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Cellular transduction gradients via vapor-deposited polymer coatings

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Summary

Spatiotemporal control of gene delivery, particularly signaling gradients, via biomaterials poses significant challenges because of the lack of efficient delivery systems for therapeutic proteins and genes. This challenge was addressed by using chemical vapor deposition (CVD) polymerization in a counterflow set-up to deposit copolymers bearing two reactive chemical gradients. FTIR spectroscopy verified the formation of compositional gradients. Adenovirus expressing a reporter gene was biotinylated and immobilized using the VBABM method (virus-biotin-avidin-biotin-materials). Sandwich ELISA confirmed selective attachment of biotinylated adenovirus onto copolymer gradients. When cultured on the adenovirus gradients, human gingival fibroblasts exhibited asymmetric transduction with full confluency. Importantly, gradient transduction occurred in both lateral directions, thus enabling more advanced delivery studies that involve gradient of multiple therapeutic genes.

Keywords

tissue engineering; gene therapy; CVD coatings; polymers; virus immobilization

1. Introduction

In development, biochemical gradients play essential roles in specifying pattern formation. For example, signaling molecule gradients from the zone of polarizing activity [1] control patterning across the antero-posterior axis of the chick limb bud.[2,3] Gradients also facilitate cell migration and differentiation during organ development as diverse as the villi [4] of the small intestine or patterning of neuronal axons.[5–7] Signaling gradients also play an important role in adult organisms where cellular and inflammatory responses relay cellular signals via cytokine gradients.[8,9] In order to guide regeneration and differentiation of cells, biomolecular gradients may be necessary to mimic their natural microenvironments,

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especially in the context of osteochondral tissue and other multi-tissue interfaces.[10] In many of these scenarios, the process could be mimicked by delivering either a therapeutic gene or protein.

In an effort to mimic normal biologic processes, a wide variety of methods have been developed to fabricate biomaterials with polymer and/or biomolecular gradients.[11,12] For example, dip-coating [13], gradients of photoreactivity [14], and microfluidic mixing [15–17] have been used to fabricate biomolecule gradients on a range of biomaterials. While such methods are robust and reproducible, there are limitations that may hinder translation of these experimental methods into clinically viable solutions. Efficient translation of microfluidic systems to a length scale appropriate for larger anatomic defects is generally challenging. While relatively easy to fabricate, diffusion-generated gradients and dip-coating procedures may rely on solutions that contain impurities that could be toxic to tissues *in vivo*. [18] Such methods also cannot yield complex microgeometries [19] that may be needed for biologically relevant scaffolds. Furthermore, methods of biomolecular attachment often rely on physical adsorption rather than a covalent reaction. Physical adsorption lacks the ability to specifically target a biomolecule for immobilization and controlled release in a spatial and temporal manner. These methods also are limited to preparing a single gradient, thus restricting control of truly mimicking normal developmental or regenerative processes.

Chemical vapor deposition (CVD) polymerization of [2,2]paracyclophanes (PCPs) into poly(*p*-xylylenes) (PPXs) [20] has been introduced into several types of biomedical and microelectromechanical devices.[21–23] These polymer coatings have been shown to be biocompatible and are FDA approved in specific applications.[24] Poly(*p*-xylylene) polymers can also accommodate reactive chemical groups in their structure [25–29], thus providing specific sites for covalent immobilization of biologic factors. Reactive PPXs have improved the performance of endovascular stents,[30] DNA microarrays [31], and microfluidic devices.[32] More recently, the CVD polymerization process was expanded to incorporate multiple monomer entry ports, allowing for deposition of well-defined copolymers[33] as well as reactive copolymer gradients.[34] These gradients have been used to immobilize molecules in two gradients, allowing for the possibility of further biochemical modification after deposition.

Based on the CVD co-polymerization technology, we now report on a new method for generating cell signaling gradients over a biomaterial substrate with potential use in regenerative medicine. We hypothesized that a cell signaling adenovirus could be immobilized selectively towards multiple copolymer gradients, allowing for cell growth and transduction along these molecular gradients.

