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FULL PAPER

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Polyelectrolyte-Clay-Protein Layer Films on Microfluidic PDMS Bioreactor Surfaces for Primary Murine Bone Marrow Culture**

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Poly(dimethylsiloxane) (PDMS) microbioreactors with computerized perfusion controls would be useful for engineering the bone marrow microenvironment. However, previous efforts to grow primary bone marrow cells on PDMS substrates have not been successful due to the weak attachment of cells to the PDMS surface even with adsorption of cell adhesive proteins such as collagen or fibronectin. In this work, modification of the surface of PDMS with biofunctional multilayer coatings is shown to promote marrow cell attachment and spreading. An automated microfluidic perfusion system is used to create multiple types of polyelectrolyte nanoscale coatings simultaneously in multiple channels based on layer-by-layer deposition of PDDA (poly(diallyldimethyl ammonium chloride)), clay, type IV collagen and fibronectin. Adherent primary bone marrow cells attached and spread best on a surface with composition of $(PDDA/clay)_5$ $(Collagen/Fibronectin)_2$ with negatively charged fibronectin exposed on the top, remaining well spread and proliferating for at least two weeks. Compared to traditional more macroscopic layer-by-layer methods, this microfluidic nanocomposite process has advantages of greater flow control, automatic processing, multiplexed fabrication, and use of lesser amounts of polymers and protein solutions.

1. Introduction

The in vivo cellular microenvironment is composed of an intricate blend of extracellular matrix proteins, soluble protein factors, immobilized protein factors, proteoglycans, small molecule signals, mineralized tissue, and numerous adjacent cell types, all of which may vary in space and time. These components present the cells with biochemical signals, and the cells are continually faced with sensing these inputs, processing the signals through signal transduction and gene regulation networks, and executing cell behavioral or fate choices.[1,2] Two of the most important microenvironment components are oxygen concentrations and the extracellular matrix (ECM). Recently, we demonstrated that oxygen concentrations can be regulated in microfluidic bioreactors made of poly(dimethylsiloxane) (PDMS).[3] Here, we focus on modulating the surface chemistry of PDMS bioreactors to create advantageous adhesive microenvironments for the culture of bone marrow cells in vitro. Although the focus of this paper is mainly the enhancement of bone marrow cell attachment, similar ECM engineering strategies will also be useful for other cell types and for regulating more complex cellular responses such as intracellular signaling[4,5] and adhesion-mediated increase in cellular secretions.[5,6]

Many types of cells that attach well to surfaces such as tissue culture plastic do not attach well to an unmodified PDMS surface, even when the PDMS surface is coated with cell-adhesive

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proteins.[7] For example, primary murine bone marrow stromal cells do not adhere well to PDMS surfaces even when coated with fibronectin or collagen (see Sec. 2.5 for details). Various approaches have been used to modify PDMS surfaces for particular applications. Modification procedures include exposure to energy sources such as plasma, corona discharge, and ultraviolet light, formation of polyelectrolyte multilayers (PEMs), radiation induced graft polymerization, silanization, atom-transfer radical polymerization, chemical vapor deposition, cerium(IV) catalysis, phospholipid bilayer modification, and sol-gel modifications.[8–12] Most of these modifications do not provide long term stability, in part because hydrophobic groups in silicones migrate up to the surface mediating hydrophobic recovery and loss of the effect of surface modification.[13] The long-term stability of biomedical devices made of PDMS is thus still questionable for applications where long-term cell attachment is required. Improved biofunctionalization methods for PDMS surfaces are needed to improve reliability.

A promising way to impart functionality to PDMS surfaces is by layer-by-layer (LBL) self assembly of polyelectrolytes and nanocomposites. LBL is a simple method that allows creation of nanoscale fuzzy assemblies or structures by alternate adsorption of macromolecular substances and most often polyanions and polycations on virtually any substrate to produce PEMs, and it has been used extensively for cell culture.[14–22] However, most PEM coatings are known to deteriorate in performance with time.[23–25] Many factors, such as the ionic strength, type of solvents, temperature, concentration of the solution, and pH of the solution (for weak polyelectrolytes) determine the multilayer structure, surface functionality, and PEM stability. Moreover, although there have been attempts to make the top surface of the PEMs biofunctional to mimic the cell-ECM interaction found in living systems and make the PEMs more versatile, more studies are needed to elucidate the interactions of the cells with the surfaces, which potentially can influence cellular processes and cell fates. Proteins are often adsorbed on PDMS to prime the surface for cell adherence; however, there are concerns that the quaternary structure of proteins, which provides them their functionality, might not be optimal when adsorbed onto PDMS.[26] However, using only synthetic polyelectrolytes will likely not produce a biofunctional surface for cell attachment; thus, inclusion of biomolecules and inorganic compounds to avoid deterioration of the properties in the cell culture media is important. The ability of LBL assembly to produce stratified coatings[27,28] can be particularly useful in this case.

Recently, the Kotov group has shown that organic-inorganic layered composites made from montmorillonite clay platelets (clay) and poly(diallydimethylammonium) chloride (PDDA) simulate morphological and mechanical properties of nacre and other biocomposites[29] and has cultured various types of mammalian cells on different compositions of PDDA/clay PEMs or PDDA/nanoparticle PEMs, which have also been modified with proteins to create cytophilic surfaces.[30–32] Some groups have also created LBL coatings inside microfluidic devices for applications ranging from improvement of electrosmotic flows in PDMS microchannels[33–35] to assembling extracellular matrix (ECM) biopolymers,[21,36] and micropatterning in PDMS channels.[37–39] PDMS channels were also used for fast, dynamic sequential LBL deposition using PDDA/PAZO[poly(1-4-(3-carboxy-4-hydroxypheylazo)benzenesulfoamido)-1,2-ethandiyil, sodium salt)] for intended application of shear induced alignment of rod-like conjugated polymers.[40]

In this report, we use Braille display-actuated microfluidic devices to automatically create multiple types of LBL films within a single run on a single bioreactor chip to screen for ideal surfaces onto which primary bone marrow stromal cells can be cultured. The LBL films tested included PDDA/Clay layers to impart chemical and mechanical stability and the two major adhesive protein components of the ECM basement membrane of the bone marrow, fibronectin (FN) and collagen (Co), to impart biofunctionality and cell adhesiveness. The coatings were tested for the ability to support primary murine bone marrow cell adhesion, viability, and proliferation over a 15 day period. We embarked on this project because the traditional method of preparing PDMS surfaces for cell culture, i.e., adsorption of an ECM protein on the substrate, did not allow for long term culture of primary stromal cells (results and Fig. S7).

