Published in final edited form as:

Adv Mater. 2012 March 15; 24(11): 1466–1470. doi:10.1002/adma.201103501.

Density Gradient Multilayer Polymerization for Creating Complex Tissue

Jerome V. Karpik,

Biomedical Sciences Program University of California at San Diego La Jolla, CA 92093-0600, USA

Dr. Yogesh Ner, and

Skaggs School of Pharmacy and Pharmaceutical Sciences Department of NanoEngineering Materials Science and Engineering Program University of California at San Diego La Jolla, CA 92093-0600, USA

Prof. Adah Almutairi

Biomedical Sciences Program University of California at San Diego La Jolla, CA 92093-0600, USA. Skaggs School of Pharmacy and Pharmaceutical Sciences Department of NanoEngineering Materials Science and Engineering Program University of California at San Diego La Jolla, CA 92093-0600, USA

Adah Almutairi: aalmutairi@ucsd.edu

Tissue engineering has recently focused on biomimetic matrices, usually polymer hydrogels, that include multiple layers with distinct structures and chemical components.^[1a–c] Current methods of fabricating such matrices are complex or expensive to implement and often produce mechanical weaknesses between layers.^[2] Thus, an adaptable, facile, and economical multilayer polymer fabrication technique that produces continuous interfaces between layers is needed. Herein we describe exactly such a method: density gradient multilayer polymerization (DGMP). We demonstrate that DGMP, a straightforward technique that uses inexpensive reagents, yields strong hydrogels with smooth transitions between layers.

Existing strategies allow for construction of multiphase scaffolds with varying degrees of complexity. Multiphase biopolymer-based hydrogel matrices have been previously fabricated by additive photopatterning,^[3a–c] laser scanning lithography,^[4] printing,^[5] sequential functionalization,^[6] and freeze-drying.^[7a,b] Additionally, spatial control of chemical or mechanical gradients has been achieved using partial miscibility of biopolymers,^[7] gradient makers,^[8] microfluidics,^[9a–c] and centrifugation.^[10] Another approach is to attach layers after fabrication.^[11a,b] However, these techniques may not be widely adopted because they require sophisticated instrumentation and/or technical proficiency.

DGMP exploits phase separations between liquids of varying density to create layers of distinct structures and chemical compositions. Briefly, serial concentrations of an inert density modifier, such as sucrose or iodixanol, are co-dissolved with prepolymer (we used bisacrylamide and acrylamide or biocompatible c(PEGda)), crosslinkers, and ligands or

proteins (see Figure 1a and Figure 1b). Next, these prepolymer solutions are gently layered on top of each other in order of decreasing density. Varying the initial concentrations and types of each of these agents in each layer allows structures and chemical properties to be tailored. Varying the settling time before polymerization (t_s) adjusts the smoothness of gradients between layers. Bulk polymerization yields a multicompartment hydrogel. Finally, the density modifier is removed, resulting in multiphase hydrogels that are structurally uninterrupted at interfaces of chemically and mechanically diverse layers.

We chose reagents for their suitability for this application. Sucrose is a highly soluble density modifier with a linear relationship between concentration and density (see Figure 1a). Iodixanol, the main ingredient of Optiprep, is a nonionic, iso-osmotic density modifier currently used in viable cell purification.^[12a,b] Polyethylene glycol and polyacrylamide (PAM)-based hydrogels are well-suited for cell culture applications^[13] because they are biologically inert, so they are resistant to non-specific protein adsorption and cell adhesion,^[14–14c] enabling precise engineering of desired biofunctionality through the covalent addition of ligands such as RGD peptide.^[15–15d] With these components, DGMP produces structurally continuous multilayer hydrogels for tissue engineering. Furthermore, the method can be adapted to varying mold shapes, sizes, and materials (Figure 1b).

Existing techniques, including sequential photopolymerization, may yield networks that are susceptible to delamination under mechanical stress.^[7,16a,b] This mechanical instability is due to discontinuity at layer interfaces. Field emission scanning electron microscopy (FESEM) of architecturally varied PEGda layers reveals enhanced continuity at the interface of hydrogels fabricated by DGMP when compared to sequential polymerization (Figure 2a vs. Figure 2b). This enhanced continuity can also be observed macroscopically in five-layer PAM hydrogels (Figure 2c vs. Figure 2d). Moreover, perpendicular compression testing revealed that PAM hydrogels fabricated via DGMP outperform analogous sequentially polymerized hydrogels (Figure 2e vs. Figure 2f).

