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Micromolding of shape-controlled, harvestable cell-laden hydrogels

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Abstract

Encapsulation of mammalian cells within hydrogels has great utility for a variety of applications ranging from tissue engineering to cell-based assays. In this work, we present a technique to encapsulate live cells in three-dimensional (3D) microscale hydrogels (microgels) of controlled shapes and sizes in the form of harvestable free standing units. Cells were suspended in methacrylated hyaluronic acid (MeHA) or poly(ethylene glycol) diacrylate (PEGDA) hydrogel precursor solution containing photoinitiator, micromolded using a hydrophilic poly(dimethylsiloxane) (PDMS) stamp, and crosslinked using ultraviolet (UV) radiation. By controlling the features on the PDMS stamp, the size and shape of the molded hydrogels were controlled. Cells within microgels were well distributed and remained viable. These shape-specific microgels could be easily retrieved, cultured and potentially assembled to generate structures with controlled spatial distribution of multiple cell types. Further development of this technique may lead to applications in 3D co-cultures for tissue/organ regeneration and cell-based assays in which it is important to mimic the architectural intricacies of physiological cell–cell interactions.

Keywords: Microgels; Tissue engineering; Micromolding; Biomaterials; Cell encapsulation

1. Introduction

Tightly regulated and controlled *in situ* microenvironments are comprised of cells, soluble factors, and extracellular matrix molecules. [1]. Mimicking the *in vivo* microenvironment can be useful for a variety of applications such as tissue engineering [2], cell-based assays [3], and directed stem cell differentiation [4]. Hydrogels, are three-dimensional (3D) crosslinked networks of hydrophilic polymers that, resemble the physical characteristics of extracellular matrices [5] and are often used to encapsulate cells. They can be tailored to exhibit high permeability to oxygen, nutrients, and other water-soluble metabolites [6]. Cell encapsulating hydrogels can be used for the generation of 3D tissue engineering structures [7] and immunoisolation microcapsules [8,9] as well as for use in scalable bioreactors [10].

Typically, cells are encapsulated within hydrogels through mixing a cell suspension with hydrogel precursors followed by crosslinking of the network. The crosslinking reaction may be controlled by a variety of environmental factors such as temperature, pH and the addition of chelating ions. In addition, hydrogels can be photopolymerized in the presence of photoinitiators via exposure to ultraviolet (UV) light [11]. Both biological hydrogels (e.g. fibrin [12], hyaluronic acid (HA) [13], agarose [8]) and synthetic hydrogels (e.g. poly(ethylene glycol) (PEG) [14,15]) have been used to encapsulate cells. For example,

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photopolymerized PEG diacrylate hydrogels, have been explored for the transplantation of islets of Langerhans for development of a bioartificial endocrine pancreas [16–18]. Similarly, photopolymerized hyaluronic acid hydrogels have been investigated as potential implantable/injectable cell delivery vehicles for cartilage regeneration [19].

The encapsulation of cells within hydrogels has been proposed as a method of enabling the scalable expansion of anchorage dependant cells within stirred bioreactors. However, the immobilization of cells within larger structures decreases the viability of cells in the center of these structures due to the lack of appropriate levels of oxygen and nutrients [16,20]. Spherical microcapsules with high surface area to volume ratios and coated annuli of cells immobilized within polymers have therefore been generated to overcome transport difficulties [8,19]. Currently most approaches to generate such structures have been based on spherical structures because of the available technologies to generate microscale spheres based on emulsification [10] or shear-induced droplet formation from syringes [8]. These approaches have been shown to be capable of forming spherical cell-laden (microscale hydrogels) microgels of controlled sizes; however, they are not amenable for the generation of other well-defined shapes. Thus, the development of approaches to generate cell-laden hydrogels with controlled sizes and shapes in a homogeneous manner may be of benefit.

Recently, photolithography [21–23] and soft lithography [24] have been used to encapsulate live cells within microscale polymeric hydrogels (i.e. microgels) anchored onto two-dimensional (2D) surfaces, which offer great potential for diagnostics and cell screening applications. Alternatively, cell encapsulation within free floating microgels (i.e. in suspension) may be advantageous not only for immunoisolation and bioreactor applications where long term cell culture is imperative, but also for tissue engineering. Such systems allow for the creation of micron sized units of tissue that can be potentially assembled to create tissue engineering constructs of controlled microscale structural and architecture. For such applications, controlling the size and shape of cell-laden microgels is important for minimizing diffusion limitations and for exhibiting control over the macroscopic engineered tissue. Micromolding of hydrogels provides a potentially powerful method for fabricating micro- and nanostructures [25,26]. Micromolding approaches are compatible with soft lithographic technology and therefore greatly minimize the need for costly photolithographic equipment and clean room facilities.

