Published in final edited form as:

Biomaterials. 2009 September ; 30(27): 4558–4566. doi:10.1016/j.biomaterials.2009.05.029.

Cytocompatibility evaluation of amphiphilic, thermally responsive and chemically crosslinkable macromers for *in situ* forming hydrogels

Leda Klouda, Michael C. Hacker, James D. Kretlow, and Antonios G. Mikos*

Department of Bioengineering, Rice University, MS-142, P.O. Box 1892, Houston, Texas 77251-1892, USA

Abstract

The cytocompatibility of amphiphilic, thermoresponsive and chemically crosslinkable macromers was examined *in vitro*. Macromers synthesized from pentaerythritol diacrylate monostearate, *N*-isopropylacrylamide, acrylamide and hydroxyethyl acrylate in different molar ratios and with varying molecular weights and lower critical solution temperatures were evaluated for cytocompatibility with rat fibroblasts. Cell viabilities of over 60% percent for all and over 80% for most formulations were observed after 24-h incubation with macromers with molecular weights in the range of approximately 1500 to 3000 Da. The chemical modification of the macromers with a (meth)acrylate group was shown to have a time- and dose-dependent effect on cell viability. Uncrosslinked macromers with lower degrees of (meth)acrylation allowed for cell viability of over 60% for up to 6 h. (Meth)acrylated macromers with lower critical solution temperature (LCST) closer to physiological temperature allowed for higher cell viabilities as opposed to those with lower LCST. The data suggest that when the (meth)acrylated macromers are assembled into a physical gel, their cytotoxicity is diminished. After gel phase separation, cytotoxicity increased. This study gives information on the parameters that enable viable cell encapsulation for *in situ* forming hydrogel systems.

Introduction

Injectable, *in situ* forming materials represent an attractive option for cellular delivery in tissue engineering. Hydrogels in particular are excellent extracellular matrix equivalents due to their highly hydrated nature [1] and can be delivered in liquid form and then solidify *in situ*. It is highly important that the solidification takes place in a clinically relevant time frame so that the material is localized at the point of interest. The solidification mechanism should be selected as not to cause necrosis to surrounding tissue by excessive heat formation and to be tolerated by encapsulated cells and/or any potentially sensitive molecules that are encapsulated for delivery. Moreover, fast solidification allows for homogeneous cell dispersion within the hydrogel matrix [2-5]. Thermally responsive hydrogels, materials that solidify upon temperature change, form *in situ* in a mild and fast manner, but they often possess insufficient mechanical properties and stability [6]. To circumvent this problem, thermogelling polymers have been modified by the addition of reactive groups that allow for covalent crosslinking. These polymers exhibit physical gelation triggered by temperature increase, followed by an

* Corresponding author. Phone: +1-713-348-5355. Fax: +1-713-348-4244. E-mail: mikos@rice.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

irreversible chemical crosslinking after a Michael-type addition [7-9] or a photoinitiated polymerization reaction [10].

Our group has recently proposed a type of thermally responsive, chemically crosslinkable macromer for the fabrication of *in situ* forming hydrogels based on *N*-isopropylacrylamide [11]. These macromers employ a hydrophobic core molecule that increases cohesive interactions of polymeric chains, augmenting the mechanical stability of the resulting hydrogel. The addition of a methacrylate or acrylate group enables chemical crosslinking with the use of a biocompatible, water-soluble thermal initiator system. The macromers are designed to exhibit a rapid thermal gelation at temperatures slightly below physiological temperature, followed by a slower chemical crosslinking *in situ*. The advantage of this approach is that fast solidification is achieved in a mild process through thermal gelation, with the subsequent slow chemical crosslinking not requiring high amounts of initiator. The rate of the chemical crosslinking reaction is dependent on the amount of initiator used, and it has been shown that thermal initiator systems are less cytocompatible above a certain concentration [12-14].

The macromers are synthesized from pentaerythritol diacrylate monostearate, a bifunctional monomer containing a natural fatty acid as hydrophobic chain, *N*-isopropylacrylamide, which provides thermoresponsive properties, acrylamide, a hydrophilic monomer, and hydroxyethyl acrylate, a hydrophilic monomer which provides hydroxyl groups for further chemical modification [11]. The lower critical solution temperature (LCST) of macromer solutions and the resultant gel stability are shown to be a function of the comonomer ratios in the composition and the amphiphilicity of the macromers. As a further step, the modification of the macromers with the addition of a (meth)acrylate group allows for covalent crosslinking of gels with higher stability as compared to physically gelled, uncrosslinked controls.

