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Critical factors affecting cell encapsulation in superporous hydrogels

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Abstract
We recently showed that superporous hydrogel (SPH) scaffolds promote long-term stem cell viability and cell driven mineralization when cells were seeded within the pores of pre-fabricated SPH scaffolds. The possibility of cell encapsulation within SPH hydrogel matrix during its fabrication was further explored in this study. The impact of each chemical component used in SPH fabrication and each step of the fabrication process on cell viability was systematically examined. Ammonium persulfate, an initiator, and sodium bicarbonate, the gas-generating compound, were the two components having significant toxicity toward encapsulated cells at the concentrations necessary for SPH fabrication. Cell survival rates were 55.7% ± 19.3% and 88.8% ± 9.4% after 10 min exposure to ammonium persulfate and sodium bicarbonate solutions, respectively. In addition, solution pH change via the addition of sodium bicarbonate had significant toxicity toward encapsulated cells with cell survival of only 50.3% ± 2.5%. Despite toxicity of chemical components and the SPH fabrication method, cells still exhibited significant overall survival rates within SPHs of 81.2% ± 6.8% and 67.0% ± 0.9%, respectively, 48 and 72 h after encapsulation. This method of cell encapsulation holds promise for use *in vitro* an *in vivo* as a scaffold material for both hydrogel matrix encapsulation and cell seeding within the pores.

1. Introduction

Hydrogels are three-dimensional, hydrophilic, physically or chemically crosslinked polymer networks that have shown potential in tissue engineering applications. Their resemblance to extracellular matrix and ability to absorb large amounts of water make them conducive for cell growth and differentiation [1]. Their permeability allows the passage of nutrients by diffusion, while simultaneously providing an outlet for waste disposal. Hydrogels can be made of many different polymers and polymerized using different techniques, yielding a variety of mechanical and chemical properties to suit the specific application [2].

Cells can be incorporated into a nonporous hydrogel (NPH) scaffold either by attachment to the surface (figure 1(*A*)) or encapsulation (figure 1(*B*)). There are multiple advantages to encapsulating cells within the hydrogel polymer matrix. Certain polymers, including poly(ethylene glycol) diacrylate (PEGDA)-based systems, exhibit poor cell attachment, rendering cell growth on the surface difficult. The scaffold acts as a mechanical barrier, protecting encapsulating cells from the immune system in the *in vivo* environment [3]. The encapsulation process also ensures a uniform distribution of cells within the scaffold [4]. However, one of the disadvantages

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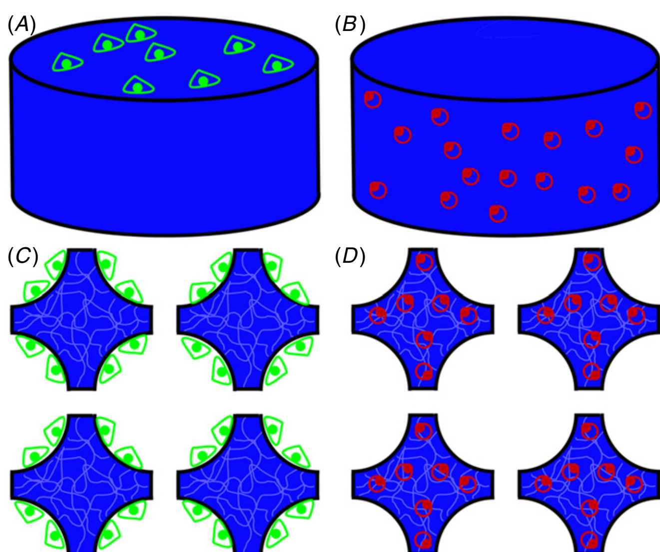

Figure 1. Schematic representation of (*A*) cells seeded upon (green) a NPH network (blue), (*B*) cells seeded within (yellow) a NPH network, (*C*) cells seeded within the porous network of SPHs, and (*D*) cells encapsulated within the hydrogel network of a SPH (not to scale).

of encapsulating cells within a hydrogel is that the conditions of polymerization, such as the initiators used, are known to exhibit cytotoxicity [5] and thick constructs restrict oxygen and nutrient transport [6].

One approach to overcoming transport limitations is to make porous hydrogels, including superporous hydrogels (SPHs). SPHs absorb water in a very short period of time due to the presence of continuously interconnected pores with diameters in the micron to millimeter scale [7]. The large pore size and a highly interconnected pore network [8] leave short diffusion path lengths (∼100 μm) making it possible to reach equilibrium swelling within minutes [9]. In addition, these short diffusion lengths within the hydrogel matrix would be expected to allow significant transport of nutrients and oxygen. SPHs were recently used as a scaffold for human mesenchymal stem cells and shown to exhibit mineralization in the presence of osteogenic media when grown on the interior pore surface of the SPH [10] (figure 1(*C*)). SPHs also have potential for the challenging task of vascularizing engineered tissue. Acellular SPHs implanted in mice were vascularized within the pores within 2 weeks [11]. However, the functionality and versatility of SPHs could be enhanced if cells were encapsulated within the hydrogel matrix (figure 1(*D*)). Of particular interest is the potential to co-culture cells of different types necessary for the formation of heterogeneous tissues.