2. Materials and Methods

2.1 – CVD Polymerization

A detailed description of the two-source CVD system has been published.[34] Briefly, 4-formyl[2,2]paracyclophane dimer (**1**) was synthesized using an established synthesis route. [35] 4-Amino[2,2]paracyclophane dimer (**2**) was purchased from Uniglobe Kisco, Inc. (White Plains, NY) and used as-received. Dimers **1** and **2** were loaded separately into two feed dishes, with each dish loaded into its own source. (Fig. 1) A system pressure of 0.22 Torr and sublimation temperatures between 80–110 °C were used to ensure sublimation. Argon carrier gas was used to independently control the flow velocities of sublimated dimers **1** and **2**. The following process condition was used to deposit poly(*p*-xylylene) composition gradients for *in vitro* studies: $M_1 = 270$ mg, $M_2 = 160$ mg, $Ar_1 = 25$ sccm, $Ar_2 = 19$ sccm. The sample holder was set 10 centimeters below the position of both sources, and the sample was set halfway between source 1 and the center point of the sample holder. To

ensure gradient formation, the sample holder was not rotated. Although the sample holder remained at 15 °C, a copper plate was set on top of the sample holder to control the angle of inclination of the sample. Deposition was successful on silicon, gold, and glass substrates. To minimize wall deposition, the chamber wall was heated to 120 °C.

2.2. – Surface Characterization

Infrared spectroscopy was performed on a Nicolet 6700 spectrometer utilizing the grazing angle accessory (Smart SAGA) at a grazing angle of 85°. FTIR spectra were corrected for any residual baseline drift. Au-coated Si substrates were used for FTIR measurements, using blank Au-coated Si as a reference. Fluorescent micrographs were captured using a Nikon Eclipse TE-2000-S fluorescence microscope (Melville, NY, USA). Quantification of AP-assays was performed with a BioTek uQuant spectrophotometer (Bio-Tek Instruments, Winooski, VT).

2.3 – Biotin-AP Immobilization Assay

CVD homopolymers **6** and **7** (Fig. 1) were deposited onto 24-well tissue culture plates (Costar). MilliQ water was used to rinse the wells prior to immobilization. 250 µL of 5mM biotin-LC-hydrazide or sulfo-NHS-LC-biotin (Pierce Inc) was applied to different wells for 10 minutes and 1 hour, respectively, at room temperature. Wells were subsequently rinsed several times with PBS. Avidin was suspended at 66 µg/mL in PBS containing 0.02% Tween 20 and 0.1% BSA. 300 µL avidin solution was then applied to each well for 1 hour, followed by a wash with the PBS/Tween/BSA solution for 45 minutes, followed by several short PBS rinses. 250 µL biotin-AP (1/3000 dilution in TBS) then reacted in each well, followed by TBS rinses, then application of 200 µL PNPP substrate for 3 minutes. Absorbance was read at OD405.

2.4 – Virus Biotinylation

Adenovirus encoding blue fluorescence protein under the control of the CMV promoter (Ad-CMV-BFP) was prepared by the University of Michigan Vector Core. SulfoNHS-LC-biotin was diluted to 37.5 µg/µL in PBS and added to the virus stock at 0.75mg/10¹² viral particles. Biotinylation was carried out at 4 °C for 2 hours. Next, 1M glycine of equal volume was added to quench the reaction. The mixture was then subjected to microcentrifugation in an Amicon Ultra Centrifugal Filter (100K membrane, Millipore). The filtrate was reconstituted in PBS to the original stock concentration (4E12 particles/mL).

2.5 – Adenovirus Immobilization and Sandwich ELISA Assay

Following immobilization of avidin (see section 2.3), surfaces were blocked with 5% BSA (w:v) in PBS overnight at 4 °C. Biotinylated Ad-CMV-BFP was reconstituted in 3% BSA (w:v) in PBS and allowed to react with surfaces for 1 hour at 4 °C. After virus immobilization, surfaces were rinsed repeatedly with cold PBS.

For sandwich ELISAs, immobilized virus was exposed to rabbit anti-adenovirus IgG (1/1100 dilution in PBS/tween/BSA; 300 µL/well) for 1 hr at room temperature, followed by a 1hr rinse with a PBS/tween/BSA solution and 3 PBS rinses. Surfaces were further blocked (3% BSA 25 °C) for 2 hours, then labeled with goat anti-rabbit IgG (1/3700 dilution 1% BSA 0.1% Gelatin in TBS, 250 µL/well) for 1 hour, and finally developed with pNPP liquid substrate for 3 minutes.

2.6 – In vitro Cell Culture

Human gingival fibroblasts (hGFs) (ATCC; Manassas, VA) were cultured using Dulbecco's Minimal Essential Medium (DMEM, 10% FBS, 1% Penicillin/Streptomycin) in standard

conditions (37 °C humidified atmosphere, 5% CO₂). Cells were passaged and seeded onto tissue culture plates at a density of 2 X 10⁴ cells/cm², using 200 µL medium/cm². Cells transduced with adenovirus were examined for expression of blue fluorescence protein at 48 hours post-seeding. For gradient transduction, rectangular tissue-culture polystyrene dishes (Nunc, Rochester, NY) were filled with a 10:1 mixture of poly(dimethylsiloxane):curing agent (Dow Corning, Midland, MI) and allowed to cure. Sections of hardened PDMS were removed from the plate to form multiple lanes within the same dish. The plate was then coated with the polymer gradient (5) (Fig. 1) via CVD gradient deposition. After immobilization of biotin/avidin (in proportion to the lane surface area), the dishes were sterilized in 70% ethanol for 1 hour, followed by virus immobilization and cell seeding.