The scope of this study is to evaluate the *in vitro* cytocompatibility of this type of macromer. Specifically, properties of unmodified macromers such as the molecular weight, the composition and the transition temperature are assessed for their effect on cytocompatibility. After chemical modification, (meth)acrylated macromers are evaluated to determine the optimal variables for viable cell delivery. The degree of modification, the concentration and the effects of acrylation versus methacrylation on cell viability are examined. Furthermore, cell viability is examined at intervals over 24 h in order to determine the length of exposure to (meth)acrylated macromers tolerable by cells.

Materials and Methods

Unmodified thermogelling macromers

Amphiphilic, water-soluble thermogelling macromers (TGMs) were synthesized from pentaerythritol monostearate diacrylate (PEDAS), *N*-isopropylacrylamide (NiPAAm) and varying contents of acrylamide (AAm) and 2-hydroxyethyl acrylate (HEA) via a free radical polymerization reaction as previously described [11]. The macromers were subsequently purified by precipitation in diethyl ether and were characterized by gel permeation chromatography (GPC), proton nuclear magnetic resonance spectroscopy ($^1\text{H-NMR}$), rheology and differential scanning calorimetry (DSC) [11]. Table 1 summarizes the composition of the macromers evaluated in the study, denoted by their molar feed composition, and their respective properties. The number-average molecular weights (M_n) and polydispersity indices (PI) were obtained by GPC for $n=3$. The lower critical solution temperature (T_η) had been previously determined rheologically as the inflection point of the temperature-complex viscosity curve ($n=3$). The structure of the TGMs is illustrated in Figure 1.

Cell culture

A rat fibroblast cell line (ATCC, CRL-1764) was cultured on T-75 flasks using Dulbecco's modified Eagle medium (DMEM; Gibco Life, Grand Island, NY) supplemented with 10 % (v/v) fetal bovine serum (FBS; Cambrex BioScience, Walkersville, MD) and 1% (v/v) antibiotics containing penicillin, streptomycin and amphotericin (Gibco Life). Cells were cultured in a humidified incubator at 37 °C and 5% CO₂. Cells of passage numbers 7-9 were used in this study.

Cytocompatibility assays of unmodified thermogelling macromers

Leachables assay—Solutions of thermogelling macromers were prepared at a 10% (w/v) concentration in cell culture media (DMEM, supplemented with antibiotics) without the addition of serum. The solutions were left on a shaker table overnight to dissolve. In the case of macromer solutions with LCST below room temperature, the solutions were left to dissolve at a temperature of 4 °C. After dissolution, macromer solutions were incubated at 37 °C for 24 h. During this time interval, the solutions first gel and subsequently phase-separate and collapse. After 24 h in the incubator, the supernatant of the macromer solutions was aspirated, sterile-filtered through a Nalgene 0.2 µm filter (Nalge, Rochester, NY) and diluted 10 times in cell culture media. Cells were seeded on a 96-well plate at a density of 28,300 cells/cm² using a working volume of 100 µL cell suspension per well. The cells were incubated for 24 hours to achieve 80-90% confluence and were then exposed to 100 µL of the solution supernatant. Two experimental groups were used, one without and one with 10-fold dilution (n=5). Following 24 h of incubation, the macromer-conditioned media were removed, and the cells were rinsed three times with phosphate-buffered saline (PBS, Gibco), followed by the addition of calcein AM and ethidium homodimer-1 in 2 µM and 4 µM concentrations in PBS, respectively (Live/Dead viability/cytotoxicity kit, Molecular Probes, Eugene, OR). The cells were incubated with the Live/Dead reagents for 30 min in the dark at room temperature. Cell viability was quantified using a fluorescence plate reader (Biotek Instrument FLx800, Winooski, VT) equipped with filter sets of 485/528 nm (excitation/emission) for calcein AM (live cells) and 528/620 nm (excitation/emission) for EthD-1 (dead cells). Untreated cells that were cultured with primary media only served as the positive (live) control, whereas cells exposed to 70% ethanol for 15 min served as the negative (dead) control. The fluorescence of the cell populations was recorded and the fractions of live and dead cells were calculated relatively to the controls as previously described [14]. Following quantification, images of the cells were obtained with a fluorescence microscope (LSM 510 META, Carl Zeiss, Germany).

