Evaluation of Photocrosslinked Lutrol Hydrogel for Tissue Printing Applications

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Application of hydrogels in tissue engineering and innovative strategies such as organ printing, which is based on layered 3D deposition of cell-laden hydrogels, requires design of novel hydrogel matrices. Hydrogel demands for 3D printing include: 1) preservation of the printed shape after the deposition; 2) maintaining cell viability and cell function and 3) easy handling of the printed construct. In this study we analyze the applicability of a novel, photosensitive hydrogel (Lutrol) for printing of 3D structured bone grafts. We benefit from the fast temperature-responsive gelation ability of thermosensitive Lutrol-F127, ensuring organized 3D extrusion, and the additional stability provided by covalent photocrosslinking allows handling of the printed scaffolds. We studied the cytotoxicity of the hydrogel and osteogenic differentiation of embedded osteogenic progenitor cells. After photopolymerization of the modified Lutrol hydrogel, cells remain viable for up to three weeks and retain the ability to differentiate. Encapsulation of cells does not compromise the mechanical properties of the formed gels and multilayered porous Lutrol structures were successfully printed.

Introduction

Organ printing is a novel approach in tissue engineering, based on layered strand- or dropwise deposition of cell-laden hydrogels.[1] This rapid prototyping-derived approach yields structured 3D scaffolds, with predetermined external shape and internal morphology, and can ensure defined cell placement. By printing different cell types at defined locations within a single scaffold, one could mimic natural cell distribution. So far, the organ printing approach has been used to make tubular-like structures of hydrogel laden with endothelial cells[2] and to design various coculture systems.[3−5] To use organ printing to construct vascularized bone grafts, endothelial cells together with bone progenitors might be a good combination, as both cell types showed enhanced functionality in cocultures.[6]

Hydrogels can be processed by a manifold of organ printing techniques and allow homogeneous encapsulation of cells and bioactive molecules.[7] For embedded cells, hydrogels provide a support matrix with a highly hydrated microenvironment that is amenable to nutrient and oxygen diffusion. For optimal use in organ printing, fast gelation is necessary during stacking of subsequent layers. Furthermore, the gel must be noncytotoxic and have an adequate stability and mechanical properties for in vitro culture and in vivo implantation. Depending on the type of tissue that is printed, the gel should ideally provide the embedded cells with the proper biochemical and physical stimuli to guide cellular processes such as migration, proliferation, and differentiation. Hydrogels used for organ printing so far include sodium alginates,[2,8] collagen, and Pluronics,[9] each suffering from their own drawbacks like lack of adhesive/biomimetic sequences, low mechanical properties, and instability in culture, respectively. The development of more suitable, tailored hydrogel matrices is therefore highly desirable.

Solutions of thermosensitive polymers undergo a temperature-dependent gel formation, based on physical interactions between the polymer units. Particularly, inverse thermogelling polymers exhibiting lower critical solution temperature (LCST) behavior are popular as injectable matrices for tissue engineering (TE)[10] but may also be used for organ printing. Such gels can preserve their shape very well during printing, but they dissolve relatively fast after gelation.[11−13] Crosslinking by photopolymerization of water-soluble polymers containing methacrylate groups using UV or visible light in the presence of photoinitiators offers spatial and temporal control over polymerization, with high polymerization rates at physiological temperatures and minimal heat production.[14] Some of the photoinitiators, including Irgacure 2959, were shown to exhibit adequate toxicological profile, with good cytocompatibility measured through analysis of cellular metabolic activity at concentrations $\leq 0.05\%$ (w/v).[15,16] Therefore, the addition of photosensitive groups to a thermosensitive hydrogel would be ideal for organ printing, with additional covalent crosslinks allowing manipulation and subsequent culture of the printed scaffolds. Low photoinitiator concentrations and low intensity UV-light should be used to minimize possible adverse effects of exposure of the embedded cells to free radicals. Findings from earlier studies indicate that higher doses of photoexposure compromises proliferation and cell cycle progression of the exposed MSC.[17] Therefore, a balance between

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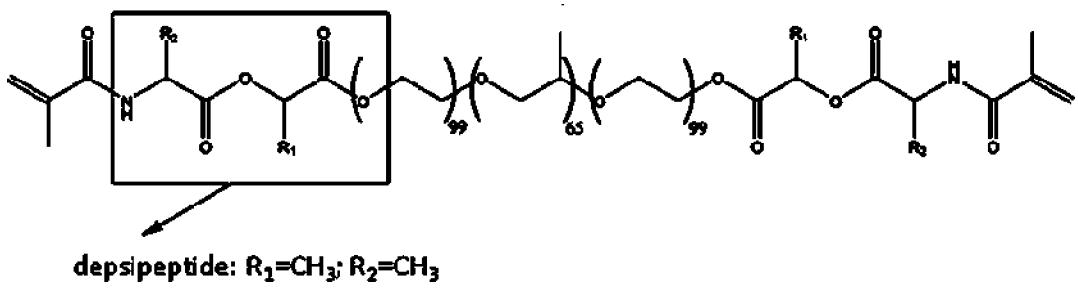

Figure 1. Structure of Lutrol F127 AlaL.

UV dosage ensuring adequate photocrosslinking of the polymer while maintaining functionality of the exposed cells is required.

