Capillary Flow Layer-by-Layer: A Microfluidic Platform for the High Throughput Assembly and Screening of Nanolayered Film Libraries

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**Abstract**

Layer-by-layer (LbL) assembly is a powerful tool with increasing real world applications in energy, biomaterials, active surfaces, and membranes; however, the current state of the art requires individual sample construction using large quantities of material. Here we describe a technique using capillary flow within a microfluidic device to drive high-throughput assembly of LbL film libraries. This capillary flow layer-by-layer (CF-LbL) method significantly reduces material waste, improves quality control, and expands the potential applications of LbL into new research spaces. The method can be operated as a simple lab bench top apparatus or combined with liquid handling robotics to extend library size. Here we describe and demonstrate the technique and establish its ability to recreate and expand on known literature for film growth and morphology. We use the same platform to assay biological properties such as cell adhesion and proliferation, and ultimately provide an example of the use of this approach to identify LbL films for surface-based DNA transfection of commonly used cell types.

**Keywords**

Layer-by-Layer; LbL; High-Throughput; Polyelectrolyte Multilayers; PEM; Screening; Ultra-thin films

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Layer-by-layer (LbL) assembly is the alternating adsorption of materials onto a surface using complementary interactions, one layer of material at a time, creating nanometer-scale thin films.\(^1\)\(^-\)\(^4\) This process enables fine control over the assembly of functional materials into ultra-thin coatings which exhibit a range of interesting properties and have found diverse applications in reactive membranes, drug delivery systems, and electrochemical and sensing devices.\(^5\)\(^-\)\(^8\) Over the past decade, exciting new developments have demonstrated the power of LbL assembly in biomedical applications, with examples ranging from bone tissue engineering\(^9\), \(^10\) to vaccine delivery\(^11\) and creating biological interfaces.\(^12\), \(^13\)

Several recent advances have led to the adaptation of LbL toward commercial translation, including the use of convection in automated Spray-LbL\(^14\), \(^15\) to coat a broad range of surfaces with accelerated cycle times and the use of Spin-Assisted LbL\(^16\)\(^-\)\(^18\) to generate thin films on planar surfaces with control of molecular orientation. Researchers have also explored using microfluidics to coat channels\(^19\) and microparticles\(^20\) via alternating flow of polyelectrolytes. These contributions are important as they demonstrate the generalizability of the LbL process and provide insight into the assembly of uniform films of a given composition. These advances enable the implementation of promising LbL systems at the speed and throughput needed for commercial products and processes; however, to date there is no approach capable of assembling libraries of LbL films for research, development, and optimization.

Attempts to create and examine libraries of different LbL film systems can yield understanding of the impact of simple compositional changes in the polymers incorporated on final film structure and property.\(^21\)\(^-\)\(^23\) The existing methods for LbL assembly for new materials discovery, however, rely on the individual production of film samples using large reservoirs of material, and must generally be facilitated using specialized or modified lab equipment.\(^24\)\(^-\)\(^26\) The limitations of current LbL techniques are particularly significant when precious, rare, and costly materials are incorporated in the film assembly. For biomaterials applications in particular, optimizing incorporation and/or release of siRNA, DNA, active growth factors, engineered peptides or other therapeutics would require significant quantities of material, thus severely limiting investigations and potential discoveries in these areas.

As research into LbL film technology continues to expand and researchers pursue new discoveries along with commercial translation in the pharmaceutical industry, several challenges need to be overcome. These include simplifying in vitro analysis of films, improving material conservation, and making the LbL process more accessible to a broader scientific community. To address these challenges and enable more thorough investigations of LbL film assemblies, we have developed a simple microfluidic approach for the high-throughput construction of multiple LbL films in parallel. We have termed this approach “Capillary Flow Layer-by-Layer” (CF-LbL) as we harness capillary action to fill microchannels in which LbL films are assembled. The CF-LbL device consists of an array of these microchannels formed by bonding a polydimethylsiloxane (PDMS) mold to an oxygen plasma treated substrate (e.g., glass, silicon, etc.). This approach requires as little as 0.1% as much material per film compared to conventional methods, while enabling the simultaneous assembly of nearly 400 times as many independent films. LbL assemblies of
varying compositions, morphologies, and architectures can be rapidly produced in a format that suits a number of physico-chemical and in vitro biological assays, and screened for meaningful material properties using this method.

