Methylation of Poloxamine for Enhanced Cell Adhesion

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Aiming at producing a synthetic collagen-mimetic material that is stiffer than collagen but that like collagen allows both cell encapsulation and cell growth on the surface, a positively charged poloxamine hydrogel was prepared by methylating the tertiary amine groups of a four-arm poly(ethylene oxide)−poly(propylene oxide) block copolymer derivative (Tetronic 1107). This derivative was subsequently reacted with methacryloyl isocyanate, rendering positively charged materials that are further cross-linkable by a photointiated free radical polymerization. Different hydrogels containing methylated poloxamine methacrylate concentrations between 6% and 18% were produced and characterized by means of water uptake and viscoelastic properties. A sharp increase in water content was observed in distilled water during the first week; some of the gels showed water uptakes as high as 2 times the initial wet weight. In PBS, this effect was less prominent due to the decrease in the osmotic gradient. Also, a gradual increase of both the storage modulus (G') and the loss modulus (G'') resulted from increasing the polymer concentration: for example, G' values ranged between 70 and 23000 Pa for 6% and 18% methylated poloxamine methacrylate hydrogels (at 1 Hz, 100 Pa of oscillatory stress). HepG2 cells embedded in different compositions and exposed to UV light displayed good viability levels after the cross-linking, unlike a previously reported attempt at creating a synthetic collagen-mimetic material. A well-spread endothelial cell morphology was apparent on methylated poloxamine films after preincubation in serum-containing medium, while on unmodified poloxamine methacrylate hydrogels cells attached poorly. However, EC did not attach well to the same material when fabricated not as films but as cylindrical modules as needed for the modular construct for which this material was intended. Thus, for this apparently more challenging geometry, it was necessary to combine collagen with the methylated poloxamine to have good attachment of EC on the surface of modules as well as films. While the challenge of creating a synthetic alternative to collagen as a stiffer cell-compatible substrate remains, methylated poloxamine displays many of the attributes that make it a useful material for tissue engineering.

Introduction

Collagen in the form of a gel is a useful material for tissue engineering because it can be used to both encapsulate cells and act as a substrate for cell attachment on the surface. The gels are, though, mechanically weak. To overcome this limitation, we have prepared a number of alternatives,[1−3] using poly(ethylene oxide)−poly(propylene oxide) block copolymers (PEO−PPO) and specifically Tetronic 1107 (poloxamine), a four-arm variant of the more conventional Pluronic linear triblock copolymer. PEO−PPO copolymers display good compatibility in contact with many different cells and tissues,[4−6] and the cross-linked derivatives improved the mechanical properties.[7] Thus, collagen/poloxamine semi-interpenetrating networks displayed higher stiffness, and more importantly, embedded human hepatoma HepG2 cells showed high viability levels and retained functional features such as α_1-antitrypsin secretion ability.[2] However, even when formulated in the form of a semi-interpenetrating network with collagen,[1,2] the PEO-rich surface still prevents protein adsorption and cell attachment, resulting in the detachment of initially adhered endothelial cells and leading to a decrease in surface coverage within 5 days.[2]

A secondary goal was to prepare a gel that had the cytocompatibility of collagen but that did not contain any collagen or biologically active components such as Arg-Gly-Asp (RGD) or similar cell adhesion peptides.[8] The latter may be particularly useful as a means of engaging particular integrin receptors and controlling the cell phenotype, but we hypothesised that neither the biochemical sophistication of immobilized RGD peptides nor the presence of collagen was necessary for our purposes. Tissue culture polystyrene does not have RGD peptides, yet it often remains the substrate of choice for cell attachment. Hence, we chose to pursue a fully synthetic approach to enable cell adhesion to poloxamine.[3] Positively charged gels were produced by grafting quaternary ammonium groups in the poloxamine network through a photoinitiated free radical copolymerization of different poloxamine methacrylate/([2-(methacryloyloxy)ethyl]trimethylammonium chloride (MAETAC) mixtures.[3] The modification resulted in good cell attachment properties, even though much of the material was composed of a PEO copolymer. While this approach appeared to overcome the limited cell adhesion properties of poloxamine-based systems, the modification was not suitable for cell encapsulation due to acute cell death with increasing MAETAC concentrations. This decreased viability probably derived from the high concentration of reactive precursors (methacrylate derivatives) during the production of the cell-containing matrix. On the other hand, the MAETAC approach proved the feasibility of the strategy for better cell adhesion.

Following a similar conceptual strategy, the present study was aimed at tailoring poloxamine derivatives to display two functional features in the backbone of the molecule: (a) positive

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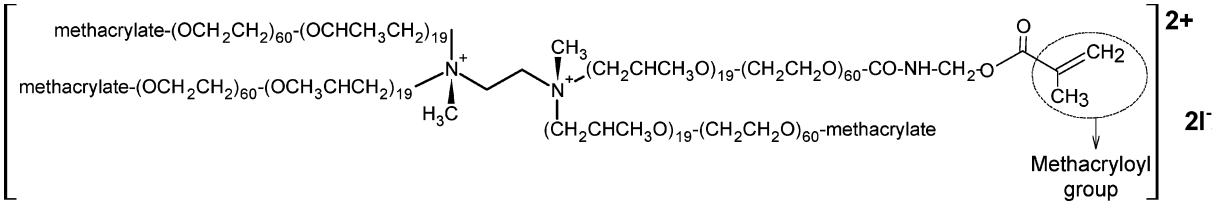

Figure 1. Structure of methylated poloxamine methacrylate iodide salt displaying positive charges. A 60% methylation extent was attained (100% for two methyl groups per poloxamine molecule), as determined by argentometric titration of the chloride salt (after dialysis). Accordingly, the methylated poloxamine derivative was a mixture of mono- and dimethylated species.

charges that improve the cell−matrix interaction and lead to enhanced cell compatibility, without the need for an apparently toxic compound such as MAETAC, and (b) unsaturated bonds susceptible to cross-linking by a free radical polymerization (as used previously by us,[1−3] Hubbell et al.,[9] and others[10,11]). The introduction of positively charged groups in the poloxamine backbone and the reduction of methacryloyl derivatives during the cross-linking were expected to improve the survival levels of encapsulated cells. We capitalized on the presence of two tertiary amine groups in the poloxamine molecule by methylating them with iodomethane and producing cationic groups (quaternary ammonium) in the central block of the poloxamine molecule. Then the positively charged polymer was reacted with a methacryloyl isocyanate derivative, rendering the bifunctional derivative.