2. Results and Discussion

We grew custom LBL nanocomposite coatings in microfluidic bioreactor chips using combinations of different building blocks. Figure 1 shows the design and set up of the microfluidic bioreactor chip and its alignment with the pin actuator module. The Braille peristaltic pumps and valves were used to direct flow in specific channels at different times to make LBL coatings with varying composition in four outlet channels as shown in Figure 2a–c.

2.1. Layered Nanocomposite Coatings were Produced inside PDMS Microfluidic Bioreactor Chips

The surface of cured PDMS was made reactive by exposure to oxygen plasma, which created a negative charge on the surface. The bioreactor chips with oxidized PDMS channels were aligned on the Braille pumps and the channel inlets were filled with PDDA, clay or DI water. PDDA and clay are both strong polyelectrolytes; therefore their ionic strength changes little within the pH ranges of interest. The negatively charged PDMS surface was exposed to a PDDA stream for 10–15 minutes (Fig. 2a shows the process of PDDA adsorption). Subsequently, loosely attached PDDA was removed by pumping DI water to rinse the channels for 15 minutes. In the third step, the clay inlet was valved open and pumped for 10–15 minutes. Finally, clay inlet was valved off and the channels were again washed by flow of DI water. This process created one PDDA/Clay bilayers inside the microfluidic bioreactor chip, and was repeated to get the desired number of bilayers in different outlet channels. Taking advantage of the computerized microfluidic system, all four channels could be patterned at once, or alternatively, the composition of the LBL in each channel could be

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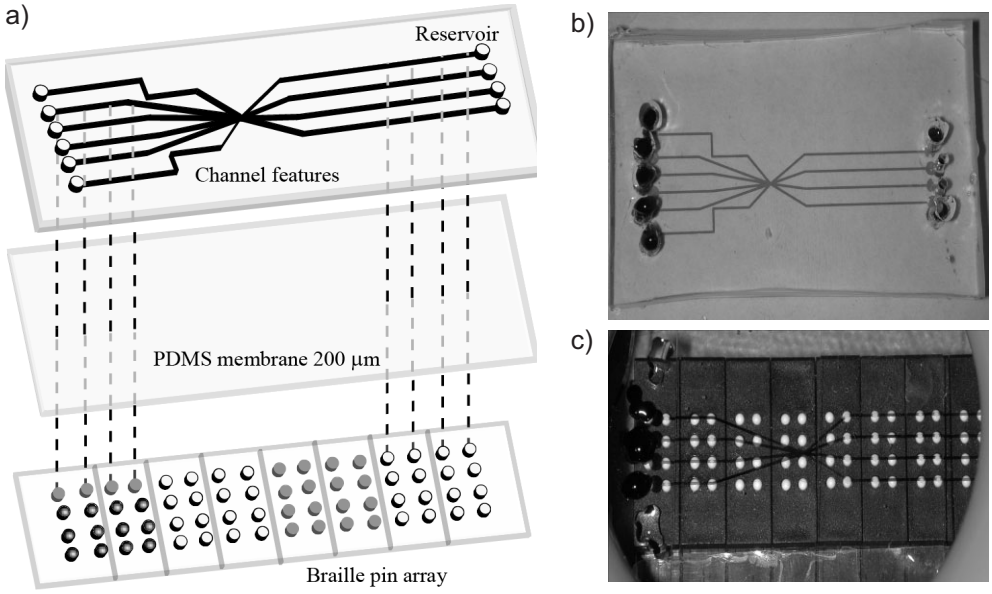

Figure 1. Microfluidic bioreactor chip design and set up. a) The bioreactor chip is composed of two components. The top component of the microfluidic device contains channel features and has punched holes (black circles) acting as reservoirs. The bottom component of the microbioreactor chip is a 200 µm thick spincoated PDMS membrane. A "horizontal" pump (four orange pins) drives cell culture media from left to right, all other channels are valved closed (gray pins are raised to close channels by deformation), and rest of the pins are in valve open position and allow fluid to pass (white pins are down), b) an actual device filled with red food dye, c) bioreactor chip aligned on Braille pins to facilitate pumping and valving.

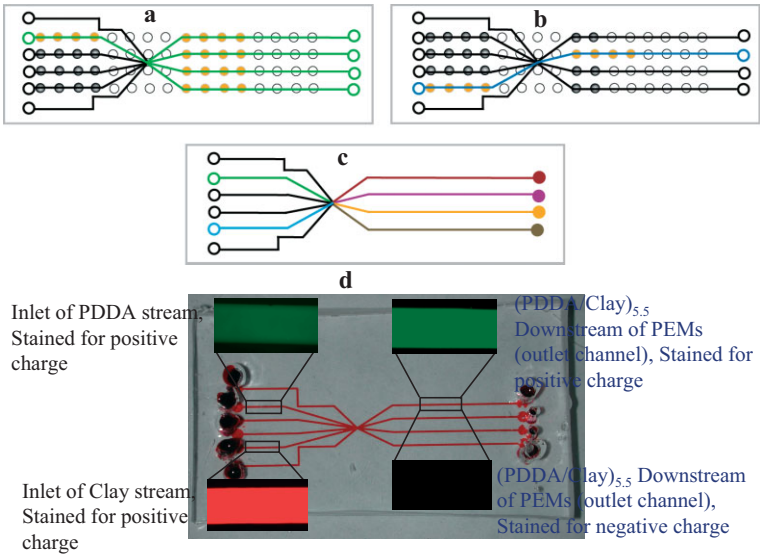

Figure 2. The creation of layered nanocomposite coatings. a) Step 1 of the process: PDDA flow and adsorption. The green channels have PDDA flowing from left to right. The black channels are valved off to avoid mingling of different streams, b) delivery of clay stream to only the second outlet channel. The orange Braille pins are acting as a pump, white are in 'valve off' position and grey are 'valve on' position. c) Schematic representation of the completed multifunctional device with different nanocomposite coatings in the four outlet channels (four different colors in outlet channels represent different composition of coating). Cell seeding is through the outlet channels. d) Fluorescent micrographs of inlet and outlet regions of PDMS bioreactor (all taken with a 10× dry objective, NA=0.25). The top nanocomposite coating layer is positively charged, as shown by pictures on the right, which show a green fluorescence for negative charge but no red fluorescence for positive charge.

varied by regulating the valving and pumping sequences that determine reagent flows. Figure 2b shows delivery of clay stream to only second outlet channel, while Figure 2c shows the bioreactor chip with four different nanocomposite coatings in the same chip.

