

Methods for Photocrosslinking Alginate Hydrogel Scaffolds with High Cell Viability

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Methods for seeding high-viability (>85%) three-dimensional (3D) alginate–chondrocyte hydrogel scaffolds are presented that employ photocrosslinking of methacrylate-modified alginate with the photoinitiator VA-086. Comparison with results from several other photoinitiators, including Irgacure 2959, highlights the role of solvent, ultraviolet exposure, and photoinitiator cytotoxicity on process viability of bovine chondrocytes in two-dimensional culture. The radicals generated from VA-086 photodissociation are shown to be noncytotoxic at w/v concentrations up to 1.5%, enabling photocrosslinking without significant cell death. The applicability of these photoinitiators for generating 3D tissue-engineered constructs is evaluated by measuring cell viability in 3D constructs with aggregate moduli in the 10–20 kPa range. Hydrogels with encapsulated bovine chondrocytes were constructed with >85% viability using VA-086. While the commonly used Irgacure 2959 is noncytotoxic in its native state and crosslinks the alginate at weight fractions much lower than VA-086, the cytotoxicity of IRG2959's photogenerated radical leads to viabilities below 70% in the conditions tested.

Introduction

BIODEGRADABLE SCAFFOLDS play an important role in tissue-engineered systems, owing to their ability to provide therapeutic function while degrading as the cellular system replaces the artificial matrix with a physiological one. Alginate is one of many materials that have been used for tissue-engineered systems, as it combines relatively low cost, convenient ionic crosslinking techniques, and hydrolytic degradation to noninflammatory byproducts; alginate has thus been used as a matrix for chondrocytes,¹ fibroblasts,^{2,3} and many other cell types.^{4,5} The biocompatibility of alginate has also enabled its use in clinical trials as a cell delivery vehicle for bone formation⁶ and diabetes therapy.^{7,8}

The most common realization of alginate scaffolds is through ionic crosslinking with multivalent cations, most often calcium.^{1,9–11} Ionic crosslinking offers control of material properties through the concentration of calcium and alginate as well as the alginate molecular weight, and high process viabilities have been demonstrated in many studies.

A number of potential advantages may be gained by photocrosslinking alginate instead of ionically crosslinking it. While calcium levels are a convenient means of controlling the material properties of ionically crosslinked alginate gels, the physiological role of calcium is important in many systems¹² and the effects of material properties,¹³ fixed charge density, and ion levels in the culture media cannot be in-

vestigated independently in an ionically crosslinked system. By comparison, photocrosslinking allows creation of alginate hydrogels independent of calcium levels. Photocrosslinking is also a convenient means for controlling gelation timing and kinetics; such techniques have been implemented in many materials, for example chondroitin sulfate,¹⁴ collagen^{15–17} and poly(ethylene glycol diacrylate).^{18–21} Previous work in photocrosslinked alginate has employed methacrylate substitution for the secondary alcohols in alginate via anhydride chemistry²² and, as in work with many other polymers, has largely employed crosslinking with Irgacure 2959.^{23–26} While Irgacure 2959 has been the primary tool in the generation of viable photocrosslinked constructs, other photoinitiators have been used for other applications.^{27,28} These photoinitiators have been largely untested in tissue engineering studies, regardless of the polymer.

The key limitation of photocrosslinking techniques for creating tissue-engineered scaffolds is that the photocrosslinking process exposes the seeded cells to ultraviolet (UV) light, chemical photoinitiators that may be cytotoxic in precursor or radical form, and organic solvents that may be required to dissolve the photoinitiators in the polymer precursor solution; photogenerated molecules can also perturb the construct by generation of temperature changes or gas bubbles. Photoinitiators that minimize the UV light and organic solvent requirements as well as the photoinitiator cytotoxicity have the potential to greatly impact the

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applicability of photocrosslinked hydrogel tissue engineering scaffolds. As such, the use of water-soluble photoinitiators such as VA-086 or V-50 may present an advantage for therapeutic cell encapsulation. The goal of this study was to compare the action of five photocrosslinking reagents during photoencapsulation of primary bovine chondrocytes.