3. Results

3.1 – CVD Copolymerization

In traditional CVD polymerization methods, monomers enter the chamber from a single entry port and are then uniformly deposited and polymerized over a rotating sample holder. [36] To facilitate formation of a continuous chemical surface gradient, a custom-made CVD deposition system accommodated two sources for monomer entry (Fig. 1). To ensure anisotropic deposition, the sample holder was not rotated. Functionalized [2,2]paracyclophanes underwent sublimation and subsequent pyrolysis prior to chamber entry. In this study, 4-formyl[2,2]paracyclophane (1) and 4-amino[2,2]paracyclophane (2) were chosen as the copolymer starting materials due to the orthogonal nature of their functional groups. No chemical reaction occurred *in situ* between the two groups based on FTIR analysis. Furthermore, the reaction pathways for each functional group remained orthogonal to each other; aldehydes reacted strongly with hydrazides, which in turn, did not react with amines. These characteristics were required to ensure production of a two-component chemically reactive gradient. Upon entry into the chamber, the monomers deposited onto the substrate at room temperature, a detail that is essential to preserving delicate scaffold materials. Processing conditions were adjusted to optimize gradient characteristics, such as coating thickness, uniformity, and gradient slope (Fig. 1).

FTIR spectroscopy was used to assess the composition of the copolymer and confirm the fidelity of the CVD coating procedure. FTIR can detect relatively small changes in bulk composition by scanning small amounts of material. Gold substrates were placed at specified positions flanking the cell culture dishes on the sample holder. After deposition, samples were examined and their peak intensities were compared. Quantitative comparisons were made using peak stretches that are characteristic of the respective monomers. Peak intensities at 3461 and 3361 cm⁻¹ (-N-H) were the strongest for samples close to the source of dimer 2 (0 cm), while the peaks at 1688 cm⁻¹ (C=O) were strongest for samples close to the entry port of dimer 1 (12.7 cm) (Fig. 2). No indication of cross-reaction between monomers was noted, as would be identified by an imine stretch at 1620 cm⁻¹. In general, surface compositions of CVD copolymers have been found to be nearly identical to compositions determined via FTIR spectroscopy.[33]

3.2 – Primary ELISA

Although viruses can be conjugated to biomaterial surfaces by several methods, the virus-biotin-avidin-biotin-materials (VBABM) method was employed for use in CVD gradient coatings.[37] This is an efficient method to immobilize adenoviruses onto homopolymer coatings, due to the binding strength of the biotin-streptavidin interaction.[38] In the case of PPX co-polymers, either the aldehyde- (6) or amino- (7) functionalized polymers are capable of reacting with biotin, depending on specific type of biotin used. Application of the VBABM method of immobilization in gradient systems requires the protocol to selectively

react with one of the two functional groups, or else the adenovirus will not be immobilized as a surface gradient. Two distinct protocols were formulated with the goal of targeting either of the two reactive groups.

Prior to immobilizing adenovirus onto CVD coatings, an assessment of binding efficiency was performed for each step. To this end, both sulfo-NHS-lc-biotin and biotin hydrazide were allowed to react with homopolymer surfaces **6** and **7** under aqueous conditions (Fig. 3). Using an avidin- alkaline phosphatase (AP) assay, an optimal avidin titration of 20 µg/well was found to preserve selective attachment onto the surface-bound biotin (data not shown). After subsequent exposure to avidin, an AP-labeled biotin complex was applied to the immobilized avidin. The biotin-AP moiety was used to simulate attachment of biotinylated adenovirus to avidin. Both types of modified biotin efficiently bound their respective target groups, without causing non-specific binding on the second functional group (Fig. 3).

Therefore, to determine the specificity of binding, biotinylated adenovirus was immobilized using a reaction medium consisting of 3% BSA. This particular BSA concentration blocked non-specific binding while allowing the reaction with immobilized avidin to proceed. Using biotinylated adenovirus encoding blue fluorescence protein (Ad5-CMV-BFP), sandwich ELISAs were performed (Fig. 4). For each titration curve, titers exist at which non-specific virus attachment is minimized, while targeted attachment is maintained. Above this optimal titer, non-specific interactions occurred, indicating failure of the blocking procedure. A titer of $3.3\text{E}8$ pfu/cm² was found to exhibit maximum adenovirus activity on the target surface while minimizing activity on the second homopolymer. When the applied titer decreased below this value, the overall activity was decreased across both surfaces, resulting in zero non-specific adhesion (Fig. 4).

