Chapter 9

3D Polymer Scaffold Arrays

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Abstract

We have developed a combinatorial platform for fabricating tissue scaffold arrays that can be used for screening cell–material interactions. Traditional research involves preparing samples one at a time for characterization and testing. Combinatorial and high-throughput (CHT) methods lower the cost of research by reducing the amount of time and material required for experiments by combining many samples into miniaturized specimens. In order to help accelerate biomaterials research, many new CHT methods have been developed for screening cell–material interactions where materials are presented to cells as a 2D film or surface. However, biomaterials are frequently used to fabricate 3D scaffolds, cells exist in vivo in a 3D environment and cells cultured in a 3D environment in vitro typically behave more physiologically than those cultured on a 2D surface. Thus, we have developed a platform for fabricating tissue scaffold libraries where biomaterials can be presented to cells in a 3D format.

Key words: Array, Biomaterials, Cell adhesion, Cell proliferation, Combinatorial methods, Polymer scaffold

1. Introduction

Combinatorial methods, which have been successful at accelerating pharmaceutical research (1, 2), are being developed and applied to accelerate biomaterials research (3–11; reviewed in 11). Meredith et al. (3) fabricated combinatorial polymer libraries containing orthogonal gradients of poly(ε-caprolactone):poly (D,L-lactic acid) (PCL:PDLLA) blend composition and annealing temperature. Osteoblasts cultured on the libraries displayed "hot spots" where the blend morphology enhanced differentiation. Anderson et al. (4) used an automated spotter to deposit monomers onto glass slides, which were photopolymerized to create polymer arrays. Polymers that supported human embryonic stem

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adhesion and proliferation were identified. Disney and Seeberger (5) coupled monosaccharides to slides for combinatorial screening of bacterial adhesion and found mannose to be the best surface. Smith et al. (6) cultured fibroblasts on polymer arrays made in 96-well plates and observed a range of cellular metabolic activities that were used in computational modeling that identified polymer descriptors that were predictive of cell response. Anderson et al. (7) used an array spotter for making polymer library arrays of several polymers and their blends and observed that blends containing poly(ethylene glycol) tended to inhibit cell adhesion. Flaim et al. (8) robot-spotted several extracellular matrix proteins and their blends into arrays and observed that hepatocyte differentiation was enhanced when collagen IV was present. Simon et al. (9) made gradients of polymer composition and used an automated image acquisition and analysis system to determine that cell adhesion and proliferation were enhanced in specific regions of the libraries. Finally, Gallant et al. (10) used "click" chemistry to synthesize gradients of surface-coupled RGD cell adhesive peptides to identify optimal coupling densities for cell adhesion and proliferation. These studies demonstrate many approaches for fabricating biomaterial arrays and how they can be used to identify the material formulations that promote different cellular behaviors.

Although previous methods for rapid screening of cell–biomaterial interactions have primarily focused on 2D films or surfaces (3–11), biomaterials are frequently used to fabricate 3D scaffolds (12), cells exist in vivo in a 3D environment and cells cultured in a 3D environment in vitro typically behave more physiologically than those cultured on a 2D surface (13–15). For these reasons, a combinatorial approach in which cell–biomaterial interactions are screened using a 3D polymer scaffold configuration will provide more relevant information regarding cell responses to test biomaterials. Toward this end, we have developed methods for fabricating combinatorial arrays of polymer scaffolds where scaffold composition and properties are varied (16, 17). The scaffold arrays can be used for rapid screening of cell response to identify scaffold compositions that best support cell adhesion, proliferation, and differentiation. Herein, we provide protocols for fabricating the polymer scaffold arrays and how to use them in screening cell response.