In this paper, we present a micromolding approach for generating cell-encapsulating 3D hydrogels of controlled shapes and sizes in the form of harvestable free units. Cells were suspended in a hydrogel precursor solution containing photoinitiator, deposited onto hydrophilic poly(dimethylsiloxane) (PDMS) patterns, crosslinked under UV radiation, and retrieved upon hydration. Two common photocrosslinkable hydrogel materials, methacrylated hyaluronic acid (MeHA) and poly(ethylene glycol) diacrylate (PEGDA), were tested using this technique, yielding shape-controlled microgels with homogeneous cell distribution at various viable cell densities. Hyaluronic acid is a natural component of the extracellular matrix known for its biodegradable, bioresistant properties [27] and its role in facilitating cellular functions such as adhesion, proliferation, and migration [28], while PEG is an inert, non-biofouling synthetic material often used as templates for immobilizing cells on 2D surfaces [25,26] or within microfluidic channels [25]. These two diverse materials were both shown to be compatible with this micromolding approach, suggesting the versatility of this technique and the feasibility of developing it further for tissue engineering applications in mimicking the architectural intricacies of physiological cell–cell interactions.

2. Materials and methods

2.1. Cell culture

All cells were manipulated under sterile tissue culture hoods and maintained in a 95% air/5% CO_2 humidified incubator at 37 °C. NIH-3T3 mouse embryonic fibroblast cells were maintained in Dulbecco's modified Eagle media (DMEM) supplemented with 10% FBS. Confluent dishes of NIH-3T3 cells were passaged and fed every 3–4 days. Murine embryonic stem (ES) cells (R1 strain) were maintained on gelatin treated dishes with media comprised of 15% ES qualified FBS in DMEM knockout medium. ES cells were fed daily and passaged every 3 days at a subculture ratio of 1:4.

2.2. Prepolymer solution

Two macromers were used: poly(ethylene glycol) and hyaluronic acid. The synthesis of MeHA was previously described [29]. In brief, the synthesis was performed by the addition of 1 wt% methacrylic anhydride (Sigma) to a solution of 1 wt% HA (Lifecore, MW = 67 kDa) in deionized water. The reaction was performed for 24 h on ice and maintained at a pH of 8–9 through the addition of 5 N NaOH. The macromer solution was then purified by dialyzing (Pierce Biotechnology, MW cutoff 7 kDa) for 48 h in deionized water and lyophilized for 3 days, resulting in a final dry form which was frozen for storage. The prepolymer form of MeHA was created by dissolving dry MeHA in PBS (Gibco) at 37 °C for 24 h to facilitate full dissolution. Immediately prior to UV photopolymerization, varying concentrations of photoinitiator solution was added to the prepared prepolymer solution. The photoinitiator solution used was 33 wt% 2-hydroxy-1-(4-(hydroxyethoxy)phenyl)-2-methyl-1-propanone (Irgacure 2959, CIBA Chemicals) in methanol.

To generate PEG hydrogels, a solution containing 10% (w/w) poly(ethylene glycol)-diacrylate polymer, PEGDA, (MW 575, Sigma) in phosphate-buffered saline (PBS, Gibco) was prepared prior to experiments in order to allow the PEGDA to adequately dissolve into solution. Immediately prior to UV photopolymerization, photoinitiator solution was added to the prepolymer solution at 1 wt%. The photoinitiator solution used was also 33% (w/w) 2-hydroxy-1-(4-(hydroxyethoxy)phenyl)-2-methyl-1-propanone (Irgacure 2959, CIBA Chemicals) in methanol.

2.3. PDMS mold fabrication

PDMS micropatterns of various shapes were fabricated by curing prepolymer (Sylgard 184, Essex Chemical) on silicon masters patterned with SU-8 photoresist. The patterns on the masters had protruding shapes (squares, circles, long rectangles) of various sizes (ranging from 50 to

400 μm), which allowed for the formation of shaped wells in PDMS replicas. PDMS molds were generated by pouring 1:10 curing agent to silicon elastomer onto the master and curing for 2 h at 37 °C. Finally, the PDMS molds were peeled from the silicon masters, cut into small rectangular shapes, and placed over glass slides to facilitate ease of manipulation. The use of glass slides allowed direct manipulation of the slides, thereby minimizing possibility of damaging the molds. Before stamping the molds were rendered hydrophilic by plasma cleaning for 45 s on medium power (PDC-001, Harrick Scientific). Untreated (hydrophobic) non-patterned sections of PDMS were similarly placed over glass slides and used as coverslides to reversibly seal the micropatterns into individual volumes (Schematic 1) during the stamping procedure.