In this work, we present methods for seeding high-viability (>85%) chondrocyte scaffolds by modifying alginate with methacrylate anhydride followed by photocrosslinking with the photoinitiator VA-086. By comparison with results from several other readily available photoinitiators, including Irgacure 2959, we highlight the role of solvent, UV exposure, and photoinitiator cytotoxicity on process viability, and evaluate the applicability of these photoinitiators for generating tissue-engineered constructs.

Materials and Methods

Chondrocyte isolation

Bovine articular chondrocytes were harvested from the stifle joint from calf legs acquired from a local abattoir (Gold Medal Packing). Articular cartilage was harvested from the joint and washed in phosphate-buffered saline (PBS; Invitrogen) supplemented with antibiotic/antimycotic (Mediatech; "antibiotic/antimycotic" throughout this section implies 10,000 U/mL penicillin G sodium, 10,000 µg/mL streptomycin sulfate, and 25 µg/mL amphotericin B). The cartilage was then diced into small pieces to maximize surface area, and washed twice in PBS supplemented with antibiotic/antimycotic. Chondrocytes were released from the washed cartilage by enzymatically digesting for 12–16 h in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 0.3% collagenase type II (Worthington) and antibiotic/antimycotic. The digest was filtered through a 180-µm filter (VWR) to remove any undigested cartilage, and the filtered solution was centrifuged at 7000 rpm for 7 min. Pelleted cells were washed by PBS supplemented by antibiotic/antimycotic and spun at 7000 rpm for 5 min. Chondrocytes were resuspended in DMEM supplemented with antibiotic/antimycotic. Process viability of cell extraction was determined using a trypan blue dye exclusion assay (Mediatech), and cell counts were determined via hemocytometer. Only samples with a cell viability of 88% or higher were used in cytotoxicity assays or in the fabrication of chondrocyte-seeded tissue-engineered constructs.

Determination of cytotoxicity of various photoinitiators in two-dimensional culture

Chondrocytes were seeded in a two-dimensional (2D) monolayer culture in six-well polystyrene (VWR) plates at a density of 10,000 cells/cm² with 5 mL of DMEM supplemented with 10% fetal bovine serum (Gemini Bioproducts) and antibiotic/antimycotic in each well. The chondrocytes were incubated at 37°C and 5% CO₂ for 24 h to allow attachment to the well bottom. An extra set of wells was prepared as a negative control for all experiments; all negative controls report >98% viability in the negative control group. Groups of three wells were subjected to a treatment of a particular photoinitiator (VA-086, V-50, Irgacure 2959, VA-044, or Irgacure 1870), photoinitiator concentration (0%–1.5% [w/v]), and UV exposure time (0–10 min). Irgacures were

acquired from Ciba; all other photoinitiators were obtained from Wako Chemicals. Aliquots of 70% (v/v) ethanol were added to wells where necessary to maintain equal ethanol concentrations across all treatments. Thirty minutes after the addition of photoinitiator, well plates were placed in the center of a UV oven (Spectroline) and exposed to UV light (365 nm longwave, 2 µW/cm²), and then returned to culture. After 48 h of incubation, viability was assessed via calcein-AM and ethidium homodimer-1 staining (Invitrogen Live/Dead Kit). A room-temperature 1 µM ethidium and 0.05 µM calcein solution was prepared for each experiment from Live/Dead Kit stock solutions. Stock solutions over 6 months old were discarded. The medium was removed from the wells, and 500 µL of the staining solution was added to each well. The sample was incubated in the dark at room temperature for 45 min. The wells were rinsed with PBS and immediately imaged using epifluorescent microscopy. Viability was measured via cell counting and automated analysis using ImageJ. A study (data not shown) of automated analysis showed that observed viabilities were insensitive to threshold levels, and varying threshold levels by a factor of two led to changes in viability smaller than the scatter in the observed data. Human interpretation of viabilities was also found to be statistically insignificant from automated analysis for both of two human observers. Three treatments were sampled for each stain for a total of at least 300 cells counted.

Methacrylic modification of alginate and photocrosslinking of constructs

A photocrosslinking technique was used to make covalently crosslinked alginate hydrogels based on chemical functionalization techniques reported previously.^{29,30} Photocrosslinking was accomplished in a two-step process. First, anhydride chemistry was used to replace secondary alcohols on the alginate backbone with methacrylate groups to create methacrylate-alginate. Second, a solution of methacrylate-alginate was photocrosslinked in the presence of a photoinitiator under exposure to UV light.