Based upon the multitude of publications that have clearly shown that cells can be easily and reproducibly incorporated into PEGDA hydrogels [5, 12–15], the hypothesis that cells could survive the encapsulation process in SPHs was investigated (figure 1(*D*)). It was thought that a fabrication process should be possible for creation of three-dimensional porous scaffolds if the parameters for foaming could be controlled. Thus, in this study, the effect of the concentration of each critical chemical component and the foaming mechanism were examined. The current study also provides an understanding of the parameters that must be controlled to allow cell encapsulation within SPHs. Despite the relative toxicity of some of the components, cells survive polymerization and encapsulation within SPHs.

2. Materials and methods

2.1. Materials

Chemicals were purchased from Fischer Scientific (Pittsburgh, PA) as reagent grade and used as received unless otherwise specified. PEGDA (MW = 3400 g mol^{-1}) was purchased from Glycosan Biosystems (Salt Lake City, UT), citric acid from spectrum chemicals, and Pluronic® F-127 (PF127) from Sigma (St Louis, MO).

2.2. Fibroblast culture

NIH-3T3 fibroblast cells (fibroblasts derived from Mus musculus; CRL-1658) were obtained from American Type Culture Collection (ATCC; Manassas, VA). Cells were cultured in 150 cm^2 flasks (Corning Inc., Corning, NY) using Dulbecco's modified essential medium (Mediatech, Manassas, VA) supplemented with 5% bovine serum (Mediatech, Manassas, VA) at 37 °C and in the presence of 5% carbon dioxide. The medium was changed twice weekly. Prior to confluence, cells were trypsinized using 0.5% trypsin-EDTA (Mediatech, Manassas, VA), counted using a Coulter counter, and plated on 48 well plates (Corning Inc., Corning, NY) at a density of 10^5 cells mL^{-1} unless otherwise noted.

2.3. Cell viability

For cells grown on culture plates, the CellTiter 96® AQueous non-radioactive cell proliferation assay (MTS assay) [16] was purchased from Promega (Madison, WI) and performed as per the manufacturer's protocol. A standard curve was obtained by plotting cell number seeded against measured absorbance value. The controls used for background absorbance were acellular SPHs in Dulbecco's phosphate buffered saline (DPBS; Invitrogen) and empty wells. The MTS assay was found to be unsuitable for encapsulated cell measurement due to background interference caused by the hydrogel. However, the MTS assay has been previously shown to be applicable to measuring viability of cells adhering to the surface of the hydrogels [10].

Therefore, for encapsulated cells, total DNA was used to quantify the number of cells present. The Quant-iT™ picogreen® dsDNA assay kit [17] was purchased from Molecular Probes (Carlsbad, CA) and the manufacturer's protocol followed after homogenizing the hydrogel samples in DPBS. A standard curve was obtained using cells directly encapsulated in PEGDA hydrogels (without foaming) and then homogenized. In addition, it was confirmed that dead cells, i.e. ethanol treated, showed limited background in the assay.

2.4. Epifluorescent microscopy

The LIVE/DEAD® cell viability assay kit, comprising ethidium homodimer [18] and calcein acetomethoxy ester

(calcein AM), was purchased from Invitrogen (Carlsbad, CA) and the assay performed as per manufacturer's protocol. Optical images were obtained using an Olympus IX-70 inverted microscope and captured using a Retiga 1300 CCD camera. Images were then processed using the IPLab software.

2.5. Component cytotoxicity

NIH-3T3 cells were treated with each of the components used in the macromer mix for SPH fabrication and incubated for 10 min, 24 h, 48 h, or 72 h. The treatment groups were compared to the control group consisting of cells incubated with 250 μL of the medium in order to assess cell viability using MTS assay and LIVE/DEAD staining. As noted, cells in both treated and control groups were washed in DPBS with calcium and magnesium prior to viability determination.

2.6. pH cytotoxicity

NIH-3T3 cells were treated with the medium pre-adjusted to a pH of 5 and then either 100 mg mL^{-1} of sodium bicarbonate or 5 M sodium hydroxide was added until medium pH rose to an alkaline pH of 8. This pH change was conducted within the span of 10 min in order to simulate the pH transition in the foaming step in the SPH fabrication process. Two control groups consisting of cells at the original densities in the medium adjusted to either pH 5 or 8 were used for comparison. Following treatment, the medium was aspirated and cells were washed in DPBS with Ca and Mg prior to viability determination or microscopic imaging.