2.4. Microgel polymerization

To encapsulate NIH/3T3 or murine ES cells within the prepolymer solution, the cells were trypsinized with 0.23% trypsin and 0.13% EDTA in PBS (Gibco). The suspension was then centrifuged at 1000 rpm for 2 min to produce a cell pellet. The pellet was then resuspended in the prepolymer solution, yielding differing concentrations of cell suspensions; 20–25 μl cm^{-2} of this cell/polymer mixture was then pipetted onto freshly plasma oxidized PDMS micropatterns. The tip of the pipette was gently brushed on the micropattern surface to remove any bubbles. A PDMS coverslide was then carefully applied on top of the pattern and gently rotated under slight finger pressure to ensure PDMS/PDMS contact. The micropattern/polymer-solution/coverslide assembly was then exposed to ~1 W cm^{-2} 360–480 nm UV light for various durations. The coverslide was then carefully removed and PBS was immediately pipetted onto the coverslide surface upon which the microgels were adhered to hydrate the newly formed hydrogels.

2.5. Microgel harvesting

After photopolymerization, the coverslide was removed from the microwell substrate to retrieve the microgels. In this process, a fraction of the microgels adhered to the microwell surface while the other fraction adhered to the PDMS coverslide. For convenience, those microgels which adhered to the PDMS coverslide upon removal of the coverslide from the PDMS micropattern were then harvested while those which remained adhered within the microwells were discarded. After hydrating the microgels upon the coverslide, a number of individual microgels spontaneously detached from the coverslide while a number remained adhered. A pipette tip was gently brushed over the coverslide to mechanically detach the remaining microgels.

2.6. Analysis of encapsulated cells

Initial encapsulated cell viability was assessed by applying a live/dead fluorescence assay to a model polymer system, consisting of cells encapsulated in thin layers of HA hydrogels made by deposition of 20 μl cell/prepolymer mixture between a glass slide and a flat PDMS coverslide. After photopolymerization, the PDMS coverslide was removed, leaving a thin layer of polymer/cell adhered to the glass slide. The thin layer was subsequently hydrated with 200 μl PBS solution containing 2 μg/ml calcein AM and 4 μg/ml ethidium homodimer-1 (Molecular Probes) and visualized under a fluorescent microscope (Zeiss, Axiovert 200). Initial cell viability assessments were made using NIH-3T3 cells for MeHA prepolymer solutions in which the macromer concentration, UV exposure duration, and photoinitiator concentrations were varied.

Two recognized parameters of cell viability—intracellular esterase activity and plasma membrane integrity—were tracked. Live cells fluoresced green, showing intracellular esterase activity that hydrolyzed the fluorogenic esterase substrate (calcein AM) to a green fluorescent product, and dead cells fluoresced red, their plasma membrane being compromised and therefore permeable to the high-affinity, red fluorescent nucleic acid stain (ethidium homodimer-1). Percent viability values were calculated by counting the number of live (green) cells and the number of dead (red) cells in a representative 400 μm × 400 μm square area magnified at 40 × and dividing the number of live cells by the number of total cells (live plus dead). Measurements were taken in triplicates, and error bars were based on standard deviation values for $n = 3$.

For confocal microscopy, cells were stained with Vybrant DiD (Molecular Probes) at 20 μl/ml in PBS, fixed with Fluoromount-G, and covered with a No. 1 thickness coverslip. Confocal images were taken at 40 × magnifications through a Rhodamine filter with a maximum focal depth of 248 μm. CFSE and PKH26 staining were performed as previously described [30] at room temperature prior to microscopy.

3. Results and discussion

3.1. Microgel fabrication

The procedure illustrated in Fig. 1 was used to fabricate homogenous microgels of specific shapes and sizes. In this process cells were suspended in a hydrogel precursor solution and molded using a PDMS stamp. Subsequently, the hydrogel precursor solution was induced to photocrosslink to form a gel. The mold was then removed to generate an array of micromolded hydrogels that could be harvested into the solution using a simple wash. The techniques can be used to generate microgel suspensions of virtually any shape as long as the desired pattern can be fabricated in the PDMS molds. Proof-of-concept square prisms, disks, and strings were fabricated with high pattern fidelity (Fig. 2). In addition, both MeHA and PEGDA polymer solutions were successfully used to fabricate hydrogels, suggesting that the micromolding technique is potentially compatible with other hydrogels such as collagen, agarose and dextran.

