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High-throughput cellular screening of engineered ECM based on combinatorial polyelectrolyte multilayer films

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ABSTRACT

The capacity to engineer the extracellular matrix is critical to better understand cell function and to design optimal cellular environments to support tissue engineering, transplantation and repair. Stacks of adsorbed polymers can be engineered as soft wet three dimensional matrices, with properties tailored to support cell survival and growth. Here, we have developed a combinatorial method to generate coatings that self assemble from solutions of polyelectrolytes in water, layer by layer, to produce a polyelectrolyte multilayer (PEM) coating that has enabled high-throughput screening for cellular biocompatibility. Two dimensional combinatorial PEMs were used to rapidly identify assembly conditions that promote optimal cell survival and viability. Conditions were first piloted using a cell line, human embryonic kidney 293 cells (HEK 293), and subsequently tested using primary cultures of embryonic rat spinal commissural neurons. Cell viability was correlated with surface energy (wettability), modulus (matrix stiffness), and surface charge of the coatings.Our findings indicate that the modulus is a crucial determinant of the capacity of a surface to inhibit or support cell survival.

1. Introduction

The extracellular matrix (ECM) is a complex mixture of proteins, polysaccharides, and growth factors that provide structural support and mediate cellular function. Synthetically mimicking natural ECM is a major goal of tissue engineering for therapeutic applications such as restoring, maintaining, or enhancing tissue function and for *in-vivo* diagnostic applications such as testing for drug toxicity and measuring metabolism. The complexity of cell-ECM interactions makes understanding the underlying principles of matrix function paramount to advancing the field [1]. Two main categories important for anchorage-dependent cellular development have been identified; the biological ECM protein interactions [2,3] that function through *specific* receptor-ligand signaling mechanisms, and physical *non-specific* interactions [4] that are dependent on the structural properties of the substrate. Although the bulk of research has focused on specific signaling mechanisms, there is growing evidence suggesting that non-specific physical properties of the substrate such as surface charge [5–7], surface energy [8–12], and the modulus of the coating [13–20], play crucial roles in cellular structure and function. However, the effects of these physical properties on cell behavior are usually measured independently of each other. This inherently leads to confounding effects as some properties are not accounted for, limiting the value of the result. The challenge to achieving a better understanding of substrate property effects stems from the large parameter space; however, it is tedious to handle such complexity with a conventional one sample approach. High-throughput combinatorial experimental strategies allow for a large number of variables to be addressed simultaneously and have been used to develop materials such as biodegradable polymers [21], polymeric supports for organic synthesis [22], sensors for herbicides [23], and non-cytotoxic materials [24,25].

Ideal candidates for making combinatorial substrates are charged polymers called polyelectrolytes that can be sequentially layered through an alternating layer-by-layer method to make stacks of polyelectrolytes called a polyelectrolyte multilayer (PEM) [26,27]. A major advantage of PEMs is that they can be made from any charged water-soluble polymer with any number of layers, and each layer can have a tunable internal architecture and density. The two most basic and well-studied polyelectrolytes used to make PEMs are poly (allylamine hydrochloride) (PAH), a carbon backbone polymer with pendent amine groups (Fig. 1A), and poly (acrylic acid) (PAA), which has pendent carboxylic acid groups (Fig. 1B). The build-up is initiated by submerging a negatively charged substrate into a solution of PAH. The positively charged