2. Materials

1. NaCl (Sigma).
2. Sudan IV (Sigma).
3. 1,4-Dioxane (Sigma).

4. Poly(D,L-lactic acid) (PDLLA), M_w 100,000 g/mol (Lactel-Birmingham Polymers).
5. Poly(D,L-lactic acid), M_w 103,000 g/mol (Absorbable Polymers International).
6. Poly(D,L-lactic acid), M_w 109,000 g/mol (Medisorb-Alkermes).
7. Poly(desaminotyrosyl-tyrosine ethyl ester carbonate) (pDTEc), M_w 183,000 g/mol (gift from Joachim Kohn at New Jersey Center for Biomaterials).
8. Poly(desaminotyrosyl-tyrosine octyl ester carbonate) (pDTOc), M_w 122,800 g/mol (gift from Joachim Kohn at New Jersey Center for Biomaterials).
9. Poly(ε-caprolactone) (PCL), M_w 80,000 g/mol (Sigma).
10. MC3T3-E1 mouse osteoblast cell line (Riken Cell Bank, Hirosaka, Japan).
11. α-Modification of Eagle's minimum essential medium (Cambrex Bio Science).
12. Fetal bovine serum (Gibco).
13. Kanamycin sulfate (Sigma).
14. Paraformaldehyde (Sigma).
15. Triton X-100 detergent (Sigma).
16. Bovine serum albumin (Sigma).
17. Sytox green cell nucleus stain (Invitrogen).
18. Ethylene oxide sterilizer (Anderson Products).
19. T-junction (Cole-Parmer).
20. Static mixer (Cole-Parmer).
21. Polypropylene 96-well plates (Sigma).
22. Female Leur connectors (Cole-Parmer).
23. Gradient syringe pumps (New Era Pump Systems).

3. Methods

3.1. Preparing the 96-Well Plates

1. Sieve NaCl using #60 and #40 sieves to yield NaCl of size range 0.250–0.425 mm.
2. Use polypropylene 96-well plates of standard dimensions (6.5 mm well diameter). Polypropylene plates have better solvent resistance than standard polystyrene plates.
3. Put 0.13 g of the sieved NaCl into the top four rows of a 96-well plate (48 wells total). If there is not enough salt in the wells, then the polymer solution will cover the salt and

form a polymer skin on top of the salt during freeze drying. The salt should be higher than the polymer solution so that the scaffolds have open pores on their top surface.

4. Level the surface of the salt in each well, so that uniform scaffolds are fabricated. A tool can be machined from plastic for this purpose and inserted in each well to level the salt (Fig. 1a, b).

3.2. Preparing Polymer Solutions

1. The protocol described herein yields a scaffold array where the composition of Sudan IV red dye is varied. This is for system demonstration only. The red dye can be omitted when fabricating scaffold arrays for cell screening experiments when scaffold formulations are being varied and tested.
2. Make two vials of polymer solution where 1 g of polymer is dissolved in 10 mL of dioxane in each vial (see Note 1). Label one vial RED and the other CLEAR. Depending on the density of the polymer used, this will yield a solution of approximately 9.4% (mass/volume).
3. To the RED vial, add 1 mL of Sudan IV solution [0.3% Sudan IV (mass/volume) in dioxane]. This will yield a RED polymer solution of approximately 8.5% (mass/volume) with 0.03% Sudan IV (mass/volume).
4. To the CLEAR vial, add 1 mL of dioxane as a control. This will yield a CLEAR polymer solution of approximately 8.5% (mass/volume).

3.3. Preparing the Two-Syringe Pump System

1. The parts shown in Fig. 1a must be assembled into the mixing apparatus shown in Fig. 1c. First, a section of Tygon tubing (2 cm long, 3.2 mm internal diameter) should be placed on each end of the static mixer. The plastic T-junction should be connected to one end of the mixer, and the end of a pipette tip (2.5 cm long) should be inserted into the tubing on the opposite end of the mixer. Tubing should be placed on the open ends of the T-junction, and then the female Luer connectors should be inserted into the tubing connected to the T-junction. The Luer connectors will provide connections for the syringes.
2. The static mixer is made of steel, is 135 mm long (Fig. 1a, c), and has an internal diameter g of 4.8 mm with a volume of 1.1 mL (Cole-Parmer). The mixer contains 18 mixing elements (helices). The mixing elements are helices that mix and fold the polymer solutions 2^{18} times (2^{18}=262,144; the exponent of “18” comes from the 18 mixing elements) (Fig. 1d).
3. The tubing and syringes should be wetted and rinsed with dioxane. Syringes filled with dioxane can be attached to the Luer connectors and used to inject dioxane into the tubing.

