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Polymeric Aqueous Biphasic Systems for Non-Contact Cell Printing on Cells: Engineering Heterocellular Embryonic Stem Cell Niches

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The ability to generate heterocellular niches through direct spatial patterning of one type of cells onto a living layer of another cell type would benefit a wide range of cell biological studies including stem cell research. Most cell patterning approaches, however, rely on patterned material adhesiveness to indirectly guide cell patterning,[1–5] and cannot position cells on an already existing cell monolayer. Cells can be embedded directly into soft supported membranes and gels using solid pins,[6,7] but the physical contact involved may damage delicate substrates such as living cells. Individual cells or cell sheets have been gently placed onto a cell monolayer, but with limitations in control of positioning and resolution.[8–10] Thermal or piezoelectric inkjet printers utilize thermal energy or applied electric voltage to eject the cell suspension from a nozzle and direct it toward the substrate. [11–13] Although this approach is non-contact and can potentially accommodate several cell types, concentrated buffer solutions[11] and special additives[14] are required and still often induce chemical, mechanical, or thermal stresses during cell suspension preparation and printing stages. Furthermore, dedicated cartridges are needed for each cell type, and pattern fidelity is an issue because cells have to be printed on a gel immersed in aqueous media.[14] Pressure-mediated extrusion of cell-seeded hydrogels through needles also creates continuous pattern of cells,[15] though with impaired direct cell-cell contact due to gel encapsulation. Therefore, existing approaches face limitations in terms of contact-free printing with and onto live cells for engineering heterocellular stem cell niches.

We address this unmet need by using polymeric aqueous two-phase systems (ATPS) to enable non-contact printing of high-fidelity cellular patterns onto delicate surfaces of living cells in fully aqueous environments. Aqueous solutions of polyethylene glycol (PEG) and Dextran (DEX) above certain concentrations segregate and form an ATPS with DEX always forming the bottom phase (Supporting Information, Fig. S1).[16] We have previously described the utility of a PEG-DEX aqueous biphasic system to efficiently micropattern genetic materials on cells for gene overexpression and RNA interference gene silencing studies.[17] However due to physicochemical differences between cells and gene delivery constructs, that formulation could not be applied to cell printing. Here we define design principles to select optimized ATPS formulations for efficient cell printing. Using this approach, we engineer cellular niches to support neuronal differentiation of mESC and show

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that the density of printed mouse embryonic stem cells (mESC) is an important factor for guiding mESC differentiation to neurons.

To understand how cells can efficiently be printed using the ATPS approach, we invoke the theory of cell partition in ATPS. Thermodynamically speaking, spontaneous partition of a cell to either of the two aqueous phases or to their interface is determined by the interfacial tension between the cell and bottom phase, γ_{2c} , compared to that between the cell and the top phase, γ_{1c} (Fig. 1a). Three scenarios are possible: (i) $\Delta\gamma=\gamma_{1c}-\gamma_{2c}>0$ will favor cell partition to the DEX phase, (ii) $\Delta\gamma=\gamma_{1c}-\gamma_{2c}=0$ will cause cells to partition mainly to the interface, and (iii) [18] $\Delta\gamma=\gamma_{1c}-\gamma_{2c}<0$ will favor partition of cells to the PEG phase. The classical Young's equation relates $\Delta\gamma$ to the contact angle, θ , of the bottom phase droplet formed on a cell layer immersed in the top phase and the interfacial tension between the two aqueous phases, γ_{12} :

$$((\gamma_{12}\cos\theta=\Delta\gamma)) \quad (1)$$

Thus, the interfacial tension γ_{12} and the contact angle can serve as a measure of cell partition in ATPS: (i) if $0<\theta<90^\circ$, cells have more affinity to the DEX phase especially as contact angle becomes smaller and approaches zero, (ii) if $\theta=90^\circ$, cells have equal affinity to both phases and partition mainly to the interface, and (iii) if $\theta>90^\circ$, cells favor the PEG phase as contact angle becomes larger (Fig. 1b).