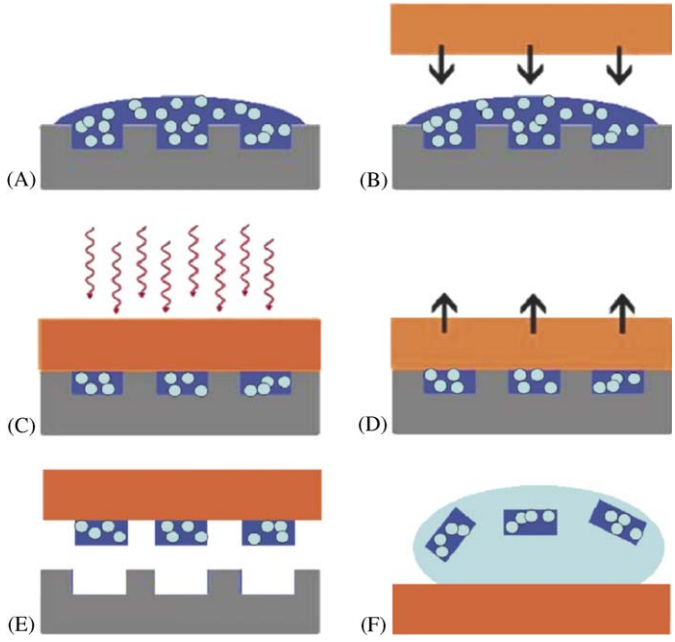

Fig. 1. Process of cell encapsulation and microgel formation. (A) Cells are suspended in prepolymer solution and deposited onto a plasma-cleaned PDMS pattern. (B) A PDMS coverslide is placed on top, forming a reversible watertight seal. (C) Polymer liquid is photopolymerized via exposure to UV light. (D) The PDMS coverslide is lifted, (E) removing the microgels which are then (F) hydrated and harvested.

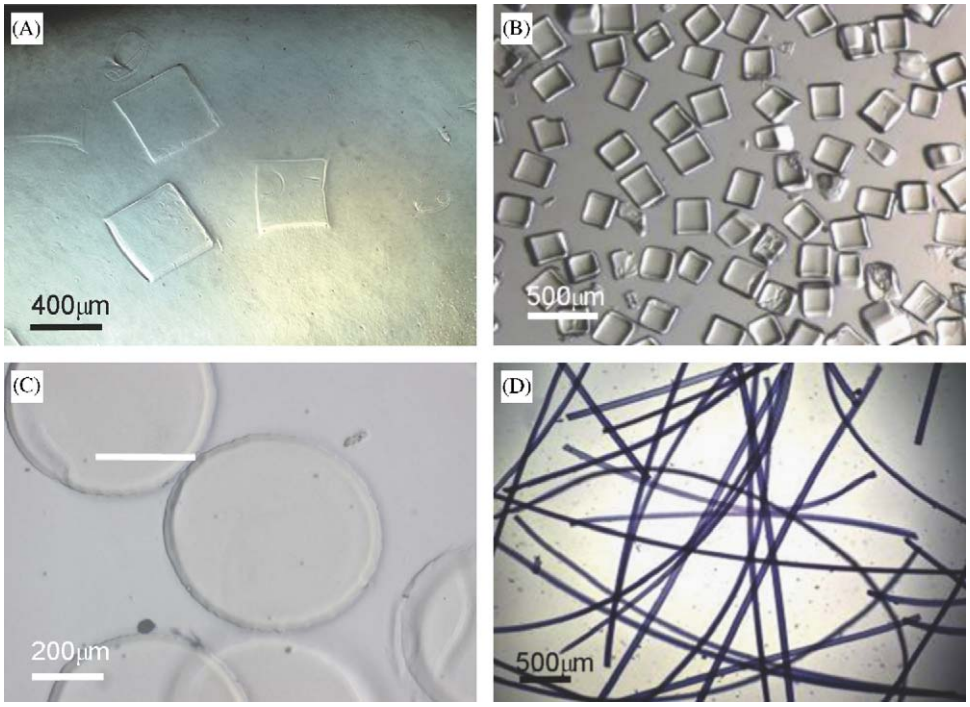

Fig. 2. Versatility in microgel shapes. Microgels can be molded into various shapes: square prisms (A), (B), disks (C), and strings ((D), stained with trypan blue to facilitate visualization) using different prepolymer solutions: (A) MeHA and (B), (C), (D) PEGDA.