RESULTS AND DISCUSSION

Each microchannel is comprised of a main channel where material from solution adsorbs onto the substrate, as well as three openings: an inlet well (1), a capillary flow break well (2), and an exit well (3) (Fig. 1a). Capillary flow within the channel is controlled by covering select regions of the PDMS mold during plasma treatment (Fig. 1c and Supplementary Fig. 1). This leaves these regions hydrophobic, while the uncovered areas receive plasma treatment and become hydrophilic. Material held within the channel is cleared by vacuum which is selectively applied by covering both holes (2) and (3) simultaneously (Fig. 1b). The layout of channels is modular by design and can be based on 96- and 384-well plate dimensions for high-throughput screening or consist of only a few microchannels for easy bench top use (Supplementary Fig. 2). The characterization of films assembled within the microchannels is performed similar to those created by existing LbL methods using ellipsometry, profilometry, light microscopy, and atomic force microscopy.

To demonstrate the application of this technology in the construction and evaluation of LbL films, we began by assembling a simple bilayer film architecture of poly(acrylic acid) (PAA) and poly(allylamine hydrochloride) (PAH). PAH was labeled with a fluorescent dye and the growth of the film was measured by profilometry. The film grew linearly and resembles similar films assembled on glass slides constructed via Dip LbL technique (Fig. 1d) with regard to thickness per bilayer. For current LbL assembly techniques, investigating libraries of LbL films is a very time and material intensive task, as it requires up to multiple days to construct a single sample while using milliliters of material solution. To demonstrate this capability with CF-LbL we assessed the incorporation of a labeled polyanion (PAA) with candidate polycations in simple bilayer assemblies (Fig. 1e). This experiment demonstrates that by using common lab imaging modalities, such as a gel imager, arrays of LbL films can be assessed for material incorporation. This type of approach could be very useful in determining the best polymer pairing for incorporation of a material of interest, such as a small molecule drug or protein therapeutic.

The layout and design of the microchannels within the device are easily customized for different applications. Channel parameters such as length, width, and shape can be modified by designing different lithographic masks for creating the PDMS mold; however, using more recent advances in soft lithography techniques we can incorporate even more diverse designs. This includes the capability to incorporate microstructures as a part of the PDMS mold that forms the channel, which can be accomplished via multilayer soft lithography. Introducing microstructures within the channel opens many interesting avenues for investigation, particularly in the area of in vitro experiments where the structures could be used to influence cell behavior.
Microstructures contained within the channels of the device can be uniformly coated with LbL films, similar to coating flat surfaces (Fig. 2a). These structures can be used to increase the active surface area within the channel, and influence cell-surface interactions and adhesion, allowing for the generation of patterned cell surfaces. To demonstrate the capability of CF-LbL to coat these microstructures we assembled a simple bilayer film of PAA/PAH using a fluorescently labeled PAH. Fluorescent imaging of the coated channels shows the uniformity of the film coating, with well reproduced films assembling within and around the microstructure features (Fig. 2b).

The use of microstructures, such as micro-wells, in cell sampling and small-scale cell culture has been well described by other research groups. The micro-well features contained within the CF-LbL device are recessed into the PDMS mold and as such are positioned outside of the flow of the main channel and can thus enable cell seeding on the recessed surface features. This is accomplished by first filling the channel with a cell suspension and flipping the device such that gravity directs the cells into the PDMS wells. After thirty minutes fresh media is flowed through the channel by pipette and the cells not contained within the micro-wells are swept away. Using CF-LbL, these micro-wells can be coated with films that contain cell binding moieties or have interesting cell response behaviors to examine cell viability, adhesion, mobility, proliferation, and other cellular activity. To demonstrate this application we used a fluorescent NIH-3T3 cell line and captured cells within a micro-well design (Fig. 2c1). Cells were observed to preferentially seed within the micro-well structures and to spread within these features after one day in culture (Fig. 2c2).