The hydrogels were characterized by means of water uptake and viscoelastic properties (G' and G''). The behavior of human hepatoma HepG2 cells embedded in the matrix and endothelial cells grown on the surface of cross-linked methylated poloxamine methacrylate films was assessed. Then the study was extended to cylindrical modules with and without additional collagen. The cylindrical modules are relevant to a new modular tissue engineering strategy that was introduced by our group.[12,13] The modular strategy is aimed at overcoming nutrient diffusion constraints in large tissue-engineered scaffolds (which usually results in low cell viability over time). This approach requires a mechanically robust, cell-compatible matrix material to both encapsulate cells and act as a substrate for endothelial cells, and the material reported here was directed toward this goal.

Materials and Methods

Synthesis of Methylated Poloxamine Methacrylate. To methylate poloxamine, Tetronic T1107 (10.1 g, 0.67 mmol, a gift from BASF, New Jersey) was poured into a round-bottom flask, dried at 100 °C under vacuum for 2 h before use, and dissolved in 50 mL of methanol (Sigma, St. Louis, MO). Then 0.84 mL of iodomethane (1000% molar excess, Sigma) was added, and the reaction was allowed to continue for 24 h at room temperature. To obtain the iodide salt, the reaction mixture was precipitated in diethyl ether (BDH, Toronto, ON, Canada), filtered, and dried. For the replacement of iodide by chloride (to improve the cross-linking; see the Results), the reaction mixture was diluted with 100 mL of distilled water and the solution successively dialyzed (regenerated cellulose tubing, MWCO = 3500, Fisher Scientific, Nepean, ON, Canada) against deionized water (24 h), NaCl 0.5% (24 h), and distilled water (24 h) and finally freeze-dried (−50 °C). Afterward, the methylated poloxamine was reacted with isocyanatoethyl methacrylate (IEM; used as received, Sigma) at 70 °C in the presence of stannous(II) 2-ethylhexanoate (catalyst, Sigma) and hydroquinone (Sigma).[1,3] The structure of the product (methylated poloxamine methacrylate) is exemplified in Figure 1 for the iodide salt.

Characterization. ^{1}H nuclear magnetic resonance spectra were recorded in a Mercury 300 MHz NMR. Spectra were obtained at room temperature from 15 wt % $CDCl_3$ solutions (Sigma).

The degree of methylation was estimated from the concentration of chloride ions in the modified polymer (on the chloride salt after dialysis) measured by the argentometric method. The pH of methylated poloxamine methacrylate (chloride derivative) water solutions was adjusted to 7−10 with H_2SO_4 (0.1 M, Fisher Scientific, Nepean, ON, Canada) or NaOH (0.1 M, Aldrich). Then K_2CrO_4 indicator (1 mL, 5 wt %, Sigma) was added and the solution titrated with standard $AgNO_3$ solution (0.11 M, $AgNO_3$, Aldrich) to a pink-yellow end point. $AgNO_3$ titrant was previously standardized with NaCl standard solution (0.015 N, ACP, Montreal, QC, Canada). The degree of methylation was calculated from the equation

$$\text{degree of substitution (\%)} = \frac{(A - B)N(\text{MW})}{2m_{\text{sample}}} \times 100$$

where A is the $AgNO_3$ titration solution volume for the sample, B is the $AgNO_3$ titration solution volume for the blank (distilled water), N is the normality of $AgNO_3$, MW is the estimated molecular weight of methylated poloxamine methacrylate chloride derivative (15100), m_{sample} is the weight of the sample, and the "2" accounts for the two tertiary amine groups present in one poloxamine molecule.

Hydrogels. Methylated poloxamine methacrylate hydrogels were produced by mixing an aliquot of methylated poloxamine methacrylate (25 wt %) containing the photoinitiator (0.06−0.07% final concentration, (2-hydroxy-1-[4-(2-hydroxyethoxy)phenyl]-2-methylpropanone, Irgacure 2959, I2959, donated by Ciba, Tarrytown, NY, used as received) with phosphate buffer solution (PBS, pH 7.4, without calcium chloride and magnesium chloride, Gibco, Carlsbad, CA). The mixture was incubated (30 min, 37 °C) and cross-linked by exposure to UV light for 5−10 min (Spectroline EN-180 lamp, one 8 W 365 nm tube, with an intensity of 1.850 mW/cm^2) at 15 cm.[10,11] The different samples are named by the weight percent concentration of the polymer. After cross-linking, the hydrogels were washed thoroughly (3 × 10 min) with PBS and incubated with the corresponding medium. For collagen/methylated poloxamine methacrylate semi-IPN matrices, PBS was replaced with collagen type I solution (3.1 mg/mL, Vitrogen 100, Cohesion Technologies, Inc., Palo Alto, CA), following the previously described protocol.[1,2] The concentration of collagen in 7% and 9% methylated poloxamine matrixes was 0.20% and 0.17%, respectively. According to the preparation methodology, a homogeneous distribution of collagen within the matrix was expected.