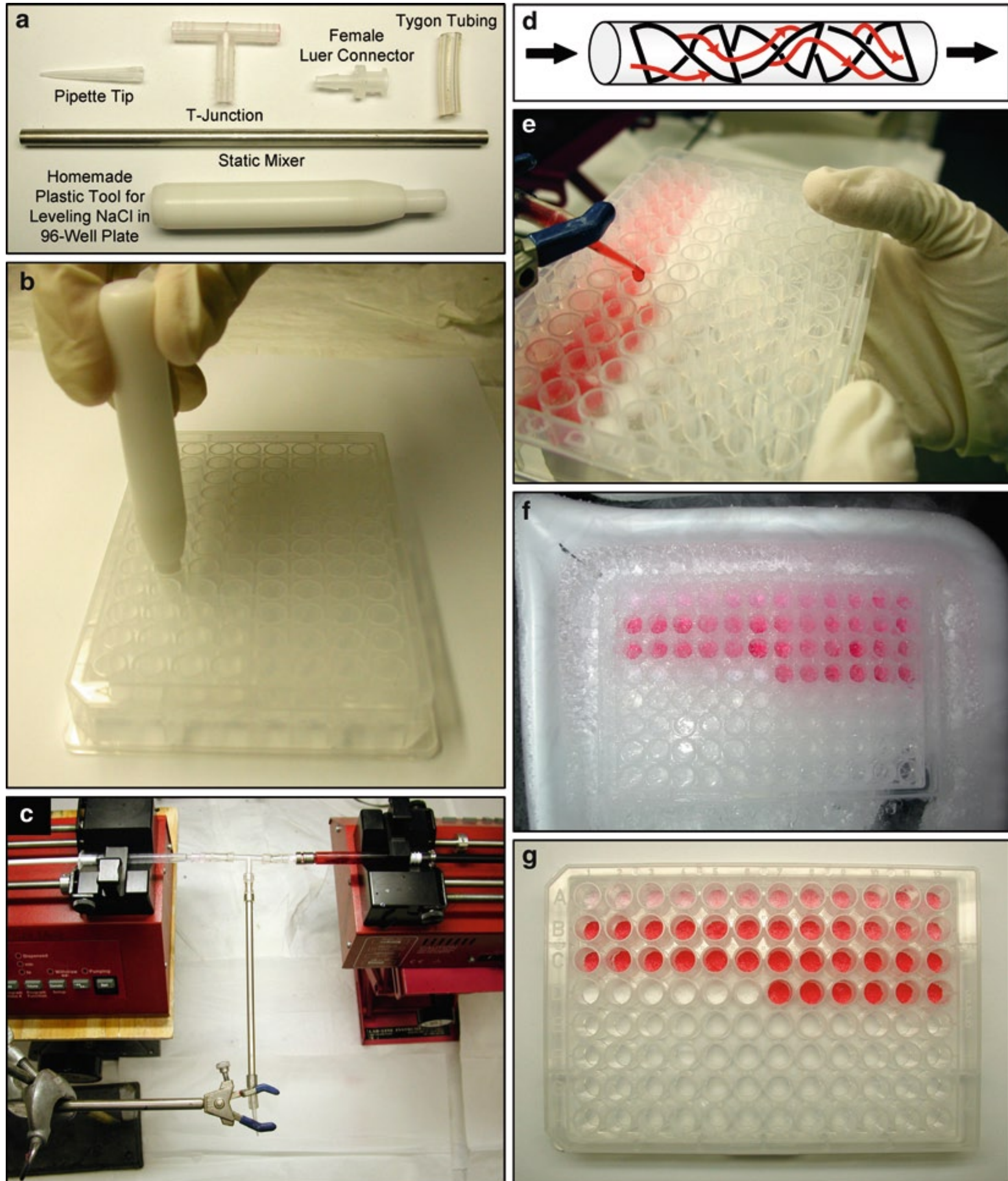

Fig. 1. (**a**) Parts required for making scaffold arrays. (**b**) NaCl porogen in 96-well plates is leveled/flattened using a home-made plastic tool. (**c**) The two-syringe pump system for making scaffold arrays is shown. The CLEAR polymer solution is in the left pump in a 1-mL syringe. The RED polymer solution is in the right pump in a 2.5-mL syringe. Flow from the syringe pumps comes together at a T-junction in the middle of the image, goes into the steel static mixer, and comes out of the pipette tip at the bottom of the image. (**d**) A diagram of the interior of the static mixer. The helices in *black* are made of metal and are part of the mixer. The helices mix and fold the flowing polymer solution depicted by the *red flow lines*. (**e**) Polymer solution being deposited into a 96-well plate as it elutes from the static mixer. The 96-well plate contains NaCl porogen in each well. (**f**) After the polymer solution is deposited into the 96-well plate, the plate is frozen in liquid nitrogen for 5 min before being freeze-dried. (**g**) A completed PDLLA scaffold array after freeze-drying and salt leaching where Sudan IV red dye is varied is shown.

Shake out the tubing and syringes so that they are wet but not holding excess dioxane.

4. Two syringe pumps should be set up to face each other, so that the syringes feed into the opposing ends of the T-junction as shown in Fig. 1c. The left syringe pump should hold the 1 mL of CLEAR polymer solution in a 1 mL syringe (4.5 mm internal diameter). The right syringe pump should hold 2.5 mL of RED polymer solution in a 2.5 mL syringe (7.25 mm internal diameter).