To demonstrate the capability of CF-LbL to investigate large LbL film libraries we performed a classic experiment first described by Rubner and coworkers, wherein the weak polyelectrolyte bilayer film of (PAA/PAH) is assembled using polymer solutions that range in pH from 2.5 to 9.0 (Fig. 3a). Changing the pH of a polyelectrolyte solution alters the degree of ionization of the adsorbing polymer and as such alters the adsorption of the polyelectrolyte to the surface. It was observed that by increasing the degree of ionization of either PAA or PAH in solution led to a decreased average bilayer thickness, whereas conditions where both polyelectrolytes are weakly ionized lead to the thickest films.

These trends in pH and film thickness closely resemble those previously reported, demonstrating the applicability of this method to traditional LbL film systems; furthermore, we were able to expand the range of pH values and conditions used in the original study.

Creating biological interfaces that modify cell behavior is an important and growing application for LbL films. Investigating the myriad of potential iterative film assemblies around one simple film architecture however can be a very time and material intensive task, especially when considering the need to sterilize each sample prior to in vitro evaluation. In addition to preparing the samples for in vitro experimentation, actually conducting experiments in situ with large sample numbers presents many logistical issues and complicates investigations significantly. A major advantage of the CF-LbL method is that the sterility of the device in which the films are assembled is easily maintained throughout the assembly process. Further, in vitro tests can be readily performed directly within the
assembled device, greatly simplifying the setup for investigations and limiting complications from film and sample handling.

To demonstrate the application of CF-LbL assembly in performing \textit{in vitro} investigations, we evaluated 32 different film architectures from the previously investigated PAA/PAH bilayer system, covering a wide range of assembly conditions. NIH-3T3 cells were seeded directly onto the film and cultured for one week. Response to the different films was evaluated by measuring cell adherence to the surface along with proliferation. Cell density and average cell spread area were measured daily using phase contrast light microscopy (Fig. 3b-c). Increasing bilayer thickness correlated significantly to decreased cell density, while no trends were apparent in regards to cell spread area (Fig. 3d-e). It was also observed that the pH of PAA and PAH had significant impact on cell density (Fig. 3f).

We were interested to determine how well the CF-LbL assembled films approximated LbL films assembled \textit{via} the traditional dip LbL technique. To do this we chose four films from the previous experiment and assembled them by both dip LbL and the CF-LbL technique. We assessed both the physical characteristics of the assembled films and cell response to them. Atomic force micrographs (AFMs) of the films assembled by either method were seen to have very similar topographies and surface roughness (Fig. 4a). The average bilayer thickness and roughness of each film was measured after 10 bilayers were assembled. There was no significant difference between the films assembled by either technique (Fig. 4b-c).

We used NIH-3T3 cells to assess cell response. The total number of cells seeded for each group was corrected for the area upon which we were seeding. Cells were observed to respond similarly on both surfaces, with thick films leading to reduced cell density. These findings closely resemble those presented in previous reports for PAA/PAH bilayer systems, for which it was found that the mechanical stiffness of the thicker films was a primary determinant of cell adhesion.  

Locally delivering nucleic acids from surfaces is a relatively simple and highly adaptable approach to alter nearby gene expression that opens significant opportunities in fields ranging from fundamental molecular biology to tissue engineering.\textsuperscript{28, 43, 44} There are numerous factors that impact transfection of material, including: nucleic acid packaging, material release, endosomal escape, and if necessary nuclear transport. In particular, the type of polycation used, including primary, secondary or tertiary amines and relative hydrophobicity of the backbone, affects the loading of DNA and RNA, the nature of ionic crosslinking and complexation, and the ability of the nucleic acid to be released from the endosome and to access cells.\textsuperscript{45, 46} Due to this complexity, investigations into LbL systems for these applications face very substantial challenges.

Previously, research groups have described LbL systems capable of the local delivery of DNA from coated surfaces.\textsuperscript{47, 48} These reports provide a great deal of promise for future applications of LbL in combination with medical devices\textsuperscript{49, 50} and as DNA vaccines depots.\textsuperscript{11, 51} At this time, however, investigations can only reasonably study individual LbL systems, often assembled using only one set of assembly conditions.\textsuperscript{47, 48} To demonstrate the use of the CF-LbL assembly for screening film libraries we decided to apply the technique to investigate a focused group of candidate films consisting of a broad range of
polycations identified from literature that have been used for DNA transfection (Fig. 5a). We assembled bilayer films of the polycations with a DNA plasmid for green fluorescent protein (GFP). HeLa cells were seeded directly onto the films within the device and were monitored for GFP expression, cell density, and average cell spread area every day for seven days in culture (Fig. 5b-d).