To measure the change in water content, specimens were produced using 100 μL of precursor methylated poloxamine methacrylate solution in 96-well plates, cross-linking, and washing (as above). Then, the hydrogels were weighed and incubated in distilled water or PBS (Tissue Culture Medium Preparation facility, Faculty of Medicine, University of Toronto) at 37 °C for different periods of time. The Δ(swelling) was calculated from the equation

$$\Delta(\text{swelling}) = \frac{W_t - W_o}{W_o} \times 100$$

where W_t is the weight of the sample at a defined time and W_o is the initial weight of the cross-linked hydrogel including the water of the initial solution. The results are represented as means ± SD (n = 3).

For the rheological analysis, the gels (2 cm in diameter) were preincubated in PBS until equilibrium. A temperature-controlled rheometer (Carri-Med TA Instruments, New Castle, DE), using 2 cm diameter parallel plates, was used to determine the viscoelastic parameters (G' and G'') at varying oscillating stress and 1 Hz constant frequency.

Cells. HepG2 cells (American Tissue Culture Collection, ATCC, Rockville, MD) were maintained in RPMI 1640 medium (Gibco) supplemented with penicillin−streptomycin (2%, Gibco) and fetal bovine serum (FBS; 15%, HyClone, Logan, UT) at 37 °C and 5% CO_2. Human umbilical vein endothelial cells (HUVECs; Cambrex Corp., East Rutherford, NJ) were maintained in EBM-2 medium (Cambrex Corp.). Cells were harvested by trypsination, and the live cell number was determined via trypan blue and a hemocytometer.

For cell adhesion studies, 1 mL of methylated poloxamine methacrylate solution with the desired concentration was pipetted into 12-well plates, preincubated at 37 °C for 30 min, cross-linked by exposure to UV, and washed with PBS (3 × 10 min). Then 2 mL of the corresponding medium was added to each gel, and the samples were incubated at 37 °C (5% CO_2) for between 7 and 10 days (depending on the cell type). Finally, the medium was removed, and the cell suspensions (1×10^6 cells/well for HepG2s and 3×10^5 cells/well for HUVECs) were added to each well.

The viability of HUVECs was estimated using a LIVE/DEAD Viability Kit (calcein AM and ethidium homodimer-1 (EthD-1), Molecular Probes, Eugene, OR). Samples of hydrogels were incubated for 25 min at 37 °C with 300 μL of calcein AM (4 μM)/EthD-1 (4 μM). Cells were seeded on the surface of the matrix, incubated 1 day prior to the assay to allow cell attachment or death, and examined with a fluorescence microscope (Zeiss, Axiovert 135, Germany).

Vybrant CFDA SE (carbofluorescein diacetate succinimidyl ester; Molecular Probes) was used as a marker for HepG2 cells embedded in methylated poloxamine methacrylate specimens. Trypsinized cells were centrifuged, and the pellet was resuspended in 10 μM Vybrant CFDA SE solution in PBS (Gibco), incubated at 37 °C for 15 min, and centrifuged. The supernatant was removed, and the cells were resuspended in fresh medium, incubated for 30 min at 37 °C, and again resuspended in fresh medium. The polymer solutions (0.5 mL, collagen-free or collagen-containing methylated poloxamine methacrylate) were mixed with CFDA-labeled cells (~2×10^7 Pcells/mL) and the resulting solutions preincubated at 37 °C for 30 min, cross-linked (UV light, 5 min) in the lumen of a polyethylene tube (2 m long, 0.76 mm i.d., 1.22 mm o.d., Intramedic, Clay Adams, Becton Dickinson, Sparks, MD), and cut to form cylindrical modules (2 mm length) using an automatic tube cutter.[2,12,13] Then the tube pieces containing the hydrogel were suspended in the corresponding medium and shaken to release the modules. Samples containing positively charged derivatives were preincubated in serum-containing medium for 7−10 days (see the films). Finally, to seed cells on the surface, the modules were suspended in about 4 mL of HUVEC suspension ($(2-4) \times 10^6$ cells) and periodically (every 30 min) resuspended by shaking the suspension for about 2 h.

Results

Quaternary Ammonium Derivative. Methylation of the tertiary amine groups present in the poloxamine molecule with iodomethane yielded the methylated poloxamine derivative. Then the methylated poloxamine was reacted with methacryloyl isocyanate[1] to produce the methylated poloxamine methacrylate iodide salt (Figure 1). The degree of modification with methacrylate was calculated from the integration of the protons of the vinyl group at 5.62 ppm (double doublet, four protons) and 6.12 ppm (singlet, four protons) and the methyl group of the poly(propylene oxide) blocks at 1.14 ppm (multiplet, 233 protons).[1] The values obtained indicated degrees of substitution close to 100%. In preliminary swelling studies, the iodide derivative was used. However, due to limitations in the cross-linking process (see the next section), iodide was interchanged with chloride by dialysis. Thus, the extent of methylation was estimated from the chloride concentration (determined by argentometric titration) in the chloride salt and was around 60% of the maximum attainable (1.2 mol of chloride ion/mol of poloxamine); a maximum of 100% corresponds to methylation of both amine groups in each poloxamine molecule. Accordingly, the modified material was a mixture of mono- and dimethylated derivatives.

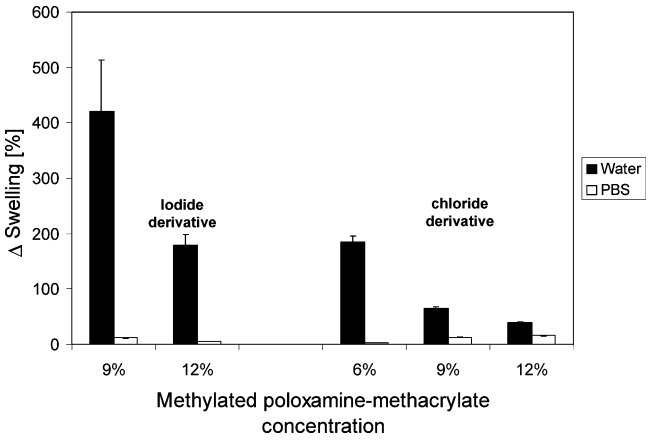

Figure 2. Change in swelling (%) in distilled water or PBS at 37 °C. The two sets of bars on the left give the data for 9% and 12% methylated poloxamine methacrylate matrixes prepared with iodide counterion. Lower water uptakes were seen with an increase in polymer concentration and therefore cross-link density. In addition, the decrease of the osmotic gradient in PBS resulted in a decrease in swelling. The three sets of bars on the right give the data for 6%, 9%, and 12% methylated poloxamine methacrylate matrices with chloride counterion. A higher cross-linking density was achieved by replacing iodide by chloride, as expressed by the decrease in changes in swelling. In PBS, only very slight changes were apparent. The data are given as means ± SD ($n = 3$).