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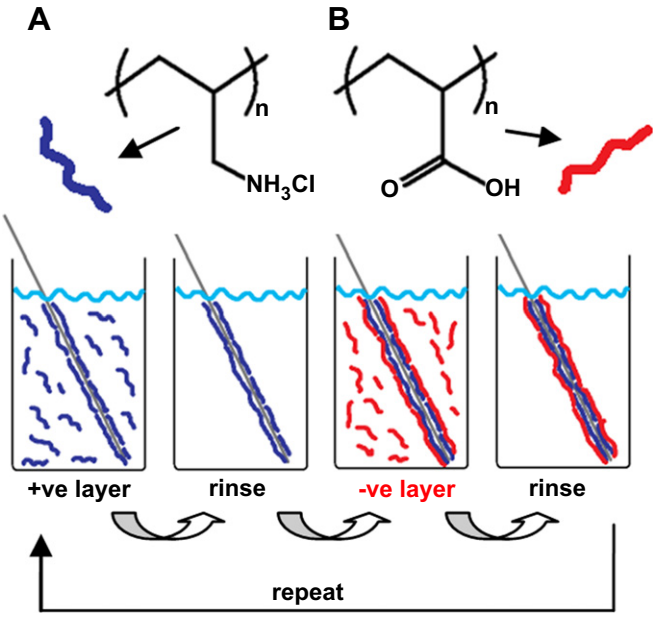

Fig. 1. Molecular structures of A) Poly(allylamine hydrochloride) and B) Poly(acrylic acid) and C) an illustration of a general 1-b-l PEM fabrication process.

polymer self-assembles onto the surface, masking and reversing the negative charge and making the surface positively charged (Fig. 1C). The positively charged coated substrate is then submerged into a solution of PAA and similarly a second layer of polymer is deposited onto the substrate, reverting the charge back to negative. This process is repeated to generate a coating that is held strongly together by many electrostatic cross-links. Additionally, since PAA and PAH are weak polyions, the degree of charge per chain (i.e. $-COO^-/-COOH$ and $-NH_2/-NH_3^+$ ratios) is influenced by the pH of the deposition solution, which provides control over the conformation (e.g. linear or loopy) of the polymer chains making up the coatings. By changing the pH of the two polymer solutions, the layer-by-layer method provides control over physical properties such as the surface charge, surface energy, thickness, water content, and the modulus of the coatings that are important for 'bioactivity'. Such tunability makes PEMs attractive materials that have found their way into a wide range of applications, including optical coatings [28], macromolecular encapsulation [29,30], and non-cytotoxic films [31]. PEMs with these specific properties are typically made individually; however, due to the large assembly parameter space, optimizing these precisely tailored coatings can be prohibitively time-consuming and expensive.

As an initial step toward engineering enhanced extracellular environments, we developed a high-throughput combinatorial instrument for the fabrication of gradient PEM films. The instrument works by continuously altering the amount of charge of solvated PAA and/or PAH chains as the film is layered across the plane, which is done by varying the pH of the adsorption bath. By rotating the substrate ±90 ° after each layer is deposited, 2D thickness-gradient films were made, representing on just a few square centimeters the equivalent parameter space of many thousands of individual uniform films. The films were then characterized under water by determining the average thickness, surface energy, and modulus across the entire area of the film. The capacity of a surface to support cell growth was then determined by examining the survival of HEK 293 cells and embryonic rat neurons, and related to the physical properties at that *x* and *y* location on the film.

2. Methods

2.1. Assembly of 2D gradient pH films

7 × 7 cm cleaved silicon wafers (University Wafer, San Jose, CA) were gradually immersed into a solution of poly(allyl amine hydrochloride) (PAH, MW 65,000, Sigma Aldrich) at a varying pHs with stirring. The resultant film was then rinsed, rotated by 90°, and gradually immersed into a solution of poly(acrylic acid) (PAA, MW = 100,000, Sigma Aldrich) at a varying pHs. The film was rinsed and the process was repeated until the desired number of layers was deposited.

2.2. Thickness measurements

The thickness of the gradient multilayer films was measured using single wavelength (633 nm) null-ellipsometry (Optrel Multiskop, Germany) fixed at 70 ° to the normal. For underwater ellipsometry, films were submerged in water for 24 h prior to measurement to ensure full hydration, as per the *in-situ* techniques described previously by our group [32–34]. Films were placed on a mobile stage (Δ 1 mm) and Δ and ψ measurements were taken at 5 mm intervals. These measurements were then processed using an appropriate model (water ($n = 1.33$)// film ($t = x$, $n = x$)//SiO_2 ($t = 2.3$ nm, $n = 1.54$)//Si ($n = 3.42$, $k = -0.011$), to obtain thickness and refractive index values.