In order to test the toxicity of the precursor solution for SPH fabrication in combination with sudden pH change, NIH-3T3 cells (1×10^5 cells mL^{-1}) were suspended in the medium containing the precursor solution. This precursor solution consisted of PEGDA replaced with poly(ethylene glycol) (PEG; MW 4000) which prevented polymerization and allowed for cell recovery. A second experimental group was established with similar conditions except for sodium bicarbonate being substituted with 10 μL of 5.0 M sodium hydroxide (no foam, but pH change). Both treatment groups were compared to the control (substituted with an equivalent amount of DPBS with Ca and Mg for the precursor solution) for viability determination or microscopic imaging.

2.7. Synthesis of SPHs

Stock solutions of PEGDA, tetramethylethylenediamine (TEMED), PF127, and citric acid were prepared and combined as previously described [10] and a cell suspension solution of 1×10^6 cells mL^{-1} in the medium was added to the precursor mix. Ammonium persulfate (APS) was then added to the precursor solution and the entire macromer solution vortexed to obtain a homogeneous solution. This solution was heated by holding the vial in a water bath at 37 °C for 2 min with intermittent vortexing of the vial. To ensure proper foaming, the solution was confirmed to be at a pH of approximately 5. Sodium bicarbonate was then added immediately to the vial and the solution allowed to stand for 5 min in order to obtain complete polymerization. A wet, sterile spatula was used to remove the newly formed SPHs. These SPHs were allowed to swell in the medium for 30 min.

As comparator groups, two NPHs were formed. First, sodium hydroxide (non-foaming; 10 μL of 5.0 M) was substituted for sodium bicarbonate in order to adjust the pH and allow for the formation of NPHs (NPH-A/T). In this case, all components were equivalent with only the absence of foaming and the particles of sodium bicarbonate. With this comparator, we were able to examine the hydrogel formed without foaming, while maintaining the pH change. In the second NPH (NPH-I), a conventional formulation [12, 19] was used in which PEGDA and Irgacure® 2959 were the only components of the precursor mixture, i.e. no PF127, citric acid, APS, or TEMED were added. Irgacure® 2959 (0.075 (w/v)%) was used as the photoinitiator with exposure to UV light at wavelength of 365 nm (5 mW cm^{-2}) for 10 min. With this comparator, we were able to assess the system compared to a conventional PEGDA hydrogel system of similar composition.

2.8. Statistical analysis

Data were analyzed using one-way ANOVA followed by post hoc unpaired *t*-tests when appropriate. All results are reported as mean plus or minus ($\pm$) standard deviation. To confirm the reproducibility of the data, a minimum of three identical, independent replicates were performed.

3. Results and discussion

To investigate if cells can survive the fabrication of SPHs, the effect of each chemical constituent in the precursor solution was separately tested on NIH-3T3 cells. These experiments were designed based on the actual SPH fabrication conditions that the cells would be exposed to for varying periods of time. Our experimental evaluation also took into account the possibility of unreacted macromer and initiator remaining in solution after polymerization. The pH for all cell treatment solutions was maintained at 5 for the 10 min experiment, replicating the original precursor solution pH (before polymerization) during SPH fabrication, and at normal cell culture pH for the remainder of the time points. In addition to the conditions described in this manuscript, other concentrations were examined over this time period. The majority of the components of the SPH show toxicity, but these same components are typically used for other PEGDA encapsulation systems with limited toxicity during polymerization. The primary new components, citric acid, Pluronic PF127, and sodium bicarbonate, are the primary contributors to individual toxicity.

3.1. Component cytotoxicity

3.1.1. PEGDA cytotoxicity. As the predominant component of hydrogels, PEGDA was used in the synthesis of SPHs at a final concentration of 15 (w/v)% [10]. In order to test for the influence of PEGDA on cell growth, cells were treated with 15 (w/v)% PEGDA at varying time points (figure 2, row 1). Compared to untreated controls, statistically significant