In this study, the concept of organ printing is combined with a recently developed photopolymerizable Lutrol hydrogel to benefit from its fast temperature-responsive gelation properties and from the additional stability provided by covalent crosslinking. This photopolymerizable thermosensitive hydrogel is a modification of thermosensitive Lutrol F127 (Lutrol-T in this paper), a pharmaceutical grade of Pluronic F127. The hydrogel is based on triblock of polyethylene-oxide and polypropylene-oxide modified with methacrylamide groups: Lutrol F127 AlaL polymer (Lutrol-TP from hereon). Photopolymerization of Lutrol-TP yields hydrogels with enhanced mechanical properties, while the degradation profile of this hydrogel can be tuned by varying the chemical composition of the depsi-peptide sequence groups. In vitro biological studies, including viability studies, have demonstrated that the materials are well tolerated by human cells.[35,36]

Here, we analyze the applicability of Lutrol-TP hydrogel for bone tissue printing, by investigating the cytotoxicity of original (Lutrol-T) and photosensitive Lutrol-TP hydrogels and by printing cell-laden Lutrol-TP scaffolds. We compare the effect of photopolymerization conditions on cell behavior, both for seeded and hydrogel-embedded goat multipotent stromal cells (MSC). Special focus is put on survival and differentiation of the embedded cells, assessed by viability/cytotoxicity assay and (immuno)cytochemical detection of early and late osteogenic markers, respectively. Further, we demonstrate the feasibility of printing the (cell-laden) Lutrol-TP scaffolds and analyze the mechanical properties of the formed gels.

Materials and Methods

Hydrogel. Lutrol F127 (Poloxamer 407; Pluronic F127) was obtained from BASF, ExAct, and is termed Lutrol-T in this paper. Lutrol F127 AlaL (Figure 1), termed Lutrol-TP, was synthesized as described in detail.[35] In short, a solution of dehydrated Lutrol F127 (BASF, Germany) in dichloromethane (DCM) was reacted with bromoacetyl-bromide or 2-bromopropanoylbromide in the presence of poly(4-vinylpyridine) as proton acceptor. The purified F127-di(α-bromoesters), designated as F127-L-Br and F127-G-Br, were used in a reaction with *N*-2,3-dimethylmaleimidoalanine to obtain the modified, photosensitive Lutrol F127-AlaL.

Photopolymerization Presets. The photoinitiator used was 1-[4-(2-hydroxyethoxy)-phenyl]-2-hydroxy-2-methyl-1-propane-1-one (Irgacure 2959, Ciba Specialty Chemicals, Basel, Switzerland). A stock solution of 5% (w/v) in PBS (obtained by 30 min incubation at 70 °C) was added to the culture medium or hydrogel to create final concentrations of 0, 0.05, or 0.1% (w/v). A Superlite S-UV 2001AV lamp (Lumatec, Munchen, Germany), which emits UVA and blue light (320−500 nm) was used to expose the cells and (cell-laden) hydrogels to the UVA intensity of ~6 mW/cm^2 (X9$_2$ photometer, Gigahertz-Optik GmbH, Puchheim, Germany), for periods up to 300 s.

Cells. Multipotent stromal cells (MSCs) were obtained from iliac bone marrow aspirates of Dutch milk goats, and isolated by adherence to tissue culture plastic. The cells (passage 2−6) were culture-expanded as described previously.[18] Briefly, aspirates were resuspended by using 20-gauge needles, plated at a density of 5×10^5 cells per square centimeter and cultured in MSC culture medium consisting of αMEM (Gibco) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin (Gibco), 2 mM L-glutamine (Glutamax, Gibco) and 15% v/v fetal calf serum (Cambrex). Cells were maintained in a humidified incubator at 5% CO_2 and 37 °C. Medium was refreshed twice a week and cells were used for further subculturing or cryopreservation. The cells from passage 2−6 were used in the experiments.

Cell Seeding on Top of Hydrogels. Upon trypsinization, MSCs were seeded on hydrogels discs ($n = 4$; 150 μL) at 10^4 cells/cm^2 in a total volume of 50 μL. The gels were prepared in 16-well chamber slides by 1800 mJ/cm^2 UV exposure of 25% (w/v) Lutrol TP polymer in the presence of 0.1% (w/v) Irgacure 2959. As a control, we used MSCs seeded on Lutrol-T gel surfaces and Matrigel (Growth Factor Reduced, BD Biosciences) discs. A total of 6 h after seeding, spreading of the seeded cells with and without the additional coating with poly-L-lysine (gel 5 min preincubated with 0.1 mg/mL poly-L-lysine in water, Sigma P4832) was analyzed by light microscopy. The viability of the seeded cells was studied 1 day after seeding with a LIVE/DEAD Viability/Cytotoxicity Kit (Molecular Probes, U.S.A.). The samples were measured using a microscope equipped with an epifluorescence setup, excitation/emission setting of 488/530 nm to detect green fluorescence (living cells), and 530/580 nm to detect red (dead) cells (Leica DM IRBE, Germany). Three randomly selected fields per sample were counted under the fluorescence microscope (Leica DM IRBE, Germany) using analysis software (Soft Imaging System, Germany). The cells' viability was calculated as the average ratio of vital over total cells per sample.

Cell Encapsulation in Lutrol-T and -TP. To study viability of encapsulated cells in Lutrol-T, the polymer was dissolved in culture medium with or without glycerol (10 mM, Merck) and hydrocortisone (60 nM, H6909, Sigma) as membrane stabilizing agents, at 20% (w/v) overnight at 4 °C. MSCs were harvested by trypsinization and encapsulated in hydrogels at 3.0×10^5 cells/ml, two samples per condition. Cells cultured on polystyrene tissue culture slides were used as control.

