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POLYELECTROLYTE MULTILAYER STAMPING IN AQUEOUS PHASE AND NON-CONTACT MODE

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Abstract

Polyelectrolyte multilayer (PEM) transfer printing has been previously achieved by stamping under dry conditions. Here, we show for the first time, that PEM can be transferred from a stamp to the base substrate under aqueous conditions whereby the two surfaces are in a non-contact mode. Degradable multilayers of (PAA/PEG)_{10,5} followed by non-degradable multilayers of (PDAC/SPS)_{80,5} were fabricated under acidic pH conditions on either PDMS or glass (stamp), and subsequently transferred over top of another multilayer prepared on a different substrate (base substrate), with a spacing of ~ 200 μm between the stamping surface and the base substrate. This multilayer transfer was performed under physiological pH conditions. This process is referred to herein as non-contact, aqueous-phase multilayer (NAM) transfer. NAM transfer can be useful for applications such as fabricating three-dimensional (3-D) cellular scaffolds. We attempted to create a 3-D cellular scaffold using NAM transfer, and characterized the scaffolds with conventional and fluorescence microscopy.

Keywords

polyelectrolyte multilayer transfer; non-contact stamping; aqueous phase stamping; cellular scaffold

1 Introduction

Given the shortage of donor supply for surgical transplantation, engineered tissue constructs to replace damaged organs is potentially attractive¹. Organs and tissues, *in-vivo*, exhibit three dimensional (3-D) cellular structures, thus requiring successful tissue constructs to exhibit a layered cellular structure similar to *in vivo* tissues and organs². Traditional methods to create 3-D cellular environments are generally based on approaches that place cells on or within polymeric matrices from natural, such as collagen, or synthetic polymers^{1, 3, 4}. Another type of 3-D culture is a sandwiched co-culture, where the homotypic or heterotypic cell-cell contact of two types of cells are sandwiched within a layer of polymer, to enhance the biological activity of the cells^{2, 5}.

Recently, layer-by-layer (LbL) assembly^{6–14} of polyelectrolytes over a monolayer of cells to create double or multiple strata of cells has been shown as an alternative method to obtaining a sandwiched 3-D cell culture system^{2, 5}. Fournier and co-workers found

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poly(diallyldimethylammonium) chloride (PDAC)/poly(styrene sulfonate) (SPS) multilayer films to be one of the most suitable PEMs (in terms of film porosity and cell viability) when deposited as the capping layer in the LbL process over a layer of cultured cells¹⁵. However, a disadvantage of using LbL technology, as illustrated in these reports^{2, 5, 15}, is that cells are intermittently deprived of cell culture media for long periods, which are required for polyelectrolyte depositions during the assembly process. Application of such harsh fabrication conditions during the LbL process is not conducive to the health of the cells.

In this study, we demonstrate an alternative method to creating a sandwiched 3-D cell co-culture by transferring polyelectrolyte multilayers onto a charged “base” substrate under aqueous conditions, in a non-contact mode. In this non-contact transfer mode, the base substrate and the stamp do not contact each other during the multilayer transfer. Non-contact multilayer transfer can be useful for creating a 3-D cellular co-culture with a permeable polymer layer sandwiched between two monolayers of cells. An advantage is the deposition of the polymer over the cells is achieved without the need for removing the culture media. PEM transfer has been shown previously by Hammond and co-workers^{16, 17}; whereby the multilayers are transferred upon contact of the stamp to the base substrate under dry conditions. The strong electrostatic interactions between the substrate and multilayer on the stamp, and the weak hydrophobic interactions between the surface of the stamp and the hydrophobic PAH multilayer facilitated the transfer^{16, 17}. However, the pressure upon contact of the stamp onto the cultured cells and further, the stamping under dry conditions are not conducive to the cells. Here, we report a multilayer transfer process performed under physiological conditions that involves transferring an assembly of PDAC and SPS onto a charged base-substrate without the stamp being in full contact with the base substrate. The successful transfer of the non-contact, aqueous-phase multilayer (NAM) depends upon the number of bilayers in the PEM to be transferred, and on the height of the stamping surface from the base substrate.