In order to create a stratified LBL structure with proteins on top of the PDDA/Clay bilayers, inlet reservoirs of PDDA and clay were rinsed with water and protein solutions containing fibronectin or type IV collagen were introduced. Processing procedures similar to the formation of PDDA/clay multilayers were used to get microchannels with varying number of protein patterns in the outlet regions of the bioreactor chip. It should be noted that the proteins collagen and fibronectin were added as separate layers in sequential steps and not as a mixture.

LBL assembly inside microfluidic devices has been established earlier by multiple groups using laminar flow, as discussed in the introduction.[21,35,38,39] In the current study, however, with the use of Braille peristaltic actuation, it was important to not have two streams mixing with each other. When the two polyelectrolyte streams mix together due to backflow, solid debris is formed (Fig. S1). The backflow problem was countered by adding a small step in the software program, i.e., after a particular polyelectrolyte stream had completed its run, the water wash streams began flowing, and the polyelectrolyte stream was

pumped back in the opposite direction to its reservoir for 1–1.5 minutes. This back-pumping ensured that no polyelectrolyte was left in the mixing region (Fig. S2).

The resulting coatings were stable, hydrophilic, biofunctional and customizable (Fig. 2c and d). For example, it was possible to create a device with $(PDDA/Clay)_7$ in all 4 outlet channels, $(PDDA/Clay)_3$ $(Co/FN)_4$ in all 4 outlet channels, or $(PDDA/Clay)_3$ in 1st outlet channel, $(PDDA/Clay)_3$ $(Co/FN)_1$ in 2nd outlet channel, $(PDDA/Clay)_3$ $(Co/FN)_2$ in 3rd outlet channel, $(PDDA/Clay)_3$ $(Co/FN)_3$ in 4th outlet channel. Therefore, in a multifunctional device, four different compositions of PEMs were simultaneously deposited inside one single device.

2.2. Microfluidic-Made Nanocomposite Multilayers are Homogenous

Fluorescent images of staining by positively and negatively charged dyes were used to verify the charges on the topmost exposed layer, stability of the layers and homogeneity of the coatings. The inlet PDDA stream is positively charged, as shown by green fluorescence of negatively charged 6-carboxyflourescein, whereas, the inlet clay stream is negatively charged as shown by the red fluorescence picture on the bottom left panel stained by the positively-charged sulphorhodamine (Fig. 2d left inlay images). The right-side inlay images in Figure 2d shows green carboxyfluorescein staining of the outlet regions where a nanocomposite coatings of $(PDDA/Clay)_{5.5}$ with PDDA on the outermost layer have been formed confirming the existence of a positive charged surface. When the same outlet is stained for negative charge, no fluorescence was observed. This study illustrates that the outlet stream has one charge on its exposed surface, and that the nanocomposite coating formed is uniform.

Figure S5 shows the fluorescence intensity of charged dyes after deposition of each layer. The fluorescence intensity is very weak in the earlier layers, and increases with layer number. At five bilayers, the intensity is very uniform, and well distributed. No change in intensity or intensity distribution was seen between five and seven layers. Thus, we conclude that five bilayers are sufficient to cover the entire area of the surface under investigation and create a homogenous surface.

The topographical similarity of the PEMs deposited $((PDDA/Clay)_8)$ on flat PDMS substrate and microfluidic PDMS device is shown in AFM images (Fig. S6). The PEMs in microfluidic bioreactors were very similar to the ones deposited on flat PDMS in terms of morphology, topology and surface roughness. Thus both surfaces are structurally and morphologically similar. These surface characteristics also match well with previously reported multilayers formed from these components.[29–31]

2.3. Multifunctional LBL Nanocomposite Coating

To demonstrate the efficient creation of LBL coatings with the capacity to support cells, we created four very different LBL coatings in one single bioreactor chip and compared their ability to support cell attachment and proliferation. These four layers were: oxidized PDMS substrate without any LBL coating, a PDDA-topped LBL coating $((PDDA/Clay)_{3.5})$, a clay-topped LBL coating $((PDDA/Clay)_4)$, and a protein-topped LBL coating $((PDDA/Clay)_4$ $(Co/FN)_5)$.

Figure 3 shows the results from the microbioreactor chip with multifunctional LBL nanocomposite coatings. The fluorescent micrographs of the inlet and outlet regions are laid on a schematic of the bioreactor chip and show top surface charge and functionality. For each region (inlet or outlet), the pictures are stained to fluoresce green for positive charge (PDDA, Co), or red for negative charge (Clay, FN). As seen in Figure 2d, the fluorescent pictures for the outlet regions in Figure 3 also show homogenous coatings of a specific charge depending on the nanocomposite coating fabrication steps. Even in the case of protein coating, the fluorescence signal (Fig. 3d outlet channels patterned with proteins) is strong and not mixed with the other dye, consistent with the presence of a homogenous coating.