Alginate (FMC Biopolymer) was dissolved into 20 mL of deionized (DI) H₂O (Labconco) to make a 2.5% (w/v) alginate solution, and 20 mL of methacrylic anhydride (Alfa Aesar) was added to the solution. The solution was maintained at room temperature and pH = 7 as ~40 mL of 5M NaOH (Sigma) was added dropwise over 72 h.²⁹ After the 3-day reaction period, the mixture was poured into 100 mL of ethanol (Sigma) to precipitate the methacrylate-alginate product. The mixture was vacuum filtered in a Buchner funnel through 5 µm filter paper. The product was recovered still wet and re-dissolved in 20 mL of DI H₂O, and then precipitated and filtered a second time. The methacrylate-alginate was then spread over a glass dish and allowed to dry in a fume hood for 24 h.

A total of 50 × 10⁶ cells were centrifuged at 7000 rpm for 7 min, and 1 mL of 3% methacrylate-alginate in Dulbecco's phosphate-buffered saline (DPBS) was poured onto the cell pellet through a 0.8 µm syringe filter. The alginate and cells were gently aspirated to disperse the cells in the alginate. The suspension was then vortexed for no more than 15 s to homogenize the suspension. Photoinitiators VA-086 (Wako) or IRG2959 were dissolved in 70% (v/v) ethanol to make a 10% (w/v) solution. Photoinitiator solution was added to 1 mL of the 3% (w/v) alginate solution to make a 3% (w/v)

alginate solution with controlled photoinitiator concentration. The solution was poured between two silanized glass plates spaced 1 mm apart. The mold was placed in the center of a UV oven (Spectroline) and exposed to 5 min of UV light (365 nm longwave, $2\mu\text{W}/\text{cm}^2$). The top glass plate was removed and the hydrogel slabs were either (1) prepared for mechanical characterization; or (2) cut into 6 mm disks with a dermal biopsy punch and placed in culture with one gel per plate in six-well plates with DMEM supplemented with antibiotic/antimycotic and 10% heat-inactivated fetal bovine serum at 1 mL of media for each disk.

Determination of cytotoxicity of various photoinitiators in three-dimensional constructs

Chondrocyte viability in three-dimensional (3D) constructs was determined using a similar protocol to 2D cultures. After photocrosslinking and 48 h of incubation, viability was assessed via calcein-AM and ethidium homodimer-1 staining with $0.05\mu\text{M}$ calcein and $1\mu\text{M}$ ethidium (Invitrogen Live/Dead Kit). The medium was removed from the wells, and $500\mu\text{L}$ of the staining solution was added to each well. The sample was incubated in the dark at room temperature for 90 min. The wells were rinsed with PBS and immediately imaged using confocal microscopy. Viability was measured via cell counting and automated analysis using ImageJ. Thresholds for image interpretation for the confocal images were determined independently from those for epifluorescent images in 2D culture, but were standardized for all 3D gels in the study. Dependence of viability on human observer or automated analysis was found to be statistically insignificant.

Mechanical characterization

Before mechanical testing, gel constructs were placed in a solution of 10 mM HEPES and 150 mM NaCl at pH 7.0 for at least 1 h before testing. As compared to the size at polymerization, the hydrogels swelled 60% in volume, resulting in an approximate final alginate concentration of 1.9% (w/v). At the time of testing, 6-mm-diameter discs were prepared with a biopsy punch.

Hydrogel discs were characterized under uniaxial compression in a radially confined geometry using a mechanical test frame (ELF 3100; Bose). Stress relaxation tests were conducted to measure the stress generated by the hydrogels in response to applied strains. The data were fit to poroelastic theory^{31–33} to calculate the aggregate modulus (H_A) of the constructs. Hydrogel discs were compressed in 5% increments at 3 min intervals up to 30% strain. Each step resulted in a stress spike and an ensuing decay as fluid and solid equilibrated in the disc. The decay in stress after each step was fit to an exponential function using nonlinear least-squares regression to determine the equilibrium stress (σ_{eq}) and time constant (τ) of the decay.³³ Equilibrium stress was measured versus strain (ϵ) and fit to a second-order polynomial using nonlinear least-squares regression. Aggregate modulus H_A was reported at 15% strain by evaluating the slope of the fitted stress strain curve.³³

Statistics

Chondrocyte viabilities for photoinitiators as a function of photoinitiator concentration and UV exposure were ana-

lyzed with two-factor analysis of variance (ANOVA) and Tukey *post-hoc* test. A repeated measures ANOVA was used when two independent tests of Irgacure 2959 viability were made. Chondrocyte viabilities for 2D well cultures as compared to viabilities immediately following chondrocyte isolation were analyzed using a Tukey-Kramer *post-hoc* test. The significance level for all cases was set at $\alpha = 0.05$.