Hydrogels. Initially, the iodide derivative was used, but the lowest concentration matrixes obtainable were around 9%. The gels were washed to remove unreacted material and immersed into distilled water or PBS, and the weight was monitored until equilibrium. The water uptake values were converted into changes in swelling: samples of modified poloxamine showed a sharp swelling. The reason for this behavior was the osmotic gradient resulting from the introduction of positive charges in the matrix.[3] For example, 9% and 12% matrices had swelling increases of 420% and 180%, respectively (Figure 2). As expected, the increase in polymer concentration and consequent increase in the cross-linking density resulted in lower swelling levels. The swelling of the gels was less marked in PBS (e.g., around 12% for a 9% hydrogel), indicative of the ionic nature of these gels (Figure 2). To optimize the cross-linking reaction and to produce matrices with lower polymer concentration than 9%, the iodide counterion was replaced with chloride by dialysis. Swelling of the chloride derivative performed in water and PBS showed a trend (Figure 2) similar to that seen with the iodide derivative, although there were differences in the cross-link density. For example, whereas a 12% matrix based on the iodide derivative had a swelling increase of 179% (in water), the counterpart containing chloride as counterion increased only 39%. Only the chloride derivative was considered for further studies.

The viscoelastic properties of the different matrixes were measured in a temperature-controlled rheometer using parallel plates for hydrogels containing between 6% and 18% polymer

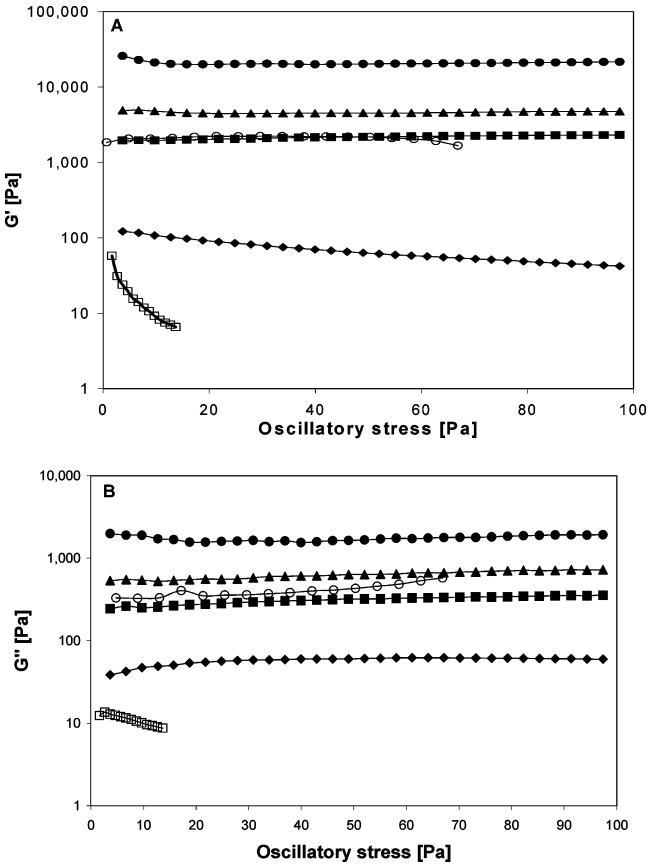

Figure 3. (A) Storage modulus (G') and (B) loss modulus (G'') of 6% (tilted squares), 9% (squares), 12% (triangles), and 18% (circles) methylated poloxamine methacrylate hydrogels. G' and G'' results of collagen (hollow squares) and 6.2% unmodified cross-linked poloxamine methacrylate (hollow circles) matrices (taken from ref 1) are included for comparison. The 6% methylated hydrogels showed inversion from a more elastic ($G' > G''$) to a more viscous ($G' < G''$) system, similar to that of collagen gel, though they appeared to be slightly stiffer and more stable at higher oscillatory stresses.

(Figure 3). The storage modulus (G') represents the elasticity of the system, while the loss modulus (G'') describes the viscous component. As expected, the increase in the polymer concentration led to denser networks and higher storage moduli. G' values increased from about the 2200 Pa level for a 9% matrix to 23000 Pa for 18% hydrogels (Figure 3A). Also the loss modulus increased with the concentration (Figure 3B). It is worth noting the low stiffness of 6% specimens, showing G' values that decreased from 120 to 45 Pa with the increase in oscillatory stress. The G'' of the 6% hydrogel steadily increased from 40 to about 60 Pa, undergoing a transition from a more elastic ($G' > G''$) to a more viscous ($G' < G''$) system at around 52 Pa of oscillatory stress.

Cells Embedded in Methylated Poloxamine Methacrylate Hydrogels. CFDA-labeled HepG2 cells were embedded in 7% and 9% methylated poloxamine methacrylate solutions before cross-linking and then subjected to photoinitiation within polyethylene tubing to produce modules.[2] Cells had higher survival levels (Figure 4A,B) than the previously reported poloxamine/MAETAC matrices.[3] However, the low viscosity of the precursor solution (at these concentrations) resulted in cell agglomeration during cross-linking, and a nonhomogeneous cell distribution was apparent.