3.3 – In vitro transduction

Once the specificity of viral immobilization was determined, the optimized protocol was applied to cell culture dishes coated with CVD copolymer gradients. Multiple lanes were fabricated in the dish using PDMS elastomer, after which the CVD process was applied to the dishes. Translation of the VBABM protocol to a larger surface area required preservation of the virus-to-surface area ratio and the viral solution concentration. After 48 hours post-seeding of human gingival fibroblasts (hGF) on the cell signaling gradient, fluorescent micrographs were taken at specified locations relative to the amine-rich end of the gradient. For all surfaces on which hGFs proliferated, cell confluency remained at least 85–90% on the entire surface. Biotin hydrazide was used to target adenoviral attachment towards the aldehyde component of the gradient. As a result, transduced cells were heavily populated on the aldehyde-rich end of the gradient (9–10 cm) (Fig. 5b). As the distance increased towards the amine-rich end, a gradual decrease in the transduced cell population occurred, until transduction was completely absent (Fig. 5b). Using a similar protocol, the gradient direction was reversed by using SulfoNHS-lc-biotin to immobilize avidin. The VBABM virus immobilization was enabled on the amine component of the gradient, which inverted the trends found in Figure 5b. Human GF transduction by Ad-CMV-BFP was significantly higher on the amine-rich end of the gradient (1.2–1.9 cm) and decreased with increasing distance towards the aldehyde-rich end (7.6 cm) (Fig. 5c).

4. Discussion

A simple procedure for depositing nanoscale coatings was developed to preserve delicate substrates of complex geometries. One reason to use localized adenovirus as a gene delivery vehicle is to enhance the efficiency of gene transfer over other delivery methods.[39] Systemic gene delivery increases the risk of infection at undesired locations. Localized delivery of replication-defective virus delivers specific proteins much more efficiently than

bolus delivery and may reduce the inflammatory responses because lower viral titers can be used. However, one challenge towards efficient gene delivery via a biomaterial has been the lack of spatiotemporal control over cell transduction.[39,40] CVD polymer gradients may allow spatiotemporal delivery of cell signaling factors from biomaterials. Long-term control of gene expression could be achieved using lentivirus, as compared to the transient expression offered by adenoviral vectors. By adjusting the adenovirus titer, the intensity of the immobilized gradient can be fine-tuned. Furthermore, simple changes in deposition processing conditions can adjust the polymer gradient slope[34], thus translating into a different adenoviral gradient. Although the copolymer gradient slope can be manipulated, the length of the gradient often surpasses length scales found in biological environments. However, this discrepancy does not hinder the desired outcome, due to the multi-step immobilization processes. Each step performs below 100% efficiency to decrease the effective gradient slope. Repetition of multiple immobilization procedures on the same gradient surface further enhance this effect, producing a final gradient that is much shorter than the initial gradient formed during CVD co-polymerization. As an added advantage, CVD does not require the use of solvents because the deposition occurs in the vapor phase. Only the purified monomer deposits onto the substrate.

The reactive monomers made by CVD technology form copolymers comprised of an identical polymer backbone, but different reactive chemical groups. This characteristic addresses a number of technological gaps found in common gradient methods. First, more advanced manipulation of surface properties remains available. For example, if a one-component virus gradient is immobilized, another biomolecule could be immobilized as a counter-gradient. The remaining functional group could also remain unreacted to provide a specific physical property, such as hydrophilicity. Second, the covalent mechanism of immobilization allows for specific targeted immobilization, as opposed to charge-charge interactions that cannot distinguish target biomolecules from other environmental constituents. This reactive targeting has found applicability in biological microarrays[33], diagnostic assays, and tailored biointerfaces. Taken together, these advantages suggest that CVD may provide a broadly applicable mechanism to functionalize controlled release devices.

We demonstrated the ability of CVD copolymerization to reproducibly generate continuous cell transduction gradients. Although only one immobilization mechanism was demonstrated for both reactive groups, formation of a dual immobilization gradient is feasible. A barrier similar to the PDMS wells could be implemented, with virus immobilization occurring on both sides of the strip. The strip could be removed after immobilization, such that two different gradients are present on both sides of the PDMS surface. A low-melting temperature wax has also been used to mask certain areas during virus immobilization.[37] Dual transduction *in vivo* would not necessitate the two gradients to be superimposed, but they should at least coexist within a relative proximity.