Results

Measured chondrocyte viabilities for the five photoinitiators tested in 2D culture in well plates are displayed in Figure 1. Viabilities were $<10\%$ for VA-044 (Fig. 1a) and Irgacure 1870 (Fig. 1b) at the levels tested, independent of UV exposure. Chondrocytes had high viability ($>80\%$) with V-50 (Fig. 1c) for UV times up to 5 min and photoinitiator concentrations up to 0.5% (w/v). VA-086 (Fig. 1d) had high viability ($>90\%$) for UV times up to 10 min and photoinitiator concentrations up to 1.5% (w/v). Chondrocyte viabilities were high ($>80\%$) for Irgacure 2959 (Fig. 1e) in the absence of UV exposure, but dropped to $\sim 10\%$ over the range from 0% to 0.2% at all nonzero UV exposures tested. Figure 2 specifically highlights the effects of UV exposure in the presence of three photoinitiators (VA-086, V-50, IRG2959) in comparison to the negative control (no photoinitiator).

In 3D constructs, viability was observed for concentrations for which photoinitiators and concentrations were selected to lead to aggregate moduli in the 10–20 kPa range (Figs. 3 and 4). For these conditions, VA-086 constructs had mean viabilities over 90%, whereas IRG2959 constructs had mean viabilities below 70%.

Discussion

Chondrocyte viability as measured by the presented tests gives some insight into the relative toxicity of (a) solvents used to dissolve the photoinitiator, (b) the photoinitiator itself, (c) UV exposure, and (d) UV-initiated radicals. These insights allow projection of the applicability of the photoinitiators.

Measured viabilities with neither photoinitiator nor UV exposure highlight the effects of solvent on chondrocyte viabilities. Ethanol was used for all 2D well plate viability measurements, yet the viability of chondrocytes with no UV exposure and no photoinitiator ($93\% \pm 3\%$; $n = 15$) was not statistically different (Tukey-Kramer *post hoc*) from viabilities measured following chondrocyte isolation ($92\% \pm 3\%$; $n = 5$). Thus, these data provide no evidence that the presence of this amount of ethanol is a primary factor in determining cell viability in these systems. The final ethanol concentration was proportional to the photoinitiator concentration, and was as high as 7% for the 1% Irgacure 2959 case.

The measured viabilities with dissolved photoinitiator but in the absence of UV exposure (lines with circles in Fig. 1a–e) indicate the cytotoxicity of the photoinitiator itself. IRG2959 and VA-086 are the least cytotoxic in their native form (i.e., in the absence of a thermal or photonic dissociation mechanism), with $>80\%$ viabilities at all tested concentrations. IRG1870 and VA-044 are clearly cytotoxic at all levels at or above 0.125% (w/v). V-50 shows $>90\%$ viability in 2D culture up to 0.5% (w/v). IRG2959, VA-086, and V-50 were all statistically less cytotoxic than IRG1870 and VA-044.