2 Materials and Methods

2.1 Materials

Poly(acrylic acid, sodium salt) solution (PAA, M_w 15,000; catalog no. 416037), Poly(ethylene glycol) (PEG, M_w 10,000; catalog no. P6667), sulfonated poly(styrene), sodium salt (SPS) ($M_w \sim 70,000$), poly(diallyldimethylammonium chloride) (PDAC) ($M_w \sim 100,000 - 200,000$) as a 20 wt% solution, branched polyethylenimine (BPEI, M_w 25,000; catalog no. 408727), carboxyfluorescein (6-CF) (green fluorescence dye) and sodium chloride (NaCl) were purchased from Sigma-Aldrich (USA). Poly(allylamine hydrochloride) (PAH) and carboxylated polystyrene latex particles (3 μ m diameter) were purchased from Polysciences, Inc (USA). The mdPEG acid molecule (M_w 236) was purchased from Quanta Biodesign. Poly(dimethylsiloxane) (PDMS) from the Sylgard 184 silicone elastomer kit (Dow Corning, Midland, MI) or glass (Corning Glass Works, Corning, NY) were used to prepare stamps. Barnstead Nanopure Diamond (Barnstead International, Dubuque, IA) purification system was used as a source for deionized (DI) water with a resistivity of 18.2 M Ω cm. Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, 0.25% trypsin-EDTA, 1X-phosphate buffered saline (PBS), and immunostaining components (rabbit anti-paxillin antibody, Alexa Fluor 488 goat anti-rabbit IgG secondary antibody, Texas Red-X phalloidin, and DAPI) were purchased from Invitrogen (Carlsbad, CA).

2.2 Stamps for NAM Transfer

Either PDMS or glass was used as the stamp for NAM transfer of BPEI(PAA/PEG)_{10.5}(PDAC/SPS)_{80.5}. The design of the stamp was in such a way that it allowed for the

formation of a capping structure upon inversion over the base substrate (plane stamp with two posts at the extreme ends), to minimize the stress applied to cells. The edge of the stamps was at a height of $\sim 200\mu\text{m}$ above the rest of the surface of the stamp (see Figure 1b). We also tested a few other stamp heights, namely $100\mu\text{m}$, $150\mu\text{m}$, $250\mu\text{m}$, and $300\mu\text{m}$. Cells after the NAM transfer with stamp heights of $100\mu\text{m}$ and $150\mu\text{m}$ appeared to be affected by the stress due to the stamping and the intermediate layer of liquid, namely, we observed more cells died as the spacing was reduced. However, the cells after the NAM transfer with stamp heights of $200\mu\text{m}$ and more did not appear to be affected by contact stress. Given that $200\mu\text{m}$ was sufficient to minimize the stress due to stamping, we characterized the NAM transfer with the $200\mu\text{m}$ stamp.

To prepare the *PDMS stamps*, 10:1 ratio of pre-polymer to initiator was cured at 60°C overnight, over a stack of covers-slips with a total thickness of $\sim 200\mu\text{m}$ adhered on a planar substrate, which served as a master for the PDMS stamps. For patterned stamping (used in the characterization), a microfabricated silicon master with photolithographically etched patterns was used. A *glass stamp* of appropriate height was also used and consisted simply of a long glass slide with a stack of cover-slips that gave a total thickness of $\sim 200\mu\text{m}$. The stacks of cover-slips were adhered onto the two ends of a long glass slide to provide a depth or clearance of $\sim 200\mu\text{m}$ in the middle of the stamping glass region.

2.3 Polyelectrolyte Multilayer Fabrication for non-contact aqueous-phase multilayer (NAM) transfer

BPEI(PAA/PEG)_{10.5}(PDAC/SPS)_{80.5} multilayers were prepared over plasma treated PDMS or glass stamp (2 min plasma treatment at 0.15 torr and 50 sccm flow of O₂ using a plasma cleaner from Harrick Scientific Corporation, NY) as the substrate. BEPI, PAA and PEG solutions were prepared at a concentration of 1mg/ml. PDAC and SPS polyelectrolyte solutions were prepared to final concentrations of 3mM each with respect to the repeat unit of the polyelectrolytes. All the polymer solutions were prepared in DI water. PDAC and SPS polyelectrolyte solutions were supplemented with sodium chloride concentration of 0.5M. All polymer solutions (PAA, PEG, PDAC, SPS), except BPEI, as well as bath washes (pure DI water) were adjusted to a deposition pH of 2.0. PAA, PEG, PDAC, and SPS solutions were then filtered using a $0.22\mu\text{m}$ cellulose acetate filter (Corning, NY) prior to use. BPEI was the first initial layer for LbL assembly and was deposited on plasma treated PDMS or glass at the pH of 10.5, and washed with unadjusted pH DI water. A Carl Zeiss slide stainer was used for subsequent LbL deposition. Deposition time for each polymer (PAA, PEG, PDAC, SPS) was set to 15 sec (actual run time 30 sec) and wash time at each step was set to 20 sec with agitation (actual run time 1 min).