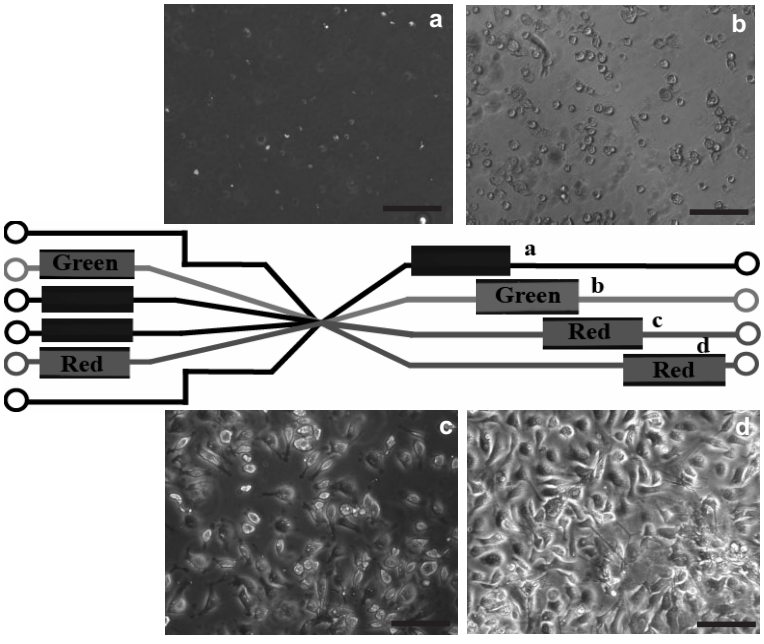

Figure 3. PDMS microbioreactor chip with multifunctional nanocomposite coatings. Four channels with compositions: a) no coating, b) $(PDDA/Clay)_{3.5}$ (PDDA topped), c) $(PDDA/Clay)_4$ coating (clay topped), and d) $(PDDA/Clay)_4$ $(Co/FN)_5$ were made by our process. The fluorescent micrographs of inlet and outlet regions (all with a 10X objective, gain = 2, exposure time = 0.10 s) are shown placed on specific points in the schematic of the microbioreactor chip to showcase different surface functionalities. For each region (inlet or outlet), the pictures are stained to fluoresce for positive charge (PDDA, Co) shown by green color and for negative charge (Clay, FN) shown by red color. The PDDA inlet stream has a green fluorescent micrograph and the DI water inlet streams have no green or red fluorescence. The clay stream inlet stream has a red fluorescent micrograph. In addition, phase contrast images (20X dry objective, all scale bars = 50 μm) compare cell cultures at day 15 of culture on the four different surfaces inside the same microbioreactor chip. For composition notation, please see Materials and Methods.

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Figure 3 also shows a comparison of cell spreading and attachment at day 15 of culture on the four different multifunctional nanocoated surfaces inside the same microbioreactor observed using a 20× dry objective (NA = 0.45, plan fluor). The cells are clearly most spread out and covering the entire surface in the last outlet channel (Fig. 3d), which had a nanocomposite coating of the composition $(PDDA/Clay)_4$ $(Co/FN)_5$. Cell adherence and spreading were reasonable in the third outlet channel (Fig. 3c) but poor on the other two surfaces. Thus by fabricating a single chip with different multifunctional nanocomposite coatings, we have compared primary bone marrow culture on four surfaces and seen distinct cellular responses. To confirm these results independently in more depth and to look at a wider range of surfaces, we performed additional experiments using multiple chips as described in the next two sections.

2.4. Number of Bilayers and Exposed Polyelectrolyte Affect Primary Bone Marrow Cells Culture on PEMs inside PDMS Bioreactors Chips

We screened a total of 30 nanocomposite coatings (different numbers of layers and combinations of components) of which we present data for 14 in this and the next sections (and Figs. 4 and 5). The 14 different nanocomposite coatings were deposited either without protein coating (this section) or with additional protein coating (next section) and tested for bone marrow stromal cell attachment. The surfaces on which we tested cell culture were either positively or negatively charged; had no proteins or had proteins adsorbed on them; and were composed of 3 to 12 bilayers. The following are the compositions of the 14 PEMs on which whole bone marrow cell culture was tested: $(PDDA/Clay)_{7.5}$, $(PDDA/Clay)_7$, $(PDDA/Clay)_3$ $(Co/FN)_{0,1,2,3}$, $(PDDA/Clay)_4$ $(Co/FN)_{0,2,4,6}$, $(PDDA/Clay)_5$ $(Co/FN)_{0,2,4,7}$. We tested three device samples per variable, and cultured cells in these devices for more than 15 days.