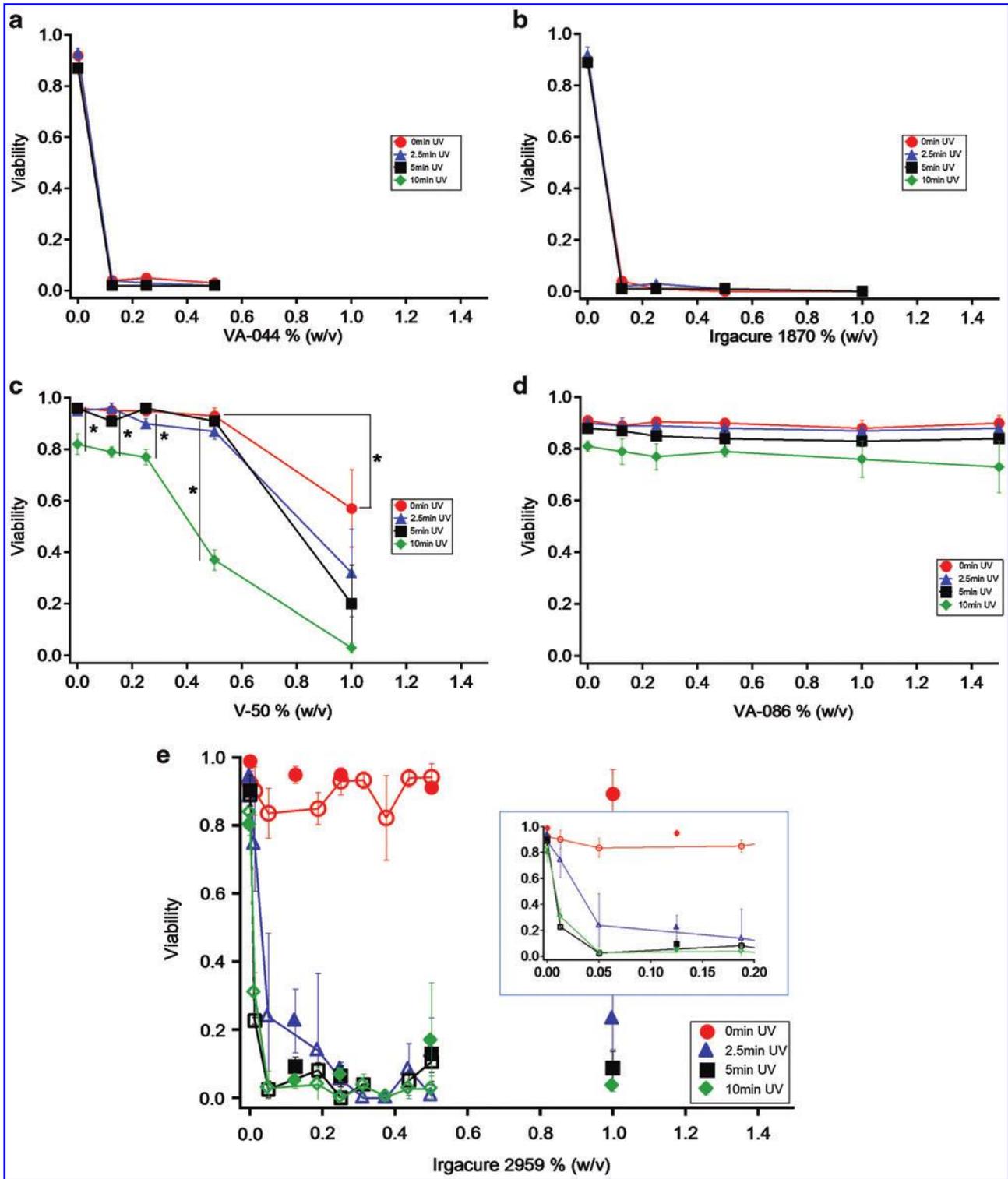


FIG. 1. Viability of primary bovine articular chondrocytes plated at a density of 1×10^4 cells/cm² into six-well plates, exposed to several different UV exposure times and different concentrations of (a) VA-044, (b) Irgacure 1870, (c) V-50, (d) VA-086, (e) Irgacure 2959. End viabilities measured after 48 h of culture. Bars are mean \pm standard deviation. * $p < 0.05$. Inset in (e) highlights results at low Irgacure 2959 concentration. Effects of UV exposure alone are seen in the results at 0% (w/v) of each photoinitiator. UV, ultraviolet. Color images available online at www.liebertonline.com/ten.