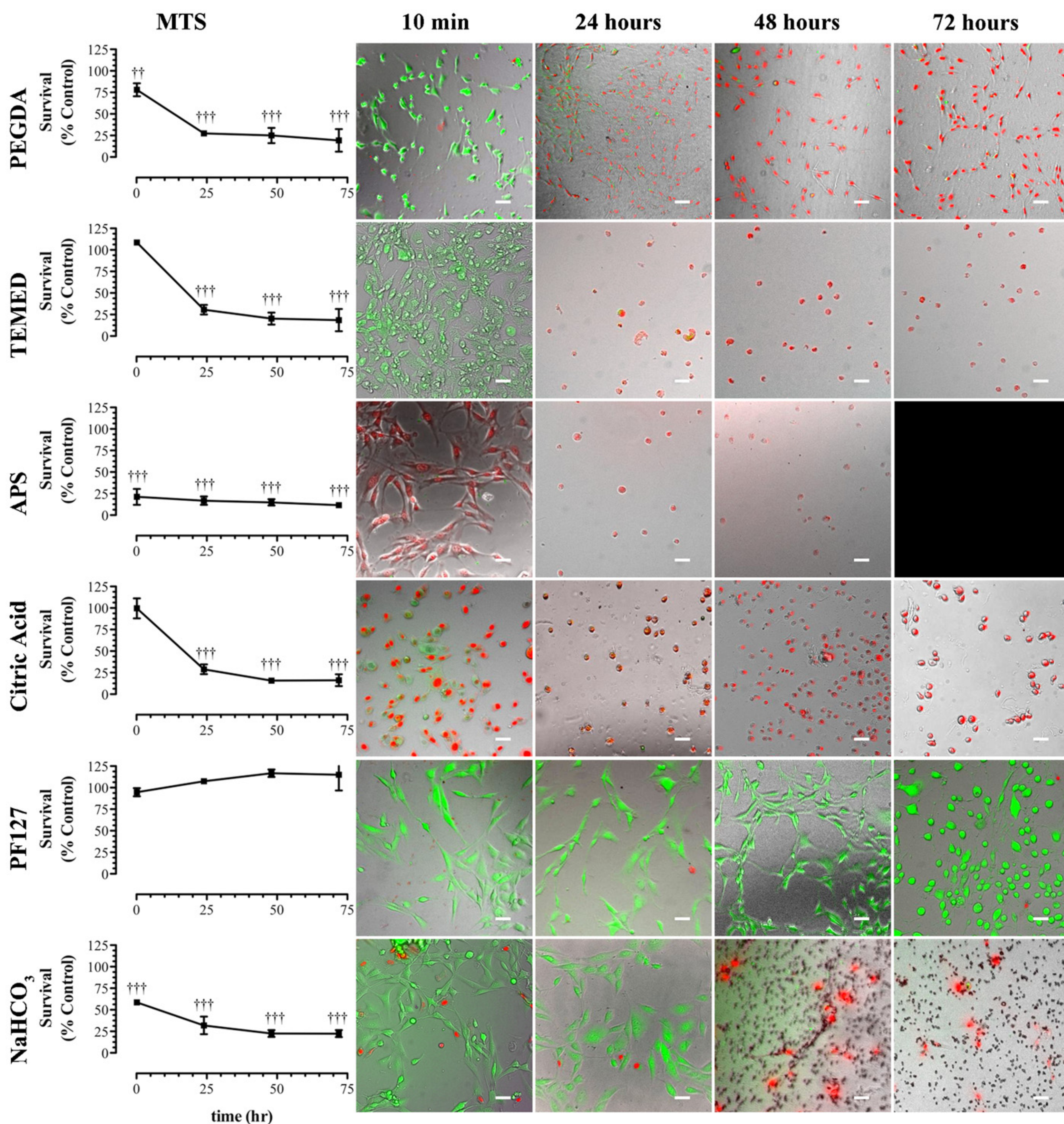

Figure 2. Component cytotoxicity. The effect of 15% PEGDA (row 1), 0.9% TEMED (row 2), 0.7% APS (row 3), 2.4% citric acid (row 4), 0.6% Pluronic® PF127 (row 5), and 200 mg mL^{-1} sodium bicarbonate (row 6) on cell viability (% control) as assessed by (graphs left) the MTS assay ($n = 3$, mean $\pm$ SD) and by (columns 2 through 5) the LIVE (green)–DEAD (red) cell assay with representative micrographs of cells exposed to the specified component for (column 2) 10 min at a pH of 5.0 (column 3) and 24 h, (column 4) 48 h, and (column 5) 72 h at a pH of 7.4 where †† and ††† indicate a statistically significant difference of $p < 0.01$ and $p < 0.001$, respectively, compared to untreated controls as determined by one-way ANOVA followed by post hoc unpaired Student's *t*-test. Scale bars in all images are 50 μm.

toxicity and morphological changes were observed when NIH-3T3s were incubated with 15 (w/v)% PEGDA at 24, 48, and 72 h ($p < 0.001$). However, during the formation of SPHs, the cells were exposured to 15 (w/v)% PEGDA for only 10 min and for this condition the toxicity was not substantial (greater than 75% viability).

3.1.2. TEMED cytotoxicity. After confirmation of minimal PEGDA toxicity on cells at 10 min exposure, each of the other chemical components in the precursor solution was then separately examined. TEMED was used at a concentration of 0.90 (w/v)% in the synthesis of SPHs. As an initiation catalyst, TEMED is known to accelerate the scission of

APS yielding sulfate, hemi-TEMED, and hydroxyl radicals, which initiate polymerization [20]. There was no significant toxicity observed (figure 2, row 2) when cells were treated with 0.9 (w/v)% TEMED for 10 min when compared to the untreated control. However, at incubation times of 24, 48, and 72 h, massive cell death and a rounded, non-adherent morphology from the few remaining live cells were observed. This demonstrated that exposure to TEMED for an extended period of time is a significant source of toxicity. This is particularly a concern since TEMED is not consumed during the polymerization process. As will be discussed further, it is recommended that the initiator system be changed from APS/TEMED to a less toxic initiator system.