2.4 NAM Transfer process

(PDAC/SPS)_n multilayers were prepared on glass or TCPS substrate, and separately multilayers were fabricated onto a stamp as described above in section 2.3. After fabricating the multilayers on the stamp, a square or rectangular cut was made in the middle of the film using a sharp razor to facilitate the release of the multilayers during NAM transfer. Also, four notches at each corner of the stamp were made by cutting out a small portion of the corners of the stamps so that the cell culture media can flow freely underneath the stamp during NAM transfer. The stamp was inverted onto the multilayer substrate (see Figure 1a) under physiological pH, aqueous media for 24 hrs. Aqueous media was never left to dry during the NAM transfer process. The stamp was removed after 24 hrs and the multilayer transferred onto the substrate was analyzed. For the NAM transfer over cells, a layer of cells was cultured onto the (PDAC/SPS)_n multilayer substrate and allowed to grow to 70% confluency. Subsequently, the multilayer coated stamps (after UV exposure for at least 20 min) were inverted over the cells, similar to above (see Figure 1b).

2.5 Cell Culture

NIH3T3 fibroblasts cells were purchased from American Type Culture Collection (USA). Cells were maintained in DMEM with 10% FBS and 100U/ml penicillin plus 100µg/ml streptomycin (P/S) at 37°C and 10%CO₂. Cells grown to 80% confluency were detached by 0.25% trypsin-EDTA and plated at a density of 2×10^5 cells per ml with 2 ml added to all substrates studied.

2.5.1 Primary Hepatocytes Isolation and Culture—Primary rat hepatocytes were isolated from 2-month-old adult female Sprague-Dawley rats (Charles River Laboratories, Boston, MA), using a two-step collagenase perfusion technique described by Seglen¹⁸ and modified by Dunn¹⁹. The liver isolations yielded 150×10^6 to 300×10^6 hepatocytes. Using trypan blue exclusion, the viability ranged from 90% to 98%. Cells were cultured in DMEM supplemented with 10% FBS, 14 ng/ml glucagon, 20 ng/ml epidermal growth factor, 7.5 µg/ml hydrocortisone, 100 µg/ml streptomycin, 100U/ml penicillin solution, and 0.5 U/ml insulin, and placed in the incubator with a humidified atmosphere containing 10% CO₂ at 37°C. Freshly isolated hepatocytes were seeded at a concentration of 2.5×10^5 cells per ml with 2ml added to the stamped multilayers. Cell culture media was replaced daily with fresh 2ml media for up to a period of week.

2.6 Cell Immunostaining

Immunocytochemistry was performed 48 hrs post cell seeding on the surfaces at room temperature. Cells were rinsed with PBS, followed by fixation with 4.0% paraformaldehyde in PBS for 15 min, rinsed 3 times in PBS, then permeabilized with 0.1% Triton X-100 in PBS for 15 min and washed 3 times with PBS. After washing, the cells were blocked in 1% Bovine Serum Albumin (BSA, US Biological) for 30 minutes. Cells were incubated with rabbit anti-paxillin primary antibody (1:50 dilution in 1% BSA solution) for 1 hour followed by three washes in 1X PBS, and then incubated with Alexa Fluor 488 goat anti-rabbit IgG secondary antibody (1:500 dilution in 1% BSA solution) for 1 hour. Cells were washed further, three times in 1X PBS. During the secondary antibody incubation, the cells were incubated with Texas Red-X phalloidin (5µl stock per 200µl of 1% BSA solution) to visualize actin filaments. Cells were then incubated for 5 minutes in 300nM DAPI (Invitrogen) to visualize the nucleus. After two final washes with 1X PBS, the cells were imaged with fluorescence microscopy.

2.7 6-carboxyfluorescein (6-CF) Dye Staining

The 6-carboxyfluorescein (6-CF) dye was used to visualize the stamps and base substrate before and after the NAM transfer process. The dye was dissolved in 0.1M NaOH and samples were stained by immersing them into the dye solution, according to previous studies^{20–22}. The 6-CF dye is negatively charged and it preferentially stains the positively charged substrates. The stained regions appear green and the unstained regions appear black when viewed under fluorescence optical microscopy with a FITC filter.

2.8 mdPEG Stamping

mdPEG acid molecule has a carboxylic group at one end and a methoxy group at the other end of the PEG monomer chain and is a relatively small molecule (M_w 236 in the present study)^{21, 22}. The activated carboxylate functional group on one end of the PEG monomer can ionically bind to a positively charged surface and the other end (methoxy terminal group) can resist further deposition any other charged dyes or polyelectrolytes on top of the mdPEG acid^{21, 22}. mdPEG acid was dissolved in a solution of 75:25 ethanol/water (pH 6.5) mixture at a concentration of 100 µM. To create mdPEG patterns, mdPEG acid was stamped onto the sample substrates, which consist of a patterned PDMS stamp (prepared as explained

above in section 2.2), using the dip-inking method of inking the PDMS stamps and 15 min of stamping, as explained previously²¹.