Direct contact assay—The macromers were vacuum-dried and sterilized under UV irradiation for 3 h. Macromer solutions were prepared in 10% (w/v) concentration in DMEM without FBS as described above. Cells were seeded on 48-well plates at a seeding density of 28,300 cells/cm² with a working volume of 500 µL cell suspension per well. After 24-h incubation, the media were aspirated and 150 µL of macromer solution was added to the cells (n=4-5). This quantity was calculated so that after gelation, a thin gel film would form above the cells. After 30-minute incubation at 37 °C to ensure gel formation, 300 µL DMEM without FBS was carefully added on top of the gel and the cells were incubated with the macromers for 24 h. Following this incubation period, the gel pellet was carefully removed with tweezers at room temperature, and cells were rinsed three times with PBS before adding the Live/Dead reagents and evaluating cell viability as described above.

Modified (acrylated and methacrylated) thermogelling macromers

In order to add chemically crosslinkable domains, the purified macromers were modified with the addition of an acrylate or methacrylate group through a reaction between the hydroxyl group of 2-hydroxyethyl acrylate (HEA) and (meth)acryloyl chloride. The hydroxyl group on

PEDAS was not modified during this reaction. The reaction, purification and characterization processes were performed as previously described by Hacker et al. [11]. The structure of modified macromers can be seen in Figure 1. Macromers with varying acrylate or methacrylate group contents were synthesized by feeding different volumes of acryloyl chloride or methacryloyl chloride respectively. Due to the lower reactivity of methacryloyl chloride, different hydroxyl conversions were observed in comparison to formulations that were supplied with an equivalent amount of acryloyl chloride. Table 2 denotes the composition of the modified TGMs and the conversion of the hydroxyls to (meth)acrylate groups as determined by $^1\text{H-NMR}$. The thermal transition temperatures are also included in Table 2, as the substitution of the hydroxyl group of HEA by the more hydrophobic (meth)acrylate moieties has an effect on the lower critical solution temperature of these macromers. LCST was previously obtained by differential scanning calorimetry for $n=3$.

Cytocompatibility of modified (acrylated and methacrylated) thermogelling macromers

The macromers were vacuum-dried and sterilized under UV irradiation for 3 h. Macromer solutions were prepared in 10, 1 and 0.1% (w/v) concentrations in DMEM without FBS as described above, which will be denoted throughout the text also as 100, 10 and 1 mg/mL respectively. Cells were seeded on 96-well plates at a seeding density of 28,300 cells/cm². After 24-h incubation, the media were aspirated. For the 1 and 0.1% solutions, 100 μL of macromer solution was added to the cells. These concentrations showed minimal gel formation at 37 °C and therefore the addition of media for nutrient supply was not required. For the 10% solutions, 40 μL of macromer solution was applied, followed by 30-min incubation at 37 °C to ensure gel formation, and 60 μL DMEM without FBS was carefully added on top of the gel. For all groups, a sample size of five was used ($n=5$). Cell viability was evaluated after 2, 6 and 24-hour incubation using the Live/Dead assay as described above.

Osmolality measurements

The osmolality of the solutions was measured with an Osmette automatic osmometer (Precision Systems, Natick, MA). In order to create a standard curve for cell viability versus osmolality of solution, the osmolalities of dextran (average molecular weight 68.8 kDa, Sigma, St. Louis, MO) solutions in concentrations from 0 to 30% (w/v) in DMEM were measured. Cells were then incubated with dextran solutions in that concentration range for 24 h, and their viability was recorded. Dextran was chosen because it is a known biocompatible polysaccharide and thus in case any toxic effects were observed, they would be due to changes in osmolality of the solution and not molecular composition.

The osmolality of the 10% (w/v) macromer solutions as used in the study was measured. Moreover, the osmolality of the supernatant solutions was measured. The supernatant was obtained after incubating the macromer solutions for 24 h at 37 °C and following gel phase separation in order to simulate the properties of the solutions to which cells are exposed during the direct contact assay.

Statistical analysis

Statistical analysis of the data was performed with a single-factor analysis of variance (ANOVA) with a 95% confidence interval ($p<0.05$). In the case of statistically significant differences, Tukey's post hoc test was conducted. Data are expressed as mean $\pm$ standard deviation.

Results

Effect of molecular weight on unmodified macromer cytocompatibility

Viability of over 80% was observed for cells incubated with the supernatant of hydrogels composed of macromers with number-average weights ranging from 1750 to 2830 Da and polydispersity indices from 2.9 to 3.6 (Figure 2A). In this experimental group, no statistically significant differences were found between undiluted and 10-fold diluted samples. For the direct contact assay, viabilities of above 80% were found for the lower molecular weight groups (Figure 2B). The higher molecular weight group showed cell viability of $69 \pm 3\%$.