3.1.3. APS cytotoxicity. APS was used at a concentration of 0.70 (w/v)% in the formula of SPHs and is an initiation partner with TEMED. Cells treated with 0.70 (w/v)% APS also showed significant toxicity (figure 2, row 3) at all time points, i.e. 10 min, 24 h, 48 h, and 72 h, with morphological changes occurring in remaining living cells. The most possible explanation for such toxicity is the decomposition of APS which releases reactive free radicals which can cause cell damage and subsequent death [21]. However, it is important to note that during the encapsulation process, cells are exposed to a lower initiator concentration. Despite this toxicity and as this will be discussed later, it is expected that the free radicals are, at least in part, consumed by the growing polymer chains leading to the observed higher survival rate for encapsulated cells. As is now clear, the APS/TEMED initiator pair has substantial toxicity toward cells and the TEMED, which is not consumed in the reaction, has lasting toxicity. There is clear need to identify more appropriate initiators.

3.1.4. Citric acid cytotoxicity. The buffering agent citric acid was used to adjust the pH to 5 at a concentration of 2.4 (w/v)% during SPH fabrication. There was a statistically significant difference in cell viability with 2.4 (w/v)% citric acid treatment at 10 min, 24 h, 48 h, and 72 h compared to the DPBS control group (figure 2, row 4). Morphologically, there is clear rounding of cells and permeablization of the cells (as shown by red, DNA-stained, cells) beginning at 10 min and at later times any remaining live cells appeared rounded and loosely adherent. There is an apparent discrepancy between the MTS data and microscopic observations. If one thinks about the mitochondrial activity measure in the MTS activity, a cell that has been perturbed (pharmacologically) will take some time to respond and decrease mitochondrial activity. In this case, there is apparent cell toxicity by the LIVE/DEAD assay since most cells are permeable and many were morphologically altered. Also, the MTS assay examined the entire well which allows local regions of live cells, which may not be visualized or captured in a representative image, to be counted as alive. In this way, the MTS assay is unbiased compared to imaging techniques.

The pH of medium was originally adjusted to 7.4 when cells were treated with citric acid over 24, 48, and 72 h. However the pH cannot be maintained because incubation with citric acid over longer periods of time caused a drop back to acidity. It is believed that this acidic pH may be one cause of cellular death. However, based on metabolic activity (MTS), cell morphology, and permeability after 72 h, citric acid itself was also deemed toxic toward NIH-3T3s. Tighter control on pH may minimize this effect, but a system where the pH does not have to be acidic would allow for the removal of this component altogether. Citric acid is not one of the more critical components, but it is clear that it has influence on cell survival.

3.1.5. Pluronic cytotoxicity. Pluronic® stabilizes the foam during SPH formation by spontaneously adsorbing at the air–water interface, reducing surface energy, and thus preventing foam collapse before polymerization can take place. Pluronic® has already been shown to be a superior stabilizer for a variety of monomer systems (e.g. acrylamide, hydroxyethyl methacrylate, hydroxypropyl methacrylate, potassium salt of 3-sulfopropyl acrylate) used in SPH fabrication compared to other alternatives including albumin, gelatin, Pluronic® 105, Silwet®, sodium dodecyl sulfate, Span®, Triton®, Tween® [7]. Cells treated with 0.6 (w/v)% Pluronic, the same used in the formation of SPH, showed neither cell death nor significant morphological changes at most time points (10 min, 24 h, and 48 h) (figure 2, row 5). It should be noted that at 72 h there was no observable toxicity, but significant morphological changes that indicate possible negative implications. The toxicity of Pluronic® has been shown to be concentration dependent [22]. Pluronic® caused little hindrance to cell proliferation in the concentration range of 0.1 to 5 (w/v)% for 5 days after treatment [22]. However, HMEC-1 (endothelial), L6 (muscle) cell lines and human liver carcinoma cells (HepG2) treated with 10 (w/v)% Pluronic were less viable than untreated cells and Pluronic in the range of 15 to 20 (w/v)% caused complete cell death [22]. These concentration levels are well above the levels used in SPH fabrication. Therefore, it can be concluded that Pluronic® at the concentration used in our SPH fabrication system is a not a cause of cellular toxicity.