2.9 Optical Microscopy

Confocal laser scanning microscopy (CLSM) images were obtained with Olympus Fluoview 1000 laser scanning confocal microscope. Phase contrast images of the cells were collected with Leica DM IL inverted microscope (Bannockburn, IL) equipped with SPOT RT color camera (Diagnostics Instruments, MI). Brightfield optical images of films were obtained using Nikon Eclipse ME 600 microscope, and conventional fluorescence microscopy images were collected using Nikon Eclipse E 600 microscope (Nikon, NY).

3 Results and Discussion

3.1 Non-Contact Aqueous-Phase Multilayer (NAM) Transfer

We show the transfer of (PDAC/SPS) multilayers from the stamp substrate onto a charged base substrate, where the multilayer transfer was performed without fully contacting the stamp to the base substrate, hereby denoted as non-contact aqueous-phase multilayer (NAM) transfer. NAM transfer can be achieved using stamps of various materials, such as polydimethylsiloxane (PDMS) or glass, provided the stamp is maintained at a desired height of $\sim 200\ \mu\text{m}$ from the base substrate. Stamps were made such that only the edges of the stamp rested on the base substrate. Thus, the transfer of the multilayer from the surface of the stamp to the base substrate occurs without the stamping area touching the base substrate (Figure 1a). The NAM transfer was demonstrated using multilayer assemblies of BPEI(PAA/PEG)_{10.5}(PDAC/SPS)_{80.5} fabricated on a stamp surface. NAM transfer can be used to encapsulate layers of cells between the polyelectrolyte multilayers (PEMs), and does not require aspirating the culture media for LbL deposition. Furthermore, the second layer of cells can be efficiently cultured and grown over the deposited PEM (Figure 1b).

Transfer of the multilayer assembly BPEI(PAA/PEG)_{10.5}(PDAC/SPS)_{80.5} is based on the concept of multilayer release from a hybrid assembly of degradable and non-degradable film, introduced by Ono and Decher²³. Non-degradable PEMs built on top of degradable multilayers can be released as a result of decomposition of the underlying degradable layers, called the sacrificial PEM. A sacrificial film breaks down by virtue of a change in external stimuli, such as pH²³, salt concentration²⁴ or temperature²⁵, resulting in the break-down of the sacrificial film, and the release of a self-standing PEM^{23–26}. The utility of degradable sacrificial films have been demonstrated in numerous applications, e.g. for tissue repair defects²⁶, patterned delivery of nucleic acids to cells⁸, controlled drug delivery^{27, 28}, etc. Poly(acrylic acid) (PAA) and poly(ethylene glycol) (PEG) are two suitable biocompatible components that can be used to make up the sacrificial PEMs, since they can form films through hydrogen bonds at low pH that decompose at high/physiological pH²³.

3.2 NAM Transfer Characterization

3.2.1 NAM Transfer Characterization: Optical and Atomic Force Microscopy—

Figure 2 shows the optical micrographs of the PDMS stamp surfaces before and after NAM transfer of BPEI(PAA/PEG)_{10.5}(PDAC/SPS)_{80.5} onto (PDAC/SPS)₁₀ multilayers, as illustrated in Figure 1. Negatively charged 6-carboxyfluorescein (6-CF) dye was used to visualize the PDMS stamps before and after the NAM transfer process. BPEI(PAA/PEG)_{10.5}(PDAC/SPS)_{80.5} multilayers were prepared on PDMS stamps and these stamps were exposed to 6-CF dye before and after NAM transfer, as shown in Figures 2a and 2b, respectively. 6-CF dye attached to the topmost positively charged PDAC layer on the stamp before NAM transfer, and to the partially exposed positively charged BPEI after NAM transfer to the base substrate. At high pH, PAA/PEG multilayers degrade slowly resulting in

a rougher surface over time^{28–30}. The partial degradation of PAA/PEG multilayers during NAM transfer could have exposed the bottom-most layer of the BPEI to the 6-CF dye. BPEI(PAA/PEG)_{10.5}(PDAC/SPS)_{80.5} multilayers when exposed to 6-CF dye after NAM transfer, resulted in non-uniform staining of the stamp. Partial degradation would cause the 6-CF dye to attach to BPEI while other regions that have not degraded the (PAA/PEG) multilayers would show no 6-CF attachment, i.e. some of the (PAA/PEG) multilayers remained on the surface. This is reflected in the highly rough film morphology (irregularities) on the stamps exposed to 6-CF dye after NAM transfer. Figures 2c and 2d show the corresponding dark field images of the PDMS stamps before and after NAM transfer, respectively, of BPEI(PAA/PEG)_{10.5}(PDAC/SPS)_{80.5} onto (PDAC/SPS)₁₀ multilayers.