Therefore efficient cell printing requires that the first condition ($0<\theta<90^\circ$) must be satisfied and that the contact angle attains values as small as possible. This in turn requires that the interfacial tension, γ_{12} , should be very small so that its horizontal component that contributes to increase in the contact angle is minimized (Fig. 1b). The interfacial tension in ATPS is determined by the concentration of the phase forming polymers (Supporting Information, Fig. S2). We selected two different ATPS pairs to test this principle. Pair 1 consists of 2.5% PEG (Mw: 35,000) and 3.2% DEX (Mw: 500,000) and pair 2 comprises 4.0% PEG (Mw: 8,000) and 5.0% DEX (Mw: 500,000), which was used before to print genetic materials on cells.[17] We performed interfacial tension and contact angle measurements with both pairs (Fig. 1c, Supporting Information, Fig. S3) and used them for printing mouse myoblast cells of similar densities (Fig. 1d). With both systems, cells remain confined to the DEX droplet; however, the pattern is more uniform with the ATPS 1 that has a lower interfacial tension and a smaller contact angle of 25° (Fig. 1d). The non-uniform printed cell spot from ATPS 2 is due to the tendency of the cells to partition to the interface as contact angle increases more towards 90° and poorer cell attachment at higher DEX concentrations. Independent cell partition experiments with both ATPS confirmed this point. Cells partitioned mainly to the DEX phase of the ATPS 1 and yielded a partition coefficient, K , of 78%, whereas with the ATPS 2, cells distributed between the DEX phase and the interface of the two phases and resulted in a partition coefficient of 42% (Supporting Information, Fig. S3). Thus, ATPS 1, which has very low phase-forming polymer concentrations and hence an extremely small γ_{12} , is the more effective formulation for high fidelity cell printing. This thermodynamic principle for selecting ATPS as a cell printing tool is general and applicable to other aqueous biphasic systems. As a rule of thumb, we suggest that the selected ATPS should have an interfacial tension of $\gamma_{12}\leq 0.01 \text{ mJ/m}^2$ to result in uniform printed cellular patterns.

Using this formulation, we demonstrate the utility of the ATPS strategy for utilizing cells both as “bioink” and “biopaper” and print cells on an existing cell layer in user-defined shapes. Cell suspension is mixed with the DEX phase and loaded into an extruded pipette tip of $300 \mu\text{m}$ tip diameter. The pipette tip is mounted on a micromanipulator and moved close

to, typically <500 μm , a monolayer of cells immersed in the PEG phase. Horizontal movement of the pipette tip results in the formation of a continuous pattern of the dispensing DEX phase on the monolayer (Fig. 1e). Figure 1f illustrates an example of spelling “cell” using green fluorescing cells on a monolayer of red fluorescing cells. Technically, inkjet printers could position cells onto a moist but substantially dry cell monolayer but subsequent media overlay necessary for cell attachment during incubation would wash away printed cell patterns.

Next we show formation of heterocellular microarrays (Fig. 2a). To generate a microarray, a cell suspension is mixed with the DEX phase and transferred into a microwell plate. Nanoliter volume dispensing pins mounted on a 1536-array fixture are dipped into the plate to load with the DEX phase. Pins are slowly withdrawn from the source plate and dipped into a culture plate containing a support cell monolayer covered with a PEG phase. The DEX phase dispenses and form distinct droplets on the support cell layer. The culture plate is incubated for 3 hrs to allow cell attachment. We note that the fully aqueous two-phase media environment enables long incubation (3hrs or more) without the need for further media addition and disturbance of the printed patterns. After cells attach, the two-phase media is washed out and replaced with the regular culture media. An array of green C2C12 cells printed onto a confluent monolayer of red fluorescent C2C12 cells using this strategy is shown in Figure 2b–d. The non-contact and gentle mechanism of dispensing the cell suspension and the extremely low interfacial tension of the ATPS supports full cellular viability of ~100% (Supporting Information, Fig. S4). This is another advantage over existing techniques that compromise cellular integrity due to mechanical forces.[19]

