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Microfluidic Synthesis of Composite Cross-Gradient Materials for Investigating Cell–Biomaterial Interactions

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Abstract

Combinatorial material synthesis is a powerful approach for creating composite material libraries for the high-throughput screening of cell–material interactions. Although current combinatorial screening platforms have been tremendously successful in identifying target (termed “hit”) materials from composite material libraries, new material synthesis approaches are needed to further optimize the concentrations and blending ratios of the component materials. Here we employed a microfluidic platform to rapidly synthesize composite materials containing cross-gradients of gelatin and chitosan for investigating cell–biomaterial interactions. The microfluidic synthesis of the cross-gradient was optimized experimentally and theoretically to produce quantitatively controllable variations in the concentrations and blending ratios of the two components. The anisotropic chemical compositions of the gelatin/chitosan cross-gradients were characterized by Fourier transform infrared spectrometry and X-ray photoelectron spectrometry. The three-dimensional (3D) porous gelatin/chitosan cross-gradient materials were shown to regulate the cellular morphology and proliferation of smooth muscle cells (SMCs) in a gradient-dependent manner. We envision that our microfluidic cross-gradient platform may accelerate the material development processes involved in a wide range of biomedical applications.

Keywords

cross gradient; microfluidics; cell–biomaterial interactions

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Jiankang He and Yanan Du contributed equally to this work.

Author contributions: JH, YD, DL, and AK designed the study; JH, YD, and YG performed the experiments; MJH developed the theoretical model and simulations; BW performed SEM and HS performed FTIR; JH, YD, and JW analyzed the data. JH and YD contributed equally as lead authors. JH, YD, MJH, and AK wrote the article. All authors discussed the results and commented on the manuscript.

Additional Supporting Information may be found in the online version of this article.

Introduction

The discovery of new materials with properties tailored to meet specific needs has the potential to lead to advances in biomedicine (Slaughter et al., 2009). An effective approach to material discovery is to create composite materials that combine the desired properties of two or more constituents. Nearly one-quarter of all manufactured polymeric materials can be categorized as composite materials (Simon et al., 2005). Combinatorial and high-throughput approaches revolutionized the design and synthesis of composite materials with accelerated discovery times and reduced costs compared to conventional low-throughput approaches (Meredith, 2009). Currently, the best methods for combinatorial material screening use microarrays or microwells to synthesize and analyze material libraries of hundreds to thousands of combinations on microscopic slides or titer plates (Yang et al., 2008; Meredith, 2009; Hook et al., 2010). These methods have rapidly screened the cell–material interactions from established material libraries to identify target (so-called “hit”) materials offering the desired regulation of cellular functions and behaviors (Anderson et al., 2004, 2005; Flaim et al., 2005; Meredith, 2009). To date, the majority of combinatorial material screening methods involved the synthesis and analysis of many discrete mixtures, each with different constituents but few variations in their concentrations (Hook et al., 2010). The concentration of a pure material and the blending ratio of multiple material components can dramatically affect the composite material properties (e.g., surface chemistry, modulus, roughness, and crystallinity) and the corresponding cell behavior (e.g., cell morphology, migration, function, and stem cell differentiation) (Pelham and Wang, 1997; Meredith et al., 2003; Engler et al., 2006; Liu et al., 2009). Therefore, the lack of variation in the material concentrations and blending ratios of existing screening platforms may limit their effectiveness. New combinatorial material synthesis approaches are needed to further optimize the concentrations and blending ratios of the target materials.

Here we employed a microfluidic gradient platform that previously produced gradients of polymers and microparticles (Du et al., 2010) to rapidly synthesize gelatin/chitosan cross-gradient materials that exhibit gradients in chemical composition and cell response. Chitosan and gelatin were chosen since they are widely used natural biomaterials with complimentary properties. Chitosan exhibits good biocompatibility and biodegradability, but restricts cell spreading and cytoskeletal stretching due to strong electrostatic interaction (Huang et al., 2005a). In contrast, gelatin is bioadhesive and promotes cell spreading and proliferation, but it is mechanically weak and easily degraded by enzymes. Positively charged chitosan can interact with negatively charged gelatin at pH values higher than 4.7 to form polyelectrolyte complexes (Yin et al., 1999) with improved mechanical, biological, and biodegradability properties compared to those of gelatin and chitosan alone (Huang et al., 2005a; Thein-Han et al., 2009). The porous structures in the gelatin/chitosan cross-gradients were created upon lyophilization. We analyzed the chemical composition along the cross-gradient materials by Fourier transform infrared spectrometry (FTIR) and X-ray photoelectron spectrometry (XPS). The chemical compositions of the cross-gradient materials were statistically correlated with the concentrations and blending ratios of the two components. Smooth muscle cells (SMCs) were cultured on the porous gelatin/chitosan cross-gradient materials and exhibited gradients in morphology and proliferation. We envision that our microfluidic platform could produce a wide range of biomaterials with gradients in chemical, mechanical, and biological properties, which could prove useful for the rapid discovery of target materials.