The PDMS molds containing negative patterns of desired shapes were made using soft lithographic techniques, and the prepolymer mixtures were photopolymerized within the corresponding positive patterns. In order to assess the effect of microgel concentration on the formation of the hydrogels and its resulting mechanical properties we tested a series of macromer concentrations (i.e. hydrogel precursor solution). It was observed that the properties of the hydrogels could be significantly altered based on the polymer concentration. For example, different concentrations of MeHA macromer in PBS (2%, 5%, 7.5%, 10% w/w) were tested, and it was found that the uptake of PBS solution and the subsequent swelling of the MeHA microgels increased with higher macromer concentrations. Also, the mechanical robustness of the microgels increased with increasing macromer concentrations. In particular, mechanical stimulation of MeHA microgels of low macromer concentration tended to break them into debris while microgels of higher macromer concentration remained relatively intact. In addition, it was noted that the larger microgels swelled more upon hydration (data not shown). This phenomenon presumably occurs because the swelling ratio of the gels was proportional to gel volume, which scales as a cubic function of length, while the surface area of the gels scales as a square function of length.

3.2. Optimization for cell viability

To optimize the various crosslinking parameters (macromer concentration, photoinitiator concentration, UV exposure duration) for cell viability prior to encapsulating cells within microgels, initial cell viability data was obtained through the utilization of a thin-layer model. Though this setup may be an imperfect model for the viability of cells loaded within microgels, it was useful for screening relevant parameters. The setup consisted of cells encapsulated in thin layers of HA hydrogels sandwiched between a glass slide and a flat PDMS coverslide. Following photopolymerization and subsequent removal of the PDMS coverslide, a thin layer of polymer/cell solution was formed on the glass slide, allowing easy access for the application of viability assays and visualization under fluorescent microscopy.

It is known that photoinitiator concentration, UV exposure duration, and macromer concentration may affect the viability of cells encapsulated within photopolymerized hydrogels [13]. To optimize these parameters for our studies, we measured the viability of encapsulated cells as a function of each parameter. As expected, cell viability decreased with increasing UV exposure duration for all photoinitiator and macromer concentration conditions (Fig. 3). Furthermore, increased photoinitator and macromer concentrations individually and in combination also decreased cell viability. Based on these results 5% MeHA in PBS with 1% photoinitiator UV-exposed for 60 s was determined to be optimal for maintaining high cell viability within hydrogel films. This is not unexpected since previous work indicated that cell viability was higher for lower macromer concentrations, lower photoinitiator concentrations, and shorter UV exposure lengths. Although even lower parameter values would likely have further increased

cell viability, it was determined that lowering any of these three parameters resulted in incomplete polymerization of the prepolymer/cell mixture.

This technique is amenable for use with many different types of materials. In the future, materials which polymerize through alternative chemistries that do not require UV exposure but rather the exposure to (for example) nontoxic 450 nm visible light [31] may be beneficial for enhancing cell viability. We have here used PEG, a synthetic nonbiodegradable material, and HA, a natural biodegradable material to demonstrate the flexibility of the technique. Upon crosslinking, we anticipate based on previous work [32] that the HA byproducts will be safe and biocompatible. For future applications, it may be useful to use other materials that may have different and possibly superior biocompatibility properties.

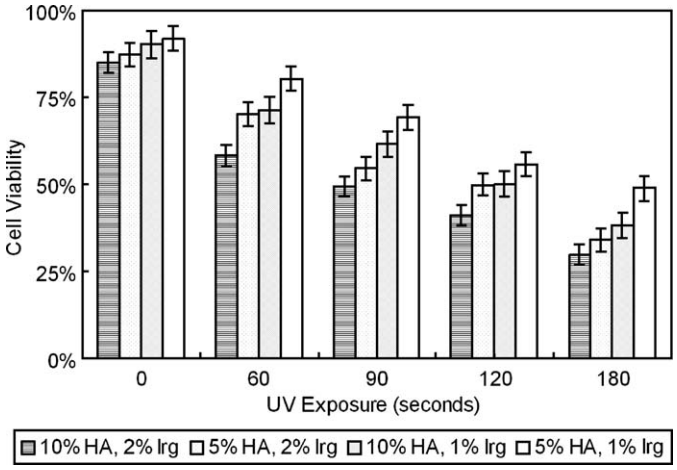

Fig. 3. Optimization for initial cell viability. Varied parameters include macromer (HA) concentrations, photoinitiator (Irg for Irgacure) concentrations, and UV exposure durations. Cell viability was observed to be optimal ($92 \pm 4\%$) for 5 wt% HA in PBS with 1 wt% photoinitiator. Error bars indicate standard deviation values for $n = 3$.

3.3. Cell encapsulation within microgels

Using optimized parameters found through the thin-layer model as a starting point, cells were successfully encapsulated within microgels and shown to be viable ($>85\%$) after the photocrosslinking step (Fig. 4). Uniform cell distribution was demonstrated throughout the depth of individual microgels (Fig. 4E) as well as across different microgels (Fig. 5A), and easy retrieval of these microgels was achieved through the subsequent hydration and suspension step (Fig. 5). Moreover, cell density is a parameter that can be finely controlled (Fig. 6), in addition to the size, shape, and uniformity of these microgels.