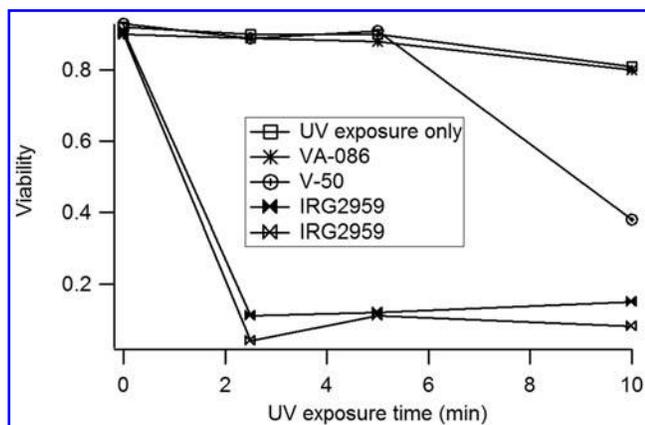


FIG. 2. Viability of primary bovine articular chondrocytes plated at a density of 1×10^4 cells/cm² into six-well plates, graphed as a function of UV exposure times for 0.5% (w/v) photoinitiator. Two measurements of Irgacure 2959 are shown. The effect of the cytotoxicity of the photoinitiated radical is clear from the viability change for Irgacure 2959 at 2.5–10 min and for V-50 at 10 min; in comparison, the VA-086 radical appears to not be cytotoxic, and viability with VA-086 is not significantly different from UV exposure alone.

The measured viabilities with UV exposure but no photoinitiator (data points on the y-axis in Fig. 1a–e) indicate the phototoxic effect of the UV exposure. UV exposure alone led to a statistically significant reduction in chondrocyte viability for 10 min exposures as compared to each exposure time point of 5 min and below ($n = 25$; $p < 0.05$).

The measured viabilities with both photoinitiator and UV exposure indicate the combined effect of UV and photo-

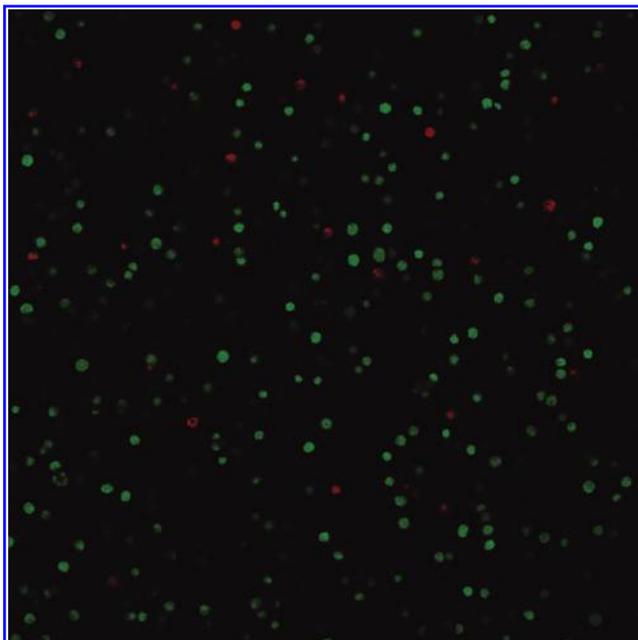


FIG. 3. Confocal micrograph of primary bovine articular chondrocytes seeded at a density of 50×10^6 cells/cm³ into photocrosslinked alginate by use of VA-086. Image acquired following 48 h of culture. Color images available online at www.liebertonline.com/ten.

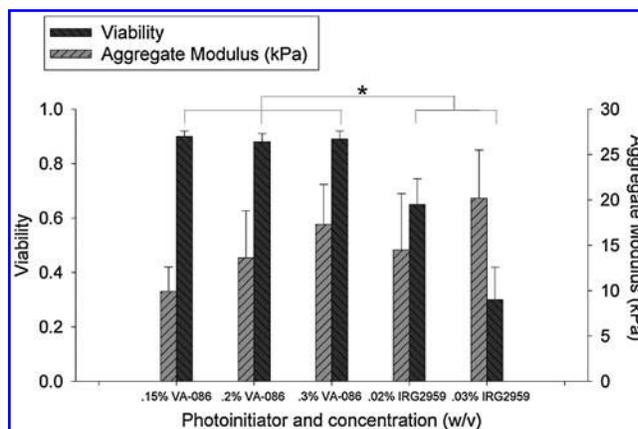


FIG. 4. Viability of primary bovine articular chondrocytes and aggregate modulus of three-dimensional alginate hydrogels fabricated using IRG2959 and VA-086 photoinitiators of varying concentration and 5 min UV exposure times ($n = 3$). End viabilities and material properties measured after 48 h of culture. Bars are mean \pm standard deviation. * $p < 0.05$.