Materials and Methods

Materials

All reagents were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise noted. Type I collagen (Invitrogen, Carlsbad, CA) was labeled with amine-reactive DyLight 549 and 649 (Thermo Scientific, Waltham, MA). The deacetylation of chitosan was 85%. Chitosan (1.5%, w/v) and gelatin (1.5%, w/v) solutions were dissolved in 0.4M acetic acid solution (pH $\approx$ 3) and then prepared as previously described (He et al., 2009), sterilized using 0.2- μ m pore filter and either used directly or stored at 4°C.

Generation of a Cross-Gradient of Two Species in a Microfluidic Device

The microfluidic device consisted of a poly(dimethylsiloxane) (PDMS) upper layer with a straight fluidic channel (50 mm $\times$ 2.0 mm $\times$ 100 μ m) attached to a glass slide (Du et al., 2009; He et al., 2010). In a previous work, we related the gradient length and evolution to the flow speed and rest times before flow reversal (Du et al., 2010). Here we shortened the duration of the gradient protocol by avoiding rest times, and instead optimized the volume of fluid pumped back and forth a fixed number of times in the channel at a fixed flow rate to yield the longest possible gradient. We chose a flow rate of 0.0143 mL min⁻¹ as a tradeoff between better pump control and high speed flow. Specifically, our “cross-gradient protocol” proceeded as follows: the channel was filled with solution 1 containing the first species at a flow rate of 0.0143 mL min⁻¹ (forward flow). The flow was generated by a syringe pump (World, Precision Instruments Aladdin, 1000, WPI, Sarasota, FL). Two hundred microliters of solution 2 containing the second species was pipetted onto the inlet opening, and a pre-defined volume was drawn into the channel (backward flow). A specified volume of fluid, called the “pumped volume,” was pumped back and forth five times (backward, forward, backward, forward, backward) to generate a cross-gradient of the two species. The microfluidic system containing the cross-gradient solutions then stood at rest for at least 30 s before being disconnected from the syringe pump and stood at rest for another several minutes to allow for further diffusive mixing before freezing. Note that the timescale for diffusion across the $H=100\mu\text{m}$ height of the microchannel is $H^2/(\pi^2 D)=10\text{--}100$ s for molecular diffusivities $D=10^{-7}\text{--}10^{-6}\text{ cm}^2\text{ s}^{-1}$, typical for the types of biomolecules used in this study. Thus, we expect that our cross-gradient solution is vertically well mixed prior to freezing.

The syringe pump was calibrated to pump accurate volumes of fluid through the channel. Small lags in the pump's mechanical system led to small differences in the volumes pumped in different directions. To compensate for the lag, a calibration between the pre-set and pumped volumes was carried out experimentally; the linear calibration curve specific to our pump is shown in Figure S1.

Computational Simulations of Cross-Gradient Generation

The evolution of the cross-gradient was predicted with a previously developed computational model (Du et al., 2010). The initial sharp transition between species was assumed to start at $x=0$, where x is the center axis of the channel. Though the channel itself was 5 cm long, the portion between the ports where the usable material was produced was $L=4.5$ cm long. Thus, we took $x=0$ and $x=L$ to denote the ends of this usable portion of the channel. We defined the cross-gradient length (CGL) as the length of the region within $0<x<L$ where the concentrations of each species were between 10% and 90%. CGL was computed from our experiments and computer simulations as shown in Figure S2. In our experiments, the channel width and height were $W=2\text{mm}$ and $H=100\mu\text{m}$, respectively. The flow rate was $Q=0.0143\text{ mL min}^{-1}$ and the average flow speed was $U=Q/(WH)=1.19\text{ mm s}^{-1}$. The model input parameters were the channel cross-sectional aspect ratio W/H 20 and the

Péclet number $Pe=UH/D=917$, where the molecular and diffusivity $D = 1.3 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ is for 10 kDa FITC-dextran and 10 kDa RITC-dextran (Du et al., 2010). To model the experiments, the simulations used a flow sequence in which a volume V , called the “pumped volume,” was pumped back and forth five times (backward, forward, backward, forward, backward) with average speed U . Simulations were run for V ranging from 0.25 to 10 μL in increments of 0.25 μL .