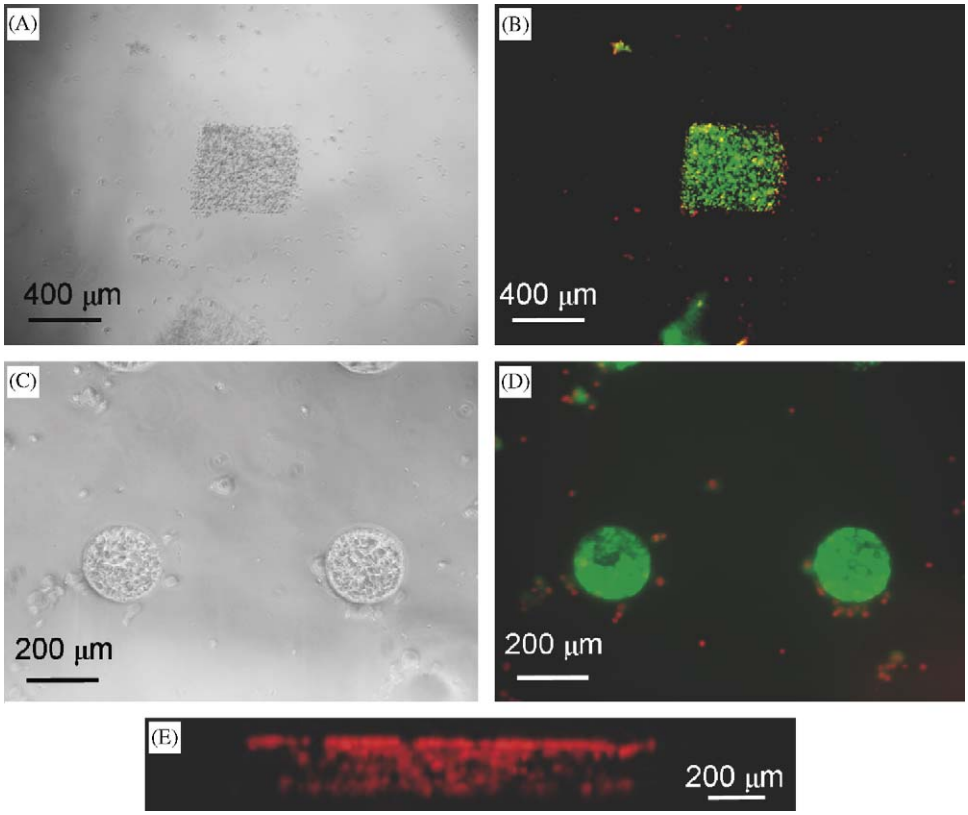

Fig. 4. Cell encapsulation, viability, and distribution. Cells were encapsulated in MeHA (A) and PEGDA (C) microgels and stained with viability markers ((B), (D): ethidium homodimer permeabilizes dead cells, showing up as red; calcein AM is metabolized by live cells, showing up as green). (E) Confocal imaging shows an even distribution of cells (rhodamine-stained) throughout the depth of microgels.