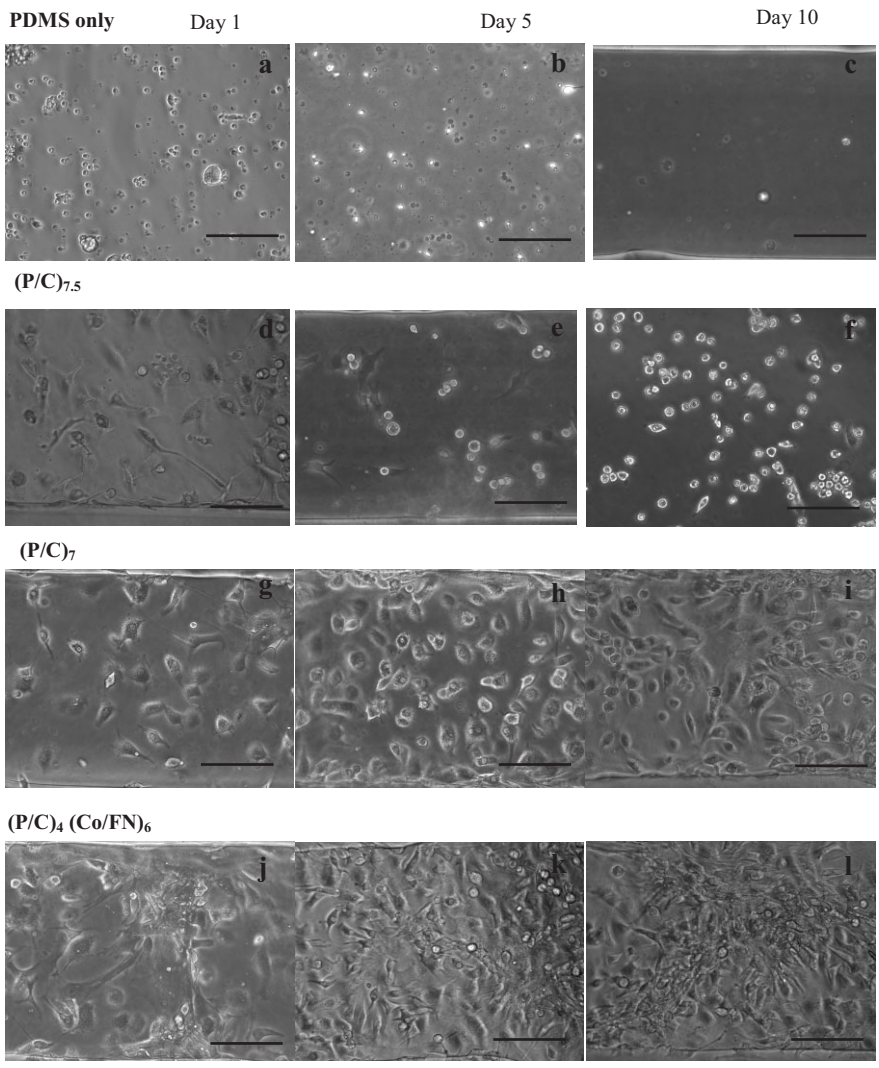

Figure 4. Spreading and attachment of bone marrow stromal cells on different nanocomposite coatings over 1 to 10 days (all scale bars = 50 μm). Bone marrow stromal cells from femurs and tibias of C57BL/6 mice were cultured on different surfaces. Micrographs depict the outlet regions of a microfluidic PDMS bioreactor chip where nanocomposite coatings were deposited and bone marrow stromal cells from femurs and tibias of C57BL/6 mice were seeded. The pictures are from representative samples at day 1 (left column), day 5 (middle column), and day 10 (right column). The cells do not attach on an oxidized PDMS surface on any day (a, b, and c). The cells attach first (day 1 and 5) and then lift off from a substrate with PDDA layer exposed to cells (d, e, f, $(PDDA/Clay)_{7.5}$, PDDA on top). The cells attach well on clay topped surfaces (g, h, i, $(PDDA/Clay)_7$, Clay on top). The cells attach, spread and proliferate best on a nanocomposite coating with proteins and larger number of (PDDA/Clay)(Co/FN) bilayers (k, L, and m, $(PDDA/Clay)_4$ $(Co/FN)_6$).

We first examined the effect of increasing the number of PDDA/Clay bilayers using 4 of the 14 different nanocomposite coatings. Figure 4 shows bone marrow stromal cells growing on different PEMs inside microchannels at day 1, 5, and 10, and quantitative measures of spreading, proliferation, and viability are given in Figure 5. The cells do not attach on an oxidized PDMS surface (Fig. 4a–c). Similarly, cells did not remain attached on PDMS surfaces with 3–4 bilayers of PDDA and clay (image data not shown). Among surfaces that do support some degree of cell attachment, cell spreading was lowest (520 μm^2/cell on day 15, condition A in Fig. 5a) when cells were cultured on a $(PDDA/Clay)_3$ nanocomposite multilayer, and increased for cells on $(PDDA/Clay)_4$ (816 μm^2/cell on day 15, condition B in Fig. 5a) and for cells on $(PDDA/Clay)_5$ (1035 μm^2/cell on day 15; condition C in Fig. 5a). On further increasing the number of bilayers to $(PDDA/Clay)_7$, cell spreading did not change significantly (1021 μm^2/cell on day 15, condition E in Fig. 5a). This may be an indication that at least 5 bilayers are needed to

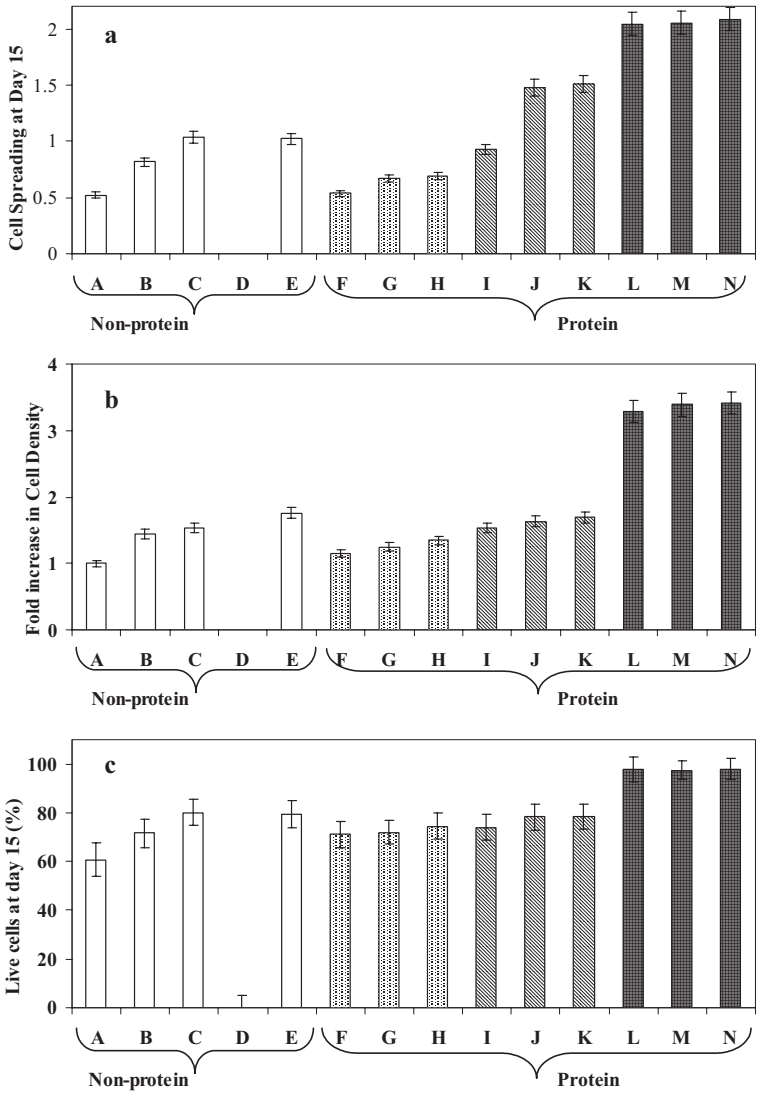

Figure 5. Cell spreading, proliferation and viability on 14 different nanocomposite coatings. a) cell spreading at day 15 ($\times 10^3$ μm^2), b) fold increase in cell density at day 15 ($\times 10^9$ cells m^{-2}), c) cell viability at day 15 (%). Legend: A= $(PDDA/Clay)_3$ $(Co/FN)_0$, B = $(PDDA/Clay)_4$ $(Co/FN)_0$, C = $(PDDA/Clay)_5$ $(Co/FN)_0$, D = $(PDDA/Clay)_{7.5}$ $(Co/FN)_0$, E = $(PDDA/Clay)_7$ $(Co/FN)_0$, F = $(PDDA/Clay)_3$ $(Co/FN)_1$, G = $(PDDA/Clay)_3$ $(Co/FN)_2$, H = $(PDDA/Clay)_3$ $(Co/FN)_3$, I = $(PDDA/Clay)_4$ $(Co/FN)_2$, J = $(PDDA/Clay)_4$ $(Co/FN)_4$, K = $(PDDA/Clay)_4$ $(Co/FN)_6$, L = $(PDDA/Clay)_5$ $(Co/FN)_2$, M = $(PDDA/Clay)_5$ $(Co/FN)_4$, N = $(PDDA/Clay)_5$ $(Co/FN)_7$. Surfaces topped with PDDA are not cytophilic for primary murine bone marrow cells.