We utilized this capability to print mouse embryonic stem cells (mESC) onto a monolayer of support PA6 stroma cells to evaluate the effect of stem cell colony size on their neuronal differentiation. mESC were arrayed at four different densities of 20, 50, 150, and 500 cells per spot and with a center-to-center spacing of 4.5 mm on a confluent monolayer of PA6 cells (Fig. 3a). The culture system was maintained for 8 days, resulting in the formation of single mESC colonies of ~0.6–2.8 mm in diameter. mESC were examined immunohistochemically for the neuron-specific class III β tubulin TuJ1 differentiation marker (Fig. 3b). We quantified the results by calculating the fluorescent intensity of TuJ1 expression for each colony (Fig. 3c). Differentiation was minimal for the smallest colony but enhanced with increase in the mESC colony size. The finding is consistent with a recent work that suggests niche size as an important parameter in determining the fate of stem cells maintained on micropatterned extracellular matrices.[20] Normalizing the data by the area of each colony still resulted in disproportionately higher TuJ1 expression intensity for larger colonies (Fig. 3d), indicating that the efficiency of mESC neuronal differentiation enhances non-linearly with increase in the colony size. The neurogenesis in mESC colonies, which was induced in serum-free conditions and without using differentiation-inducing chemicals such as retinoic acid, was mediated by direct contact with the support PA6 cell layer.[21] However, direct heterocellular contact alone cannot explain the non-linear effect we observed. The exact mechanisms remain undefined. Cell surface-bound factors on PA6 cells, secreted factors secondarily tethered to PA6 cell surface,[22] soluble factor signaling, [23,24] and presence of other cells such as glia[25] and astrocytes[26] may all, at least in part, support mESC differentiation toward a neuronal phenotype.

In summary, we presented an optimized polymeric aqueous biphasic system for direct non-contact printing of cells on living cell layers in a single step while supporting full cell viability and functionality. The technology is simple to implement, requires only inexpensive materials and off-the-shelf equipment, and is easily accessible to a wide range of researchers without microfabrication expertise. The utility of this approach was demonstrated by creating physiologic heterocellular microenvironments to support mESC

differentiation to neurons. We identified niche size as an important regulator of the differentiation efficiency. This strategy will be useful to study inductive or inhibitory mechanisms responsible for the differentiation of mouse and human stem cells in heterocellular environments. The capability of printing cells in multi-layers and in user-defined shapes also makes the technology potentially useful for a broad range of tissue engineering applications.

Experimental

ATPS preparation and cell microarray printing procedure

PEG (2.5% w/w) (Mw:35000, Fluka) and DEX (6.4% w/w) (Mw:500000, Pharmacosmos) were selected as phase forming polymers of the ATPS based on the measurements of their interfacial properties detailed in Fig. S3. Both aqueous phases were prepared in culture media one day before use and kept at 4°C. Cells were grown to desired confluence, trypsinized, centrifuged down, and resuspended in appropriate volume of culture media. The cell suspension was thoroughly mixed with an equal volume of the DEX phase to give a 3.2% phase concentration and the resulting solution was transferred into a 1536-well plate. A final C2C12 cell density of $1.5\text{--}2.5 \times 10^6$ was used for microarray experiments. Prior to experiments, slot pins (V&P Scientific, FP3S500H) were mounted on a pin tool fixture (V&P Scientific, AFIX1536FP3), which itself was assembled with a micromanipulator. Pins were dipped into a cleaning solution (V&P Scientific), DI water, and isopropyl alcohol, as indicated by the manufacturer. Clean pins were dipped into the wells and filled with the cell suspension. Retraction of pins from the solution was done slowly to minimize residue on the outer surface of the pins. Next, pins were lowered into the close vicinity of the cell monolayer covered with the PEG phase solution and were allowed to dispense the cell suspension. After formation of DEX droplets containing cells, pins were slowly retracted and moved out of the culture dish. The culture system was incubated for 3 hrs to allow cells attach. Then, the two-phase media was washed out using PBS and replaced with regular culture media.

