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Shrink Wrapping Cells in a Defined Extracellular Matrix to Modulate the Chemo-Mechanical Microenvironment

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Abstract

Cell-matrix interactions are important for the physical integration of cells into tissues and the function of insoluble, mechanosensitive signaling networks. Studying these interactions *in vitro* can be difficult because the extracellular matrix (ECM) proteins that adsorb to *in vitro* cell culture surfaces do not fully recapitulate the ECM-dense basement membranes to which cells such as cardiomyocytes and endothelial cells adhere to *in vivo*. Towards addressing this limitation, we have developed a surface-initiated assembly process to engineer ECM proteins into nanostructured, microscale sheets that can be shrink wrapped around single cells and small cell ensembles to provide a functional and instructive matrix niche. Unlike current cell encapsulation technology using alginate, fibrin or other hydrogels, our engineered ECM is similar in density and thickness to native basal lamina and can be tailored in structure and composition using the proteins fibronectin, laminin, fibrinogen, and/or collagen type IV. A range of cells including C2C12 myoblasts, bovine corneal endothelial cells and cardiomyocytes survive the shrink wrapping process with high viability. Further, we demonstrate that, compared to non-encapsulated controls, the engineered ECM modulates cytoskeletal structure, stability of cell-matrix adhesions and cell behavior in 2D and 3D microenvironments.

Key Terms

Fibronectin; Laminin; Collagen Type IV; Fibrinogen; Myocyte; encapsulation; surface-initiated assembly; c2c12

Introduction

The extracellular matrix (ECM) is a fibrillar network of proteins, glycosaminoglycans and other biomolecules, which forms a scaffold around cells that provides structural support,

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CONFLICTS OF INTEREST

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ETHICAL STANDARDS

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growth factor sequestration, a network for adhesion and mechanical signalling and a host of other functions.^{4, 8, 9, 13} For example, the adult stem cell niche is thought to contain a unique ECM protein structure, composition, support cell population and set of soluble and insoluble signalling molecules that help maintain the multipotent state of the stem cells.¹⁶ In contrast, in 2D culture cells are typically grown on rigid tissue culture treated polystyrene (TCPS) that is pre-coated with an ECM protein or coated with ECM proteins that adsorb from serum supplemented into the media.^{15, 29} While these ECM proteins enable adhesion of cells to the TCPS and subsequent proliferation, many primary cell types can only be passaged a limited number of times before becoming senescent of changing phenotype, such as undergoing epithelial to mesenchymal transition (EMT).^{1, 26} Culture in 3D using synthetic and/or natural hydrogels can address some of these limitations by altering the chemo-mechanical environment to better replicate in vivo conditions and have been effective for culturing a wide range of cell types.^{7, 24} However, these hydrogels are typically isotropic in structure, do not recreate ECM dense structures such as basement membranes and have compositions (e.g., collagen, fibrin, matrigel, PEG) that typically differ from that of the complex in vivo environment. Further, passaging these cells, whether in 2D or 3D, often requires using enzymes and calcium chelators that disrupt cell-matrix and cell-cell adhesion to produce a single cell suspension. When re-seeded the cells must expend energy to reestablish cell matrix and cell-cell adhesions in the new environment into which they are placed. Researchers have developed a number of micro- and nano-fabricated approaches to engineer the cell microenvironment to mimic that found in vivo.^{12, 18, 19} Here we sought to develop a set of unique capabilities to (i) encapsulate cells in a defined ECM that better mimics the native ECM structure and (ii) do so while minimally disrupting cell-matrix and cell-cell adhesions.

A wide range of cell encapsulation techniques have been developed to engineer a defined microenvironment that can protect cells from the surrounding environment, sequester growth factors or drugs with the cells and increase the retention of cells injected into tissues.^{21, 28} For example, researchers have demonstrated the use of microfluidics to encapsulate suspended cells within a gelatin core surrounded by a silica-gel shell that provides protection from oxidative and mechanical stress.⁶ Similar to many encapsulation approaches, after a defined period of time the gel breaks down, enabling the cells to migrate out into the surrounding environment. In another approach, micropatterned surfaces were used to encapsulate cells in a pyrrole-alginate hydrogel that simultaneously could perform controlled release of protein.³⁰ This system enabled the controlled presentation of soluble and in soluble factors while maintaining high cell viability. Recent work has also demonstrated that encapsulation materials based on polydimethylsiloxane (PDMS) and calcium peroxide can actively release oxygen to support metabolic activity in larger constructs that would otherwise suffer from hypoxia-induced necrotic cores.²⁵ However, while all of these examples encapsulate cells, to date none have done so in an ECM that is similar in density, structure or composition to the native ECM these cells are surrounded by in vivo. While it is not established that such an encapsulation technique is required, we propose that doing so may provide a unique microenvironment that more closely matches that found in vivo and thus improve our ability to modulate cell behavior.

Here we describe an approach to engineer ECM sheets that can be used to partially encapsulate cells in order to modulate the chemo-mechanical microenvironment. Using an adaptation of surface-initiated assembly (SIA),^{11, 33} we are able to engineer well-defined, nano-scaffolds of assembled ECM proteins into free standing structures. By adhering cells prior to the release of these ECM nano-scaffolds, we can effectively shrink wrap the cells, or SHELL them, in a layer of assembled protein matrix. Uniquely, these ECM nano-scaffolds are engineered at the size scale of the cell, ~75 μm in lateral dimensions and ~50 nm thick. Further, by using the SIA approach we can SHELL a variety of cell types in defined ECM consisting of fibronectin (FN), laminin (LAM), fibrinogen (FIB) and/or collagen type IV (Col IV), representing the major protein composition of the native peri-cellular matrix. The long-term goal is that these ECM nano-scaffolds and the SHELL process will enhance therapeutic cell delivery by supporting survival and functional integration of cells in an otherwise diseased matrix environment, such as that found in infarcted myocardium.