initiator, indicating the cytotoxic effect of UV-generated radical species. For IRG1870 and VA-044, viabilities measured with varying UV exposure provide little additional information; however, for photoinitiators IRG2959, VA-086, and V-50, reduction in viabilities measured with varying UV exposure indicates the toxicity of the radicals generated by exposure of these photoinitiators to UV light (Fig. 2). This combined effect is significant for IRG2959 and V-50 ($n = 5$; two-factor ANOVA; Tukey *post-hoc*; $p < 0.01$), but not for VA-086, and the cytotoxic effect is significantly higher for IRG2959 than for V-50 ($n = 5$; $p < 0.05$). Collectively, these data indicate that photo generated radicals from V-50 and IRG2959 are cytotoxic, whereas those generated from VA-086 are not.

Measurements in 3D constructs are consistent with those in 2D cultures. In 3D, VA-086 constructs led to statistically higher viability ($\sim 90\%$) than IRG2959 constructs ($< 70\%$) within the range tested ($n_{VA086} = 6$; $n_{IRG2959} = 6$; $p < 0.05$). While direct statistical comparison is not possible between the 2D cultures and 3D constructs, these viability results in the 3D constructs are consistent with the trends observed in 2D culture.

The goal of this study was to compare the action of five photocrosslinking reagents on methacrylate-modified alginate. These photocrosslinkers were chosen owing to availability, established success in photocrosslinking in oxygenated environments, water solubility, or chemical similarity to crosslinkers having one or more of these properties. The photoinitiator VA-086 was shown to have the most desirable properties, enabling high viability and high concentrations and long UV exposures. The statistically significant factors controlling chondrocyte viability in the samples tested were UV exposure of 10 min; the presence of IRG1870 or V-044 photoinitiators; and the presence of photodissociated radicals from V-50 and IRG2959 photoinitiators. Because of its mild effect, UV exposure at the levels tested is of little practical significance, though a statistical significance was measured at 10 min. By far the most important factor in cell viability was the cytotoxicity of the UV-photodissociated radical species. The results lead to the

conclusion that IRG1870 and V-044 are cytotoxic and unsuitable for tissue engineering applications, whereas IRG2959, V-50 and VA-086 are viable potential photoinitiators. VA-086 leads to higher viabilities as compared to V-50, whereas generating similar levels of photocrosslinking, and thus V-50 is likely an inferior candidate as compared to VA-086. VA-086 was unique among the tested photoinitiators in that none of the observed viabilities showed a statistically significant effect of photoinitiator concentration, regardless of the presence or absence of UV exposure.

Irgacure 2959 has been used extensively for photocrosslinking of tissue-engineered scaffolds owing to its efficient photodissociated radical and low cytotoxicity in native form.^{23–26,34–37} However, the Irgacure 2959 radical does exhibit cytotoxicity, and while construct viabilities >50% have been reported,³⁰ Irgacure 2959 is of limited applicability if ~90% end viability is expected. The current results in 3D constructs, which are consistent with the 2D well plate viability measurements, indicate that for creating photopolymerized alginate-chondrocyte constructs in the 10–20 kPa modulus range, VA-086 photoinitiator leads to significantly higher viabilities, measured near 90% in these studies.

These results make VA-086 a promising initiator for photocrosslinking procedures for tissue engineering, and the use of VA-086 may make photocrosslinked alginate suitable for generating high-viability constructs. Although the current study focuses on the use of VA-086 with alginate, it is likely applicable to other photocrosslinking-based tissue engineering systems such as polyethylene glycol (PEG),³⁸ chondroitin sulfate,³⁰ and collagen.³⁹

To date, photocrosslinked polymers have been used in tissue primarily for the creation of relatively small constructs. This may be due in part to cytotoxicity concerns highlighted in this study. VA-086, either alone or in conjunction with other photoinitiators (e.g., Irgacure 2959), may enable photocrosslinkable polymers to be used more widely in applications such as tissue injection molding⁴⁰ and 3D tissue printing.⁴¹ Further, VA-086 may enhance efforts to create cell-seeded microscale devices⁹ that have previously relied on ionic crosslinking.

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Disclosure Statement

No competing financial interests exist.

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