From a screen of 16 films, we found a diversity of cellular response and transfection efficacies that show some interesting trends. Cell density, measured as the number of live cells per square millimeter, was highest on the (PAH/pEGFP)$_{10}$ film, which reached near confluence after only three days of culture. The only two other films to reach confluence within one week were (Gelatin/pEGFP)$_{10}$ and (LPEI 25kDa/pEGFP)$_{10}$. Initial cell seeding density was observed to correlate inversely to film thickness, which was largely maintained after one week in culture (Fig. 5e). Film thickness however was not observed to correlate with cell spreading, similar to what was observed for the PAA/PAH systems (Fig. 5f). The primary goal of screening a diverse group of polycations was to elucidate characteristics that correlate with transfection efficacy. The major trait that was seen to correlate with transfection was the type of amine, where films that consisted of polycations which contained tertiary amines achieved the highest average transfection efficacy, while those with only primary amines achieved the lowest on average (Fig. 5g).

The best performing film architecture from this screen was determined to be (BPEI/pEGFP)$_{10}$ as it reached the highest fraction of GFP expressing cells with relatively high cell density. BPEI, which presents primary, secondary and tertiary amines, is known for its ability to both effectively complex nucleic acids, and to buffer at endosomal pH, which can cause endosomal osmolytic swelling and subsequent nucleic acid release to the cytosol. This film was evaluated on a larger scale by assembling it on microscope slides. HeLa cells were cultured on these large LbL coated slides for seven days (Fig. 5a-b). Cell behavior and GFP expression was then analyzed. The fraction of GFP expressing cells as well as average cell density and average cell spread area on the larger film closely resembled that of cells cultured within the CF-LbL microchannels (Fig. 5c-e). Together these data provide strong support for the capability of CF-LbL to investigate LbL films for use in applications on larger scales. The films assembled by CF-LbL within microchannels performed identically to their counterparts assembled by Dip LbL on a scale about a thousand times larger in surface area.

**CONCLUSIONS**

In summary, we have described a method for the high-throughput assembly and screening of LbL films. We used this method for three demonstrations: (1) a study on the effect of pH on weak polyelectrolytes in LbL film assembly, (2) the in-situ examination of cell adhesion and viability on LbL assemblies, and (3) the investigation of a library of films for DNA transfection from surfaces. This method is paired with a simple modular microfluidic device that can be used for the simultaneous assembly of hundreds of independent films or for smaller more focused bench top investigations. The device is fabricated via common soft lithography techniques and when assembled provides a sterile environment where sensitive biological analysis can be performed on each film independently.
The potential applications of LbL film assemblies are incredibly diverse and growing. Here we have provided a method using a simple microfluidic device that makes possible the high-throughput screening of LbL assemblies while significantly reducing the amount of material used. This method represents a simple yet significant advance for the investigation of LbL films while expanding the ability to study these coatings to laboratories that previously could not. We anticipate that this approach could have enormous impact in a broad range of fields that include developing drug delivery systems and creating biological interfaces.

METHODS AND MATERIALS

Materials

Linear polyethylenimine (LPEI, $M_w$ of 2.5k, 25k, 250k), and branched polyethylenimine (BPEI, $M_w$ of 1.2k, 10k, 50-100k) were purchased from Polysciences; low molecular weight chitosan (LMW Chitosan, $M_w$ of 15-30k), high molecular chitosan (HMW Chitosan, $M_w$ of 300k), gelatin (pH=2.5), diethylaminoethyl-dextran (DEAE Dextran), protamine sulfate (PrS), poly-L-lysine (PLL, $M_w$ of 15-30k and 30-70k) and poly(allylamine hydrochloride) (PAH, $M_w$ of 60k) were purchased from Sigma Aldrich; and the degradable poly(beta-aminoesters) (designated Poly 1, and Poly 2) were synthesized using the method that was previously published\textsuperscript{56}.