3.1.6. Sodium bicarbonate cytotoxicity. Sodium bicarbonate has two functions in the SPH formulation, the primary of which is to generate gas for foam creation which is then stabilized by surfactant, i.e. Pluronic®. It is the escape of carbon dioxide during this reaction that gives the SPH its interconnected network. Sodium bicarbonate also neutralizes the pH of the reaction mixture to a point where TEMED can become active as a co-initiator with APS [7]. The concentration of sodium bicarbonate used in the formation of our SPHs was 200 mg mL^{-1} and in this experiment, significant cell death and morphological changes occurred upon sodium bicarbonate treatment/incubation (figure 2, row 6). At 10 min, most cells were still alive as indicated by a few cells exhibiting red fluorescent staining and this was further supported by the MTS assay indicating more than 50% of control viability. Cells treated with sodium bicarbonate were slower growing at 24 h as indicated by a significant drop in MTS value, but maintained their morphology. Some dead (red) cells were also observed at this time. However, with longer incubation times,

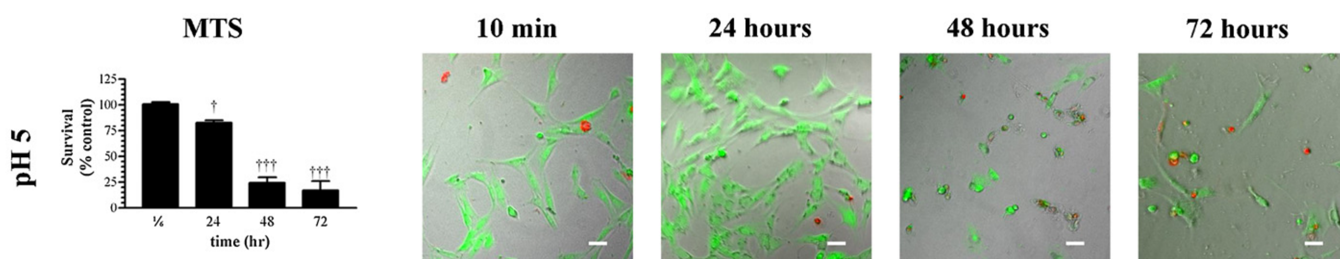

Figure 3. Effect of pH 5.0 on cell viability. Toxicity was assessed by (graph left) the MTS assay ($n = 3$, mean $\pm$ SD) and by (columns 2 through 5) the LIVE (green)–DEAD (red) cell assay with representative micrographs of cells exposed to pH 5.0 for (column 2) 10 min, (column 3) 24 h, (column 4) 48 h, and (column 5) 72 h where † and ·††† indicate a statistically significant difference of $p < 0.05$ and $p < 0.001$, respectively, compared to untreated controls as determined by one-way ANOVA followed by post hoc unpaired Student's *t*-test. Scale bars in all images are 50 μm.

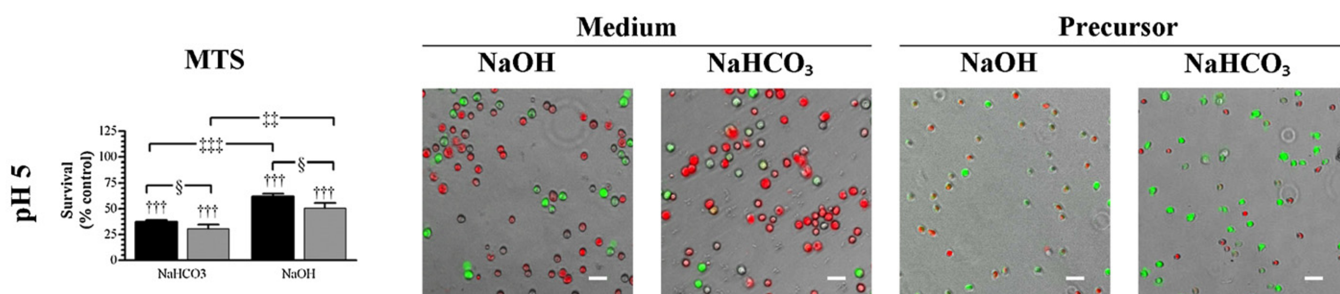

Figure 4. Effect of foaming on cell viability. Toxicity was determined by MTS assay after incubating cells at pH 5 in the (black bars) medium or (gray bars) precursor solution (PEG, APS, TEMED, and Pluronic® F-127) for 10 min; either sodium hydroxide (10 μL; NaOH) or sodium bicarbonate (100 mg; $NaHCO_3$) were then added to foam or change pH of the solution ($n = 3$, mean $\pm$ SD) where ††† indicates statistically significant difference ($p < 0.001$) compared to untreated controls, § indicates statistically significant difference ($p < 0.05$) comparing medium and precursor solution conditions, and ‡‡ and ‡‡‡ indicate statistically significant difference ($p < 0.01$ and 0.001, respectively) comparing $NaHCO_3$ and NaOH treated groups as determined by one-way ANOVA and post hoc unpaired Student's *t*-test. Cells grown in culture media were also imaged by (columns 2 through 5) the LIVE (green)–DEAD (red) cell assay. Scale bars in all images are 50 μm.

cells became rounded and non-adherent leading to death to the majority at 72 h. Clear sodium bicarbonate crystals are visible on the culture plate, and these are the predominant feature at later times. It has been reported in the literature that concentrations of 2.1, 4.2, and 8.4 mg mL^{-1} of sodium bicarbonate do not affect the viability of endothelial derived EA.hy 926 cell line for 6 h [23]. However, the concentration of sodium bicarbonate used in our experiment was significantly higher. At such high exposure levels, it can be concluded that sodium bicarbonate is a significant source of cell toxicity.