Materials and Methods

ECM Nano-Scaffold Fabrication and Release

The ECM nano-scaffolds for SHELL were fabricated using SIA based on adaptation of previously described methods.^{11, 33} Briefly, PDMS stamps for microcontact printing were prepared using established soft lithography techniques^{10, 32} and used to pattern 75 μm squares of ECM protein onto PIPAAm coated glass cover slips. Prior to use, the PDMS stamps were sonicated in a 50% ethanol solution for 60 minutes and then dried under a stream of nitrogen. Dedicated PDMS stamps were used for each ECM protein to prevent cross-contamination. The stamps were incubated for 60 minutes with either fibronectin (FN, BD Biosciences), laminin (LAM, BD Biosciences), fibrinogen (FIB, MP Biomedical), or collagen IV (Col IV, BD Biosciences) at a concentration of 50 $\mu\text{g}/\text{ml}$ (FN, LAM, Fib) or 500 $\mu\text{g}/\text{ml}$ (Col IV) in sterile distilled water (Figure 1, step 1). After incubation, PDMS stamps were washed in sterile water to remove excess protein and then dried under a stream of nitrogen. The ECM protein coated PDMS stamps were then used to microcontact print PIPAAm coated coverslips, with contact maintained for 30 minutes to ensure transfer of the 75 μm square pattern (Figure 1, step 2). Upon removal of the PDMS stamps, the quality of the ECM squares on the PIPAAm was inspected using phase contrast microscopy. For studies examining release of the ECM nano-scaffolds without cells, coverslips were placed in a Petri dish and ~42°C phosphate buffered saline (PBS) was added and allowed to cool to room temperature. As the temperature dropped below the lower critical solution temperature (LCST) of the PIPAAm (~32°C) the PIPAAm swelled and dissolved, resulting in the non-destructive release of the square ECM nano-scaffolds.

Cell Culture and Shrink wrapping of Cells in ECM Nano-Scaffolds

Three different cells types were studied to understand how the release and partial encapsulation process affected cell viability and behavior. The murine skeletal myoblast C2C12 cell line (ATCC) was cultured in high glucose Dulbecco's modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin and 2 mM L-glutamine in an incubator at 37°C and 10% CO₂. Bovine corneal endothelial cells (CECs) were isolated from fresh whole bovine eyes (Pel-Freez Biologicals). The corneas

were excised from whole globes and soaked for 20 minutes in PBS containing 1% penicillin-streptomycin-amphotericin B (Life Technologies, Grand Island, NY, USA) and 0.5% gentamicin (Life Technologies). Corneas were incubated endothelial side up in a 12-well spot plate with approximately 300 μ L of Tryple Express (Life Technologies) at 37 °C for 20 minutes. CECs were released into the Tryple Express by gently scraping with a rubber scalpel, combined and centrifuged for 5 minutes at 1500 rpm. The cells were designated as passage 0 (P0), resuspended in low glucose DMEM with 10% FBS, 1% penicillin-streptomycin-amphotericin B and 0.5% gentamicin and cultured in flasks until passage 2. Embryonic chick cardiomyocytes were isolated from the ventricles of day 7 chicken embryos based on published methods.¹⁷ Cardiomyocytes were diluted to a density of 250,000 cells/mL in M-199 media supplemented with 10% heat-inactivated FBS and 1% penicillin-streptomycin and 2 mL of cell suspension was seeded onto the samples for the SHELL process. Note that the seeding concentration of the cardiomyocytes was higher than for the other cell types, we found this to be necessary to achieve adequate cell adhesion to the FN squares, and may be due to the lower adhesivity of cardiomyocytes as compared to other cell types such as fibroblasts.

To shrink wrap cells in the ECM nano-scaffolds, the coverslips patterned with the ECM protein squares were first placed in 35 mm diameter tissue culture Petri dishes and sterilized under UV light in the biosafety cabinet for 30 minutes. Next, the Petri dishes were placed on a hot plate and the coverslips inside were warmed to 40°C. The cells were then suspended in culture media at a concentration of 25,000 cells/mL for the C2C12 and CECs or a concentration of 250,000 cells/mL for the cardiomyocytes. Cell solutions were placed in a 15 mL centrifuge tube and heated in a dry block to 42°C. It was important that once the cell solution reaches 42°C it was immediately seeded onto the warm coverslips in order to minimize the time cells are exposed to this elevated temperature. After cells were seeded onto the coverslips they were immediately transferred to the 37°C cell culture incubator. C2C12 and CECs were incubated with the ECM protein squares for 2.5 hours to ensure cell attachment and provide time for initial spreading (Figure 1, step 3). Cardiomyocytes were incubated for 4 hours to allow additional time for attachment because these cells take longer to establish focal adhesions after isolation from the heart. After incubation, the Petri dishes were removed from the incubator, the media was aspirated and samples were rinsed with 40°C PBS to remove non-adherent cells. At this point any cells adhered to the coverslip were attached and spread on the ECM protein squares (Figure 1, step 4). To shrink wrap cells 2 mL of 40°C PBS was added to the Petri dish and allowed to cool to room temperature. As the PIPAAm passed through its LCST the ECM protein squares were released and contracted around the adhered cells, partially encapsulating them in an ECM protein nano-scaffold (Figure 1, step 5). After release these shrink wrapped cells (SHELLs) were handled in a manner equivalent to standard cells in suspension using serological and micropipettes.