Cell Attachment. The aim of methylation was to improve the cell adhesion characteristics relative to those of pure cross-linked poloxamine methacrylate hydrogels, while still allowing for high cell viability for embedded cells after encapsulation. The introduction of positive charges was expected to increase the direct electrostatic interactions of the matrix with the negatively charged cell membrane and also result in the adsorption of adhesion proteins present in the culture medium.[3] HepG2 cells seeded on methylated hydrogels adhered better than on the unmodified poloxamine methacrylate, as shown for a 9% hydrogel, 1 day after seeding (Figure 5); also, some spreading was apparent. Endothelial cells showed good attachment and a characteristic well-spread pattern on films, even 7 h after seeding (Figure 6A). The cells proliferated and formed an almost confluent monolayer within 1 day (Figure 6B). A live/dead cell assay revealed a high number of live cells (Figure 6C,D) and very few dead cells after 1 day (Figure 6E).

In contrast to the results on films, poor cell adhesion was apparent on methylated poloxamine methacrylate cylindrical rods (modules; see below). These findings were similar to those observed on the previously described collagen/poloxamine matrices: poor attachment on modules (Figure 7A) but good attachment to films.[1,2] MAETAC-modified materials preincubated in serum-containing medium behaved differently, having strong attachment and proliferation even on modules (Figure 7B), indicating that the adhesive interactions were strong enough even for the cylindrical rods. Methylated poloxamine was more like the collagen/poloxamine modules than the MAETAC modules, and only poor cell attachment (Figure 7C) was seen on modules.

Table 1 summarizes the advantages and disadvantages observed with the different modifications of poloxamine hydrogels that we have explored regarding cell attachment and embedding.[1−3] Due to the advantageous features of a collagen-containing network (in terms of cell distribution) and increased cell attachment on films by introducing collagen or positively charged groups in the matrix, the synergistic effect of collagen and methylated poloxamine was evaluated. A hybrid combining both modifications showed enhanced attachment and spreading of HUVECs even on a modular construct (Figures 7D,E), suggesting a synergistic behavior. Also, after encapsulation there was a better distribution and higher survival levels (fewer dead HepG2 cells) (Figure 8) with this combined material.

Discussion

Aiming at overcoming nutrient diffusion limitations in large tissue-engineered scaffolds, our group has introduced a new modular approach to tissue engineering.[12,13] The modular strategy requires a unique combination of highly stiff and cell-compatible matrix material to both encapsulate cells and act as a substrate for endothelial cell attachment on the surface. The use of unmodified poloxamine hydrogels to produce a modular construct with stiff modules, containing embedded cells and cells grown on the surface, is limited by the high content of PEO (~70%) that results in low protein and cell adhesion. Previously, we reported on the addition of positive charges as a strategy to enhance presumably both primary electrostatic cell−surface interactions and enhanced adsorption of proteins present in the culture medium.[3] In that example, the quaternary ammonium moieties were grafted in the matrix by co-cross-linking poloxamine methacrylate and MAETAC. These positively charged hydrogels displayed better cell adhesion properties on the surface of both films[3] and modules (Figure 7B). Due to the high concentration of methacrylates (from poloxamine methacrylate and MAETAC) during encapsulation and cross-linking, acute death of embedded HepG2 cells (after exposure to UV) was

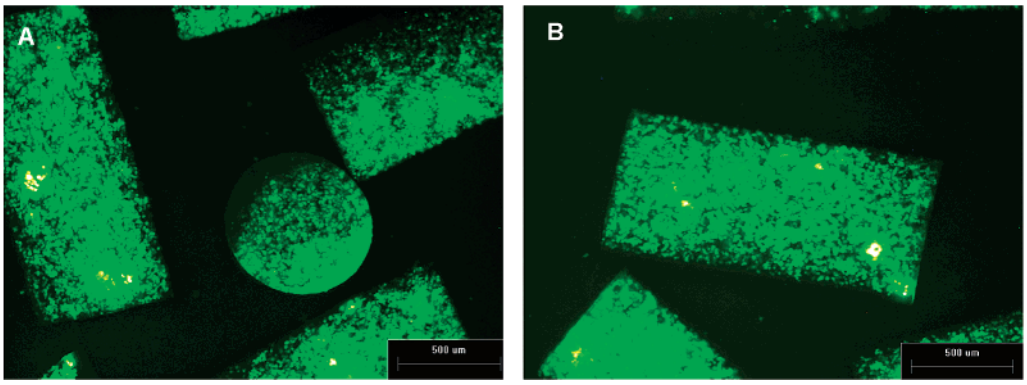

Figure 4. Fluorescent micrographs of fluorescently labeled (Vybrant CFDA SE, green) HepG2 cells (~2 × 10^7 cells/mL) embedded in 7% (A) and 9% (B) methylated poloxamine methacrylate modules, immediately after cross-linking. Due to the lower concentration of methacrylates a higher survival was attained relative to that of the previously reported MAETAC-containing gels.[3] The low viscosity of the methylated poloxamine methacrylate solution however led to a nonhomogeneous distribution. The scale bar is equal to 500 μm (magnification 25×).

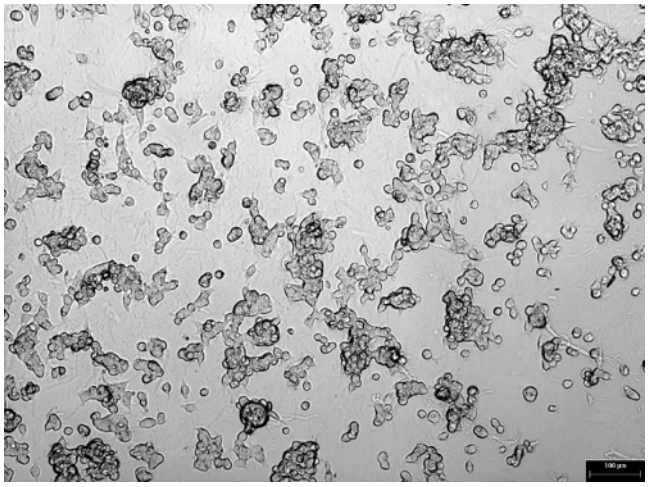

Figure 5. Micrograph of HepG2 cells seeded on a 9% poloxamine methacrylate matrix, at day 1. The film was preincubated in serum-containing medium for 1 week before seeding. The modification of the poloxamine by methylation, thereby introducing quaternary ammonium groups, resulted in enhanced attachment, compared to that of the cross-linked pure poloxamine matrix. The scale bar is equal to 100 μm (magnification 50×).