get a homogenous nanocomposite coverage, as has been illustrated previously for some LBL pairs although not in microfluidic devices.[41]

There is some evidence in the literature that cells need a positively charged surface to attach well, since all vertebrate cells possess unevenly distributed negative surface charges.[42] However, bone marrow stromal cells did not attach well to the positively charged PDDA-topped surface, initially attaching but later shrinking in size, detaching, and dying (Fig. 4d–f, $(PDDA/Clay)_{7.5}$, PDDA on top, condition D in Fig. 5). $(PDDA/Clay)_{7.5}$ was worse for cell attachment because PDDA is exposed to cells, and PDDA has been proven to be unfavorable for primary cell attachment.[19,37,43] Therefore, even though this surface had 7.5 bilayers, the chemistry of the top exposed surface affects the cell behavior, and when polymers which are cytophobic are exposed to cells, the cells will not attach and spread for long periods of time. Other primary cells also do not attach to PDDA surfaces.[19,37,43]

In contrast, the same bone marrow stromal cells adhered to a negatively charged clay-topped surface (Fig. 4g–i, $(PDDA/Clay)_7$, Clay on top), spreading (1021 μm^2/cell on day 15, condition E in Fig. 5a) and remaining viable (79.5 % cells alive on day 15, Fig. 5c). This might be due to the higher surface area, higher roughness, and/or composition (SiO_2, $AlO_4(OH)_4$, Na+) of the clay. This montmorillonite clay is composed of materials that the cells contact in their natural environment, and thus should be biocompatible.

As evidenced from Figure S7 as well as results from cell viability and cell density assays (data not shown), there are no apparent differences between bone marrow stromal cell responses to PEMs created on flat PDMS substrates or PEMs deposited in microfluidic devices (see Fig. 5). However, the physiological ratio of cells to fluids found in microfluidic devices is expected to create a more physiological microenvironment, compared to conventional cells culture. Such differences will be advantageous for enhanced autocrine and paracrine effects, for example, and are expected to affect cell function.[2,3,44]

2.5. Protein-Topped Nanocomposite Coatings are Better at Supporting Bone Marrow Stromal Cells

The following 10 protein-topped LBL films deposited in microfluidic bioreactors were also tested for their ability to support bone marrow stromal cells: $(PDDA/Clay)_3$ $(Co/FN)_{1,2,3}$, $(PDDA/Clay)_4$ $(Co/FN)_{2,4,6}$, and $(PDDA/Clay)_5$ $(Co/FN)_{0,2,4,7}$. Figure 4k–m show the bone marrow stromal cells growing on PEMs with protein coating ($(PDDA/Clay)_4$ $(Co/FN)_6$) at day 1, 5, and 10. As compared to cells on $(PDDA/Clay)_{7.5}$ or $(PDDA/Clay)_7$ surfaces, the cells appear more spread.

On adding 2, 5, or 7 bilayers of Co/FN to $(PDDA/Clay)_5$, the cell spreading on day 15 increased from 1035 (no Co/FN) to 2039, 2053, and 2085 μm^2/cell, respectively (Fig. 5a, condition C, L, M, N). The cell density increased from 2.35×10^9 cells m^{-2} (no Co/FN) to 2.61×10^9 cells m^{-2} (Fig. 5b, condition C, L, M, N), and the percentage of live cells increased from 80 % (no Co/FN) to 98 % (Fig. 5c, condition C, L, M, N), when two to seven bilayers of Co/FN were added to five bilayers of PDDA/Clay. Differences between 2, 5, and 7 bilayers of Co/FN were not statistically significant, suggesting that two layers of Co/Fn were sufficient to coat the PDDA/Clay surface completely and provide sufficient adhesive ligands on the exposed surface. There may be a synergistic

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contribution of having both (PDDA/Clay) and (Co/FN) layers, as the stiff mechanical properties of (PDDA/Clay) ($E_{(P/C)1} = 0.11$ GPa[29]) compared to the relatively soft PDMS ($E_{PDMS} = 2$ MPa[7]) are also known to be beneficial for cellular adhesion.[30,32]

For comparison with PEMs that have PDDA/Clay coatings, bone marrow stromal cells were also cultured on flat PDMS substrates on which either Co, or FN or a 1:1 mixture of Co and FN was adsorbed for 30 minutes. On these surfaces, the stromal cells detached after only a few days of seeding. The FN adsorption control was also performed in a microfluidic device surface, and the same result was found. Lack of cell spreading on these surfaces at day 15 is shown in Figure S7.

The composition of the multilayers affects cell behavior as evidenced by cell spreading, attachment, proliferation and viability on biofunctional nanocomposite coatings. The cell sizes became larger with time on these cytophilic coatings. The bone marrow stromal cells achieved 80 % confluence by day 3 of culturing in these nanocomposite coatings. These nanocomposite coatings had the most spreading, proliferation and density, and the largest number of live cells. Among the biofunctional nanocomposite coatings, the best ones for culture of bone marrow stromal cells were $(PDDA/Clay)_5(Co/FN)_{2,4,7}$. These results are consistent with our earlier observation about the effect of composition of nanocomposite coating on its ability to support primary bone marrow culture.