Experimental Optimization of Cross-Gradient Generation

The effect of the “pumped volume” on the length of the cross-gradient was studied experimentally. The cross-gradient protocol listed above was employed with solutions 1 and 2 containing 1 wt% gelatin and 1 wt% RITC-dextran, respectively, and pumped volumes 1, 2, 3, 4, 5, 6, 7 μL . A second set of cross-gradients were produced with solutions 1 and 2 containing 1 wt% FITC-dextran and 1 wt% gelatin, respectively. The fluorescently labeled dextran gradients were imaged separately using a Kodak Gel Logic 100 Imaging System, superposed digitally, and quantified with ImageJ to estimate the cross-gradient length, as defined in Figure S2.

Characterization of Chemical Composition of Gelatin/Chitosan Cross-Gradients

Gelatin/chitosan cross-gradients were created using the cross-gradient protocol with solutions 1 and 2 containing 1.5% chitosan and 1.5% gelatin, respectively. The cross-gradients were stabilized by freezing at -80°C , and then air-dried to obtain gelatin/chitosan composite films.

FTIR analysis characterized the chemical composition along the gelatin/chitosan cross-gradient film. The sample (5 cm in length) was divided into ten consecutive sections and analyzed on an ALPHA FTIR spectrometer (Bruker Optics, Billerica, MA). The spectrum of each section was recorded from 400 to 4,000 cm^{-1} with a resolution of 4 cm^{-1} . The spectra were analyzed using OMNIC (Thermo Electron, Waltham, MA). For chitosan, gelatin and their blends, the absorption bands at 1,654–1,640 cm^{-1} for Amide I correspond to C=O stretching, while the absorption bands at 1,580–1,534 cm^{-1} for Amide II denote N-H bending (Thein-Han et al., 2009). The ratio of the peak heights was calculated for eight discrete blends (gelatin ratio: 0%, 12.5%, 25%, 37.5%, 50%, 62.5%, 75%, and 100%) and five samples for each blend. The chemical compositions along three cross-gradient samples were quantified using a calibration curve based on the first six blends. Due to the effects of the inlet/outlet ports, sections 1 and 10 were not used for FTIR characterization.

XPS enabled further characterization of the surface chemistry of the gelatin/chitosan cross-gradient film. Analyses were performed on a Kratos Axis Ultra XPS instrument using a monochromatic Al Ka radiation source operating at 15 kV and 10 mA. The elements in the sample surface were identified from a survey spectrum at a pass energy of 160 eV. The areas under the specific peaks were used to calculate the atomic percentages. High-resolution spectra were also recorded at a pass energy of 20 eV, and overlapping peaks were resolved into their individual components by CasaXPS software. Eight points along the sample were measured at 0.5 cm intervals.

Structural Characterization of Gelatin/Chitosan Cross-Gradients

To fabricate porous composite structures, the gelatin/chitosan cross-gradients were freeze-dried under different conditions. The effects of the pre-freeze temperature and duration on pore morphology were quantified by a scanning electron microscope (SEM, ULTRA 55, ZEISS, Thornwood, NY). Before visualization, the scaffold was sputter coated with gold. Overlapping phase images were taken along the channel using a phase microscope (Nikon Eclipse TE2000-U, Avon, MA) and stitched together.