5. The syringe pumps should be placed on "lab jacks" so that the syringes, tubing, and T-junction can be leveled. A ring stand with a clamp can be used to support the end of the static mixer with the pipette tip as shown in Fig. 1c. There should be a slight decline (5°) in the static mixer as it goes from the T-junction toward the pipette tip to help prevent bubbles. However, if there is too much of a decline, then the polymer solution will drip out ahead of the back pressure, causing bubbles and inconsistencies in composition.
6. The pipette tip itself should also be angled downward at approximately 30°C. The down angle helps drops to form and fall cleanly into the 96-well plates. If there is not enough down angle, then the droplets can run back along the pipette tip and be difficult to get into the wells of the 96-well plate. If the pipette tip has too much down angle, then the polymer solution will drip out ahead of the back pressure causing bubbles and inconsistencies in composition.
7. The syringe pumps must be programmable and must be able to ramp up or down between two different velocities. The left pump holding the CLEAR polymer should be programmed to start at 0.5 mL/min and ramp down linearly to 0 mL/min over 72 s (0.3 mL). The right pump holding the RED polymer should be programmed to start at 0 mL/min and ramp up linearly to 0.5 mL/min over 72 s (0.3 mL) and then hold at 0.5 mL/min for 5 min. The RED polymer pump continues to pump after the CLEAR polymer pump has stopped in order to push the mixed polymers out of the mixer, through the pipette tip and into the 96-well plate.

3.4. Mixing and Deposition of the Scaffold Arrays

1. The CLEAR and RED polymer solutions should both be primed down the tubing up to the T-junction. This can be achieved by turning the syringe pumps on and off as appropriate. Next, an additional 325 μL of the CLEAR polymer should be pumped from the left pump (the solution will flow into the static mixer a little bit). There is cross-mixing during the fabrication process, and leading with a bolt of 325 μL of the CLEAR makes it possible to get the widest possible range of compositions in the library.