To study the fate of encapsulated cells in Lutrol-TP hydrogels, the polymer was either dissolved in culture medium, for viability studies, or in osteogenic medium, to study differentiation, at 25% (w/v) o/n at 4 °C. MSCs were harvested by trypsinization and resuspended in the triblock copolymer solutions at 4.4×10^5 cells/ml gel at room temperature. Hydrogel discs (6 mm ∅ and 1 mm thick) were prepared in 16-well chamber slides by 60 or 300 s exposure of 50 μL Lutrol-TP in the presence of Irgacure 2959. The total UV doses used were 0, 360, or 1800 mJ/cm^2. For viability analysis, a nonphotopolymerized hydrogel was used as control. To promote cell distribution inside the gels, polymer solutions in culture medium were supplemented with poly-L-lysine (0.05 mg/mL Sigma) or fibronectin (human, 2.5 μg/mL, Harbor Bio-Products, 0172003). To study the osteogenic differentiation potential of MSCs inside the gels, cells were mixed with Lutrol-TP at

5×10^6 cells/ml gel. The gel discs ($n = 3$ per condition, 150 μL) were covered with 50 μL medium and cultured at 5% CO_2 and 37 °C. After the indicated period of incubation (6 h to 3 weeks), the viability of cells was measured with the use of LIVE/DEAD Viability/Cytotoxicity Kit, as described above.

Analysis of Osteogenic Differentiation. The activity of alkaline phosphatase (ALP), present early during osteogenic differentiation, was determined for the MSCs encapsulated in the Lutrol-TP hydrogels exposed to 1800 mJ/cm^2 UV-light in the presence of 0.1% Irgacure 2959 ($n = 4$). After 1 week of culture in osteogenic medium, the medium was discarded and the gels were washed in PBS and embedded in TissueTek. Cryosections of 10 μm were fixed in 100% acetone, washed with PBS-0.1% Tween, and Fuchsin Substrate-Chromogen System (DakoCytomation, Carpinteria, U.S.A.) staining was applied according to manufacturer's recommendations to detect alkaline phosphatase positive cells (pink staining). Hematoxylin was used as counter stain. The presence of ALP positive cells was analyzed with an Olympus BX50 light microscope equipped with an Olympus DP 70 camera, by calculating the average ratio of ALP positive over total cells from four randomly selected fields per sample.

For detection of collagen I production, hydrogel samples were imbedded in TissueTek after 2 weeks of incubation, and 10 μm cryosections were cut. The sections were blocked with 5% (w/v) BSA in PBS and incubated with mouse anticollagen type I antibody (2 μg/mL in 5% (w/v) BSA in PBS, clone I-8H5, Merck, Japan) for 1 h, followed by incubation with goat antimouse-Alexa488 (20 μg/mL in 5% (w/v) BSA in PBS, Molecular Probes, U.S.A.) for 30 min. Wash steps with 0.1% Tween20 in PBS were performed between the incubations. The sections were mounted with Vectashield containing DAPI for nuclear staining (Vector Laboratories) and analyzed under the fluorescence microscope.

Mechanical Properties of Photopolymerized Hydrogels. To test how cellular encapsulation affects mechanical properties of the formed gels, Lutrol-TP hydrogel discs (25% (w/v) polymer in culture medium) with ($n = 3$; 1×10^6 cells/mL gel) or without cells ($n = 4$) were prepared by exposure of 250 μL triblock solution in the presence of 0.1% (w/v) Irgacure 2959 to UV light at 1800 mJ/cm^2. The stiffness of the hydrogel samples was measured at room temperature 4 h after photopolymerization, using a dynamic mechanical analyzer (DMA 2980, TA Instruments, Etten-Leur, The Netherlands) in controlled force mode. Hydrogels of 6.4 × 6 mm (height × diameter) were placed between the parallel plates (diameter upper plate 6 mm, diameter lower plate 45 mm) and a static force was applied between 0 and 1 N, and varied at a rate of 0.05 N/min. The Young's modulus (E) was determined as described previously,[19] by measuring the variation of stress/strain ratio.

Printing of Lutrol-TP Scaffolds. The Bioplotter pneumatic dispensing system (Envisiontec GmbH, Germany) was used for 3D printing of hydrogel scaffolds. This system was previously employed for extrusion of hydrogels and is described in more detail elsewhere.[20] Briefly, the Bioplotter is a three-axis dispensing machine, which builds up 3D constructs by coordinating the motion of a pneumatic syringe dispenser. The dispenser deposits extrudate consisting of empty or cell-laden hydrogel on a stationary platform. Models of the scaffolds are loaded via the Bioplotter CAD/CAM software, which translates this information for the layer-by-layer fiber deposition by the Bioplotter. In the current study, the speed of deposition was set at 16 mm/sec and the pneumatic pressure that was applied to the dispensing syringe (containing 25% (w/v) Lutrol-TP in culture medium, without cells, at 4 °C) was set at 2 bar to yield uniform, continuous extrusion of fibers. An inner nozzle diameter of 210 μm was used. Two different configurations of deposited fibers (0/90 and 0/45:0/90 configuration) were tested for Lutrol-TP. Rectangular 3D scaffolds of 20 × 20 mm with spacing between fibers of 300 μm and a layer thickness of 150 μm were constructed in a Petri dish. Ten-layer scaffolds of Lutrol-TP were constructed and subsequently photopolymerized under the UV lamp as indicated.

Statistical Analysis. Statistical analysis was performed with SPSS 12.0.1 software. A two-way analysis of variance (ANOVA) was used to evaluate the viability measurements of seeded and encapsulated MSCs. LSD and Bonferroni post hoc tests were used to compare the groups. A Student's t test was used to compare the mechanical properties of Lutrol-TP gels cast with and without the cells. P-values of less than 0.05 were considered statistically significant. All values are reported as mean ± standard deviation.