Figures 2e and 2f show the optical micrographs of the surfaces of the glass stamps before and after NAM transfer, respectively. They illustrate that irregular surface features are not present on the BPEI(PAA/PEG)_{10.5}(PDAC/SPS)_{80.5} films before stamping. However similar characteristics of partial erosion of the (PAA/PEG) multilayers are visible on the glass stamp after NAM transfer, as shown Figure 2f.

Figure 3 shows the optical micrographs of the charged base substrates before and after NAM transfer of the BPEI(PAA/PEG)_{10.5}(PDAC/SPS)_{80.5} onto the (PDAC/SPS)₁₀ multilayers on the base substrate. As shown in Figure 3a, the negatively charged (PDAC/SPS)₁₀ coated substrate was exposed to negatively charged 6-CF dye before NAM transfer and as expected, no staining was observed. However, exposure of the negatively charged substrate (i.e. (PDAC/SPS)₁₀) to 6-CF dye after the NAM transfer of BPEI(PAA/PEG)_{10.5}(PDAC/SPS)_{80.5} resulted in the positive PDAC being on top and thus a uniform staining of the substrate (Figure 3b). The negative SPS charge on the glass base-substrate provides the opposite charge needed to interact with the topmost layer of the stamp (which is PDAC), required for proper NAM transfer to occur.

Similar to 6-CF staining, negatively charged 3µm carboxylated polystyrene latex particles (beads) were also used to visualize the NAM transfer. As shown in Figure 3c and 3d, negatively charged polystyrene beads did not attach to the (PDAC/SPS)₁₀ negatively charged base-substrate, with SPS as the topmost layer prior to the transfer process (Figure 3c). However, beads attached to the base substrate after NAM transfer, suggesting the deposited (PDAC/SPS)_{80.5} multilayers likely resulted in PDAC as the topmost surface on the substrate (Figure 3d).

Further, mdPEG acid molecule, which has carboxylic group at one end and methoxy group at the other end of the PEG monomer chain, was also used to confirm the NAM transfer process. Figure 4a shows an image, where the mdPEG acid was patterned onto a positively charged substrate (with PAH coated on a plasma treated negatively charged glass substrate) and subsequently exposed to 6-CF dye. This resulted in green patterns indicating the 6-CF dye have attached to the PAH regions on the substrate, and the black patterns are the mdPEG acid patterns that have the methoxy groups exposed on the surface, which will not interact with the 6-CF dye²¹. Similarly, Figure 4b shows an image, where the mdPEG acid was patterned onto the base-substrate, after the NAM transfer of BPEI(PAA/PEG)_{10.5}(PDAC/SPS)_{80.5} onto the (PDAC/SPS)₁₀ multilayers on the base substrate, and subsequently exposed to 6-CF dye. NAM transfer of the BPEI(PAA/PEG)_{10.5}(PDAC/SPS)_{80.5} multilayers onto a negatively charged base substrate likely resulted in the topmost surface on substrate consisting of PDAC. Thus, patterning this positive surface with mdPEG and subsequently exposing to 6-CF dye, resulted in green patterns of dye attached to the transferred PDAC regions on the base-substrate and the black patterns are where the mdPEG acid was stamped onto the base-substrate. Therefore, this further supports that the deposited

(PDAC/SPS)_{80.5} multilayers after NAM transfer resulted in PDAC as the topmost surface on the substrate.