2. Make sure both syringe pumps are reset to the beginning of their respective pumping programs. Start both pumps simultaneously to begin mixing and deposition.
3. After a couple of minutes, polymer solution will begin to elute from the pipette tip. Catch two drops of polymer solution in each well of the 96-well plate for 36 wells as shown in Fig. 1e (see Notes 2 and 3).
4. Control scaffolds of pure CLEAR and pure RED can also be prepared if desired. For the CLEAR controls, CLEAR polymer solution is pumped at constant speed (0.5 mL/min) from a single syringe pump and deposited at two drops per well into the 96-well plate containing 0.13 g of sieved salt per well. RED controls can be prepared in the same manner but using RED polymer solution. Completed control scaffolds of pure CLEAR and pure RED polymers are visible in the completed scaffold array in Fig. 1g (fourth row from top of plate, six CLEAR on left and six RED on right).
5. The wet 96-well plate containing the polymer solutions should be centrifuged for 2 min with a lid at 2,000 rpm (210 rad/s) in a swinging bucket centrifuge, using 96-well plate holders. This gently forces the polymer solutions to the bottom of each well ensuring that the scaffolds will be of uniform shape.
6. Freeze the 96-well plate in liquid nitrogen as shown in Fig. 1f (wear safety glasses!). Place the 96-well plate in a shallow pan and very gently pour in liquid nitrogen until the 96-well plate is submerged. This must be done slowly to avoid disturbing the scaffolds before they are frozen. Use a spatula to hold the plate below the surface of the liquid nitrogen (the plate will float). Leave the plate in the liquid nitrogen for 5 min to insure a thorough freeze. When ready to place on the freeze-dryer, gently pour off the liquid nitrogen and immediately place in the freeze-dryer (see Note 4).
7. Freeze-dry scaffolds overnight at 13 Pa (100 mTorr) or less using a liquid nitrogen trap to catch dioxane. The trap should be bypassed at the end of the day (before the overnight), so that the dioxane will not return to vapor phase when the liquid nitrogen is gone (see Notes 5 and 6).

3.5. Salt Leaching and Drying Scaffold Arrays

1. After overnight freeze-drying, remove scaffolds from freeze-dryer and leach salt in water. Plates can be placed in shallow pans and held under water using weights. Rubber-coated lead rings designed for holding down bottles in a water bath work very well for this. Cover pan with foil to keep dust out during leaching.

2. When submerging the plates in water for salt leaching, air bubbles may be present in the wells on top of the scaffolds, which can inhibit the salt leaching. A transfer pipette can be used to *very* gently force water into the wells to displace the bubbles. If this is done too forcefully, then the scaffolds can become dislodged from the plate.
3. Water should be changed daily for a total of 5 d of salt leaching (see Note 7). After 5 days, remove the 96-well plate from the water and gently place it upside down on clean paper laboratory towels to absorb excess water. The plates should be handled gently to prevent the scaffolds from becoming dislodged.
4. Air-dry scaffold arrays for 3 d while protected from dust (in a drawer, or wrapped lightly with a paper towel). Do *not* dry the scaffolds in an oven as this will distort them (they collapse even in a low temperature oven 37°C). A dried, completed scaffold array is shown in Fig. 1g (see Notes 8–10).

3.6. Characterizing Scaffold Arrays Using Sudan IV Dye Absorbance

1. Sudan IV absorbance can be used to track scaffold composition in the arrays in order to characterize array fabrication (see Note 11). For this analysis, sieved NaCl should be omitted from the 96-well plate. Two drops of polymer solution should be deposited directly into the wells of the 96-well plate as they elute from the pipette tip and static mixer. Add 0.1 mL of dioxane to each well, mix, and read absorbance of each well at 490 nm (Abs490) using a plate reader. Control CLEAR and RED wells can also be prepared and read for calibration.
2. The Sudan IV analysis is demonstrated in Fig. 2e using PDLLA scaffolds (16). The Abs490 values for six PDLLA 96-well scaffold arrays were averaged and plotted. Control CLEAR and RED wells were also prepared, read, averaged, and plotted. The plot shows that a linear ($R=0.99$) and reproducible change in Sudan IV concentration is present in the libraries, which spanned 90% of the composition range from pure CLEAR to pure RED (7% RED–97% RED).

3.7. Scaffold Porosity and Microstructure

1. The 96-well array scaffolds are too small for accurate determination of porosity by gravimetric analysis (weighing scaffolds to determine mass and using calipers to determine volume). Thus, gravimetric measurements to determine scaffold porosity were made using larger scaffolds (PDLLA, $M_w=103{,}000$; M_w = "relative molecular mass" for the purposes of this work) fabricated by the same process of freeze-drying and salt leaching (16). As described in Simon et al. (16), the total porosity of the scaffolds is 97% ($n=8$, SD = 0.1%) calculated using the equation, "$1-[(m/d)/v]$ = total porosity", where m is mass of the scaffold (g), d is polymer density (g/mL), and v is volume

