B3) show higher magnification separate and merged images of the tensin (green) and vinculin (red) localization in the region of the arrow in the major image. Scale Bar, 20 μ m.