Viability of Shrink wrapped Cells

To assess viability each cell type was shrink wrapped in FN as described above and the percent viability of the cells was determined using the LIVE/DEAD Mammalian Cell Cytotoxicity Kit (Life Technologies). Briefly, 4 μ L of ethidium homodimer was used to bind

to the DNA of dead cells and label them fluorescent red and 1 μ L of calcein AM was used to label live cells, which enzymatically convert the dye to fluorescent green. These dyes were incubated with the cells at 37°C for 30 min following the shrink wrapping process. Control experiments were also performed to determine if the transient exposure to slightly elevated temperatures (40–42°C) during SHELL was impacting cell viability. As a first control, C2C12 cells were taken through the same heating and cooling stages they experienced during SHELL; being heated to 42°C, seeded in a 35 mm tissue culture polystyrene Petri dish, cultured at 37°C for 2.5 hours, and then cooled from 37°C to 25°C to simulate the release process. As a second control, C2C12 cells were seeded in a 35 mm tissue culture polystyrene Petri dish and cultured for 2.5 hours while maintained at 37°C during the entire process, i.e. subjected neither to heating or cooling. For both control groups, after the prescribed process LIVE/DEAD dye was incubated with the cells for 30 minutes at 37°C prior to analysis. Cells in the experimental and control groups were then imaged using a fluorescent microscope and post-processed using ImageJ to quantify the number of live (green) and dead (red) cells per image. For experimental groups five images were taken per sample and five samples were analyzed per cell type (n = 5, endothelial, cardiomyocytes, and C2C12) and for control groups three samples were analyzed per temperature condition (n=3). Results on viability were statistically analyzed by one-way ANOVA (SigmaPlot).

Immunofluorescent Staining and Imaging

To better understand the SHELL process, both the ECM proteins and the cells were fluorescently labeled. To visualize the ECM nano-scaffolds, FN was fluorescently labeled by conjugation to Alexa Fluor 546 Maleimide (Life Technologies) following the manufacturer's instructions in order to bind free cysteines. Fluorescent FN was combined in a ratio of 2:3 with unlabeled FN and used to pattern 75 μ m squares on PIPAAm coated coverslips. These FN squares were imaged pre-release and post-release using a laser scanning confocal microscope (Zeiss LSM 700). To image cells adhered to the FN squares pre-release, C2C12s were resuspended in Opti-MEM media (no Phenol red) supplemented with 2% FBS and 1% penicillin-streptomycin and seeded onto samples mounted in a custom stage-top incubation system maintained at 40°C to prevent premature PIPAAm dissolution. After 2 hours the samples were rinsed with 40°C PBS to remove non-adherent cells and then fixed and permeabilized in a 40°C solution of 4% formaldehyde with 0.05% Triton-X for 10 minutes. The samples were then rinsed 3 times with 40°C PBS and incubated with a 1:200 dilution of 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI, Life Technologies), a 1:100 dilution of rabbit anti-vinculin primary antibody (Sigma-Aldrich) and a 1:100 dilution of Alexa Fluor 633 Phalloidin (Life Technologies) at 37°C for 2 hours. The sample were subsequently rinsed three times with 40°C PBS followed by incubation with a 1:100 dilution of Alexa Fluor 488 goat anti-rabbit secondary antibody (Life Technologies) at 37°C for 2 hours. As noted, samples and all solutions were maintained at ~40°C throughout this process to ensure the PIPAAm did not dissolve. After incubation with the secondary antibody the samples were rinsed with 40°C PBS and imaged using Epifluorescent or confocal microscopy, continually maintained at 40°C.

Fluorescent staining and imaging of shrink wrapped cells was accomplished with a similar process, but performed at room temperature since the PIPAAm was already dissolved. After

release, the shrink wrapped C2C12s were allowed to settle on to the glass coverslip for approximately 30 minutes in order to adhere. We observed that SHELLs adhered to cell culture surfaces at a rate similar to cells seeded from suspension. The samples were then rinsed 3 times in PBS and fixed and permeabilized in 4% formaldehyde and 0.05% Triton-X. During the third wash, 5 drops of NucBlue Fixed Cell Stain (Life Technologies) was added to visualize the nuclei. The samples were then incubated with 1:100 dilutions of rabbit anti-vinculin primary antibody and Alexa Fluor 633 phalloidin for 2 hours. The samples were again rinsed 3 times with PBS and then incubated with 1:100 dilution of Alexa Fluor 488 goat anti-rabbit secondary antibody for 2 hours. Finally, samples were rinsed 3 times with PBS and the coverslips were mounted to glass slides with Pro-Long Antifade reagent (Life Technologies). Individual shrink wrapped cells were imaged in 3D using confocal microscopy. The 3D imaging data was post processed using AutoQuant X software (Media Cybernetics) for deconvolution and then segmented and rendered in 3D using Imaris image processing software (Bitplane).