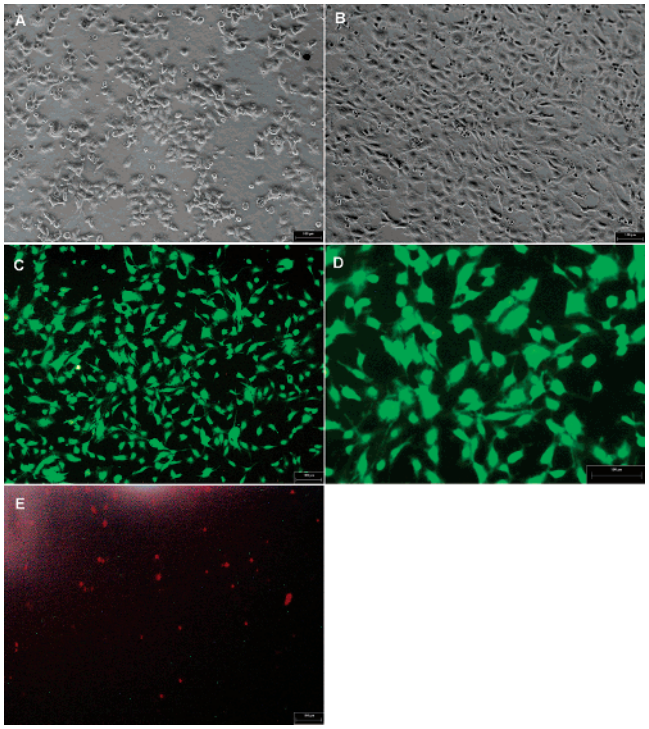

Figure 6. (A, B) Micrographs of HUVECs seeded on a 9% methylated poloxamine methacrylate matrix, after preincubation with 3% serum-containing medium for 10 days: (A) 7 h and (B) 1 day. A well-spread pattern was observed and a high surface coverage was achieved after 1 day. The scale bar is equal to 100 μm (magnification 50×). (C) Fluorescent micrograph of live (calcein AM) HUVECs seeded on top of a 9% methylated poloxamine methacrylate hydrogel, 1 day after seeding. The scale bar is equal to 100 μm (magnification 50×). (D) Same as C, higher magnification. The scale bar is equal to 100 μm (magnification 100×). (E) Fluorescent micrograph of dead (ethidium homodimer-1, red) HUVECs on top of a 9% methylated poloxamine methacrylate hydrogel (same field as micrograph D). The scale bar is equal to 100 μm (magnification 50×).

observed, making this modification inappropriate for cell encapsulation.

Following the idea of introducing cationic groups, the modification used in the present work exploits the methylation of tertiary amine groups in the central block of the poloxamine molecule and later reaction with a methacryloyl isocyanate[1,2] to render the positively charged matrix cross-linkable. Thus, the elimination of reactive precursors such as MAETAC used in the earlier work was expected to lead to higher cytocompatibility.

When using the iodide derivative, the minimal polymer concentration to attain cross-linked hydrogels was around 9%. At lower concentrations there was insufficient photoinitiator available to compensate for the quenching of the free radical polymerization by the iodide.[14] To achieve higher cross-linking extents and produce matrices containing lower polymer concentrations than 9% (and further reduce the cytotoxicity due to high methacrylate concentrations), iodide was replaced by chloride. In this way, hydrogels containing 6% methylated poloxamine methacrylate were attainable.

Since the matrices are intended for the production of constructs for tissue engineering, dimensional and shape changes due to water uptake or syneresis is relevant. Cross-linked pure PEO−PPO hydrogels tend to shrink upon heating at 37 °C due to the rearrangement of PPO blocks in the molecule, even at concentrations lower than the minimal concentration needed for gelation.[15] However, the presence of positive charges in the cross-linked poloxamine network and the subsequent increase in the osmotic gradient between the cross-linked hydrogel and the surrounding aqueous environment affected this behavior.[3] Swelling tests in water (at 37 °C) showed high water uptake values at 37 °C (Figure 2). In contrast, studies in PBS resulted only in a mild swelling (below 20%). This was in accordance