Results

Effect of Gel Surfaces on Cell Distribution and Viability. Analysis of cell distribution directly and 6 h after seeding of MSCs on top of Lutrol-T and photopolymerized Lutrol-TP hydrogel discs revealed that the cells aggregate toward the center of the hydrogel disk forming cell clusters on the hydrogel surface (Figure 2A,C,D). Coating of the hydrogel surface with poly-L-lysine resulted in more uniform distribution of the cells on the surface, although no stretching of the MSCs was observed (Figure 2E,F), as was seen when using Matrigel (Figure 2B). Viability analysis 1 day after seeding indicated that the survival on photopolymerized Lutrol-TP surface (formed by 1800 mJ/cm^2 UV exposure with 0.1% Irgacure) was significantly higher than on Lutrol-T gels: (70 ± 4% vs 30 ± 7%; $p < 0.05$*; Figure 2G). Additional coating with PLL promoted survival of the seeded MSCs on Lutrol-T (Lutrol-T vs Lutrol-T+PLL: 30 ± 7% vs 54 ± 5%; $p < 0.05$ **) and not on Lutrol-TP surfaces.

Cell Encapsulation and Viability Analysis. No significant differences in cell survival between the groups were seen after 6 h for all the tested conditions. After 1 and 3 days, TCPS-cultured MSCs were significantly more viable than the cells embedded in hydrogels. Analysis of cell survival of MSCs encapsulated inside Lutrol T hydrogels demonstrated progressive cell death of embedded cells, with around 30% survival after three days (Figure 3). It has been reported previously that the viability of cells embedded within the Pluronic hydrogel was significantly enhanced by the addition of hydrocortisone and glycerol,[21] and therefore, we used these supplements in similar concentrations in this study. Addition of hydrocortisone enhanced the survival of the cells after 1 and 3 days, although a persistent decline in cell survival over the course of three days was observed. After 1 day, viability inside gels supplemented with H was significantly higher than control Lutrol-T gels (70 ± 7% vs 52 ± 11%; $p = 0.021$). After 3 days of encapsulation, survival in the gels supplemented with either H (52 ± 0.5%) or H+G combination (47 ± 6%) was significantly higher than in control Lutrol T gels (31 ± 8%; $p = 0.009$ and $p = 0.025$, respectively). This was not the case for gels supplemented with only glycerol (39 ± 6.5%; $p = 0.06$ compared to control).

Lutrol-T hydrogels are highly unstable in culture starting to dissolve within minutes after incubation in culture medium. Cell-laden Lutrol-TP gels exposed to 360 mJ/cm^2 of UV light in the presence of 0.1% Irgacure formed gel discs, which dissolved after 6−7 days. Lutrol-TP gels polymerized by 1800 mJ/cm^2 UV exposure in the presence of 0.05 or 0.1% Irgacure were more stable, with the latter hydrogel discs remaining stable in culture for up to three weeks postpolymerization. MSCs embedded within photopolymerized Lutrol-TP were distributed evenly throughout the gels with formation of clusters (Figure 4A). Addition of poly-L-lysine (PLL) or fibronectin (FN) to the gels promoted more homogeneous dispersion of the cells, after 1 day of encapsulation, although cell clusters were still present in gels supplemented with PLL (Figure 4B,C). Initial cell survival of encapsulated MSCs in photopolymerized Lutrol-