Culture of Shrink wrapped Cells in 2D and 3D

For analysis in 2D, C2C12 myoblast cells were seeded onto fluorescently labeled FN squares (40% Alexa Fluor 546, 60% unlabeled) as described previously and incubated for 2.5 hours in a cell culture incubator to permit cell adhesion. Following incubation, the samples were rinsed with 40°C C2C12 growth media to remove non-adhered cells and then cooled to room temperature to shrink wrap the cells. To remove the dissolved PIPAAm from solution, the shrink wrapped cells were pipetted into a 15 mL centrifuge tube and centrifuged at 1100 rpm for 5 minutes. Note that after centrifugation the SHELLs formed a cell pellet that was readily broken up back into suspension using gentle agitation, comparable to that typically obtained with cells in suspension. The supernatant was aspirated and the cells were resuspended in 3 mL of growth media and seeded onto PDMS coated 25 mm diameter glass coverslips. Control samples consisted of C2C12 cells (not shrink wrapped) seeded onto PDMS coated coverslips. Samples were fixed in a 4% formaldehyde solution at 30 minutes, 12 hours, and 24 hours post-seeding ($n = 3$ per time point). After fixation, the samples were rinsed with PBS 3 times and during the third wash, 5 drops of NucBlue was added. The samples were then incubated for 2 hours with a 1:100 dilution of Alexa Fluor 633 Phalloidin. All samples were imaged with confocal microscopy consisting of 10 fields of view per sample and quantitatively analyzed using ImageJ. Briefly, a maximum intensity projection was created from each Z-stack and the actin channel was used to create a binary image. Any gaps in the signal corresponding to the cell bodies were filled in using the ‘fill holes’ command and then any remaining defects were filled in manually. Any cell clusters at the border of the image were excluded from analysis. The ‘analyze particles’ function was then used to calculate the area of each cell or cell cluster from the binary image. The cell/cluster area and nuclei per cell/cluster were compared using a Two-way ANOVA with Tukey pairwise test (SigmaPlot) to determine statistically significant differences based on $P < 0.05$.

To study cell behavior in 3D, C2C12 myoblast cells were shrink wrapped as described for the 2D studies, except after centrifugation the cells were resuspended in 1 mL of culture media. C2C12 cells were embedded in fibrin gels by mixing 600 μ L of the cell suspension

with 540 μ L of fibrinogen dissolved in sterile, distilled water at a concentration of 40 mg/mL. To initiate the formation of a fibrin gel, 60 μ L of thrombin at a concentration of 20 U/mL was added to the cell-fibrinogen solution. The solution was mixed and evenly pipetted onto 3 glass coverslips (400 μ L per coverslip) and allowed to gel. Once the fibrin gels were fully formed, they were placed in a 6-well plate with 2 mL of C2C12 growth media and cultured in the incubator. Samples were fixed in 4% formaldehyde after 30 min, 12 hours and 24 hours ($n = 3$ per time point). Control samples consisted of C2C12 cells (not shrink wrapped) embedded in fibrin gels using the same process. After fixation cells were stained with a 1:200 dilution of DAPI and a 1:100 dilution of Alexa Fluor 633 Phalloidin for 2 hours and then washed 3 times in PBS. Samples were mounted on glass slides and imaged using confocal microscopy. The 3D imaging data was post processed using AutoQuant X software for deconvolution and then segmented and rendered in 3D using Imaris image processing software.

Results

The SHELL technique requires that an ECM nano-scaffold is able to release from the PIPAAm surface during the thermally-triggered dissolution process and wrap around the adhered cell(s) (Figure 2A). To demonstrate this, we first wanted to establish that the ECM nano-scaffolds by themselves were able to release and fold over in 3D. An array of 75 μ m FN squares was patterned on the PIPAAm and when released using SIA folded over on themselves (Figure 2B). A video of 3 adjacent FN squares helped visualize this process and illustrate that the folding dynamics were consistent from square to square (Video S1). This was then repeated for 75 μ m squares composed of FIB (Figure 2D), LAM (Figure 2F) and Col IV (Figure 2H). For each ECM protein the squares released from the PIPAAm to form an ECM nano-scaffold that typically folded over along one of the diagonal axes and resulted in a rolled up, triangular shape. The ECM proteins were observed to follow different release kinetics, where FN, FIB and COL IV contracted and folded quickly (10 s to 15 s) upon thermal release while LAM contracted and folded more slowly (~35 s). The cause for this difference in folding rate is unknown and may be due to difference in the molecular structure of these ECM proteins but was not investigated further because once cells were adhered to the squares the encapsulation rate was similar for all ECM proteins.

Next, we wanted to demonstrate that we could use the SHELL technique with any of the ECM test proteins. To do this, we cultured C2C12 myoblasts on the different ECM nano-scaffolds and allowed them to release. We found that for FN, FIB, LAM and Col IV (Figure 2C, 2E, 2G and 2I, respectively) that the shrinking wrapping process occurred in a similar manner, resulting in a partially encapsulated cell, with the ECM protein appearing to be on the bottom of the cell. In all cases, tracking the release from the time the PIPAAm first started to dissolve (time = 0) to complete release was ~40 s regardless of the ECM protein type. Further, the nature of the release and partial encapsulation process was similar, with the spread cell appearing to pull the releasing ECM square in towards itself from all sides, eventually forming a ball-like structure. This release process is best observed in the video of the C2C12 cell on a FN square being shrink wrapped (Video S2). Finally, it should be noted that while the cells did ball-up to a degree, they did not adopt the highly spherical morphology observed for cells that have been trypsinized.