Biomimetic tissue engineering also requires fabrication of not only discrete multicompartment gels but also gradients of varying degrees. To demonstrate the ability of DGMP to produce a variety of gradients, both smooth and sharp, between layers with different structures, we initially created biphasic matrices with 7% and 1% (w/w) PAM precursor using sucrose solutions of distinct densities and varied the settling times. Immediately polymerized hydrogels exhibit discrete compartments, with an abrupt transition, or sharp gradient, with different susceptibilities to swelling (Figure 2g, left). However, as t_s is incrementally increased prior to bulk polymerization, the lower layer becomes less susceptible to swelling (Figure 2g, from left to right). This illustrates that increasing settling time increases the graduation in mechanical transition.

To determine whether DGMP allows spatial restriction of biological cues, we designed a simple experiment in which the biological cue would allow cell adhesion; whether the cues were separated would be obvious from whether cells attached. We fabricated tissue culture compatible two-dimensional (2D) substrates with alternating layers of covalently bound aPEG-RGDS labeled with Alexa Fluor 350 (to help visualize patterning) and seeded them with C2C12 myoblasts (Figure 3a–Figure 3b). We developed a small scale manufacturing technique using iodixanol concentration gradients to construct these multilayer disc-shaped PEGda hydrogels specifically for tissue culture with standard well plates (Figure 1b). Briefly, molds were cut from 0.8 mm silicone sheets sized to fit between glass slides and used to construct seven-layer PEGda substrates via iodixanol DGMP. The tissue-culture discs swelled, were sterilized, and then seeded with C2C12 myoblasts (Figure 3b). We observed that adhered cells co-localized with covalently grafted RGDS peptide, visualized by alternating substrate fluorescence (Figure 3c). Furthermore, we were able to

photoencapsulate C2C12 myoblasts by photopolymerization of the solutions, containing these cells, to form gels in the presence of 35% (w/v) iodixanol. Briefly, $500,000 \text{ cells cm}^{-3}$ were photoencapsulated in single compartment PEGda hydrogels with 8 mM RGDS and expanding cell colonies were confirmed viable by calcein-AM live stain after two weeks in culture (Figure S3). These results show that the iodixanol DGMP method allows fast, simple fabrication of hydrogel scaffolds with spatially defined active biological cues.

To determine whether this method allows spatial control of discretely arranged proteins, we created matrices in which small molecules (fluorescein-o-acrylate or rhodamine B) were bound (Figure S1a–S1c) or protein was encapsulated (Figure 3d and S2a–S2b). Bovine serum albumin (BSA) was encapsulated in alternate layers (Figure S2a) or in discretely stepped gradients (Figure S2b). Further, we modulated smooth protein cross-gradients in PEGda hydrogels by increasing t_S after layering sucrose solutions of ovalbumin conjugated to Alexa Fluor 488 (OVA-488, 4 mg mL^{-1} ; Figure 3d, top) and BSA conjugated to Alexa Fluor 594 (4 mg mL^{-1} ; Figure 3e, bottom). Longer t_S resulted in progressively graduating protein concentration profiles.

In conclusion, we introduce a novel multilayer single step polymerization technique that separates phases by solvent density. This method is accessible, versatile, and facilitates control of discrete, as well as continuously graduated, mechanical and chemical interfaces within structurally uninterrupted hydrogel networks. Although we demonstrate this technique in photo-polymerized model hydrogel scaffolds, this simple method can be applied to any polymer system. We apply a sucrose DGMP method to spatially control mechanics, encapsulated proteins, and covalently bound small molecules within hydrogel matrices. We use iodixanol DGMP to pattern bioactive peptides and cells on 2D tissue culture substrates. Importantly, the range of geometries and feature sizes presented throughout this communication were fabricated with common laboratory equipment and reagents. This powerful and adaptable technique is compatible with a range of polymer types (including those suitable for in vivo applications, such as hyaluronic acid) and solvents, as iodixanol is also compatible with organic solvents. DGMP could be combined with a multitude of current fabrication paradigms to increase the complexity of matrices for tissue engineering, controlled drug delivery, or biological investigation.