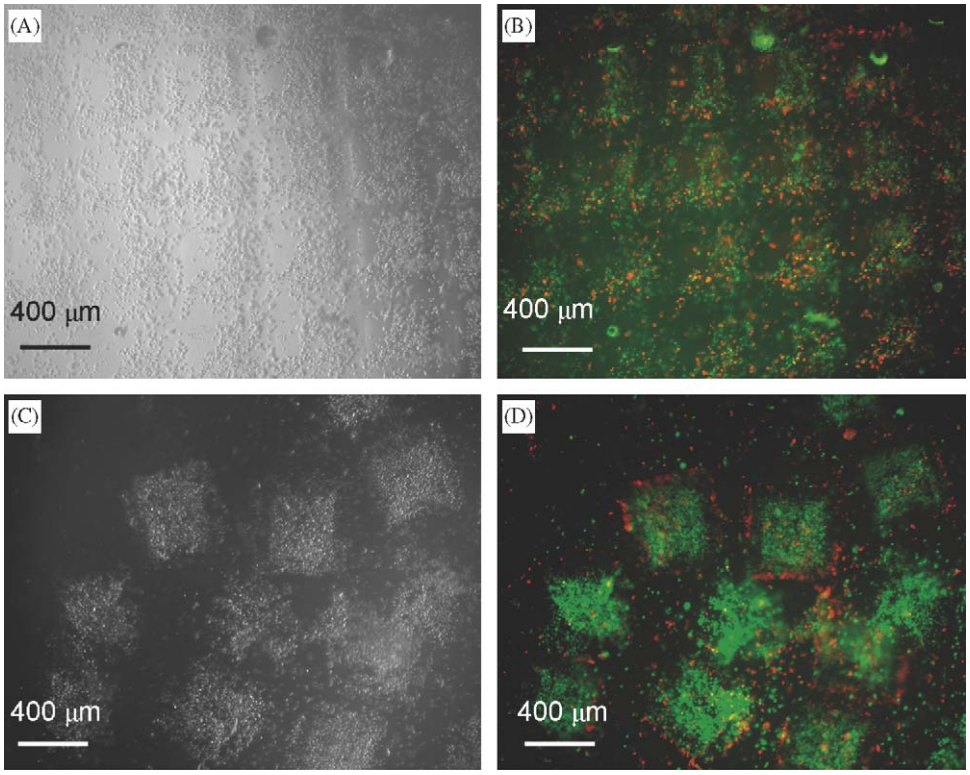

Fig. 5. Harvesting microgels. Removal of the PDMS coverslide following UV exposure yields a uniform array of HA microgels with cells encapsulated inside (A). The subsequent hydration allows these microgels to be dislodged and suspended in solution (C). Viability stains (B), (D) show >85% viability.

Additional optimization was performed in order to accommodate the encapsulation of cells within microgels. It was found, for example, that the addition of cells led to weaker mechanical stability of the gels for any given macromer concentration, photoinitiator concentration, and UV exposure duration, and that the degree of compromise was proportional to the density of cells encapsulated. In order to counter the higher tendency for debris formation, the MeHA concentration was increased from 5 to 10 wt%, which in turn allowed the UV exposure duration to be reduced from 60 to 45 s.

Another challenge was that a thin film tended to form between the PDMS coverslide and the PDMS pattern upon photopolymerization of prepolymer/cell mixture, especially at high cell densities. The thin film inhibited harvesting of the microgels because the microgels were then covalently attached to the thin film and could not be removed. This was due presumably to the cells which did not fall into the molds being sandwiched between the coverslide and non-well areas of the pattern pieces, thereby preventing complete sealing between the coverslide and pattern. The phenomenon was minimized by using PDMS patterns with small spacing between the negative features such that the cells could more easily be displaced to the microwells and decreased the likelihood that a cell would be sandwiched between the coverslide and the non-well areas of the pattern pieces. In addition, by maintaining the PDMS coverslip in a hydrophobic state it was possible to maximize dewetting of the solution from the surface and thus minimize the formation of the thin films between microgels. In the future the problem may potentially be reduced by generating microwell patterns with hydrophobic surfaces and hydrophilic wells.

An additional complication of generating photopolymerized microgels encapsulating cells versus microgels without cells is the added viscosity of prepolymer solution in which cells are suspended. This additional viscosity is presumably behind the difficulties encountered in making microgels smaller than 400 μm. Using PDMS micropatterns of smaller sizes, it was found that gels of the proper shape and size rarely formed; it appeared post-photopolymerization that the cell/prepolymer solution did not properly fill the wells and that the wells were instead filled with air bubbles. This problem was attributed to the additional viscosity of the cell/prepolymer solution compared to the prepolymer solution without cells based on the fact that microgels formed adequately under the same macromer concentration, photoinitiator concentration, and UV duration parameters within PDMS micropatterns of features as low as 50 μm. In the end, the parameter values of 10% MeHA macromer concentration, 1% photoinitiator, and 45 s UV exposure duration were found to be optimal for cell viability and microgel stability. These parameter values proved to be optimal for generating microgels with differing encapsulated cell densities (Fig. 6), keeping cells viable past 6 days of incubation in media.