Preparation of PA6 feeder cell monolayer and mESC culture

Stromal PA6 cells (Riken) derived from skull bone marrow were kindly provided by K. Hiroaki and grown in α -MEM (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Invitrogen) and 1% antibiotic (Invitrogen). For the colony size-dependent mESC differentiation experiment, a confluent monolayer of PA6 was formed and incubated in α -MEM, 10% FBS and 10 μ g/ml mitomycin C (Sigma) for 2 hrs for feeder layer formation and then incubated overnight in the conditioning media at 37°C, 5% CO₂, and 95% humidity. The conditioning media consisted of G-MEM (Invitrogen), 10% knockout serum replacement (KSR, Invitrogen), 0.1 mM non-essential amino acids (Invitrogen), 2 mM glutamax (Invitrogen), 1mM sodium pyruvate (Sigma), and 0.1 mM 2-mercaptoethanol (Sigma). mESC were kindly provided by S. O'Shea. Cells were cultured in porcine gel-coated Petri dishes using G-MEM supplemented with 10% KSR, 1% FBS, 0.1 mM non-essential amino acids, 1mM sodium pyruvate, 0.1 mM 2-mercaptoethanol, and 2000 U/ml leukemia inhibitory factor (LIF, Millipore). mESC were printed on a PA6 monolayer at different densities using ATPS prepared with the conditioning media. After mESC attached to the underlying support cell layer, the ATPS media was washed out and replaced with the conditioning media. mESC were cultured for 8 days to form colonies from single cells. Culture media was changed on day 4 and then every other day.

Immunofluorescence, imaging, and image analysis

After 8 days in culture, mESC colonies were fixed in -20°C methanol for 6 min, washed three times with PBS and twice with PBS containing 5% BSA (blocking reagent). The rabbit

neuronal class III- β -tubulin (TuJ1) mAb (Covance) in PBS/5% BSA at a concentration of 1 μ g/ml was added to fixed cells for 1 hr at room temperature. After washing, the primary antibody was visualized with fluorescently labeled goat anti-rabbit IgG (Jackson ImmunoResearch) at a concentration of 3 μ g/ml incubated for 1 hr. We imaged cell microarrays section by section using an inverted fluorescence microscope (Nikon, TE300). Images were pseudocolored, merged, and superimposed in Photoshop 10.0 CS3 (Adobe). We used SimplePCI (Compix) for the measurements of fluorescence intensity and area of differentiated mESC colonies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Design principles to select ATPS for cell printing

(a) Partition of cells in an ATPS is directly determined by difference in interfacial energies of cells with the two aqueous phases ($\Delta\gamma = \gamma_{1c} - \gamma_{2c}$). (b) Contact angle of a DEX phase droplet on cell monolayer covered with the PEG phase is a measure of the partition of cells in ATPS and efficiency of the ATPS for cell printing. (c) Interfacial tension, contact angle, and partition coefficient results with two different ATPS formulations. (d) Printed spots of C2C12 cells, the left spot was printed using the ATPS comprising 2.5%PEG35K and 3.2%DEX500K and the right spot was printed using the ATPS consisting of 4.0%PEG8K and 5.0%DEX500K. (e) Schematics of cell printing on a cell monolayer in user-defined shapes. Cell suspension in the DEX phase is printed onto an existing cell layer by continuous dispensing from the pipette tip. (f) C2C12 cells were stained using a green fluorescent cell tracker and printed onto a monolayer of C2C12 cells stained with a red fluorescent cell tracker spelling “cell”. Scale bar 200 μm in (d), 1 mm in (f).)

Figure 2. ATPS-mediated cell microarrays on living cell layers

(a) Schematics of cell microarray printing using ATPS approach. (b) An 8×4 microarray of C2C12 cells stained with green cell tracker printed on a monolayer of red fluorescent C2C12 cells. (c,d) Magnified fluorescent and brightfield images of the boxed spot in (b). Scale bar 800 μm in (b).)

Figure 3. Colony size-dependent neuronal differentiation of mESC on PA6 support cells
 Schematic and day 1 brightfield images of mESC printed on a PA6 monolayer at four different densities. (b) Day 8 fluorescent images of mESC colonies stained with anti-TuJ1 antibody. (c) TuJ1 expression increases with the colony size. (d) TuJ1 expression normalized by the colony area suggests neuronal differentiation is enhanced non-linearly with increase in the colony size. Scale bar 250 μm in (a), 500 μm in (b).))