Quantification of Cell Behavior on the Porous Gelatin/Chitosan Cross-Gradient

To quantify the cell behavior on the porous gelatin/chitosan composite materials, porous scaffolds were treated with alcohol for 2 days to remove any residual acetic acid and then washed five times in DPBS and twice in culture medium. SMCs were cultured in SMC basal medium (RPIM 1640, Gibco, Invitrogen, Carlsbad, CA) at 37°C in a humidified incubator. Upon trypsinization, the cells were seeded at a density of 1×10^4 cells/cm² on the surface of the porous structures. After 2 h of cell seeding, the samples were washed with culture medium to remove unattached cells. To visualize the cytoskeleton at days 1 and 3, the samples were fixed in 3.7% formaldehyde solution, treated with 0.1% Triton X-100 solution and stained with Alexa Fluor 594 phalloidin (Invitrogen). Cell nuclei were stained with DAPI to facilitate cell number counting. The samples were also visualized with a fluorescent microscope.

Cell count and morphology were analyzed from the resulting images with ImageJ. The cell morphology was quantified by the cell shape factor, defined (Huang et al., 2005b) as $4\pi \text{ area}/(\text{perimeter})^2$. Cell shape factors close to 1 indicate a circular shape. Statistical analyses were performed with one-way variance analysis (ANOVA) and Tukey HSD tests for post hoc comparison. Values of $P < 0.05$ were considered statistically significant.

Results

Generation and Stabilization of Composite Cross-Gradient Materials

Our composite materials were rapidly synthesized by first producing a gradient in the relative concentrations of two materials using our cross-gradient protocol and then stabilizing the gradient by freezing (Fig. 1A). The channel was filled with solution 1 containing the first material. Solution 2 containing the second material was pipetted onto the inlet and subsequently drawn into the channel. A gradient in the relative concentration of the two materials was fully formed after pumping the fluid back and forth five times, and was visualized with fluorescent labels (Fig. 1B). The concentration cross-gradient could be stabilized by freezing the sample in situ on the microfluidic chip. The PDMS upper layer was then peeled off to expose the composite material, which could be air-dried to obtain a composite film or freeze-dried to create a porous structure. Figure 1C shows a phase image of a composite film consisting of a gelatin/chitosan cross-gradient. The different optical properties of gelatin and chitosan caused the composite film to gradually change from opaque (gelatin-rich) to transparent (chitosan-rich). To facilitate the visualization of the freeze-dried porous gelatin/chitosan structures, we intentionally skipped the glutaraldehyde pre-cross-linking so the gelatin would easily dissolve in neutral or alkaline solutions (e.g., DPBS) leaving only chitosan. As shown in Figure 1D, the brightness of the porous composite material in DPBS gradually increased from the gelatin-rich to chitosan-rich sides, indicating the gradual decrease of gelatin and increase in chitosan concentrations along the sample.

Optimization of Cross-Gradient Synthesis

The length of the cross-gradients in our composite materials was controlled by adjusting the volume of fluid pumped in each direction, called the “pumped volume”. Creating cross-gradients of fluorescent molecules and measuring the fluorescent intensity along our microchannels elucidated the dependence of the cross-gradient length on the pumped volumes. Provided the transported species are non-reacting, their gradients evolve and grow independently, as if alone in the channel. The cross-gradient is the sum of the gradients of the individual species. The cross-gradient evolution was predicted by computer simulation to rationalize our experimental observations. For example, the simulations provided a direct relation between the cross-gradient length and the flow program consisting of flow cycles in

which a specified volume of fluid, the “pumped volume”, was pumped back and forth in the microchannel.

The cross-gradient length increased to ~25 mm as the pumped volumes increased from 0 to 4 μL , and then dropped as the pumped volumes increased further (Fig. 2). The reason for the apparent decrease in the cross-gradient length for large pumped volumes was rationalized from our numerical simulations, which allowed the instantaneous progression of the cross-gradient to be monitored. During the alternating flow sequence, individual gradient lengths increased while the cross-gradient length could decrease if the gradient was pumped outside the finite microchannel (Fig. 2G). As the pumped volumes increased, so did the time durations over which the gradient left the channel. The dependence of the final cross-gradient length on the pumped volume is shown in Figure 2H. The discrepancy between the observed and predicted optimal pumped volumes was likely due to effects associated with the overflow into the inlet port and inlet drop, which induced mixing that partially compressed the gradient. The simulations assumed a channel of uniform rectangular cross-section and did not include these effects. Despite this discrepancy, the maximum cross-gradient length observed in our 5 cm microfluidic channel and predicted by our simulations was approximately 25 mm.