3.2. pH cytotoxicity

During the SPH fabrication process, cells were first suspended in a solution buffered at pH 5 (compared to a typical cell culture medium which maintains a pH in the range of 7.0 to 8.0). To continue to understand how each step involved in SPH influences cell survival, we examined how cells respond to this lowered pH (figure 3). For short periods, there is limited influence of the decreased pH on cell viability. At longer times (>24 h), there is clear cell toxicity, but some cells still exhibit normal morphology. Since the pH alone does not have immediate impact, the influence of the change in pH was examined.

On addition of sodium bicarbonate, there occurs a sudden change from acidic to alkaline conditions (pH 8). To examine this, we seeded cells in tissue culture media adjusted (with HCl) to pH 5 and treated the cells with either sodium bicarbonate or sodium hydroxide to reach normal pH for culture (figure 4). In this case (figure 4), cells were rounded since they were plated and examined after 10 min accounting for the abnormal morphology and live (green), rounded cells. There was statistically greater toxicity ($p < 0.001$) for the sodium bicarbonate treatment, but both groups exhibited at least 40% toxicity after 10 min. Two reasons can be proposed for the greater toxicity with sodium bicarbonate: the large particles have surface-induced toxicity and the chemical species themselves are more toxic. While the sodium bicarbonate addition step is critical to the fabrication of SPHs, because it contributes to the porous nature of the gels, this sudden change in pH was significantly toxic to cells and was similar in toxicity compared with a single component. We are currently examining other methods of gas formation that do not mandate the rapid pH change to avoid this significant problem.

3.3. Chemical foaming cytotoxicity

The gas foaming process may be deleterious to cell viability. For this experiment, PEGDA was substituted with PEG in order to prevent the polymerization of the precursor solution and which would then allow us to study the foaming process alone without the difficulty of assessing or confounding

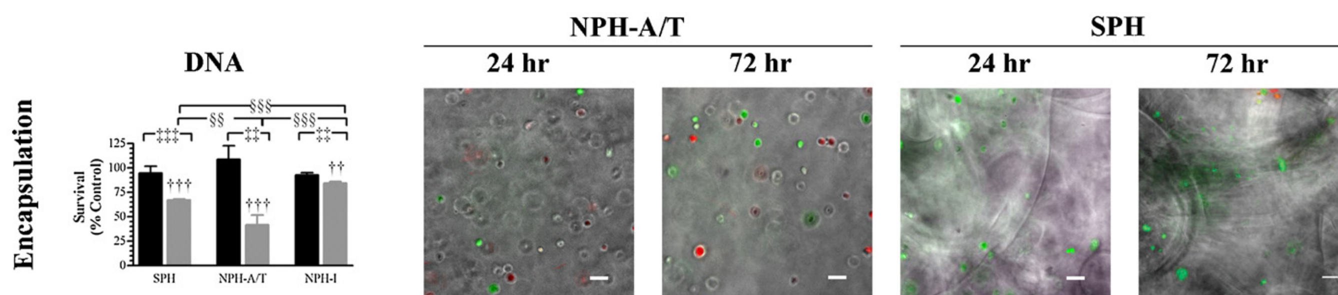

Figure 5. Cell survival after encapsulation. Cell survival encapsulated as determined by DNA using picogreen assay within SPHs, NPHs synthesized with APS and TEMED (NPH-A/T), and NPHs synthesized with Irgacure® 2959 (NPH-I) at (black bars) 24 h and (gray bars) 72 h post seeding ($n = 3$, mean ± SD) where † and †† indicate statistically significant difference ($p < 0.05$ and <0.01, respectively) compared to untreated controls, ‡, ‡‡, and ‡‡‡ indicate statistically significant difference ($p < 0.05$, 0.01, and 0.001, respectively) compared to different times within the hydrogel group, and §§ and §§§ indicate statistically significant difference ($p < 0.01$ and 0.001, respectively) compared to other hydrogel groups at a specified time. Representative images of NIH-3T3 cells encapsulated within hydrogels as determined by LIVE–DEAD cell assay at 24 and 72 h in SPHs or non-porous (NPH-A/T) hydrogels. The scale bar is 50 μm in all the images. Green fluorescence indicates live cells while red fluorescence indicates dead cells.

responses of cells growing within the hydrogel matrix. In one group, cells were treated with a precursor solution of PEG, APS with TEMED, citric acid and sodium bicarbonate that had the ability to produce foam but which could not polymerize. Sodium hydroxide was substituted for sodium bicarbonate in the second experimental group with all other conditions maintained. This prevented the foaming process (similar to the procedure used in the NPH-A/T formation). Approximately 70% cell death was observed in the group treated with the SPH precursor solution compared to approximately 50% cell death in the group treated with the NPH-A/T precursor solution (figure 4). Therefore, it can be concluded that the majority of cell toxicity was due to the composition of the macroporous hydrogels with additional cell death resulting from the rapid change in pH and the limited further toxicity foaming process itself. The foaming process is one of the minimal contributing factors toward toxicity.