Experimental Section

Cell Maintenance

C2C12 murine myoblasts (AATC) were maintained in Dulbecco's Modified Eagle's Medium supplemented with fetal bovine serum (10% v/v) and penicillin/streptomycin ($1\% \times 100\times \text{ v/v}$) at $37^\circ\text{C}/5\% \text{ CO}_2/95\%$ relative humidity. Cell culture reagents were obtained from Life Technologies.

Fluorescently labeled aPEG-RGDS Synthesis

aPEG-RGDS-350 was synthesized with slight modifications to a previously described procedure.^[17] Briefly, RGDS peptide (American Peptide, Arginine-Glycine-Aspartic Acid-Glycine) was conjugated to PEG (MW 3400 g mol^{-1}) by reaction with aPEG-SCM (Laysan Bio, SCM: Succinimidyl Carboxymethyl) at 1.2:1 molar ratio in the presence of DIPEA at 1.2:2 molar ratio overnight in DMSO under argon at room temperature. aPEG-RGDS was purified by dialysis, lyophilized and confirmed via matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) in universal matrix (Sigma) (Figure S4) and $^1\text{H-NMR}$ in D_2O (Figure S5). An equimolar amount of Alexa Fluor 350 carboxylic acid, succinimidyl ester (Molecular Probes) was then added to aPEG-RGDS dissolved in DMF overnight in DMSO under argon at room temperature and purified by dialysis, lyophilized and stored under argon at -20°C .

Multilayer Hydrogel Fabrication

For 3D sucrose DGMP (Figure 1a): aqueous solutions of ethylene glycol diacrylate (Dajac Labs, MW 4000 g mol⁻¹; 10%, 15%, or 20% w/v; Figures 2a–b and Figure 3d) or acrylamide (Promega; 10% w/v; Figures 2c–Figure 2g) were separately prepared in water. Precursor densities were individually modified with serial concentrations (0–50% w/v) of sucrose. Each solution was gently layered in transparent cylindrical polypropylene molds made from modified syringes (Figures 2a–g) or in hydrophobic silanated (Sigmacote, Sigma) glass Pasteur pipettes (Figure 3d). For 2D iodixanol DGMP (Figure 1b): aqueous precursor monomer solutions of ethylene glycol diacrylate (15% w/v, Figure 3a–c) were separately prepared in Dulbecco's phosphate buffered saline (w/Ca/Mg, HyClone). Precursor densities were modified with serial concentrations (5–40% w/v) of iodixanol (Axis-Shield OptiPrep, 60% iodixanol in water). Each solution was gently layered in molds made from 0.8 mm thick silicone spacers cut with 10 mm and 8 mm biopsy punches (Acuderm) and sandwiched between hydrophobic silanated glass slides (Figure 3a). For all precursor solutions, Durocur 2959 photoinitiator (Gibco) was held constant (10 µL of 300 mg mL⁻¹ in N-vinyl pyrrolidone per milliliter of solution). Free radical polymerization was photoinitiated under irradiation with 365 nm light for 1 min in a Luzchem Research UV chamber (~2500 mW cm⁻²) per side unless otherwise noted. Gradients were agitated in ten volumes of PBS for at least two days with two buffer exchanges per day to remove density modifiers, unreacted prepolymer, and photoinitiator.

Structural Stratification

DGMP gradients were compared to sequentially polymerized multi-layer hydrogels, in which precursors identical to those for DGMP were used, both groups were irradiated for 1 min per layer, and the process was not optimized. For microstructural examination (Figure 2a–b), bilayer PEGda hydrogels (10% w/v, upper left; 20% w/v, lower right), were snap frozen in N₂, lyophilized overnight, and coated with chromium (45 s at 130 mA), then recorded with an FEI XL30 SFEG SEM. For macrostructural examination and mechanical testing (Figure 2c–f), five-layer PAM (10% w/v, 1% w/w crosslink) hydrogels were photographed (Figure 2c–d) and compressed with a Satec materials testing machine (Figure 2e–f). To examine the effect of graduated transitions on structural properties, PAM mechanical gradients were created in biphasic sucrose gradients by diffusion of bisacrylamide from layers of 7% to 1% (w/w) crosslinker for t_s of 0, 15, 30, 60, 120, and 180 min (Figure 2g).