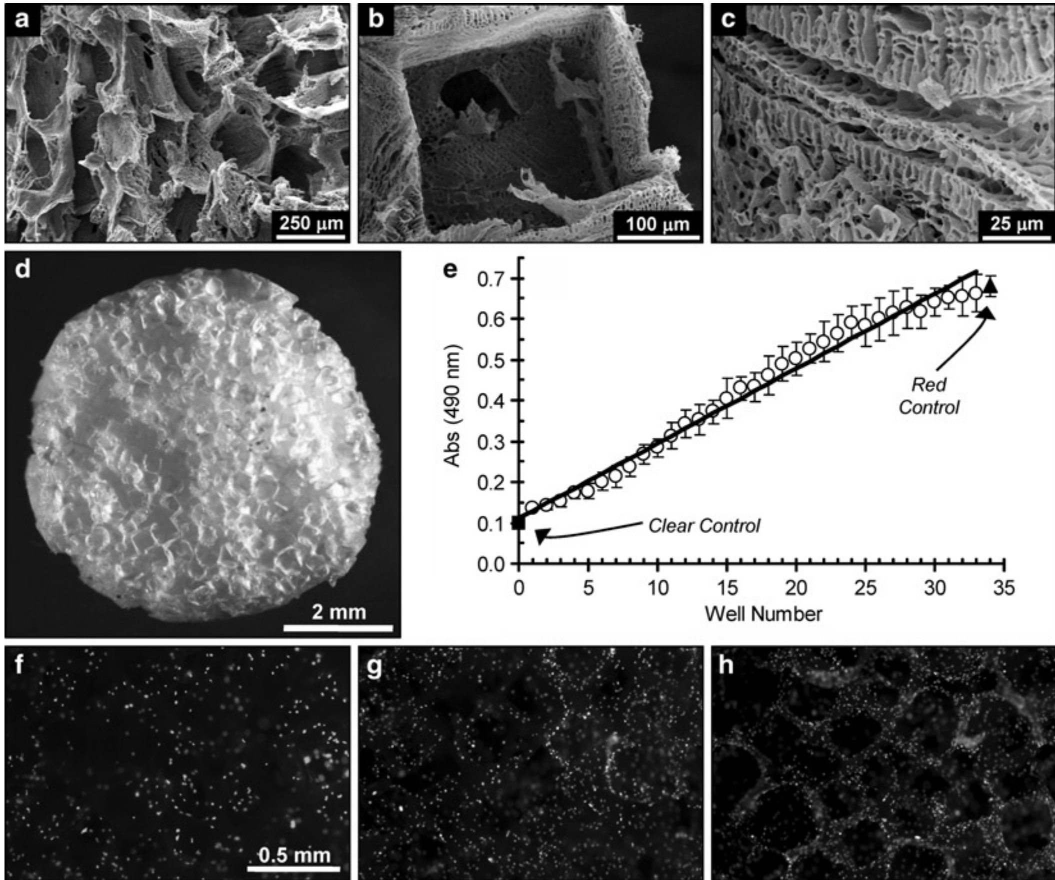

Fig. 2. (**a–c**) SEM images of the interior pores of a PCL scaffold made by the scaffold array process. Panel (**a**) is lower magnification and shows several pores, (**b**) shows a single pore formed by a NaCl crystal at the very bottom of a scaffold in the 96-well plate (scaffold bottom is on right) and (**c**) shows the small pores in the scaffold walls and struts that form during the freeze-drying process as the dioxane sublimes. (**d**) Stereomicroscope image of a PDLLA scaffold made by the scaffold array process. (**e**) The change in composition of the 96-well scaffold libraries was determined by tracking Sudan IV red dye. Six 96-well libraries were fabricated and Sudan IV was determined by measuring Abs490. Abs490 for six 96-well libraries was averaged and plotted against well number. Control clear (*black square*) and red (*black triangle*) scaffolds were also analyzed. Error bars are SDs, and the line is linear regression ($R=0.99$). Ninety percent of the range from 0 to 100% is covered (7–97% red). (**f–h**) The nuclei of MC3T3-E1 osteoblasts cultured on pure PCL scaffolds made by the library approach were stained to fluoresce green using Sytox green. Cells were cultured for 1 d (**f**), 7 d (**g**), or 14 d (**h**).

of scaffold (mL). Of this total porosity of 97%, the theoretical porosity from NaCl leaching was 83% calculated using the equation, "$(m/d)/v=$NaCl Porosity", where m is mass of NaCl used (g), d is NaCl density (2.165 g/mL), and v is scaffold volume (mL). The remaining 14% porosity (97–83%=14%) is due to voids in the scaffold walls that form as the dioxane sublimes during freeze-drying.