The SHELL process is rapid and induces changes in cell morphology, which could potentially damage the cells. To investigate this, we next evaluated the shrink wrapping of different cell types in FN square nano-scaffolds and assessed cell viability. In this study we consider the C2C12s to be the most robust because they are an established cell line, and as previously demonstrated (Figure 2C) these cells can readily be shrink wrapped (Figure 3A). We next repeated this experiment using CECs, which are a primary cell we harvested directly from bovine corneas and cultured in vitro for <5 passages. Similar to the C2C12s, the CECs could be shrink wrapped in the FN nano-scaffold and this process appeared visually identical. Finally, we evaluated whether primary cardiomyocytes harvested directly from embryonic chick hearts could adhere and be shrink wrapped. Initial experiments using a cell concentration of 25,000/mL, which typically results in 1 to 2 cells per FN square, did not result in cardiomyocytes adhering. Cardiomyocytes are known to adhere slowly compared to other cell types, thus we decided to significantly increase the seeding density and the adhesion time. Using 250,000 cardiomyocytes/mL and allowing 4 hours for initial cell adhesion we were able to get the cardiomyocytes to adhere. In contrast to the C2C12s and CECs, typically 3 to 4 cardiomyocytes adhered per FN square. However, despite this increase in cell number the cardiomyocytes could be shrink wrapped in the FN in manner similar to the other cell types. A LIVE/DEAD cytotoxicity assay was used to determine the viability of each cell type after SHELL (Figure 3B). Each of the cell types exhibited high cell viability after the shrink wrapping process (Endothelial cells = $95.8 \pm 0.8\%$; C2C12 = $98.1 \pm 2.2\%$; Cardiomyocytes = $96.9 \pm 1.8\%$). We also performed control experiments to verify that the thermal treatment, including transient heating to 42°C and cooling to room temperature, did not impact viability or bias the adhesion of cells to the ECM nano-scaffolds. For the thermal treatment control, cells experienced the same temperature changes as the shrink wrapped cells but were seeded into a TCPS Petri dish instead of undergoing the shrink wrapping process. A second constant temperature control was also performed where cells were simply maintained at 37°C, i.e. neither taken through the temperature changes nor shrink wrapped. Both the thermal treatment and constant temperature controls had high cell viabilities of $96.2 \pm 0.9\%$ and $96.1 \pm 1.8\%$, respectively. Cell viabilities for all experimental and control groups were analyzed by one-way ANOVA ($\alpha = 0.05$) and showed that there were no statistically significant differences between groups. Based on these results, we concluded that the SHELL technique is able to encapsulate multiple cell types in ECM nano-scaffolds with high viability and that the thermal treatment, including transient heating to 42°C and cooling to room temperature, did not affect cell viability.

To better understand the SHELL process and how the ECM nano-scaffold was interacting with the encapsulated cells, we fluorescently labeled both the ECM proteins and the cells and imaged them at high resolution. First, to show that the direct conjugation of fluorescent molecules to the free cysteine residues in FN did not disrupt the ability of FN to properly release and fold over itself, we micropatterned 75 μm FN squares with a 2:3 ratio of fluorescent to non-fluorescent FN (Figure 4A). We then allowed the FN nano-scaffold to thermally release and found that it was still able to properly fold over itself, establishing that it would be suitable for SHELL (Figure 4B). Next we seeded C2C12 cells, allowed them to adhere the standard 2 hours and then, while maintaining 40°C to prevent the dissolution of PIPAAm, fixed, stained and imaged them in this pre-release state. The C2C12s adhered to

the FN squares and spread to cover the surface, with clearly visible actin filaments demonstrating the formation of a typical actin cytoskeleton (Figure 4C). Staining for vinculin revealed focal adhesions at the end of actin filaments and deformation of the underlying FN square where the cell was clearly applying traction forces (white arrows, Figure 4D). It should be noted that this elevated temperature fixing, staining and imaging process did cause disruption of the underlying PIPAAm layer as visualized by holes forming in the FN square (Figure 4C and 4D). These holes do not appear to be present when performing the standard SHELL technique.

Having visualized the cells pre-release, we next fixed, stained and imaged the cells after SHELL to determine cell structure post-release. We observed that unlike cells that have been trypsinized, after SHELL cells maintain some of their cytoskeletal structure including actin filaments still tethered to the FN nano-scaffold (Figure 4E). Further, the C2C12s had a robust cortical actin cytoskeleton and retained cell-cell adhesions, based on the tight coupling of cells in the cases when two or more cells were shrink wrapped together (Figure 4F). Focal adhesions also remained, as imaging vinculin revealed preservation of adhesions between the cell and the FN nano-scaffold below (Figure 4F) and a single slice of a confocal stack mid-way through the cell revealed that these adhesions continued as far up the side of the cell as the FN nano-scaffold went (Figure 4G). Finally, to visualize how the cells are physically shrink wrapped in the nano-scaffold, the FN signal was segmented out (Figure 4H) to show that the nano-scaffold only covers a portion of the cell surface. This becomes clearer when the cell nucleus and actin cytoskeleton are also rendered to show that the cell was effectively sitting on top of the FN (Figure 4I). This demonstrates that the cells in this study were only partially encapsulated in the FN nano-scaffold, and thus represents a difference between typical cell encapsulation approaches where the whole cell is usually surrounded by the scaffold material.