Characterization of Chemical Composition Along Gelatin/Chitosan Cross-Gradient

Spatial variations in chemical composition along our composite gelatin/chitosan cross-gradient materials were measured with FTIR. Figure 3A shows the FTIR spectra of eight consecutive sections along the gelatin/chitosan cross-gradient film, which exhibited the characteristic absorption bands for Amide I and Amide II. The absorption bands of Amide I gradually decreased in strength from the gelatin-rich to chitosan-rich sides of the cross-gradient. Moreover, the absorption bands of Amide II shifted to lower wavelengths which may have been caused by intermolecular hydrogen bonding between chitosan and gelatin molecules during the formation of the polyelectrolyte complex (Thein-Han et al., 2009). The gelatin content was calculated from the FTIR measurements using a calibration curve (Fig. 3B). The calibration curve was derived by curve fitting the ratios of the Amide I and Amide II spectral peak heights for six different blends with known gelatin content. Gelatin contents lower than 62.5% were related linearly to the ratio of peak heights ($R^2 = 0.96$, Fig. 3B inset), while those above 62.5% were obtained by extrapolation using a parabolic regression ($R^2 = 0.99$, red symbols in Fig. 3B). The ratio of Amide I and II peak heights did not vary appreciably when the gelatin content was above 62.5%. Quantitative FTIR analysis enabled us to precisely correlate the composition of our cross-gradient materials with spatial position.

To further characterize the gelatin/chitosan cross-gradient, we used XPS to measure the precise elemental composition of the material surface. As expected, the common elements carbon (C), nitrogen (N), and oxygen (O) were all present in wide scanning spectra along the gelatin/chitosan cross-gradient materials (Fig. 4A). Silicon (Si) was also detected and may have been introduced during the manufacturing and purification processes of the crab shell-derived chitosan (Lopez-Perez et al., 2007). As shown in Figure 4B, the relative concentration of carbon and nitrogen gradually decreased from the gelatin-rich to chitosan-rich sides, while that of oxygen and silicon increased. The representative C 1s core-level spectra of the gelatin-rich and chitosan-rich sides showed significant differences in peak component areas associated with C–O, C–N, and C–H species (Fig. 4C). The calculated peak area ratios of C–N/C–O gradually increased along eight consecutive sections from the gelatin-rich to chitosan-rich sides (Fig. 4D). The XPS measurements provided complementary details on the gradient nature of our composite materials.

Structural Characterization of Porous Gelatin/Chitosan Cross-Gradient Materials

To characterize the structural properties of the gelatin/chitosan cross-gradient materials and to probe their dependence on the relative concentrations of the components, we freeze-dried the materials and visualized the resulting porous structures with phase microscopy and SEM. The porous structures were sensitive to the duration and temperature of pre-freezing (Fig. 5A). By reducing the pre-freeze temperature from -20 to -80°C , the film layer commonly observed in freeze-drying (Wu et al., 2010) was reduced, exposing more of the underlying porous scaffolds. In addition, reducing the pre-freeze duration increased the surface porosity and generated directional porous structures. We therefore chose to pre-freeze at -80°C for 5 min for our studies on cell–material interactions.

We observed marked differences in cell response when cultured on the gelatin/chitosan scaffolds prepared under different conditions. Cells cultured on materials covered with thin films exhibited random actin distribution (Fig. 5B top), while those cultured on scaffolds with less film and relatively high surface porosity exhibited extensive elongation (Fig. 5B bottom). The cell alignment and the extensive elongation of the cytoskeletal actin filaments mimic the SMC morphology *in vivo*, and may have been induced by the directional porous structures. Despite the gradients in blending ratios along the gelatin/chitosan cross-gradient materials, the overall porous structures were similar (Fig. 5C). Therefore, at these concentrations, the observed differences in cellular response along the cross-gradients should be attributed largely to variations in chemical composition.

Interaction of SMCs With Porous Gelatin/Chitosan Cross-Gradient Materials

To demonstrate the use of our microfluidic cross-gradient platform for cell–biomaterial interaction studies, we measured the response of SMCs cultured along gelatin/chitosan cross-gradient materials. In particular, we quantified gradients in cellular morphology, adhesion and proliferation and related these to the local properties (e.g., chemical composition) of the materials. Chitosan and gelatin are ideal constituents because of their distinct effects on cells (Fig. S3). When SMCs were cultured on a porous gelatin/chitosan cross-gradient for 3 days, the cell morphology gradually changed from elongated on the gelatin-rich side to round on the chitosan-rich side. The gradual transition in SMC cytoskeletal organization was evident in the day 1 and 3 cultures (Fig. 6A and B). The SMCs exhibited extensive elongation in regions with more than 54.3% gelatin, but ceased to spread and instead formed cellular aggregates in regions with less than 10% gelatin.