Device Fabrication

Masters templates for preparing microfluidic channels were made with an SU-8 photoresist in bas-relief on silicon wafers. The microfluidic device was fabricated in polydimethylsiloxane (PDMS) by using a standard soft-lithography method\textsuperscript{57}. In brief, masters with microchannels were prepared with features of SU-8 on silicon wafers with diameter of 4 inches by a photolithography technique. The PDMS elastomer was prepared by mixing pre-polymer with the curing agent (Sylgard 184, Dow Corning) at the weight ratio of 10:1, respectively. The mixture of the pre-polymer and curing agent was then poured onto the master, and cured in an oven at 75 °C for 4 hours. After curing, the replica was carefully peeled off from the master, and holes with various diameters were made by a Harris micro-punch at the designated positions. The patterned PDMS sheet and a glass slide were plasma treated for 90 s in a plasma cleaner chamber (PDC-3XG, Harrick, USA), and then brought to contact and immediately sealed. The thickness of the formed microchannel is approximately 100 μm. Micro-patterned surfaces inside micro channel were created by using multilayer soft-lithography as previously described\textsuperscript{58}.

LbL Film Assembly

Material solutions were prepared at a 2 mg/mL concentration and pH adjusted to desired level. All solutions were sterile filtered using a 0.2 μm syringe filter prior to use. Material solutions were introduced to the inlet of a microchannel by pipette. Solutions are drawn into the microchannel via capillary force to fill the entire channel within 1-3 seconds. After channel filling the excess material within the inlet is recovered from the inlet. Material in solution is allowed to adsorb for a pre-determined amount of time (e.g. 5, 10, or 15 min) and then the channel is cleared either by vacuum or by pressurized air. The channel is then washed three times with a washing solution to remove material that is not well adsorbed to
the channel surface. This process is then repeated with a complementary species for adsorption and the entire process can be repeated to reach the desired number of layers of LbL film. After film assembly is complete the PDMS sheet can be removed from the substrate, leaving a micro-strip of LbL film on the substrate. Dip LbL films were assembled using a Carl Zeiss HMS DS50 slide stainer. Films were either built on Si wafers or glass slides. Rinsing steps were performed using UltraPure™ DNase/RNase-Free water (Life Technologies) that was pH adjusted to match with the prior material adsorption step.

**Multilayer Film Characterization**

Film thickness was measured for films built on Si wafers using a Veeco Dektak (Plainview, NY, USA) surface profilometer. Surface roughness was measured using a Veeco Dimension 3100 atomic force microscope (AFM). Coating of microstructure containing CF-LbL devices was performed using a ZEISS Axiovert 200 fluorescent and brightfield microscope.

**Cell Culture and Transfection Studies**

NIH-3T3 and HeLa cells were used in this work; both were purchased from ATCC (Manassas VA). NIH-3T3 cells were used to evaluate cell behavior on PAA/PAH LbL films. HeLa cells were used for all transfection studies. Cells were seeded within the CF-LbL assembly device following LbL film construction at an initial density of 0.1M/mL and allowed to settle for 30 minutes, after which media was exchanged to remove unattached cells. All cell lines were cultured in Advanced-MEM with 5% FBS and 1% Pen-Strep and 2mM L-glutamine. Media was exchanged daily by placing a droplet at the inlet and removing the waste at the exit of the microchannels. Bright field and fluorescent imaging of cells was done with a ZEISS Axiovert 200. Images were analyzed using ImageJ image analysis software. Confocal imaging of cells seeded on microchannel walls was done with a Nikon A1R Ultra-Fast Spectral Confocal Microscope and three-dimensional projection was created using Velocity software. Flow cytometry was performed with FACSCalibur flow cytometer.