Having demonstrated the method and analyzed the cell structure following SHELL, we next evaluated whether there was any effect on cell behavior. To test this we cultured shrink wrapped C2C12s or non-shrink wrapped controls on PDMS coated coverslips for 30 min, 12 and 24 hours to determine if there were any differences in cell spreading and growth. For this 2D growth assay the PDMS coated coverslip was not oxidized or pre-coated with any ECM proteins, thus it was a relatively hydrophobic surface to which cells typically exhibit poor adhesion and growth. Results show that both the SHELL and control cells adhered to the PDMS and began to spread and proliferate (Figure 5A), however, the morphology and growth rate of the cells differed greatly. The control cells became flattened and spread out, mostly as single cells, and went through at most one cell division during the 24 hour culture period. In contrast, the SHELL samples had cells that spread out but remained coupled together in multicellular clusters, individual cells were rarely observed, even out to 24 hours of culture. Analysis of cell area revealed that the size of the control cells leveled out after 12 hours due to the fact that cells were typically isolated or at most connected to one other cell (Figure 5B). In contrast, cells in the SHELL sample continued to grow and spread while remaining in clusters. The number of nuclei per cell or cell cluster increased similar to the cell area data, showing that the control cells had 1 to 2 nuclei per cell/cluster while the SHELL clusters had significantly more cells at all time points (Figure 5C). Further, results showed that the FN nano-scaffold remained relatively intact during the culture and was

typically observed at the center of the SHELL clusters. These results indicate that the SHELL process can alter the growth characteristics of cells in a 2D environment, in this case causing a pronounced change from slow spreading single cells to relatively fast spreading and proliferating multicellular clusters.

To further evaluate the ability of SHELL to alter cell behavior, we next seeded shrink wrapped C2C12s or non-shrink wrapped controls inside fibrin gels to investigate the behavior in a 3D environment. Results showed that the overall growth and proliferation was similar for both conditions, suggesting that in the context of a permissive 3D ECM hydrogel the presence of the relatively small FN nano-scaffold had no measurable effect. For example, after 30 minutes both the SHELL and control cells appeared as relatively spherical with projections starting to be extended into the fibrin gel (Figure 6A and 6B). While the SHELL C2C12s appeared to have longer protrusion and more of them, this proved difficult to quantify. After 12 hours both SHELL and control cells had similar size and morphology, and in both cases had not yet appeared to begin to proliferate as most cells were still isolated and mono-nucleated (Figure 6C and 6D). At the final 24 hour time point there were still no apparent differences between the SHELL and control cells (Figure 6E and 6F). In both cases the cells had become highly elongated and formed interconnected cell networks, perhaps even beginning to fuse into nascent myotubes. While the FN nano-scaffold did not appear to have any effect in the fibrin gel, it was present throughout the 24 hour culture period and was not completely degraded by the cells. Thus, in a highly permissive 3D ECM environment, within which cells already can easily adhere and move, it appears that the ECM nano-scaffold as currently engineered has no measureable effect on cell size and morphology as a function of time, compared to control cells.

Discussion

Here we have demonstrated a methodology to shrink wrap cells in a defined ECM nano-scaffold, which partially encapsulates them in a sheet of dense matrix that preserves a degree of cytoskeletal structure, cell-matrix and cell-cell adhesions and can alter cell growth behavior in 2D. These results establish that SHELL has distinct differences from other cell encapsulation techniques as well as other cell-release approaches. For example, Okano and co-workers have pioneered the use of PIPAAm grafted to Petri dishes for cell sheet engineering.^{14, 22} While conceptually similar, cell sheet engineering relies on the cells to synthesize and assemble their own ECM over multiple days in culture. Further, cross-linked PIPAAm surfaces have been observed to actually entrap nanometer thick layers of ECM protein after cell sheet release.⁵ In contrast, for SHELL the PIPAAm is not grafted to the substrate, rather it is physically entangled when in the hydrophobic states above the LCST and then hydrates and dissolves completely below the LCST. It is this complete dissolution that enables nanometer thick ECM protein nano-scaffolds to be non-destructively released during the SIA process. SHELL is also different than other cell encapsulation techniques^{21, 28} that use hydrogels composed of ECM proteins such as collagen, fibrin or Matrigel or glycosaminoglycans such as hyaluronic acid and chitosan.^{21, 23, 27, 34} With SHELL we can use these same proteins as well as FN, further we can use the LAM and Col IV components of Matrigel individually or in combination. This can provide improved control over the composition of the ECM we are using to shrink wrap the cells.

Current cell encapsulation approaches are clearly effective in many applications, however the hydrogels used in many of these cases are by their nature mostly water. They do not match the protein dense ECM of the basement membrane. While we have not performed ultra-structural analysis of the ECM nano-scaffolds in this study, previous work has shown that the engineered FN produced by SIA is 4 to 5 nm thick when patterned on the PIPAAm and as it contracts laterally during release increases to 50 to 100 nm thick.^{11, 33} Using the pre-release thickness and 75 μm square dimensions, we can estimate the volume of FN shrink wrapped around the cells as $\sim 28 \mu\text{m}^3$. While it has proved difficult to determine the density of FN within the nano-scaffold, we can estimate an upper limit from the amount of FN that adsorbs to the PDMS stamp used for microcontact printing. For the 50 $\mu\text{g/mL}$ concentration of FN used for microcontact printing, the maximum density of FN in the nano-scaffold is $\sim 800 \text{ ng/cm}^2$.³⁵ This means that the results observed for SHELL are due to a piece of engineered FN less than 100 nm thick and weighing less than ~ 0.22 picograms. This amount of FN material is sufficient to alter cytoskeletal structure (Figure 4E), cell-cell and cell matrix adhesions (Figure 4F and 4G) and growth behavior in 2D (Figure 5). The 75 μm square used in these studies were purposely designed such that only a few cells would be able to adhere, but the result is only partial encapsulation. Future work will explore other geometric designs in order to increase the surface area of the cell covered by the ECM nano-scaffold.