The ECM combination we used in the current study is rather unique as FN has a Co binding domain.[45] In fact, FN and Co are often used together for cell culture studies.[46] Moreover, fibronectin and type IV collagen are both important members of the extracellular matrix of the bone marrow microenvironment.[47] Both positively and negatively charged proteins adsorb to some extent on multilayer surfaces regardless of the surface charge because proteins bear on their surfaces domains with both positive and negative surface excess charges. Not surprisingly, adsorbed amounts and the protein layer thicknesses are larger when the proteins and the terminating polyelectrolyte layer are oppositely charged compared to protein adsorption onto films terminating with a similarly charged polyelectrolyte layer.[48] Proteins might denature and change conformation once adsorbed on the surface,[26] which may affect biological responses and confound the data. The dominant charge of proteins depends on the pH, and pH was carefully monitored during our experiments. It is believed that other interactions, such as van der Waals and hydrophobic attraction are also likely to contribute to protein layer formation and stability.[16,29]

Our results compare well with other specific reports of culture of bone marrow cells and other primitive cells.[15,49] More broadly, PDMS substrates are widely used in medical research and in a variety of clinical devices.[50] Control of the polymer surface chemistry is a crucial aspect of such devices which rely heavily on cell-biomaterial interface. PDMS substrates are intrinsically hydrophobic due to the low surface energy backbone[13,50,51] and require a biofunctional hydrophilic film on the surface in order to promote cell attachment and interaction with substrate. The biofunctional nanocomposite coating described in this report is specifically useful for making PDMS and other substrates conducive to culture of primary bone marrow cells. Our results with stratified LBL assembled films for primary murine bone marrow culture also corroborate studies with a variety of other cells types that demonstrate the broad usefulness of biofunctional LBLs on PDMS substrates.[14,22,37,38,52] Thus, the computerized microfluidic LBL methods described should also be more broadly applicable to a wide range of cell types and with many complimentary advantages over traditional LBL film formation methods, such as computerized pumps and valves for greater flow control, automatic processing using programmable software, customized creation of different coating compositions in different microchannels in one experiment, and reduced use of polymer and protein solutions. Although this manuscripts describes these concepts using a relatively simple channel system with relatively low number and density of channels, the methods and devices are compatible for scale up to enable simultaneous preparation and testing of a much larger number of variables.[53,54]

3. Conclusion

In this work, we have made the surface of microfluidic PDMS bioreactor chips hydrophilic, stable and cytophilic for an extended time by control of surface chemistry. This is achieved by the growth of stable biofunctional nanocomposite coatings on the PDMS surface, so that the primary murine bone marrow stromal cells can attach and spread on the substrate and mimic ECM-cell interactions *ex vivo*. The nanocomposite coatings were made utilizing computerized microfluidic perfusion systems and various polyelectrolytes PDDA, clay, type IV collagen and fibronectin to optimize the PDMS channel surface for successful culture of primary murine bone marrow cells in ex vivo cultures. The adherent cells of marrow attached and spread on nanocoated PDMS microchannel surfaces for more than two weeks. The protein-topped nanocomposite coatings ($(PDDA/Clay)_5$ $(Co/FN)_{2,4,7}$) were best to achieve maximum cell spreading, proliferation and viability for these cells. Although this paper focused on optimizing attachment of bone marrow stromal cells, the process should be useful for creating distinctive ECM inside PDMS bioreactors for a wide variety of cell types.

4. Experimental

Materials and Methods: Cell Culture: Unsorted primary bone marrow cells (hence forward called bone marrow stromal cells) from femurs and tibias of C57BL/6 mice were cultured in MyeloCult (M5300, StemCell Tech.) supplemented with 1 % v/v hydrocortisone (07904, StemCell Tech.) and 1 % v/v antibiotic-antimycotic (15240, Gibco). The 25 cm^2 T flasks (3815, Corning) were placed in a humidified 5 % CO2 cell culture incubator at 33 °C. Cells were allowed to grow for two weeks in the plastic dishes in 8 mL of media before plating on PDMS substrates. Half of the media in T flasks was replaced every week.

After 2 weeks, cells were collected by washing in Hank's Balanced Salt Solution (37250, StemCell Tech) and incubated with 0.25 % Tryp-

sin/EDTA (07901, StemCell Tech). The trypsin solution was neutralized with 25 % serum in MyeloCult and spun down for 5 min at 4 °C and 1000 rpm. The supernatant was removed and the pellet was resuspended in 200 μL of MyeloCult media, and this suspension was used for seeding cells onto the PDMS substrates. The cell suspension was seeded in the outlet of the channels from which it was directed into specific areas of the device.

Preparation of the Microfluidic Bioreactor Chip: The chip consisted of two components: a channel top layer and membrane bottom layer, as seen in Figure 1A. The chips were formed from pre-polymer (Sylgard 184, Dow Corning) at a ratio of 10:1 base to curing agent. The top layer was formed using a glass wafer mold (soft lithography) [55] to form a layer with negative relief channel features ~ 30 μm in height and 300 μm in width. The positive relief features of the mold were composed of SU-8 (Microchem, Newton, MA) formed on a thin glass slide (200 μm thick) using backside diffused-light photolithography [56]. The glass slide was silanized with tridecafluoro-(1,1,2,2-tetrahydrooctyl)-1-trichlorosilane (United Chemical Technologies Inc., Bristol, PA). This channel layer was cured overnight at 120 °C and holes were punched (Dermal biopsy puncher, Miltex Inc., York, PA) in the channel layer to connect channel features to the bottom layer. The bottom layer was a thin membrane of PDMS (200 μm) prepared by spin coating (Cee 100 Spin Coater, Brewer Science Inc., Rolla, MO) and curing PDMS in an oven at 120 °C overnight. The PDMS pieces were bonded by plasma oxidation [44,54,57].

LBL Nanocomposite Fabrication: For the coating process, the surface of cured PDMS was made reactive by placing it in oxygen plasma for 60 s at 300 mTorr. The peristaltic pumping sequences of Braille pins deposited alternate layers of 0.5 wt % poly(diallyldimethylammonium chloride) (PDDA)(+) ($M_w = 200\,000$, Sigma, MO) and montmorillonite clay (–) (Aldrich, MO) onto the PDMS surface of the microfluidic bioreactor chip. Between each polyelectrolyte deposition sequence, the channels were thoroughly washed with deionized water. Four to eight bilayers of PDDA/clay were deposited. To test the charge on the top exposed surface, fluorescently labeled charged dyes were used. Sulforhodamine (Sigma, MO) detected the negative charge, and 6-carboxyfluorescein (Sigma–Aldrich, MO) detected the positive charge on the surfaces.