3.4. Effect of cell encapsulation on viability

In order to determine the effect of encapsulation within the hydrogel matrix on cell viability, NIH-3T3 cells were encapsulated within either SPHs or NPHs and observations taken over the span of 7 days (figure 5). At the end of 24 h there were $0.95 \pm 0.07 \times 10^6$ viable cells/SPH which in the next 6 days reduced ($p < 0.001$) to $2.26 \pm 0.18 \times 10^5$ viable cells/SPH. Possible reasons for cell death, as already mentioned, are toxicity of APS or sodium bicarbonate, foaming technique, or rapid change in pH of the precursor solution from acidic to alkaline during the fabrication or encapsulation processes. In the case of cells encapsulated in NPH-A/T hydrogels, viability was approximately $1.07 \pm 0.02 \times 10^6$ cells/NPH at the end of 24 h which dropped ($p < 0.001$) to $1.19 \pm 0.163 \times 10^5$ cells/NPH over the next 6 days. While the causes of cell death were similar to the reasons listed for SPH encapsulation, there was a two-fold decrease in living cells when encapsulated in NPH compared to SPH. This confirms the importance of hydrogel pore availability in promoting cell viability by allowing for both attachment and the exchange of nutrients, release of waste materials, etc [24]. We conclude that for successful encapsulation using this cell line, SPHs may be more suitable than NPHs formed using this method ($p < 0.001$). We confirmed this by using the same formula and also a formula which has been widely investigated in the literature using Irgacure® as the initiator [12, 15, 19, 25, 26]. For the earliest time point, our porous hydrogel maintains the cells similarly to that of the similarly polymerized hydrogel (NPH-A/T), but by 72 h the Irgacure® polymerized hydrogels stabilize the cell population ($1.09 \pm 0.01 \times 10^6$; $p < 0.001$ compared to SPH and NPH-A/T) while the SPHs have substantial cell loss. We hope, in the near future, to utilize the information learned through this study to improve the foaming system to eliminate the pH change and possibly the polymerization initiators. It should be noted, however, that this study was a significant success as it is counterintuitive that the cells would survive to a much higher extent when the SPH was formed than when each of the components was examined individually or even in combination.

A method for cell encapsulation in hydrogels has been developed that can be easily utilized by any investigator. Although there is only moderate advantage in terms of survival when compared to the equivalent NPHs, much was learned from this study that can be used in the future for improving and utilizing the method for pore formation and cell encapsulation. There was much surprise that the system would allow the cell survival observed since we did foresee much of the toxicity associated with specific components. Since we clearly observed survival, we are encouraged to investigate methods that will not utilize the pH change associated with sodium bicarbonate gas formation. We are also investigating alternate initiators. Although Irgacure is widely utilized in hydrogel formation in tissue engineering, it is not possible to use in the foaming systems due to the opaque nature of the foam. We are currently investigating each of these two alternatives to better produce SPHs and hope to report these in the near future. There are other systems that do not utilize the harsh chemistries of foaming and polymerization, but these systems have other limitations, including mechanical strength and structural limitations, that can be overcome with our newly developed system forming macroporous hydrogels.

Further, this technology can also be modified to contain many of the attributes currently possible with conventional NPH systems to augment the properties. The system described can be altered to incorporate alternate adhesion molecules [27, 28], chemistries [29–32], mechanics [33, 34], and biodegradability [35, 36]. These altered systems will be investigated as alternate foaming and initiation methods are also investigated to improve on cell survival. When combined with recent evidence that macroscopic SPH structures can be used to culture and differentiate mesenchymal stem cells [10] and that the structures are vascularized [11], there is great potential for applying this technology to tissue engineering and protein delivery.

An important potential application of this system is to create vascularized engineered tissue. The field of tissue engineering has only brushed the surface of being able to vascularize large tissues [37]. More research has recently been involved in allowing greater porosity for vascularization even in hydrogel scaffolds due to slow degradation [38–41]. By creating systems that will allow greater cell seeding and rapid vascular ingrowth, scaffolds may be better positioned to be the temporary support that the name scaffold implies. Cells would then have the ability to populate and remodel the scaffold in a much more rapid manner. In addition, the ability to rapidly and reproducibly seed cells in the pores may allow a scaffold that can be rapidly remodeled.

4. Conclusions

Based on the results and observations it can be concluded that the initiator APS and the mechanism for generation of foam using sodium bicarbonate as a foaming agent are toxic to cells. The acid-neutralization subjects cells to the drastic change in pH from an acidic to alkaline pH, which leads to significant cell death. However, the foaming technique can be used for cell encapsulation in SPHs with survival rates comparable to NPHs at 48 h. Alternate methods of foaming and crosslinking must be explored to optimize cell survival.

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