The SHELL technique shrink wraps ECM around cells, but the idea of folding a structure around cells is not in and of itself a new concept. For example, other “self-folding” scaffolds have been developed using SU-8 photoresist sheets connected by polycaprolactone (PCL) hinges or from PIPAAm/PCL hybrid structures.^{3, 31} However, both of these approaches have limitations compared to SHELL. The SU-8/PCL folds into boxes, but requires a temperature of $>58^\circ\text{C}$ to do so and thus cells must be loaded into the scaffold via small gaps at the edges after folding is complete. Further, release of the cells from the box requires degradation of the PCL hinges at a pH >13.0 , which can damage cells.³ The PIPAAm/PCL self-folding scaffolds fold similarly to our SHELLs, in that the folding occurs when the temperature is dropped below the LCST of PIPAAm. However, when the folded PIPAAm/PCL scaffolds are exposed to temperatures above 32°C , they spontaneously re-open, which would then release the contained cells immediately upon injection or delivery in to a physiologic environment.³¹ The tight conformal contact the ECM nano-scaffolds make with the cells is unique in terms of engineered structures that fold around cells, and this is due largely to the focal adhesions between the cell and the ECM protein. The strength of this cell-ECM adhesion is illustrated by the fact that the nano-scaffolds remain attached to single cells and small cells clusters despite being pipetted, centrifuged, and pipetted again, as done in the 2D and 3D cell growth studies (Figure 5 and 6). Additionally, once the cells have been re-seeded in a physiologic environment, the SHELLs remain intact, with the cells still attached to the nano-scaffolds after 24 hours.

Looking forward, one potential application for SHELL is for therapeutic cell delivery via injection. Two possible advantages for these cells are (i) having an assembled cytoskeleton that may help cells resist fluid shear forces during injection and (ii) having the cells delivered with a functional ECM to help modulate the local microenvironment, such as in the case of fibrotic or ischemic tissue. For example, recent work has shown that cells

injected through standard hypodermic needles are subjected to high shear forces that can significantly reduce cell viability.^{2, 36} Cells in suspension are normally released from a culture surface using trypsin and EDTA, which by design disrupts cell-matrix and cell-cell interactions, leading to cytoskeletal disassembly. Thus, the typical injected cell may be mechanically weak compared to the state it was in when adhered to the surface. Though untested, it is possible that the retained cytoskeleton in the SHELL samples (Figure 4) will increase the stiffness of the cells and enable them to better resist the shear forces during syringe based delivery. However, it should be noted that the presence of F-actin does not always indicate the presence of cell contractility and increased cell stiffness; AFM indentation or related techniques will need to be performed to verify this. As also mentioned, the SHELL samples are partially encapsulated in a defined ECM, which could help modulate the local microenvironment at the site of cell delivery. We have demonstrated that FN, LAM, FIB and Col IV can all be used for SHELL (Figure 2), and though not shown here, it is straightforward to mix these ECM proteins in well-defined combinations.³³ This can be done either by mixing the protein solutions together prior to microcontact printing or performing layered prints to create thicker, multicomponent ECM nano-scaffolds. This is important since the expression level of each protein and combinations of proteins thereof depend on tissue type and developmental stage. Finally, a major role of the ECM is to sequester growth factors and modulate their activity in conjunction with adhered cells. In this paper we have not introduced growth factors beyond what might be present in the FBS used during culture. However, growth factors such as platelet derived growth factor (PDGF), which through binding to FN is known to regulate its bioactivity,²⁰ could easily be incorporated. Thus, we envision that using SHELL to immobilize specific growth factors around cells could further modulate cell behavior, and in the case of 3D fibrin gels may produce a cell response that we could observe. In the future we plan to test this concept using shrink wrapped cells injected into ischemic tissue in vivo using myocardial infarct after scar formation as a model system.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Biography

Dr. Feinberg is the principal investigator of the Regenerative Biomaterials and Therapeutics Group at Carnegie Mellon University. He earned his BS in Materials Science and Engineering from Cornell University in 1999 with Co-op experience at Abiomed, Inc., working on total artificial hearts. He then earned MS and PhD degrees in Biomedical Engineering from the University of Florida, focused on engineering cell-material interactions to prevent and enhance adhesion. This was followed by postdoctoral training at Harvard University, developing new biomaterials and stem cell-based cardiac tissue engineering strategies. He joined Carnegie Mellon in the fall of 2010 as an Assistant Professor with joint appointments in Biomedical Engineering and Materials Science and Engineering. Dr. Feinberg has co-authored over 15 peer-reviewed publications and holds 14 US patents and patent applications. He has also been awarded the NIH Director's New Innovator Award and the American Heart Association Scientist Development Grant. Current research is focused on biomimetic strategies to fabricate ECM protein-based materials from fibronectin, laminin, and collagens type I and IV to build 2D and 3D scaffolds for corneal and cardiac tissue engineering. Further, he is working to understand the biomechanics and mechanobiology of these engineered ECM protein materials, specifically how strain modulates biological activity.