2. Large pores (0.2–0.4 mm) formed by salt leaching are shown in Fig. 2a, while small pores (<10 μm) formed by dioxane sublimation are shown in Fig. 2c. In Fig. 2b, a large, cubic pore formed by salt leaching is visible in the middle of the

panel, while smaller "dioxane-sublimation" pores are visible in the walls of this "salt-leach" pore.

3. Scaffold porosity is essentially independent of the polymers used because the scaffolds are so highly porous and because the densities of different polymers are similar [PDLLA=1.3 g/mL (Absorbable Polymers International), PCL=1.1 g/mL (Sigma–Aldrich), pDTEc=1.2 g/mL, pDTOc=1.2 g/mL] (17). Although the density of different polymers can vary slightly, the polymer accounts for only 3% of the scaffold volume. Thus, a 10% change in polymer density will result in only a 0.3% change in the total porosity of the scaffolds.

3.8. Cell Culture on Scaffold Arrays

1. Sterilize scaffold arrays using an ethylene oxide sterilizer (Anprolene AN74i, Anderson Products) and degas under vacuum in a desiccator for 2 d.
2. When ready to seed cells, add 0.2 mL of medium per well and place under vacuum for 2 min (bubbles may form). Briefly release the vacuum and reapply for 2 min. This procedure removes air from the interior scaffold pores filling them with medium.
3. Scaffolds can be seeded with up to 100,000 cells per well (in 0.2 mL of medium) and successfully cultured for 2 weeks with two medium changes per week without the medium becoming acidic (turning yellow from cell waste accumulation) (unpublished observations).
4. Standard cell assays can be performed to assess cell adhesion, morphology, and proliferation in scaffold arrays, such as fixing and staining for microscopy or soluble colorimetric assays for cell viability and counting (16, 17).
5. MC3T3-E1 osteoblasts cells cultured for 1 d, 7 d, or 14 d on control PCL scaffolds made by the library approach are shown in Fig. 2f–h (see Note 12). Ten thousand cells were seeded on each scaffold and the nuclei were stained to fluoresce green. Note that the number of cells increases with increasing incubation time, indicating that cells can adhere and proliferate on scaffolds made by the library approach. Also note that the pores of the scaffold are visible in Fig. 2h, as the cells have become confluent.

4. Notes

1. Safety glasses should be worn when fabricating scaffolds arrays. There is potential for splashing of solvent and liquid nitrogen.
2. The average drop size during deposition can be determined by counting the number of drops that fall in a given time (1 min) at a known flow rate (0.5 mL/min). When this was

done on five different days using PDLLA, the average drop size was 15.2 μL (SD = 2.0 μL, n = 5) [16]. Similar drop sizes were observed for pDTEc, pDTOc, and PCL.