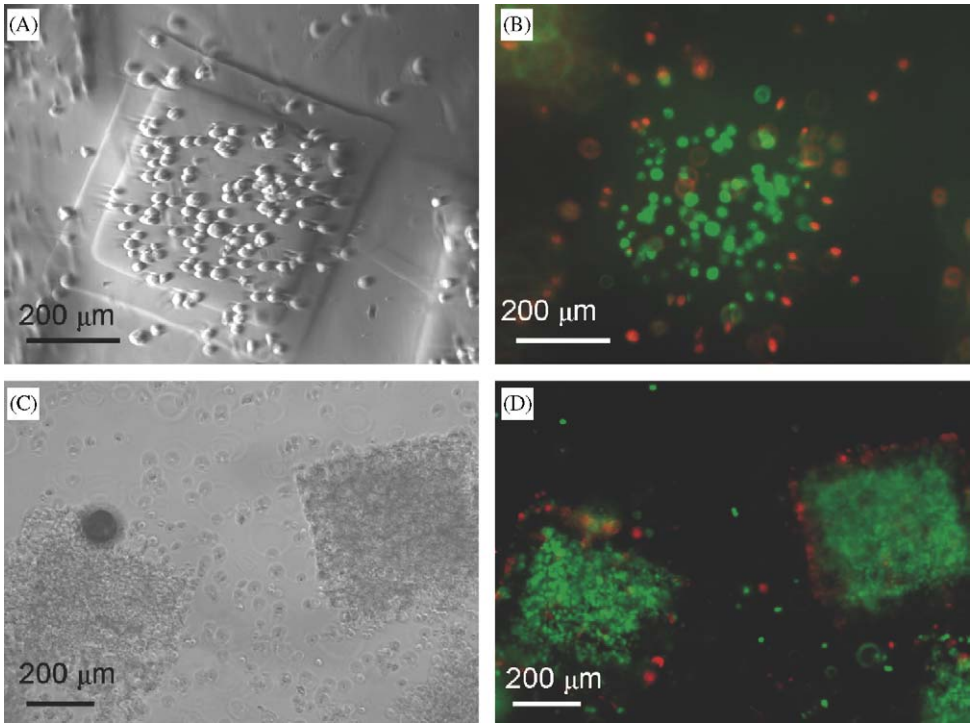

Fig. 6. Variation in cell density. (A)–(D) Cell density in microgels can be finely controlled. Variations shown range from (A), (B) 5×10^7 cells/ml to (C), (D) 20×10^7 cells/ml HA prepolymer solution. Viability stains (B), (D) show >85% viability.

Lastly, we observed that cells at the periphery of the microgels were more likely to lose their viability. This phenomenon can be seen from Figs. 4–6 in which dead cells (red) were observed around the periphery of the microgels. We hypothesize that these cells were adhered to the surface rather than embedded within the surface layers of the microgel, since they were found to be mobile and detachable from the surface upon physical contact with a micromanipulator.

3.4. Hydrogel arrangements

Taking a step beyond easy retrieval, microgels generated using this method can be arranged in specific configurations. An example is an alternating checkerboard pattern (Fig. 7), assembled with fluorescently red- and green-stained cells in separate sets of microgels. This was performed by physically manipulating individual microgels into the pattern using a micromanipulator. Although this approach is time-consuming due to difficulties encountered in manipulating individual microgels, the successful ordered arrangement of shape-specific hydrogels containing different cell types presents the possibility of reproducing physiological cellular arrangements *in vitro*.

Micromolding could therefore potentially provide a first step to a bottom-up approach to tissue engineering in which individual units of cell-encapsulating microgels are assembled into larger macrostructures of particular three-dimensional configurations. Control over the specific shape and size of microgels may be especially useful since shape-fitting microgels could then be placed adjacent to one another and assembled into larger structures or tissues. In addition, encapsulation of cells within cell-laden hydrogels using micromolding techniques offers several advantages over current systems and can be interfaced with microfluidics technology to engineer synthetic microvasculature [33].

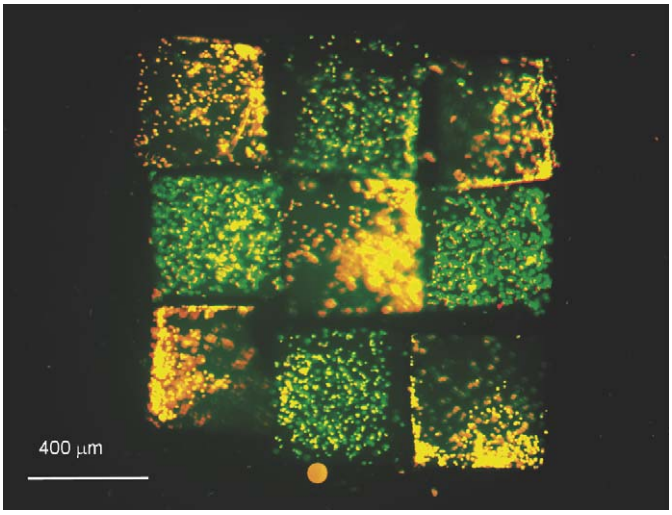

Fig. 7. Microgel arrangement and assembly. Rhodamine (red) and FITC (green) stained cells were encapsulated in separate HA microgels and subsequently arranged in an alternating checkerboard pattern.

4. Conclusion

In summary, a micromolding technique for encapsulating live cells in microscale photocrosslinkable hydrogels of controlled 3D shapes was developed. The distribution of cells successfully encapsulated in both MeHA and PEGDA hydrogels was found to be homogeneous, and the technique was shown to be amenable for various cell densities. This simple method may be a potentially useful tool for tissue engineering applications that require controlled spatial distributions of cells.

Acknowledgements

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