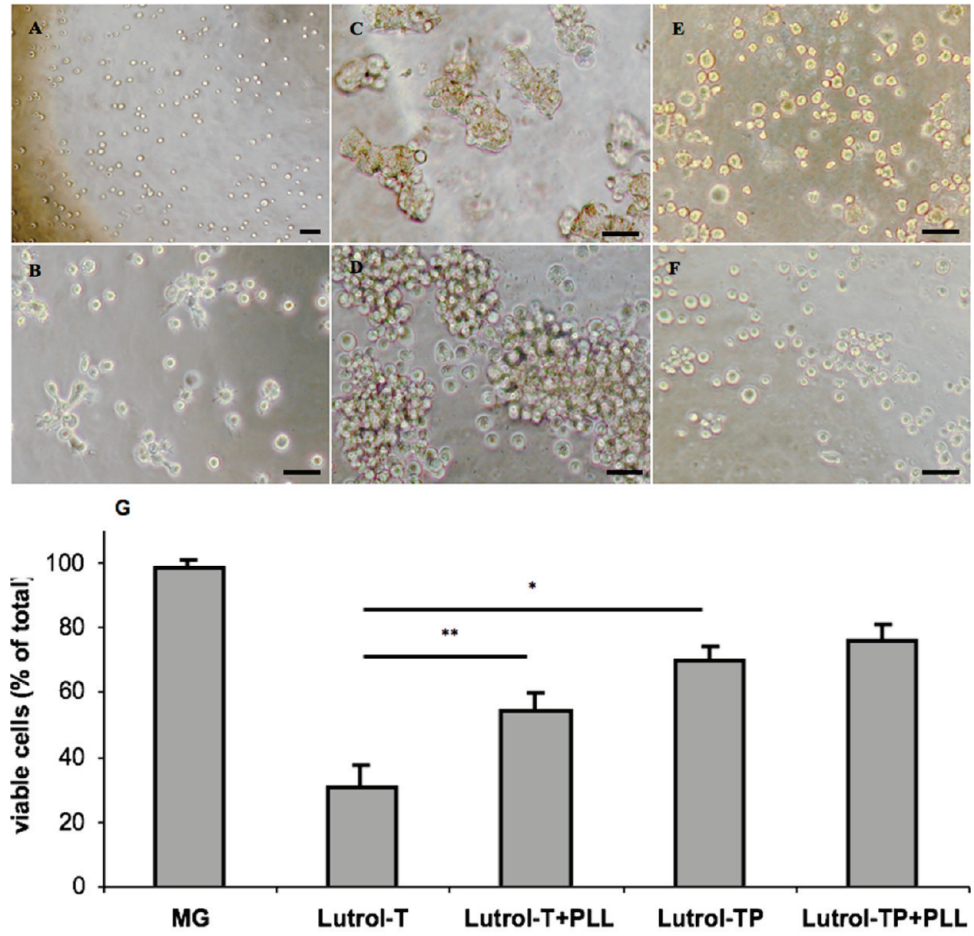

Figure 2. Effect of poly-L-lysine coating on cell morphology and viability. A−F: Cell morphology of MSCs seeded on Lutrol hydrogels; (A) directly after seeding on Lutrol-T (formed by 1800 mJ/cm^2 UV exposure with 0.1% Irgacure); (B−D) 6 h after seeding of MSC on Matrigel (B), Lutrol-T (C), and Lutrol-TP uncoated (D); (E,F) cells 6 h after seeding on Lutrol-T coated with PLL (E) and Lutrol-TP coated with PLL (F); scale bar: 50 μm; (G) Cell survival of seeded MSCs after one day on various hydrogel surfaces ($n = 4$ per condition). MG: Matrigel.

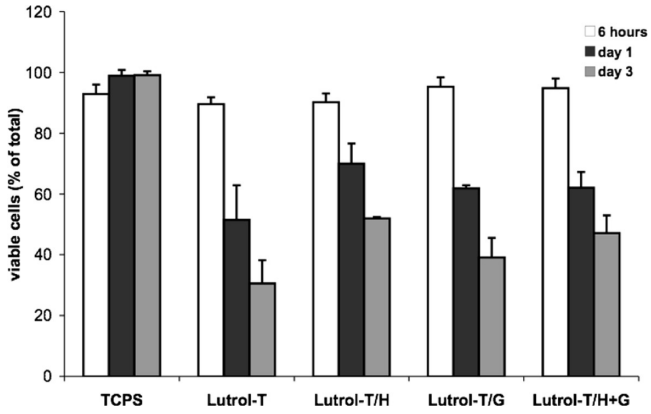

Figure 3. Viability of MSCs in Lutrol-T gels (25% w/v), control or supplemented with 60 nM hydrocortisone (H) and 10 mM glycerol (G), as compared to viability of MSCs seeded on tissue culture polystyrene surfaces (TCPS), $n = 3$ per condition, per time point.

TP hydrogels obtained at different polymerization conditions was assessed after 1 day of incubation (Figure 4D). Analysis after 1 day ($n = 3$ or 5 per condition) indicated a statistically significant difference between unphotopolymerized and photopolymerized gels ($p \leq 0.001$), without a significant effect of the different photopolymerization conditions. Addition of PLL and fibronectin did not significantly contribute to cell survival (Figure 4D). Viability in unphotopolymerized Lutrol-TP was significantly lower than in photopolymerized gels (Figure 5A), after both 2 and 7 days ($p \leq 0.001$;*), measuring 6 ± 3% after 7 days. Viability of MSC inside Lutrol-TP exposed to 360 mJ/cm^2 UV light, in the presence of 0.1% Irgacure, decreased with time and was significantly lower than in the two other photopolymerized conditions after both 2 ($p < 0.05$;**) and 7 days ($p < 0.001$;***), with 32 ± 3% viable cells after seven days, as compared to 59 ± 5% and 57 ± 1% in Lutrol-TP obtained by 1800 mJ/cm^2 UV exposure with 0.05% and 0.1% Irgacure, respectively. Survival of the embedded MSCs at 2 and 3 weeks is presented in Figure 5B, and measured around 60%.

Differentiation. Upon encapsulation, the MSCs retained a round morphology inside the gels (Figure 6) despite the addition of PLL or fibronectin to the hydrogel. To analyze the osteogenic differentiation of photoencapsulated MSCs, we determined the activity of alkaline phosphatase, an ectoenzyme produced by osteoblasts involved in ensuring a sufficiently high local concentration of phosphate for mineralization to occur. After a 1 week incubation in osteogenic medium, ALP was present in 10.2 ± 2.7% of the embedded MSCs (Figure 6). To further assess osteogenic differentiation, collagen I, a major specific marker of bone matrix highly expressed during the entire process of bone formation,[22] was measured. Figure 7 shows the presence of collagen I in a substantial amount of the embedded MSCs after 2 weeks, as determined by immunocytochemistry.