LBL deposition was continued by the assembly of bilayers of proteins onto the base PDDA/Clay coating. Collagen type IV (lyophilized from human placenta, Sigma, MO), positively charged at pH 4 [32], and fibronectin (human plasma, Sigma, MO) negatively charged at pH 7 were deposited on the PDDA/Clay bilayers. One to seven bilayers of the biomolecules were deposited on the top of PDDA/Clay bilayers. After the LBL coatings were assembled, the bioreactor chip reservoirs were rinsed multiple times with DI water, and finally sterile PBS was added to all reservoirs and the device was stored in a sealed Petri dish at 4 °C until further use. When the devices were ready for cell culture testing, they were first sterilized under UV light in a bio-safety cabinet for 30 minutes. The PBS in the reservoirs was replaced by MyeloCult, and cells were seeded from the outlet channels.

Our notation for PEM composition is as follows: $(PDDA/Clay)_5$ refers to deposition of five bilayers of PDDA and clay, and the top (exposed) surface is clay. $(PDDA/Clay)_{5.5}$ means that 5.5 bilayers of PDDA and clay were deposited, and thus the top (exposed) surface is PDDA. $(PDDA/Clay)_{5,6,7,8}$ means that a device was fabricated with composition of $(PDDA/Clay)_5$ in first outlet channel, $(PDDA/Clay)_6$ in second outlet channel, $(PDDA/Clay)_7$ in third outlet channel, and $(PDDA/Clay)_8$ in fourth outlet channel. Likewise, Co/FN refers to a PEM built with one layer of positively charged collagen overlaid with one layer of negatively charged fibronectin. It is not a mixture of two proteins.

LBL Deposition on Flat PDMS Substrates: PEMs were deposited on flat PDMS substrates using LBL technique [16,29]. Six beakers were arranged in series, and PDDA was added to the first and clay to the fourth, the rest contained DI water. Two mL of PDMS was dropped in 35 mm Petri dishes and cured in a 60 C oven for two hours. After plasma oxidation, these Petri dishes were dip coated in the beakers described above to create PEMs of different compositions. The following PEM composition were created on flat PDMS substrates: $(PDDA/Clay)_5$, $(PDDA/Clay)_{5.5}$, $(PDDA/Clay)_7$, $(PDDA/Clay)_5$ (Co/FN)$_3$.

In addition, the following surfaces were also created on flat PDMS substrates: fibronectin adsorbed on a flat PDMS substrate, collagen type IV adsorbed on flat PDMS substrate, fibronectin and collagen type IV (1:1) adsorbed on flat PDMS substrate. A control of fibronectin adsorption was also prepared in PDMS microdevice.

Device Cell Seeding: After sterilization, PBS was replaced with Myelocult media in the reservoir, and the bioreactor chip was placed on an array of pin actuators adapted from Braille displays for at least one hour to peristaltically pump fluid through the channels [57]. Bone marrow stromal cells were then seeded into the chip from the outlet channels. The cells were manipulated to attach only in the area of the bioreactor chip where nanocomposite coatings were earlier formed by using a software program to block out other parts of the device. The cells were given 1–2 h to attach under static (pins up and channels valved closed) condition in a humidified incubator (Forma 310 Series Direct Heat CO_2 Incubator, Thermo electron Corporation**,** Marietta, OH) maintained at 33 °C and 5 % CO_2.

The bioreactor chips were then taken off the pumping hardware and stored in Petri dishes inside the incubator. The Petri dishes were further stored in a larger container with DI water to prevent evaporation. The cells were grown on the nanocomposite coatings for up to 15 days, with media changes every other day. The media was changed every day by simple aspiration from the inlet and outlet reservoirs. Pictures of the cells growing in different nanocomposite coatings were taken every day. Cell densities, cell spreading, cell proliferation and cell viability were recorded over 15 days.

Fluid Actuation: An array of 64 pin actuators adapted from a Braille display module (SC9, KGS, Saitama, Japan) was used for fluid actuation [57]. The pin actuator module was controlled with a computer via Universal Serial Bus (USB) through a finger-sized stand alone custom controller circuit board (Olimex, Plovdiv, Bulgaria). The microfluidic bioreactor chip interfaced with the pin actuator module by simply holding the chip in place by using some weights such that the channels align with the pins which push upward closing the channel, as seen in Figure 2. The pin movements for valving and pumping were controlled with a custom computer program written in C #[3]. The average flow rate was controlled by changing the time delay between pin motions. The flow rate was kept constant at $(8.3 \pm 2.3) \times 10^{-2}$ μL s^{-1} for all experiments.

Cellular Assays: Cell Spreading, Cell Density, Cell Proliferation, and Live-Dead Assay: The microbioreactor chip was mounted on a glass slide and examined on an inverted phase contrast microscope (Nikon TE-300) with a 20× dry objective. Black and white images were captured with a CCD camera (Hamamatsu ORCA-100). Simple PCI software (Compix Inc; Cranberry Township, PA, ww.cimaging.com) was used for capturing and analyzing the images and calculating cell surface area, cell densities and cell viability. For cell viability, LIVE/DEAD Viability/Cytotoxicity Kit (Invitrogen, Carlsbad, CA) containing Calcein AM and ethidium bromide was used to quantify percentage of cells alive at day 15 of bone marrow stromal cell culture. The controls for these experiments were microbioreactor chips without any surface modifications, and the variables were chips with varying nanocomposite coatings. Cell proliferation was measured as increase in cell density at day 15 compared to day 1. Cell viability was measured as percentage of cells alive at day 15. For all the cellular assays, experiments were performed in triplicates, and fifteen images were analyzed to obtain one data point. The data were analyzed statistically by ANOVA and Tukey's test at a 95 percent confidence level assuming unequal variances. There were 14 different types of PEMs created and tested in microdevices plus 4 different types PEMS created and tested on flat PDMS slabs. Each PEM substrate was analyzed through micrographs of ten different regions to give ten different data points each for cell viability and cell density. These ten data points were collected every day for fifteen days to give a total of fifteen data sets for each PEM tested. In addition, all experiments were performed in triplicates. ANOVA was performed to find differences between the 14 different PEMs created inside microchannels as well as between the 4 PEMs created on flat PDMS and Tukey's

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test was performed to find differences between specific PEMs within each set of surfaces.

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