2.3. Cell survival assays

Human embryonic kidney 293 (HEK 293) cells were cultured in Dulbecco's modified Eagle medium (DMEM, Invitrogen) supplemented with 100 unit/mL penicillin G (Invitrogen), 100 μg/mL streptomycin (Invitrogen), and 10% fetal bovine serum (Invitrogen). Cultured cells were incubated in a 5% CO_2 and 37 °C humidified incubator. Spinal commissural neurons were isolated from embryonic day 13.5 (E13.5) Sprague–Dawley rat embryos and cultured in Neurobasal medium (Invitrogen) supplemented with 100 unit/mL penicillin G, 100 μg/mL streptomycin, 2 mM GlutaMAX-1 (Invitrogen) and 10% fetal bovine serum. After 16–24 h in culture, the medium was replaced with Neurobasal medium supplemented with 100 unit/mL penicillin G, 100 μg/mL streptomycin, 2 mM GlutaMAX-1 and 2% B-27 (Invitrogen). For adhesion and growth assays, 15,000 HEK 293 cells/cm^2 and ~1 million neurons were plated per PEM film coated silicon wafers. HEK 293 cells following 1 day *in vitro* (DIV) and spinal commissural neurons at 2 DIV were fixed with 4% paraformaldehyde (PFA, Fisher Scientific) and 0.1% gluteraldehyde (Sigma) for 60 s and then blocked with 3% heat inactivated horse serum (HS, Invitrogen) and 0.1% Triton X-100 (Fisher Scientific). Cells were stained with 0.8 unit/mL Alexa 488 coupled Phalloidin (Invitrogen) and 500 ng/mL Hoechst 33258 (Invitrogen). Films were cover slipped using FluoroGel (Electron Microscopy Sciences).

2.4. Cell imaging and counting

Cells were imaged using an Axiovert 100 inverted fluorescence microscope (Carl Zeiss Canada, Toronto, ON) with a Magnafire CCD camera and MagnaFire 4.1C imaging software (Optronics, Goleta, CA). Images were captured at positions equivalent to thickness measurement locations (controlled by an x-y Δ1 mm stage). The number of cells was quantified by counting Hoechst positive nuclei using ImageJ software (U. S. National Institutes of Health, Bethesda, Maryland, USA). The macro used for cell counting consisted of conversion to a 16-bit picture format, background subtraction, threshold adjustment to exclude background, conversion to binary, and a cell count/cell area calculation. The same macro was used for all images to ensure consistent counts. To measure the surface area of embryonic spinal commissural neurons, cells were stained with fluorescently labeled phalloidin to visualize F-actin and total cell area per image calculated.

2.5. Modulus measurements using Atomic force Microscopy (AFM)

Force measurements of the multilayer films were performed using an AFM in force calibration mode (Nanoscope Version 3A, Digital Instruments), using protocols previously described by our group [35]. The multilayer surface and the tip were brought together in a fluid cell at room temperature. Silicon nitride probes were used (radius = 20–60 nm) with a manufacturer specified force constant, *k*, of 0.12 N/m. All elasticity measurements of the films were performed with the same AFM tip; thus, no calibration for the absolute spring constant of the tip was done. The AFM detector sensitivity was calibrated by obtaining a force curve on a baer substrate and determining the slope of the linear portion of the data after contact. Obtaining force curves of the multilayer film involved bringing the tip in close contact with the surface in aqueous media and obtaining force measurements after allowing the system to equilibrate for 10 min, or until reproducible curves were observed. The rate of the indentation cycle was kept constant at 0.2 Hz. For elasticity measurements, four replicate measurements of the tip deflection as a function of the piezo z-position were acquired with the unmodified AFM tip.