Chemical Stratification

For fluorescence pattern images of PEGda tissue culture discs (Figure 3a and Figure 3c), co-dissolved iodixanol and prepolymer solutions were combined with either methacryloxyethyl thiocarbamoyl rhodamine B (Polysciences, 20 µM) or aPEG-RGDS-350 (8 mM). Precursor solutions were gently layered stepwise into silicone and glass molds, described above, and photopolymerized. Bioactivity of patterned integrin-binding peptide, RGDS, was evaluated by seeding C2C12 myoblasts (20,000 cells cm⁻²) onto hydrogel discs. Resulting cell patterns were observed after 24 hrs in culture via epifluorescence and phase-contrast imaging (Zeiss Axiovert 200; Figure 2c). Protein gradients in PEGda (15% w/v) were created via biphasic sucrose DGMP by counter-diffusion of OVA-488 (Figure 3d, upper) and BSA-594 (Figure 3d, lower) for t_s of 0, 30, 90, and 150 min (both proteins initially 4 mg mL⁻¹, Life Technologies).

Photography and image processing

Gross hydrogel images were obtained in a BioRad VersaDoc-4000MP. Color photographs were taken with a Canon Powershot A11000 IS. Individual field fluorescence and

transmitted light microscopy images were color composited with Image Pro Plus software. All multi-field images were manually reconstructed with ImageJ software (NIH) using the MosaicJ plugin with all corrections disabled (e.g., blending, smart color, and rotation). Average and line fluorescence intensity profiles were generated with Image J software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

J.V.K. and Y.N. contributed equally to this work. The authors gratefully acknowledge support from the NIH Directors New Innovator Award (1 DP2 OD006499-01), and King Abdulaziz City for Science and Technology. The authors thank Prof. Marc A. Meyers for assistance with mechanical measurements, Dr. Nadia Fomina for assistance with ¹H-NMR analysis, and Ryan Anderson of Calit² Nano3 for FESEM technical support.

References

1. a) Ma PX. *Adv Drug Deliv Rev.* 2008; 600:184. [PubMed: 18045729] b) Lee KY, Mooney DJ. *Chem Rev.* 2001; 101:1869. [PubMed: 11710233] c) Khademhosseini A, Langer R. *Biomaterials.* 2007; 28:5087. [PubMed: 17707502]
2. Place ES, Evans ND, Stevens MM. *Nat Mater.* 2009; 8:457. [PubMed: 19458646]
3. a) Liu VA, Bhatia SN. *Biomed Microdevices.* 2002; 4:257.b) Elisseeff JH, Sharma B, Williams CG, Kim TK, Sun DN, Malik A, Khan M, Leong K. *Tissue Eng.* 2007; 13:405. [PubMed: 17504064] c) Weber LM, Cheung CY, Anseth KS. *Cell Transplantation.* 2008; 16:1049. [PubMed: 18351021]
4. Hahn MS, Miller JS, West JL. *Adv Mater.* 2005; 17:2939.
5. Boland T, Xu T, Damon B, Cui X. *Biotechnol J.* 2006; 1:910. [PubMed: 16941443]
6. Kizile S, Sawardecker E, Teymour F, Perez-Luna VH. *Biomaterials.* 2006; 27:1209. [PubMed: 16157371]
7. a) Harley BA, Lynn AK, Wissner-Gross Z, Bonfield W, Yannas IV, Gibson LJ. *J Biomed Mater Res A.* 2010; 92:1078. [PubMed: 19301263] b) Varghese S, Hwang YS, Zhang C. *Chem Mater.* 2010; 20:345.
8. Chatterjee K, Lin-Gibson S, Wallace WE, Parekh SH, Lee YJ, Cicerone MT, Young MF, Simon CG Jr. *Biomaterials.* 2010; 31:5051. [PubMed: 20378163]
9. Cosson S, Kobel SA, Lutolf MP. *Adv Funct Mater.* 2009; 19:3411.b) He J, Du Y, Villa-Urbe JL, Hwang C, Li D, Khademhosseini A. *Adv Funct Materi.* 2010; 20:131.c) Cuchiara MP, Allen AC, Chen TM, Miller JS, West JL. *Biomaterials.* 2010; 31:5491. [PubMed: 20447685]
10. Roam JL, Xu H, Nguyen PK, Elbert DL. *Biomaterials.* 2010; 31:8642. [PubMed: 20719381]
11. a) Straky KS, Heilshorn SC. *Adv Mater.* 2009; 21:4148.b) Gleghorn JP, Lee CSD, Cabodi M, Stroock AD, Bonassar LJ. *J Biomed Mater Res - A.* 2008; 85:611. [PubMed: 17806116]
12. a) Graziani-Bowering GM, Graham JM, Filion LG. *J Immunol Methods.* 1997; 207:157. [PubMed: 9368642] b) Brewer GJ, Torricelli JR. *Nat Protoc.* 2007; 2:1490. [PubMed: 17545985]
13. Khetan S, Burdick J. *J Vis Exp.* 2009; 32:1590. [PubMed: 19855372]
14. a) Tang J, Peng R, Ding J. *Biomaterials.* 2010; 31:2470. [PubMed: 20022630] b) Peng R, Yao X, Ding J. *Biomaterials.* 2011; 32:8048. [PubMed: 21810538] c) Leslie-Barbick JE, Saik JE, Gould DJ, Dickinson ME, West JL. *Biomaterials.* 2011; 32:5782. [PubMed: 21612821]
15. a) Lutolf MP, Hubbell JA. *Nat Biotechnol.* 2005; 23:47. [PubMed: 15637621] b) Roberts MJ, Bentley MD, Harris JM. *Adv Drug Deliv Rev.* 2002; 54:459. [PubMed: 12052709] c) Peppas NA, Keys KB, Torres-Lugo M, Lowman AM. *J Controlled Release.* 1999; 62:81.d) Zhang Z, Lai Y, Yu L, Ding J. *Biomaterials.* 2010; 31:7873. [PubMed: 20674012]
16. a) Kuckling D, Pareek P. *Polymer.* 2008; 49:1435.b) Calvert P, Frechette J, Souvignier C. *MRS Proc.* 1997; 489:153.
17. West JL, Hoffmann JC. *Soft Matter.* 2010; 6:5056.