The SMC morphology and proliferation were quantified by the “cell shape factor” and the “cell number,” which were correlated with the local composition of the gelatin/chitosan cross-gradient materials. The value of “cell shape factor” increased from 0.16 to 0.76 in the direction of increasing chitosan concentration, indicating a decrease in cell spreading (Fig. 6C). However, the “cell shape factor” varied little from day 1 to day 3, indicating that the majority of cell morphology changes occurred within the first day. The number of SMCs per unit area increased steadily in the direction of increasing gelatin concentration and also increased from day 1 to day 3 (Fig. 6D), demonstrating the dependence of cell adhesion and proliferation on gelatin concentration and culture time. The proliferation rate was higher at the gelatin-rich side of the cross-gradient compared with the chitosan-rich side. Our high-throughput screening platform produced key information to correlate SMC behavior with the properties of the gelatin/chitosan composite materials.

Discussion

We have presented a microfluidic combinatorial platform to rapidly synthesize gelatin/chitosan cross-gradient materials for investigating cell–material interactions in a high-

throughput manner. The gelatin/chitosan cross-gradient materials produced by our microfluidic platform yielded abundant information elucidating the dependence of a variety of cellular behaviors on the chemical properties of the materials. A similar approach could create composite libraries from a wide range of materials, including organic, inorganic, or hybrid materials (Gomez-Romero, 2001), exhibiting diverse properties. Microfluidic convection would first generate cross-gradients of pre-polymer solutions. The cross-gradients would then be stabilized by air- or freeze-drying or chemical-, thermal-, or photo-cross-linking. The morphology of the resulting cross-gradient materials could be controlled by appropriate choice of stabilization. For example, 2D films and 3D porous scaffolds can be synthesized by air- and freeze-drying, respectively. If cells are pre-mixed with two photo-cross-linkable pre-polymer solutions such as poly(ethylene-glycol) and methacrylated gelatin, our microfluidic platform has the potential to generate cell-encapsulated hydrogels with material cross-gradients after ultraviolet irradiation. The ability for a single platform to synthesize various material morphologies enables the creation of physiologically relevant microenvironments tailored to investigate a particular set of cell–material interactions.

The convection-driven blending of two components in our microchannel could enable the rapid synthesis of combinatorial material libraries without sophisticated equipment (Potyrailo et al., 2003; Anderson et al., 2005; Hook et al., 2010) or uncontrollable mixing/deposition procedures (Simon et al., 2004, 2005). In addition, our simple microchannel design consumes only small amounts of reagent (less than 10 μL of each solution), important for reducing the costs associated with screening rare or costly materials (i.e., peptide-based or rare metal-based materials).

An additional advantage of our platform over microarray platforms is that it synthesizes composite materials incorporating continuous spatial variations in the blending ratio. In other words, a continuum of combinations of material properties can be tested simultaneously on a single sample, while microarray platforms can only test a discrete number of combinations. The continuous feature enables the precise testing of material properties and their effects on cell behavior. Thus, our platform could be used for primary “candidate-pick” screening to yield promising ranges of the concentrations and blending ratios, and subsequently as a precision secondary screening tool to quickly and precisely identify the optimal values.

The throughput of our microfluidic gradient platform could be increased by mounting parallel channels on a single chip. Combined with a multichannel pumping device, the multichannel platform could produce multiple gradient materials simultaneously.

Our gradient platform could also be used for tissue engineering applications. Assuming a uniform concentration input into the channel, the time for gradient generation of diffusible species scales as the channel height squared (Du et al., 2010). Within those constraints, the microchannel dimensions (width, length) and perhaps height could be scaled up to fabricate bulkier anisotropic composite materials such as tissue constructs to mimic the cartilage–bone interface (Singh et al., 2008).