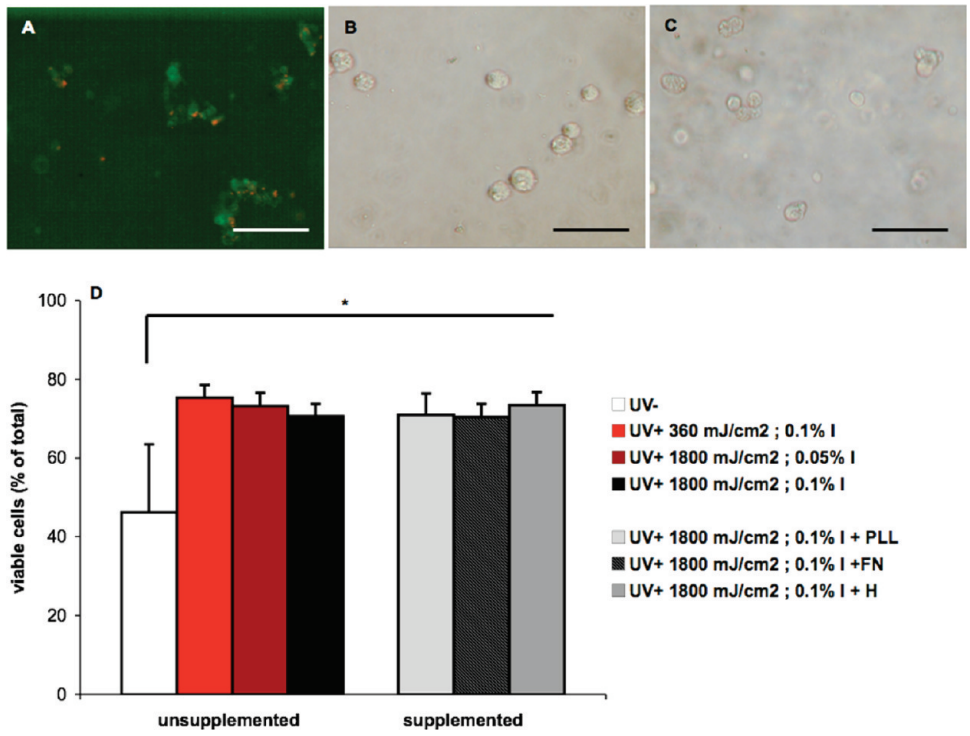

Figure 4. MSCs after 1 day of encapsulation in Lutrol-TP. (A) Live/dead assay on MSCs in Lutrol-TP polymerized in the presence of 0.05% (w/v) Irgacure and 1800 mJ/cm^2 UV exposure; live cells, green; dead cells, red; bar = 100 μm; (B,C) MSC 1 day after encapsulation in Lutrol-TP, supplemented with fibronectin (B) and PLL (C), bar = 50 μm. (D) Viability of MSCs embedded in Lutrol-TP gels obtained under different photopolymerization conditions (UV: unphotopolymerized, UV+: photopolymerized, I: Irgacure) and the effect of modified Lutrol-TP gels on cell viability, asterisk: $p < 0.05$. Lutrol-TP gels were supplemented with 0.05 mg/mL poly-L-lysine (PLL), or 2.5 μg/mL fibronectin (FN), or 60 nM hydrocortisone (H) and exposed to 1800 mJ/cm^2 UV in the presence of 0.1% Irgacure.

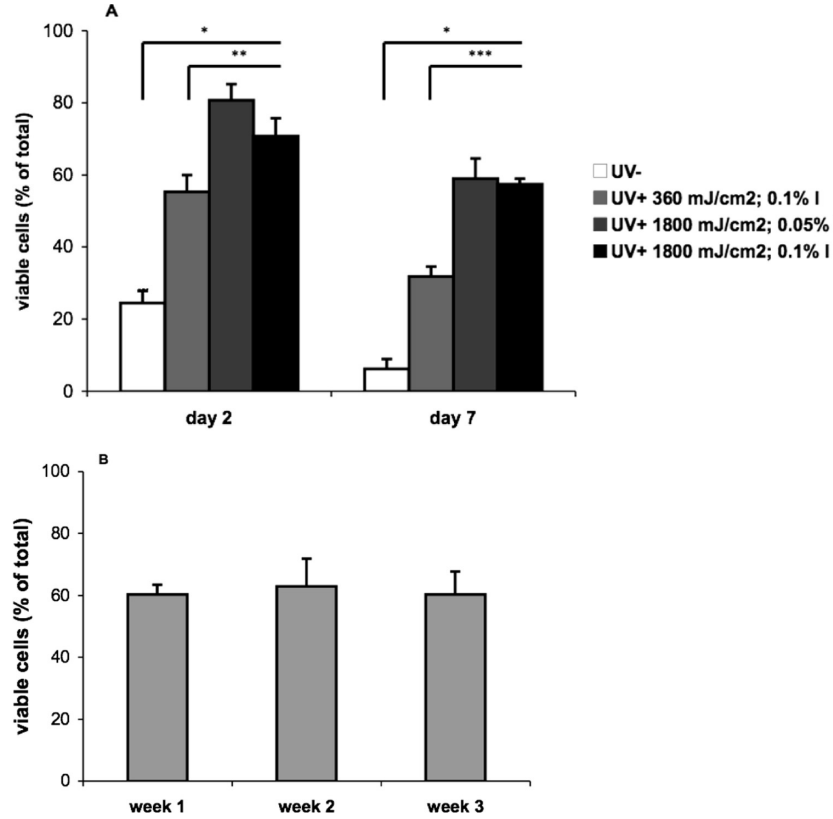

Figure 5. (A) Survival of embedded MSCs after 2 and 7 days incubation in Lutrol-TP ($n = 3$ per condition, per time point). (B) Long-term survival of MSCs embedded in Lutrol-TP gels, formed by exposure to 1800 mJ/cm^2 UV light in the presence of 0.1% (w/v) Irgacure ($n = 5$ per time point).

Mechanical Properties of the Formed Gels. The Young's modulus of the empty and cell-laden hydrogels was determined as the absolute value of the slope from the graph plotting the compression force versus the observed strain.