Figure 1.

Schematic representation of two variations of the density gradient multilayer polymerization (DGMP) method. (a) Five serially diluted solutions of sucrose are co-dissolved with prepolymer; and either fluorescently-tagged protein A or B. (b) Seven serially diluted solutions of iodixanol are co-dissolved with prepolymer and either methacrylated rhodamine or RGDS-350 (b). Then, after designated settling time (t_s), stratified hydrogels are bulk polymerized. The density modifier can be washed away, leaving stable microstructures.

Figure 2.

DGMP results in continuous structures and mechanical stability at interfaces of adjacent compartments in mechanically heterogeneous hydrogels. a–b) Multicompartament PEGda hydrogels fabricated via sucrose DGMP (a) are microstructurally more continuous at the interface between 10% (w/v, upper left) and 20% (w/v, lower right) prepolymer than sequentially photopolymerized PEGda (b). White arrows highlight interfaces. Bar indicates 50 μm . c–f) Five-layer PAM (10% w/v, 1% w/w crosslinker) hydrogels approximately 12 mm in diameter prepared by DGMP or sequential photopolymerization. DGMP produces hydrogels (c) that are macrostructurally more continuous than sequentially photopolymerized PAM (d). DGMP produces stronger hydrogels (e) than sequentially photopolymerized PAM (f) as indicated by perpendicular compression to failure and failure mode evaluation. Photos are inset in force vs. displacement curves ($n = 2$). Note that DGMP hydrogels bulk ruptured at around 90% strain while sequentially polymerized hydrogels *delaminated* at around 65% strain as indicated by white arrow. g) Increasing t_s prior to bulk polymerization modulates structural gradients in 10% (w/v) PAM bilayer hydrogels. As schematically represented (left), bisacrylamide crosslinker diffuses through the density interface to graduate the transition between 7% and 1% crosslinker (w/w monomer). Swelling in water demonstrates the transition from discrete to increasingly continuous mechanical gradients.

Figure 3.

DGMP facilitates spatial control of scaffold bioactivity. a–c) 2D seven-layer PEGda tissue culture substrates prepared as outlined in Figure 1b for 10 mm (a, left six) and 8 mm (a, right two) hydrogel discs with alternating layers of RGDS-AlexaFluor 350 (8 mM) or methacrylated rhodamine. b) After swelling, discs are added to tissue culture well plates. c) Phase contrast image of C2C12 monolayer, color composited with corresponding epifluorescence image. Note that cell spreading is restricted to two regions of PEG-RGDS, additionally indicated by the normalized average fluorescence intensity plot below image. Bar indicates 100 μm . d) Progressive cross-gradients of Ova-488 (top) and BSA-594 (bottom) encapsulated via DGMP at different t_s , as outlined in Figure 1a, with intensity plots. Scale indicates 10 mm.