We believe that PDAC/SPS multilayers were completely transferred. First, PDAC/SPS are electrostatically bonded multilayers which do not degrade due to a change in the surrounding pH. Therefore, we do not expect the PDAC/SPS multilayers to degrade when placed under physiological pH and if they transfer, they will transfer as a single multilayer structure. Second, complete transfer of the PDAC/SPS multilayers was confirmed by the characterizations shown in Figures 3 and 4. All the characterizations (6-CF dye attachment, carboxylated bead attachment, and mdPEG stamping) suggest that the topmost layer on the substrate after NAM transfer was PDAC. If there had been a partial transfer of (PDAC/SPS) multilayer, the topmost layer on the substrate could either be PDAC or SPS. However, we showed that 6-CF dye, which is negatively charged and only stains positively charged substrates, attached on the base substrate after NAM transfer (see the green image in Figure 3b) whereas it did not attach on the base substrate before NAM transfer (where SPS was the topmost layer) (see the black image in Figure 3a). Similarly, the negatively charged polystyrene beads only attached on the base substrate after NAM transfer (Figure 3d) whereas it did not attach on the base substrate before NAM transfer (where SPS was the topmost layer) (Figure 3c). Further, when mdPEG acid (which is a negatively charged molecule at one end, and neutral at other end) was stamped on the base substrate after NAM transfer, the 6-CF dye addition after mdPEG stamping provided the green/black patterns (Figure 4b), which could have not been possible had the top surface been negatively charged with SPS because there would be no mdPEG acid patterns in that case. Thus after complete transfer, the topmost layer should be PDAC. This suggests that the (PDAC/SPS)_{80.5} bilayers were completely transferred onto the base substrate with PDAC as the topmost layer which was originally the first layer built after the sacrificial (PAA/PEG) films on the stamp. This suggests that there was complete transfer of (PDAC/SPS) multilayers in the NAM transfer process. Third, Ono and Decher showed also that with (PAH/SPS)_n films formed on degradable (PAA/PEG) films were released completely as free floating films in water²³. This all support that the PDAC/SPS multilayers were completely transferred.

Further, it should be noted that there is a critical thickness of multilayers required for NAM transfer to occur successfully. All of the NAM transfers using BPEI(PAA/PEG) multilayers, as explained in this study, were carried out using 80.5 bilayers of (PDAC/SPS) assembled on top of (PAA/PEG) multilayers on the PDMS stamp. The NAM transfer process was feasible for 80.5 bilayers of (PDAC/SPS), whereas we did not observe any NAM transfer with 20.5 bilayers of (PDAC/SPS). This was confirmed using optical microscopy and ellipsometry (data not shown).

3.2.2 NAM Transfer Characterization: NAM transfer over a monolayer of cells

—PEM stamping of BPEI(PAA/PEG)_{10.5}(PDAC/SPS)_{80.5} on top of (PDAC/SPS)₁₀ multilayers under aqueous conditions in non-contact mode (NAM process) was further confirmed with cells cultured on top of the stamped surface after the NAM transfer process.

An improved *in vitro* cell culture system with enhanced hepatic function could help to supplement the current demand for liver transplantations, the main method for treating a variety of liver disease^{3, 4}. Primary hepatocyte functions are known to be enhanced when cultured in 3-D and in polymeric matrix environments^{3, 31, 32}. *In vitro* 2-D cell culture studies have shown that nonparenchymal cells, such as fibroblasts, enhance the metabolic activity of primary hepatocytes^{33, 34}. Therefore, in this study, we attempted to create an *in vitro* 3-D cell co-culture environment of primary hepatocytes and nonparenchymal cells, such as fibroblasts, to enhance the metabolic activity of hepatocytes over a 2-D culture environment. However, the limited ability of (PDAC/SPS) films to permit the transport of

large molecules precluded the formation of a successful 3-D co-culture. Nevertheless, we were able to use the co-culture as another characterization method to demonstrate the NAM transfer process.

The first layer of cells (fibroblasts) was cultured on the base-substrate of (PDAC/SPS)₁₀ multilayers (with SPS as the topmost layer). We inverted the BPEI(PAA/PEG)_{10.5}(PDAC/SPS)_{80.5} multilayers on the PDMS stamp over top of the fibroblast layer during the NAM transfer process. Multilayers when inverted over a non-confluent layer of cells in the presence of cell media (of pH ~ 7.2) degraded the sacrificial component (PAA/PEG)_{10.5} of the hybrid PEM, and thereby releasing the non-degradable multilayer (PDAC/SPS)_{80.5}. We showed previously that 25 bilayers of (PAA/PEG) degrade slowly over time and do not degrade completely for up to at least 10 days²⁸. Although it may take a long time to completely degrade a 25 bilayer PAA/PEG film, nonetheless it will release the upper layers as soon as the top layer of the (PAA/PEG) film is exposed to physiological pH, as supported by the following explanations. The degradation kinetics of (PAA/PEG) multilayers reported by Ono and Decher, showed that less than 7 bilayers of (PAA/PEG) did not release the upper films of (PAH/SPS) as self-standing, floating films, however films of greater than 7 bilayers released the upper films within 30 minutes²³. They show complete removal of (PAH/SPS) films from the (PAA/PEG) films after exposure to high or physiological pH. Further, our results (Figures 3 and 4) strongly suggest that the entire film of (PDAC/SPS) was released and transferred during the NAM transfer process (as discussed above in section 3.2.1). This suggests that the top few layers of the (PAA/PEG)_{10.5} multilayers on the stamps in this study degraded within 30 min to release the (PDAC/SPS) multilayers, even if the entire (PAA/PEG)_{10.5} multilayers are not completely degraded during the time-frame of the NAM transfer. The released non-degradable multilayer attaches to the underlying exposed base PEM (which has a negatively charged SPS on the topmost surface) and cells (which have negatively charge membrane) through electrostatic bonding. This results in a layer of cells sandwiched between two homogenous PEMs. The second layer of cells (primary hepatocytes) was subsequently cultured over top of the capped PEM film (i.e. transferred film of (PDAC/SPS)_{80.5} multilayers) (Figure 5a).