Figure 1. Schematic of the SHELL process

Step 1, PDMS stamps are coated with an ECM protein solution and dried. Step 2, the PDMS stamp is used to microcontact print the square pattern onto a PIPAAm coated glass coverslip. Step 3, cells in suspension are seeded onto the micropatterned ECM squares and allowed to adhere and spread for 2 hours at 37 °C. Step 4, once the cells have spread on the ECM squares the temperature of the media is allowed to cool below the LCST of the PIPAAm, which causes the PIPAAm to dissolve. Step 5, during the PIPAAm dissolution process the ECM square is released and a protein nano-scaffold the shrink wraps around the cells.

Figure 2. ECM nano-scaffolds are patterned onto a surface and then shrink wrapped around the adhered cell(s)

(A) Schematic showing the SHELL process. (B) Example of a 75 mm square FN nano-scaffold by itself during the thermal release process where the underlying PIPAAm dissolves into solution. The initial square shape results in spontaneous folding over along one of the diagonal axes into triangle-like form. (C) Example of a square FN ECM nano-scaffold with an adhered C2C12 myoblast. As the underlying PIPAAm dissolves the cell contracts and the FN ECM nano-scaffold partially folds around it. (D) Example of a square FIB nano-scaffold by itself during the release process. (E) Example of a square FIB nano-scaffold with an adhered C2C12 myoblast. (F) Example of a square LAM ECM nano-scaffold folding. (G) Example of a square LAM nano-scaffold with adhered C2C12

myoblast. (H) Example of a Col IV nano-scaffold folding (I) Example of a COL4 nano-scaffold with adhered C2C12 myoblast. Scale bars are 25 μm .

Figure 3. The ECM nano-scaffolds can be used to shrink wrap different cell types while maintaining cell viability
 (A) Examples of a CEC, C2C12 myoblast and cardiomyocytes before and after shrink wrapping in FN nano-scaffolds. Representative images also show the live (green) and dead (red) cells present after SHELL in the FN nano-scaffolds. (B) Graph showing the percent viability of each cell type after encapsulation in FN ECM nano-scaffolds. Scale bars are 25 μm for the phase images and 50 μm for the fluorescent images and error bars indicate standard deviation.

Figure 4. Structural imaging of the FN nano-scaffolds and the adhered cells

(A) Image of a fluorescently labeled FN nano-scaffold before thermal release. (B) Image of a fluorescently labeled FN nano-scaffold after thermal release. (C) C2C12 cells adhered to fluorescently labeled FN nano-scaffolds before thermal release. The F-actin filaments are clearly visible as the cells spread across the scaffolds. (D) Cells adhered to the FN nano-scaffolds pre-release exhibited focal adhesions as visualized by vinculin staining and indicated by the arrows. The vinculin is localized at the end of the actin filaments and are pulling on the underlying FN. (E) A 3D image of a shrink wrapped C2C12, the arrows indicate actin filaments within the cell body that are maintained. (F) Two C2C12s released together maintain their cell-cell adhesions as indicated by the close proximity of the cortical actin cytoskeletons and maintain their cell-matrix adhesion as indicated by vinculin-positive focal adhesions to the FN nano-scaffold. (G) A single confocal slice of midway through the cell shows that vinculin-positive adhesions continue up the side of the cell as far as the FN nano-scaffold goes. In (H) the FN has been segmented out and the cell is transparent. (I) is the same view with the actin visible to illustrate where the cell attached to the FN nano-scaffold. Scale bars are 10 μm for A–D and 5 μm for F and G.

Figure 5. Shrink wrapped C2C12 cells seeded onto PDMS coated cover slips displayed significantly different behaviors compared to non-shrink wrapped controls

(A) Control cells (non-shrink wrapped) and shrink wrapped cells at 30 min, 12 hours and 24 hours after seeding onto PDMS coated coverslips. Shrink wrapped cells formed cell clusters with many nuclei (blue) centered around the FN nano-scaffold (red), whereas the control cells were more spread and present as mostly individual cells, the F-actin cytoskeleton is green. (B) The area of cells or cell clusters as a function of time was different, with the shrink wrapped cells continuing to spread throughout the experiment. (C) The number of nuclei per cell/cluster was constant for the control cells, but increased for the shrink wrapped cells indicating they were proliferating. Data was analyzed by two-way ANOVA with Tukey pairwise comparison where * indicates a statistically significant difference between the shrink wrapped and control cells ($P < 0.05$) at that time point. Scale bars are 25 μm and error bars are standard deviation.

Figure 6. Shrink wrapped and control C2C12s seeded into 3D fibrin gels displayed similar growth and morphology over 24 hours in culture

At 30 minutes post-seeding, both control cells (A) and SHELLs (B) were still mostly rounded with a few cytoplasmic projections. By 12 hours post seeding, both control cells (C) and SHELLs (D) had begun to spread within the gels. By 24 hours post-seeding, control cells (E) and SHELLs (F) were highly elongated within the fibrin gel forming multicellular structures, possibly nascent myotubes. At each time point, the FN nano-scaffold can still be seen within the gels still associated with the SHELLs. Grid spacing in the 3D rendered images in 10 μm . Cells are fluorescently labeled for the nucleus (blue), F-actin (green) and FN (red).