3. The dead volume of the tubing system including the T-junction, static mixer, pipette tip, and tubing connectors is ≈ 2 mL. Approximately, 0.85 mL of CLEAR polymer (0.07 g) and 2 mL of RED polymer (0.17 g) are used to make each scaffold array, although only ≈ 1 mL is actually deposited into the 96-well plates to yield the scaffold arrays. More RED than CLEAR is required since additional RED is required to push the gradient through the mixer and into the 96-well plate. The gradient takes ≈ 4 min to pump: 72 s of mixing and ≈ 3 min of pumping for the gradient to get pushed out of the mixer and into the salt trough.
4. When preparing several scaffold arrays at once, they can be temporarily frozen in liquid nitrogen and stored in a –80°C freezer for up to 2 h so that all the arrays can be loaded onto the freeze-dryer at one time. Make sure to reimmerse the arrays for 5 min in liquid nitrogen to refreeze them before putting them on the freeze-dryer.
5. The 96-well plates are bulky and may not fit in the glass bell jars that typically come with freeze-dryers. In this case, the 96-well plates can be placed in the manifold of the freeze-dryer. However, a trap may have to be installed between the freeze-dryer manifold and the pump to catch the dioxane. Alternatively, a secondary chamber with a large opening for loading in the 96-well plates could be attached to the freeze-dryer. A vacuum oven, Parr Bomb (steel reaction vessel), or vacuum desiccator may work (make sure they can withstand the low pressure or they could implode!). A trap can be placed between the secondary chamber and the freeze-dryer to catch dioxane.
6. Dioxane sublimes quickly enough during the freeze-drying process, so the scaffolds stay frozen. If the polymers being used to make the scaffold arrays are not soluble in dioxane and a different solvent must be used, then it may be necessary to keep the scaffolds cold during freeze-drying. This can be achieved by packing the plates in dry ice. [1,4-Dioxane physical values from manufacturer (Sigma–Aldrich): density = 1.033 g/mL; boiling point = 101°C; melting point = 11°C; M_w = 88.1 g/mol].
7. Five days of salt leaching is sufficient to remove all the salt from all of the scaffolds. When scaffolds were leached for only 3 d, residual NaCl was occasionally found in some of the scaffolds.
8. If two drops of polymer solution are added to each well of the 96-well plate at an average drop size of 15.2 μL, then 30.4 μL of polymer solution will end up in each well. This

equals 2.6 mg of PDLLA using 8.5% (mass/volume) PDLLA solutions. Thus, the mass of each scaffold in a completed array is ≈ 2.6 mg and each scaffold contains ≈ 10 μg of Sudan IV (0.4% by mass). Finished scaffolds are cylinders 6.5 mm in diameter and 2 mm high.

9. The following polymers have been used successfully to make scaffold arrays as described herein:
   (a) Poly(d,l-lactic acid): PDLLA
      - M_w 100,000 g/mol, Lactel-Birmingham Polymers (unpublished results of the authors)
      - M_w 103,000 g/mol Absorbable Polymers International [16]
      - M_w 109,000 g/mol, Medisorb-Alkermes (unpublished results of the authors)
   (b) Poly(desaminotyrosyl-tyrosine ethyl ester carbonate): pDTEc, M_w 183,000 g/mol [17]
   (c) Poly(desaminotyrosyl-tyrosine octyl ester carbonate): pDTOc; M_w 122,800 g/mol [17]
   (d) Poly(ε-caprolactone): PCL, M_w 80,000 g/mol, Sigma–Aldrich (Figs. 1c, e–g and 2a–c)
10. Scaffolds can be gently removed from the 96-well plates with a needle for analysis. Gently slide the needle down the side of the scaffold against the wall of the well and pry the scaffold. Figure 2d shows a scaffold that has been removed from the plate and imaged by stereomicroscopy.
11. When making scaffold libraries where the scaffold composition is actually varied (not just red dye), FTIR can be used to characterize the composition. Control samples of known composition can be made to establish an FTIR calibration curve for assessing the scaffold arrays [9, 17–20].
12. The MC3T3-E1 murine osteoblast cell line (Riken Cell Bank, Hirosaka, Japan) was used as a model for osteoblasts [21] and cultured as described [9]. Low passage cultures (>6) at 80% confluency were passaged with trypsin for experiments. Medium was α-modification of Eagle's minimum essential medium (Cambrex Bio Science) supplemented with 10% volume fraction fetal bovine serum (Gibco) and 0.060 mg/mL kanamycin sulfate (Sigma). For staining, cells were fixed (4% volume fraction paraformaldehyde), permeabilized (0.5% volume fraction Triton X-100), and blocked (1% mass fraction bovine serum albumin). Cells were fluorescently stained in phosphate buffered saline (PBS) containing 5 μmol/L Sytox green (Invitrogen). Scaffolds were removed from 96-well plates and observed on slides while moist with PBS. Cells were imaged by epifluorescence microscopy at 100× magnification (10× objective).

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