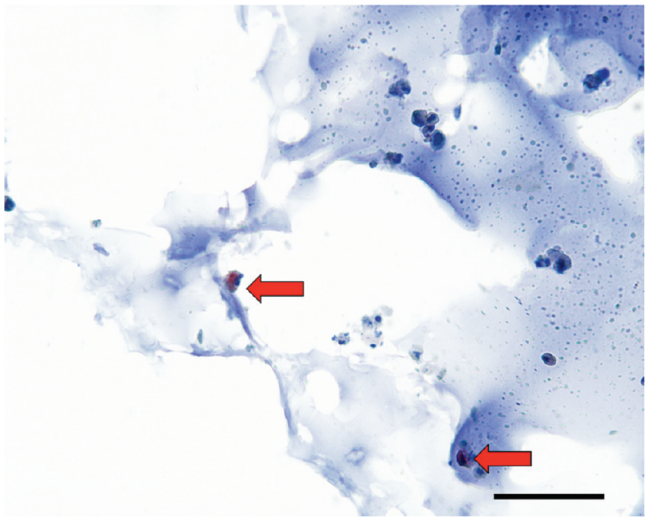

Figure 6. MSCs embedded in polymerized Lutrol-TP (light blue) for 1 week in the presence of osteogenic medium. ALP-specific staining is indicated in red (red arrow); scale bar = 100 μm.

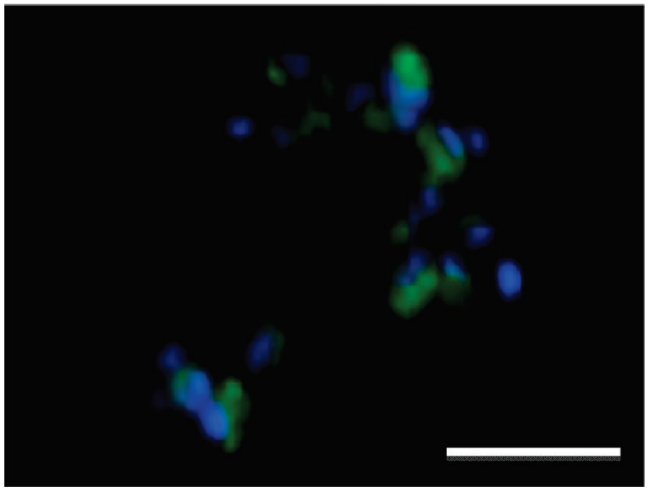

Figure 7. Collagen I immunocytochemistry, 2 weeks after encapsulation; scale bar = 50 μm. Collagen I (green); DAPI nuclear stain (blue).

Average Young's modulus of cell-laden hydrogels measured 21 ± 3 kPa, comparable to 20 ± 2 kPa of the empty hydrogels ($p = 0.55$).

Printing of the Scaffolds. Computer-controlled deposition of Lutrol-TP hydrogel resulted in scaffolds with a thickness up to 10 layers (Figure 8A) and regular vertical pores throughout the printed samples. It was possible to print the hydrogel with different fiber configurations. The weight of subsequent hydrogel layers resulted in broader strands than the original needle diameter and fusion of transversal pores during stacking of layers (Figure 8A). Prior to photopolymerization, the scaffold was soft and could be easily damaged, compromising handling (Figure 8B). After polymerization, a stabile, mechanically strong structure was formed (Figure 8C).

Discussion

In this study we analyze the applicability of a novel, thermosensitive, and photopolymerizable Lutrol hydrogel for 3D deposition of (cell-laden) hydrogel scaffolds to function as possible bone grafts. Lutrol-TP turned out to be very suitable for printing with a Bioplotter system, with thermosensitive gelation of the material ensuring easy 3D deposition and providing temporary support of the printed shape prior to photopolymerization, without collapsing. Subsequent photopolymerization resulted in organized 3D scaffolds that could be easily handled and cultured, with hydrogels retaining their stability for up to 21 days.[35] Adjusting the photopolymerization regime to photoexposure during deposition can possibly overcome the fusion of transversal pores.

The functionality of this new hydrogel as cell-supporting matrix for seeded and encapsulated cells was studied by measuring the cytotoxicity of the hydrogel and the effect of photoencapsulation on viability and osteogenic differentiation of goat osteogenic progenitor cells, MSCs. Our findings indicate that the majority of the cells are able to survive within the photopolymerized Lutrol-TP hydrogels for up to 3 weeks and retain the ability to differentiate after encapsulation.

Goat MSCs encapsulated in nonphotopolymerized Lutrol-T gels, dispersed throughout the gels with formation of clusters. Only a fraction of cells survived the encapsulation, with as minimal as 6% viable cells in 25% (w/v) Lutrol-T after 7 days of encapsulation. This low viability inside Pluronics has been reported previously also by other research groups and was in part attributed to disturbed membrane stability of the cells exposed to triblock-polymer units.[21] Therefore, we added hydrocortisone and glycerol to the polymer solutions prior to gelation, as addition of these media to Pluronics was shown to enhance cell viability through an unknown mechanism.[21] Although adding hydrocortisone could alleviate part of the cytotoxicity of the gels, an ongoing decrease in viability of MSCs remained. Furthermore, Lutrol-T gels were instable and could not be retrieved from culture after 3 days of incubation.