Figure 5a shows the phase contrast and fluorescence microscopy images where primary hepatocytes were cultured over the BPEI(PAA/PEG)_{10.5}(PDAC/SPS)_{80.5} multilayer that was transferred on top of fibroblasts, the latter cultured over the base substrate of (PDAC/SPS)₁₀ multilayers. Thus, this represents a sandwich 3-D co-culture of fibroblasts and primary hepatocytes, where the fibroblasts are sandwiched between two different sets of PEMs. The phase contrast images show both cell types, where the primary hepatocytes can be seen cultured partially on top of the fibroblasts. This 3-D co-cultured was then subjected to immunostaining, and was observed that the molecules, i.e. dye DAPI, primary and FITC labeled secondary antibodies for vinculin, and TRITC-conjugated phalloidin, stained the primary hepatocytes cells but not the fibroblasts cultured underneath. This suggests that there was indeed an intermediate layer of PEMs which acted as a substrate for the primary hepatocytes, and as a capping layer for the fibroblasts cultured on the base layer, below the intermediate layer of PEM. Figure 5b demonstrates the fibroblasts under the transferred multilayer did not stained with DAPI dye, whereas the fibroblasts outside the stamping region stained. In this experiment a second layer of cells was not added over the transferred multilayer. Therefore, in addition to the above characterizations discussed in section 3.2.1, the immunostaining characterization strongly suggests that there is an intermediate layer of PEMs between the two cell types which the dye molecules could not permeate. Therefore, the NAM transfer resulted in multilayer deposition over a layer of fibroblasts cultured on the base PEM substrate.

Fournier and co-workers encapsulated a cell line using PDAC/SPS multilayer deposition by sequential layer-by-layer process over a layer of cells in the presence of buffer solutions, and showed that up to six bilayers of films allowed a bidirectional diffusion of small molecules, preserving the cell metabolism after the deposition of multilayers¹⁵. Bruening and co-workers showed that multilayers of PDAC/SPS can be used for nanofiltration applications, since these multilayers can selectively exclude certain ions based on the ion size, film architecture and number of bilayers in the film^{35–37}. Typically 3–5 bilayers of PDAC/SPS are sufficient for nanofiltration. Therefore, a higher number of (PDAC/SPS) layers should be impermeable to ions. Our results are consistent with these previous studies since we observed that 80.5 bilayers of PDAC/SPS prevented the diffusion of dyes with molecular weights of 350.25 kDa (DAPI) and higher. A higher number of PDAC/SPS bilayers (80.5) instead of fewer layers were chosen with BPEI(PAA/PEG)_{10.5} multilayers because the NAM transfer was inefficient with fewer number of (PDAC/SPS) bilayers (as discussed above). Thus, a higher number of (PDAC/SPS) bilayers was useful for characterizing the multilayer transfer in the NAM process based on PAA/PEG degradation.

Since the dyes molecules could not penetrate the intermediate PEM layer, therefore we assumed the restricted transport of nutrients required for supporting fibroblast viability would reduce their ability to enhance the function of the primary hepatocytes. Thus, we did not further characterize the metabolic activity of the primary hepatocytes in these 3-D co-cultures. However, dyes and antibodies based staining of this co-culture provided an alternative method to characterizing the transfer of PEMs with the NAM process.

To obtain a successful 3D co-culture using NAM transfer process, further modifications are required with the present NAM transfer approach. A different set of multilayers, such as PAH/PAA or PAH/SPS which contain a more loopy configurations³⁸ could be assessed for improvements in the porosity and transport of nutrients and dyes across the multilayer. Alternatively, the dried BPEI(PAA/PEG)_{10.5}(PDAC/SPS)_{80.5} films on the stamp could be punctured mechanically using a micron-sized needle shaped brush, such that holes may be created mechanically to support transport of nutrients across the multilayer.