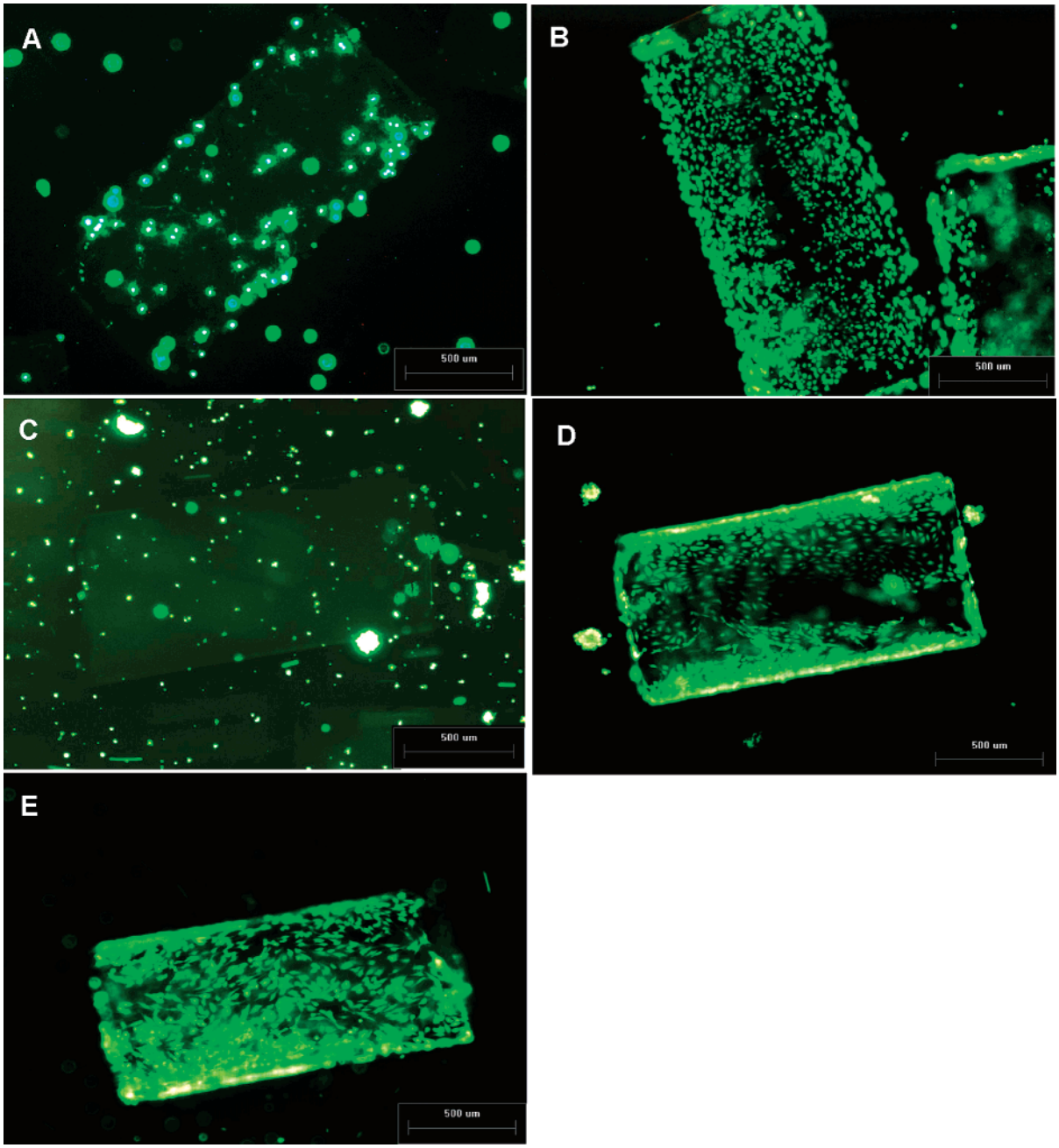

Figure 7. Fluorescent micrographs of live (calcein AM) HUVECs seeded on modules, after 1 day: (A) collagen-containing 8% poloxamine methacrylate, (B) 9%/0.48 M poloxamine methacrylate/MAETAC, (C) 9% methylated poloxamine methacrylate, (D) collagen-containing 7% methylated poloxamine methacrylate, and (E) collagen-containing 9% methylated poloxamine methacrylate modules. Whereas HUVEC poorly attached to unmethylated collagen-containing poloxamine and methylated collagen-free modules (cells detached from the matrix during the assay), improved adhesion was apparent on MAETAC-modified and hybrid modules (collagen/methylated poloxamine methacrylate). The scale bar is equal to 500 μm (magnification 25×).

with a decrease in the osmotic force due to the presence of salts and agreed with results previously reported.[3]

The viscoelastic parameters (G' and G'') were measured to determine the influence of the modification on the mechanical profile of the hydrogels. G' and G'' values were expected to be greater as the polymer concentration increased, and this was observed; the 6% samples were very soft. The rheological behavior was similar to that of collagen gel,[1] though the methylated poloxamine methacrylate matrix appeared to be slightly stiffer and more stable at higher oscillatory stresses. In addition, results suggested that lower degrees of cross-linking were attained in comparison with those of poloxamine methacrylate matrices (without methylation), as expressed by the lower G' displayed by the methylated network as compared with unmodified poloxamine,[3] for similar concentrations. It is important to note, though, that the 6.2% concentration of unmodified poloxamine is the result of an original 4% hydrogel that shrank at 37 °C, leading to an increase of the polymer concentration. In contrast, a 9% methylated poloxamine matrix swelled by about 13%, leading to a decrease of the polymer concentration to 7.8%.

Cell Compatibility. As previously reported, HepG2 and HUVECs did not attach well to pure poloxamine methacrylate gels, consistent with the PEO-rich nature of the material.[1−3] The enhancement of cell adhesion to a poloxamine-based biomaterial, through incorporation of cationic groups in the matrix, was explored.[3] While this modification increased cell attachment to the surface of hydrogel films and modules, the

Table 1. Advantages and Disadvantages of Different Modifications Intended by Our Group To Improve the Cytocompatibility of Poloxamine Hydrogels for a Modular Construct

matrix	description	advantages	disadvantages
collagen/poloxamine methacrylate	collagen/poloxamine methacrylate hydrogel (semi-IPN) produced by cross-linking collagen-containing poloxamine methacrylate mixtures (see refs 1 and 2)	good cell distribution, high viability of embedded cells, -good EC cell attachment on films	poor EC attachment on modules
poloxamine methacrylate/MAETAC	positively charged poloxamine methacrylate matrix obtained by copolymerization of a methacryloyl quaternary ammonium derivative (MAETAC) and poloxamine methacrylate (see ref 3)	good cell attachment on films and modules	poor cell distribution, low viability of embedded cells after cross-linking
methylated poloxamine methacrylate	positively charged poloxamine methacrylate hydrogel produced by methylation of tertiary amine groups of poloxamine	good EC cell attachment on films, higher viability of embedded cells than that of MAETAC derivatives after cross-linking	poor cell distribution, poor EC attachment on modules