Conclusions

We employed a microfluidic platform to rapidly synthesize gelatin/chitosan cross-gradient materials for investigating cell–material interactions. The convection-driven generation of the cross-gradient was optimized experimentally and theoretically. The chemical compositions of the gelatin/chitosan cross-gradients were characterized by FTIR and XPS, which quantified the variations in the concentrations and blending ratios of the two components. 3D porous structures were created in the gelatin/chitosan materials by in situ

lyophilization. SMC morphology and proliferation varied markedly across the gradient materials and depended strongly on the local chemical composition. The platform's ability to produce materials with continuous ranges of chemical, structural, and biological properties should make it ideal for targeted material discovery and should help provide new insights into cell–biomaterial interactions and composite material synthesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Microfluidic platform to synthesize composite cross-gradient biomaterials. **A:** Schematic for the cross-gradient generation. The channel was filled with solution 1, solution 2 was pipetted onto the opposing port and drawn into the channel. Alternating flows in the channel generated a gradient in the relative concentrations, termed here a “cross-gradient,” of the two solutions. The gradient was subsequently stabilized by freezing and then air-dried or freeze-dried to obtain a composite film or porous structures. **B:** Visualization of a cross-gradient containing green-fluorescent DyLight 549-labeled collagen and red-fluorescent DyLight 649-labeled collagen. **C:** Composite material film generated by air-drying a gelatin/chitosan concentration cross-gradient. From the gelatin-rich to chitosan-rich sides, the film gradually changed from opaque to transparent. **D:** Dissolution of gelatin from a freeze-dried porous material containing a gelatin/chitosan cross-gradient.

Figure 2.

Experimental and theoretical investigation on the effects of the pumped volume on the length of the cross-gradient produced by the cross-gradient protocol. **A–F**: Fluorescent intensities of FITC-dextran and RITC-dextran along the channel after cross-gradient generation with pumped volumes (A) 1 μL , (B) 2 μL , (C) 3 μL , (D) 4 μL , (E) 5 μL , (F) 6 μL . **G**: Simulation of the evolution of the cross-gradient length while 6 μL was pumped back and forth five times. Arrows indicate flow reversal and F/B indicate the flow direction (forward/backward). **H**: Comparison of the observed and predicted final cross-gradient length as a function of pumped volume. Each dot corresponds to a simulation with a particular pumped volume.

Figure 3.

FTIR characterization of chemical composition along the gelatin/chitosan cross-gradients.

A: FTIR spectra of gelatin/chitosan composite sections at different positions. **B:**

Quantification of gelatin content at different locations along the composite material. Black diamonds indicate experimental values obtained from linear calibration curve (inset); red diamonds indicate values extrapolated from a parabolic fit (red dashed line) to the black diamonds. Inset: Linear calibration derived from the ratios of Amide I and II peak heights (at $1,645\text{ cm}^{-1}$ and $1,547\text{ cm}^{-1}$, respectively) in six blends of known gelatin/chitosan composition.

Figure 4. XPS analysis along gelatin/chitosan composite materials. **A:** Representative XPS wide scanning spectra. **B:** Spatial quantification of elemental composition. **C:** Representative XPS carbon (C) 1s core-level spectra. **D:** Spatial quantification of the peak area ratio of C-N/C-O.

Figure 5.

Porous structures in gelatin/chitosan composite materials and their effect on SMC alignment. **A:** Effect of pre-freeze temperature and duration on porous structures and the formation of thin surface films. **B:** Effect of porous structures on SMC behavior after a 3-day culture, as quantified by F-actin staining (red) and DAPI staining (blue). SMCs elongated more on the scaffold prepared at -80°C for 5 min (bottom) than that at -20°C for 15 min (top). **C:** Porous structures in a gelatin/chitosan cross-gradient pre-frozen at -80°C for 5 min show little spatial variation along the cross-gradient (phase microscope, top and middle; SEM, bottom).

Figure 6.

Effect of porous gelatin/chitosan cross-gradients on SMC behavior. SMCs were cultured on the composite materials for (A) 1 day and (B) 3 days. Fluorescence microscope images of SMCs show cytoskeleton distributions by F-actin staining (red) and nuclei by DAPI staining (blue). Quantification of (C) cell shape factor and (D) cell number on different sections of the gelatin/chitosan cross-gradient. ** $P < 0.01$, * $P < 0.05$, NS indicates “not significant.”