4 Conclusions

Here, we demonstrate the NAM transfer process by stamping one set of polyelectrolyte multilayers over another set of multilayers. We show the multilayer transfer using BPEI(PAA/PEG)_{10.5}(PDAC/SPS)_{80.5}, where the PEM transfer was based on the degradation of PAA/PEG multilayers at physiological pH, releasing the non-degradable (PDAC/SPS)_{80.5} multilayers. The multilayer transfer in non-contact mode is important for certain applications, such as fabrication of a 3-D cellular scaffold, where the two or more different cell types could be cultured over each other with a sandwich layer of PEMs in-between. We demonstrated a non-contact transfer of PEM is possible in aqueous conditions, which we characterized using optical microscopy and cell culture. The cell culture trials indicate that the stamped multilayer of (PDAC/SPS)_{80.5} was impermeable to molecules above a molecular weight of 250 kDa. This transferable multilayer assembly configuration requires further optimization to make the transferred multilayers more permeable to nutrients and biomolecules required for cell growth beneath the transferred film.

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Figure 1.

(a) Scheme showing non-contact aqueous-phase multilayer (NAM) transfer in aqueous media. On the stamp, the first two initial layers (green and orange layers) represent degradable H-bonded (PAA/PEG)_{10.5} multilayers, and the subsequent four layers (dark blue and dark red) represent (PDAC/SPS)_{80.5} (PDAC as topmost layer) multilayers. On the substrate before NAM transfer (left image), the two layers (light red and light blue) represent (PDAC/SPS)₁₀ (SPS as topmost layer) multilayers. During the NAM transfer, the (PDAC/SPS)_{80.5} multilayers are released from the stamp and adhere onto the (PDAC/SPS)₁₀ multilayers on the base substrate, as shown in the right image. The curved lines in the surroundings represent the liquid media at physiological pH. (b) 3-D cellular assembly after NAM transfer onto a layer of cells cultured on the base PEM substrate, which is followed by a second layer of cells seeded onto the transferred NAM multilayer.

Figure 2.

(a – d) Stamps before and after NAM transfer of BPEI(PAA/PEG)_{10.5}(PDAC/SPS)_{80.5} on top of (PDAC/SPS)₁₀. Top panel: Fluorescent images of 6-CF stained PDMS stamp before and after NAM transfer. Middle panel: Dark field optical images of PDMS stamp before and after NAM transfer. Bottom panel: (e, f) Bright field optical images of glass stamps before and after NAM transfer of BPEI(PAA/PEG)_{10.5}(PDAC/SPS)_{80.5} on top of (PDAC/SPS)₁₀.

Figure 3. Substrates before (left column) and after (right column) NAM transfer of BPEI(PAA/PEG)_{10.5}(PDAC/SPS)_{80.5} on top of (PDAC/SPS)₁₀ as illustrated by: (a, b) Fluorescent images of negatively charged 6-CF dye staining of substrates, and (c, d) Optical images of negatively charged 3μm carboxylated polystyrene latex particle added to the substrates.

Figure 4. Surfaces after mdPEG stamping and 6-CF staining on: (a) PAH coated glass, (b) substrate after NAM transfer of BPEI(PAA/PEG)_{10.5}(PDAC/SPS)_{80.5} on top of (PDAC/SPS)₁₀.

Figure 5.

(a) Fluorescent (confocal and conventional microscopy) images and DIC/phase contrast images of primary hepatocytes and fibroblast co-cultured in 3-D using the NAM multilayer transfer process. DIC/phase contrast images of both primary hepatocytes and fibroblasts show only the primary hepatocytes stained. Their corresponding fluorescent images are above the DIC/phase contrast images. Successful staining of the top layer of cells, i.e. primary hepatocytes, and no staining of the bottom layer of cells, i.e. fibroblasts, suggests that the (PDAC/SPS)_{80.5} multilayers were transferred during the NAM transfer process. Fibroblasts can be seen on the bottom layer below the primary hepatocytes, indicated by the arrows in the DIC/phase contrast images. Thick (PDAC/SPS)_{80.5} films prevented the diffusion of the staining dyes from reaching the fibroblast layer. Six different images corresponding to 6 different samples/replicates are shown, illustrating the NAM transfer. (b) Conventional microscopy and phase contrast images of fibroblast cultured in 3-D using the NAM multilayer transfer process without the second layer of cells added on top of the transferred multilayer. DAPI staining was performed after NAM transfer over the cultured fibroblasts. The NAM transfer region is shown enclosed by the dashed lines. The cells outside the stamping region stained with DAPI while the cells under the transferred multilayer (the region enclosed within the dashed lines) did not stained due to the

impermeability of the transferred films. The phase contrast image shows the fibroblasts under the transferred multilayer which remained unstained.