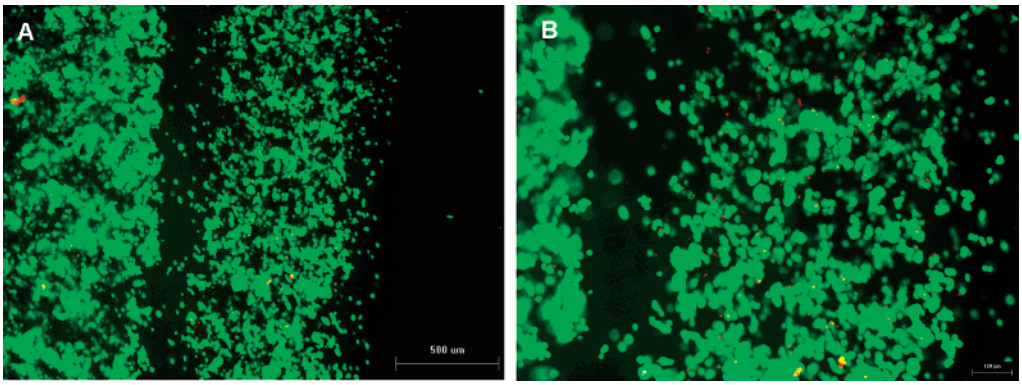

Figure 8. (A, B) Fluorescent micrographs of live (calcein AM) and dead (ethidium homodimer-1, red) HepG2 cells (~2×10^7 cells/mL) embedded in 7% methylated poloxamine methacrylate/collagen modules, immediately after cross-linking. Higher viability levels were attained than with MAETAC gels.[3] The scale bar is equal to (A) 500 μm (magnification 25×) and (B) 100 μm (magnification ×50).

high concentration of methacrylate derivatives coming from both the poloxamine methacrylate and the quaternary ammonium modifier (MAETAC) compromised the ability to encapsulate cells within the matrix by photo-cross-linking.

The present strategy was directed to reduce the concentration of free radicals (due to high methacrylate concentrations) by replacing the quaternary ammonium groups from MAETAC by positive groups introduced in the poloxamine backbone. Thus, in this modification, methacrylate moieties were only coming from poloxamine methacrylate and the reduction of these reactive precursors (due to the absence of quaternary ammonium methacrylate) was expected to improve the cytocompatibility of the encapsulation.

In contrast to the MAETAC approach, where the ratio between polymer preventing cell adhesion (PEO) and groups enabling cell adhesion (quaternary ammonium) could be adjusted, here we could not increase the cationic group concentration (to get higher adhesion) without altering the PEO content to the same extent. Thus, a lower density of positive charges in the matrix was attained in the method reported here, and this was expected to limit the extent of cell attachment that could be obtained relative to that of the MAETAC approach. On the other hand, we envisioned higher compatibility for the embedded cells because of the absence of high concentrations of reactive methacrylates. It is worth noting that the concentration of methylated poloxamine methacrylate (and consequently of methacrylate) was similar to levels that had already showed acceptable viability levels for HepG2 cells.[1,2]

Embedded HepG2 cells exposed to cross-linking had a higher viability after cross-linking than was seen with poloxamine/MAETAC materials (Figures 4A,B). While HepG2 did not attach on pure poloxamine films,[3] the modified matrix showed better adhesion (Figure 5). HUVECs adhered and had both high viabilities and good surface coverage (Figure 6). These findings supported the enhanced properties of the methylated matrix at least on films, where gravity enhances the appearance of attachment.

It is worth noting that the concentration of positive charges in the methylated poloxamine was lower than that determined as optimal in the earlier poloxamine/MAETAC system and lower cell attachment extents were expected and were so observed. Moreover, attachment to the methylated poloxamine depended on the geometry of the structure, and low attachment extents were observed on modules (Figure 7C), following a behavior similar to that on collagen-containing poloxamine hydrogels (Figure 7A). This difference probably reflects the effects of gravity and differences in the seeding process. On films, a static process is used and cells in suspension settle on the hydrogel surface (remaining on the surface, to some extent, regardless of the attachment properties of the surface). Even on nonadhesive surfaces such as pure poloxamine methacrylate samples, one can appear to have very loosely "attached" cells. Changes in surface chemistry and adhesion characteristics (e.g., by addition of collagen) are sufficient to get some attachment on films, but these adhesive forces appear to be inadequate for cylindrical modules. On modules (due to the small dimensions of the hydrogel rods), dynamic seeding is used and only a few cells are capable of attaching to the matrix (probably because of still insufficient attachment properties), resulting in very low cell density.[3,4]

To study a potential synergistic effect that could lead to improved attachment on modules while maintaining low cytotoxicity levels, a material combining the collagen and methylated poloxamine methacrylate was prepared. Endothelial cells attached and proliferated on modules, indicating the synergistic effect of the hybrid (Figures 7C,D), while embedded cells exposed to the cross-linking process had a high viability and better distribution than collagen-free samples, supporting the better cytocompatibility (Figure 8). This combination presented the advantages of collagen and methylation.

Conclusions

Four-arm PEO−PPO derivatives were modified with positively charged groups by methylating the tertiary amine groups present in the central block of the molecule. The modification resulted in good cell adhesion properties on the surface of films, while these hydrogels were more suitable for cell encapsulation than those of the previous MAETAC approach. Hence, for films, we have achieved our goal of preparing a stiffer, synthetic material that has the cell compatibility of collagen with respect to both cell attachment and cell encapsulation. On the other hand, there was still room to improve the material because cell attachment to cylindrical modules was limited. To address this problem and to improve the uniformity of distribution of cells within the new material, we combined the methylated material with collagen. This collagen/methylated poloxamine methacrylate system displayed a synergistic effect that resulted in good attachment of HUVECs to modules and better distribution profiles for embedded cells after cross-linking.

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