Although previous studies demonstrated the immobilization of adenovirus onto functionalized poly-*p*-xylylenes via a VBABM method, the specificity of virus attachment must be elucidated for two reasons: 1) multiple surface functional groups are present and could compete for covalent attachment and 2) an anisotropic gradient coating necessitates elimination of non-specific adhesion. A consistent trend observed in this study was that the biotin hydrazide reaction yielded less experimental error compared to sulfoNHS-Ic-biotin. This result could be attributed to the stronger selectivity of biotin hydrazide compared to sulfo-NHS-Ic-biotin. This characteristic biotin hydrazide resulted in stronger selectivity of adenovirus attachment during the ELISA. Subsequently, the targeting of the NH₂ functional group by sulfoNHS-Ic-biotin produced transduction gradients with greater experimental error.

Reactive CVD coatings may be useful for controlled delivery applications, provided that the reactive groups do not elicit harmful biological responses. A reactive CVD polymer inhibited restenosis and exhibited low cytotoxicity when coated on endovascular stents and implanted into live sheep.[30] More recently, RunX-2 adenovirus was immobilized onto another version of reactive poly-*p*-xylylenes, which enabled differentiation of human bone marrow stromal cells (hBMSCs) into functional osteoblasts.[41]

PPX coatings have been also been extended into 3D systems.[22,42] For example, wax can be deposited and removed at low temperatures, allowing for separate regions of immobilization.[37] Although the gradient deposition was demonstrated for 2-dimensional culture dishes, the CVD polymerization technology has been shown to deposit PPXs uniformly in high-aspect ratio microchannels[43], a characteristic that is needed for coating of three-dimensional scaffolds. Three-dimensional PCL scaffolds coated with CVD polymers were shown to immobilize adenovirus and subsequently transduce cells throughout the inner pores.[37] Together, these studies suggest that the mechanisms to form dual gradients in 2D could be extended into 3D controlled delivery devices. The CVD polymers uniformly deposit onto surfaces exposed during deposition. As such, larger substrates could be coated without eliminating the conformal characteristics of CVD. A small-sized CVD chamber limits production rate and variability, which would limit the size of scaffolds coated for *in vivo* applications. However, CVD systems are easily scaled up by using a larger chamber.

5. Conclusions

This study describes an efficient method to generate cellular transduction gradients onto scaffolds or substrates of choice. CVD co-polymer coatings deposited as gradients served as the basis for covalent immobilization of cell-signaling adenovirus onto a biomaterial substrate to mimic cell-signaling gradients observed in nature. Copolymer deposition occurred on substrates that were subsequently used for cell culture. The copolymers exhibited a continuous gradual change in composition along the length of the substrate. Immobilization of an adenovirus occurred as a gradient, which then transduced cells in the form of a designed gradient and resulted in a controlled gradient of cellular activity. The gradient direction was also reversed, allowing for multiple types of biological gradients in different directions. This approach resolves several specific shortcomings found in existing gradient fabrication techniques. Many clinical applications could be accelerated as biochemical gradients are a crucial tool for mimicking many *in vivo* environments.

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Figure 1.

(a) Chemical vapor deposition (CVD) polymerization was used to deposit copolymers bearing a composition gradient on the substrate.

Figure 2.
FTIR spectroscopy of the copolymer gradient indicates a gradual change in composition, with respect to the topological position.

Figure 3.

Approach to form biomolecule gradients using orthogonal reactive groups. (a) Schematic demonstrating the binding assay employing alkaline phosphatase-labeled biotin used to determine the binding efficiency and selectivity of avidin, with respect to reactive CVD polymer coatings. (b) Depending on the type of biotin used, avidin selectivity can be achieved with respect to either PPX-CHO or PPX-NH₂. AP represents alkaline phosphatase reported. * $p < 0.05$, $n = 4$.

Figure 4. Sandwich ELISAs were used to evaluate the binding efficiency and selectivity of the VBABM immobilization method. Using the functionalized biotins from figure 2, biotinylated adenovirus encoding BFP adhered selectively towards either (a) aldehyde or (b) amine-functionalized poly(*p*-xylylene). Y-values are plotted with respect to the reaction medium titre.

Figure 5.

(a) Schematic of gene delivery and cell transduction gradients. Adenovirus encoding BFP was immobilized onto a cell culture dish treated with the CVD copolymer gradient. Human gingival fibroblasts were then seeded onto the adenovirus gradient. A gradient of transduced cells were generated within the confluent cell population, with the gradient direction determined by (b) biotin hydrazide attachment to CHO groups, or (c) sulfoNHS-*l*c-biotin attachment to NH_2 groups.