Additional photopolymerization of the Lutrol-TP gels renders the material more stable with regard to degradation, as covalent crosslinks prevent the dissolution of the gel. Hydrogel discs, obtained by photopolymerization of Lutrol-TP gels in the presence of 0.1% Irgacure and 1800 mJ/cm2 UV exposure, persisted in culture for up to 3 weeks, and were evaluated for viability of encapsulated cells during a three-week period. Photopolymerization of the gel had a positive effect on short-term cell viability with a substantially higher amount of viable cells than in the unphotopolymerized controls (50% vs 5%). A similar effect was seen for MSCs seeded on top of photopolymerized Lutrol-TP surfaces when compared to Lutrol-T gels. The positive effect of photopolymerization on cell survival may be attributed to crosslinking of the polymer that diminishes the dissolution of the gel and reduces the amount of triblock polymer molecules in direct contact with the cells. Notably, after crosslinking of Lutrol-TP, hydrocortisone no longer promotes cell viability, in accordance with the proposed effect of monomers on plasma membrane stability.[21]

We did not detect differences in cell survival between gels obtained at different photopolymerization conditions, indicating that the more stringent conditions can be applied to form scaffolds without directly harming cell viability. The long-term effects of UV-exposure and photoinitiator on protein and DNA damage in this hydrogel setting must still be determined. Cell survival around 60% is representative for unmodified, synthetic photopolymerized hydrogels.[23] Survival of encapsulated MSCs, even in hydrogels acknowledged as fully biocompatible can be as low as 15% after one week of encapsulation,[24] largely due to minimal interaction between hydrogel matrix and cells. It has been previously shown that integrins play a central role in adhesion, resistance to apoptosis, and promotion of cell survival.[25]

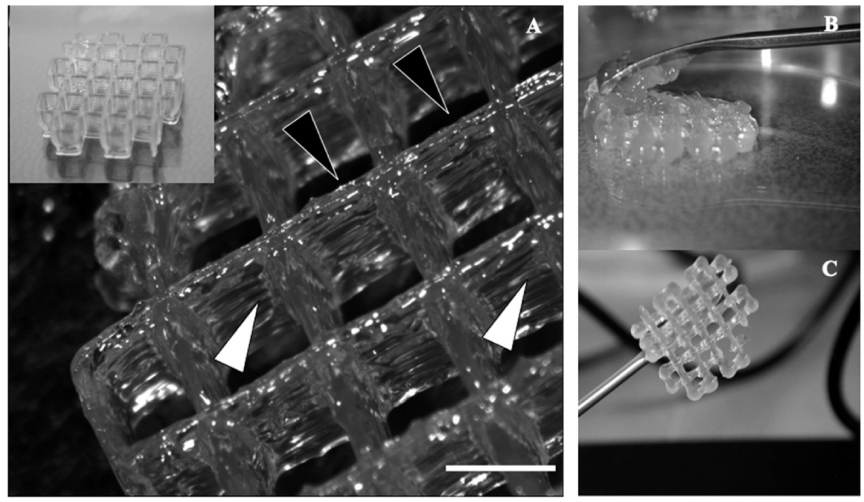

Figure 8. Printed Lutrol-TP scaffolds; (A) organized 3D structure (black arrowhead: vertical pores; white arrowhead: fused horizontal pores; bar: 2.5 mm); inset: total printed scaffold; (B) printed unphotopolymerized structure; (C) handling after photopolymerization.

When MSCs were seeded on Lutrol-T and the photopolymerized Lutrol-TP hydrogel surfaces, the cells exhibited clustering and additional coating of the surfaces with poly-L-lysine promoted more homogeneous cell distribution. Poly-L-lysine and fibronectin also enhanced the distribution of cells embedded in the gels. Polylysines, polycations that mediate integrin-independent adhesion, as well as fibronectin, a high-molecular-weight glycoprotein that interacts with cell surface expressed integrins, have been used to successfully promote cellular attachment and spreading of cells in hydrogels and on hydrogel surfaces.[26,27] Additional modification of the synthetic photopolymerizable hydrogels with the arginine-glycine-aspartate (RGD) adhesive sequence found in fibronectin and many other extracellular matrix proteins drastically enhances cell attachment and spreading of osteoblasts[28] and significantly promotes survival of the embedded hMSC.[24,29] In this study we observed no positive effect of FN or PLL on stretching or survival of cells embedded in Lutrol-TP, possibly because the supplements were simply mixed with the gels and not grafted or otherwise covalently attached.

Photoencapsulated MSCs retained the ability to differentiate toward the osteogenic lineage, with over 10% of the embedded cells exhibiting alkaline phosphatase activity and producing collagen I. A higher degree of osteogenic differentiation would be required for future use of this photopolymerizable gels in printed bone grafts. The use of high density culture contributes to the formation of vital intercellular contacts, and this is expected to enhance the differentiation of encapsulated cells:[30] the so-called "community-effect". Osteogenic differentiation can be further enhanced by grafting phosphoester groups,[31] heparin,[29] or collagen mimetic peptides[32] to the polymer chains.

Addition of the photosensitive groups to the Lutrol-T increases the mechanical properties of the formed gels after photopolymerization. The Young's modulus of the formed gels fall in the range of other photopolymerizable gels used for cellular encapsulation.[33] Addition of cells to the gels did not significantly alter the compressive modulus of the gels, indicating that initially, the embedded cells do not diminish the mechanical properties of the gels by distorting the photogel structure. Matrix stiffness is expected to have a significant effect on tissue development by the embedded cells,[34] with osteogenic differentiation possibly requiring stiffer hydrogels than the materials used in this study. Enhancing of crosslink density could be useful in this respect.

Conclusions

Modification of Lutrol F127 polymers with photosensitive groups renders thermosensitive hydrogels more stable in culture, making them attractive to design scaffolds for TE applications. MSCs embedded in photopolymerizable Lutrol-TP gels remain viable during the study period, with a significantly higher viability as compared to the same unphotopolymerized hydrogel or to Lutrol-T gels, and are able to differentiate toward the osteogenic lineage. This novel biomaterial is highly suitable for 3D fiber deposition, enabling formation of organized 3D scaffolds.

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