**Statistical Analysis**

Data analysis was performed between groups using Student’s t-test. These were rectified using ANOVA for comparisons between multiple groups. A value of p < 0.05 was used to indicate statistical significance.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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References


Figure 1. Design of capillary flow layer-by-layer (CF-LbL) device
(a) Top and side view of a single channel within a CF-LbL device, the red region is O$_2$ plasma treated. (b) The process of alternating adsorption of material inside of the microfluidic channels, (+) polycation and (−) polyanion species. (c) Multiple independent channels within a single CF-LbL device. The top image is fully O$_2$ plasma treated, the bottom image selectively treated, scale = 3mm. (d) Measurement of film thickness for a sample PAA/PAH$_{FITC}$ LbL film. (e) Screening LbL film architectures for material incorporation. Fluorescently labeled PAA is incorporated into bilayer LbL films with the polycations: PAH, branched polyethylenimine (BPEI), linear polyethylenimine (LPEI). Data shown as mean ± s.d. Channels used were 800μm wide and 2.0 mm long.
Figure 2. CF-LbL coating of microstructures contained within microchannels for diverse customized applications

(a) Schematic for the coating of microstructures within the CF-LbL channels. (b) Demonstration of patterned microstructures included within the microchannels, (1&3) Lithographic mask designs for the micro-wells, (2&4) fluorescent imaging of channels containing microstructures coated with PAA/PAH-FITC. Scale bar = 150μm. (c) Micro-patterned surface within the channel used to direct NIH-3T3 cell seeding. (1) Imaged immediately after clearing uncaptured cells from main channel with media. (2) Fluorescent imaging of spread cells after 24 hours. Scale bar = 50μm. Channels used were 800μm width and 1.2 mm length. Channels shown were 500μm wide and 5.0 mm long.
Figure 3. High throughput screening of LbL assemblies
(a) pH-dependent thickness behavior of sequentially absorbed layers of weak polyelectrolytes, 10 bilayers. (b) NIH-3T3 cell density on films. Cells were initially seeded at 0.1M/mL. Units = cells/mm². (c) The average spread area of cells on the different film architectures. Units = μm². (d) Effect of film thickness on cell density. Increasing bilayer thickness negatively impacted the total number of cells which initially seeded on the films. (e) Plot of cell spread area vs. bilayer thickness. No correlation was apparent. (f) Effect of PAA pH on the cell density on the films. Higher PAA pH corresponded with increased cell density, regardless of PAH pH. Channels used were 500μm wide and 5.0 mm long.
Figure 4.
Comparison of (PAA/PAH)$_{10}$ films prepared via Dip LbL or CF-LbL techniques. (a) AFM micrographs showing the different topographies for each of the LbL films assembled. (b) Film thickness of the assemblies as measured by profilometry. (c) Film roughness as measured by AFM. (d) NIH-3T3 cell density after three days of growth on film coated glass. (e) Average cell spread area for a cell adhered to the film surface. Data shown as mean ± s.d. Channels used were 500μm wide and 5.0 mm long.
Figure 5. Polycation library screen of DNA transfection from LbL films
(a) Chemical structures of polycation repeat units. (b) Heat maps of cell density, cell spreading area, and fraction GFP of DNA transfection of cells cultured on films. (c) Transfection from films containing similar polycations of differing molecular weights. (d) Cells on (BPEI/pEGFP)$_{10}$ film cultured within the microchannels after 5, 6, and 7 days of culture. Scale bar = 50 μm. (e) Effect of film thickness on cell density at one and seven days in culture. (f) Effect of film thickness on average cell spread area. (g) Effect of amine type on DNA transfection after one week in culture. Polycations that contained tertiary amines (3°) achieved significantly higher transfection levels than those that contained only primary amines (1°). Data shown as mean ± s.d. Channels used were 1.2 mm wide and 5.0 mm long.
Figure 6. Large-scale reproduction of best performing architecture shows similar behavior

(a) Analysis of transfection of HeLa cells cultured on (BPEI/pEGFP)_{10} coated microscope slides. BF = Bright Field. Scale bar = 500 μm, inset scale bar = 50 μm. (b) Flow cytometry analysis of GFP expression for HeLa cells cultured on (BPEI/pEGFP)_{10} for one week. (c) Fraction GFP positive HeLa cells cultured on either Dip LbL or CF-LbL coated glass after one week. (d) Average cell density on film coated substrates after one week in culture. (e) Average cell spread area for HeLa cells seeded on films prepared by either Dip LbL or CF-LbL methods. Data shown as mean ± s.d.