Effect of composition on unmodified macromer cytocompatibility

Cells incubated with hydrogel supernatant showed high viabilities (above 75%) for all but one formulation, with a decrease in live cells with increasing acrylamide content in the composition (Figure 3A). The formulation with the highest acrylamide content in the composition (20%) and no 2-hydroxyethyl acrylate showed lower viability values of $55 \pm 2\%$. No statistically significant difference was noted between undiluted and 10-fold diluted supernatant. When cells were exposed to the thermogelling macromers in a direct contact assay, cell survival was above 80% (Figure 3B).

Effect of LCST on unmodified macromer cytocompatibility

Among the formulations tested, lower cell viability was observed for the formulations with transition temperatures of 38.7 and 40.8 °C, both in the leachables ($55 \pm 2\%$ and $61 \pm 5\%$ respectively) as well as in the direct contact assay ($81 \pm 19\%$ and $60 \pm 22\%$) (Figure 4). Macromer solutions with lower or higher transition temperatures than the aforementioned showed viabilities of 70% or more for their leachable components and the direct contact assay. Characteristic fluorescence microscopy images of the cells after incubation with these macromers can be seen in Figure 5. High densities of live cells (stained green) with morphology similar to the positive control were observed for most formulations, and no obvious damage to cells that were in direct contact with the hydrogel was noted. The composition with an LCST of 40.8 °C (molecular composition PEDAS₁NiPAAm₁₆AAm₄) showed an increased number of dead cells (stained red).

Effect of macromer solution osmolality on cytocompatibility

Cell viability showed a decreasing trend after 24-h incubation with increasing osmolalities of dextran solutions in various concentrations; differences, however, were not statistically significant (Figure 6). For a solution osmolality of 451 mOsm/kg, cell viability was $85 \pm 7\%$. Thermogelling macromer solutions in 1% (w/v) concentration in cell culture media showed similar osmolalities to that of DMEM (330 mOsm/kg). Solutions of 10% (w/v) concentration had osmolalities on the order of 406–462 mOsm/kg. The supernatant of gels after 24-h incubation at 37 °C and full phase separation showed even lower values (340–408 mOsm/kg).

Cytocompatibility of (meth)acrylated thermogelling macromers

Effect of macromer concentration—At the 2 h time interval, most formulations had similar cell viabilities in 1, 10 and 100 mg/mL concentrations or showed trends of decreasing viability (Figure 7). A formulation with a 32% degree of acrylation showed values of 0.95 ± 0.09 , 0.71 ± 0.08 and 0.68 ± 0.10 at 1, 10 and 100 mg/mL respectively. A formulation with a 49% acrylation had cell viabilities of 1.23 ± 0.06 , 0.99 ± 0.05 and 0.12 ± 0.01 at the above concentrations. The effect of concentration was even more pronounced with decreasing trends in viability at the 6- and 24-h intervals. At 24 h, the cell viabilities for the 32% acrylated formulation decreased from 0.75 ± 0.02 at the 1 mg/mL concentration to 0.16 ± 0.05 at 100 mg/mL.

Effect of time—In addition to the aforementioned results, the effect of incubation time on cell viability can be seen in Figure 8 for a concentration of 100 mg/mL. An unmodified composition showed a statistically significant decrease in viability at 6 h. The fraction of live cells at 24 h was 0.61 ± 0.05 , which did not change from the viability at 6 h. For all acrylated formulations, a statistically significant decrease in viability was observed after 24 h. At 6 h, the compositions with lower degrees of acrylation showed cell viabilities of 60% and higher. A composition with low methacrylation also showed a statistically significant decrease in cytocompatibility at 24 h, whereas the macromers with higher methacrylation exhibited high toxicity as soon as 6 h after incubation.

Effect of modification—The macromers were rendered less cytocompatible with increasing degrees of modification. Figure 9 depicts cell viability after 24-h incubation with 100 mg/mL macromer solutions. Unmodified macromers showed a viability of $61 \pm 5\%$, which was very similar for macromers with 8% acrylation. For macromers with higher acrylation degrees of 32% and 49%, only $16 \pm 6\%$ and $2 \pm 1\%$ of the cells respectively were viable after 24 h. Methacrylation seemed to be less tolerable by the cells from these results. After 24-h incubation, cell viability was only $3 \pm 1\%$ for both formulations, which had methacrylation degrees of 10% and 29%, respectively.

Discussion

The effects of molecular weight, monomer ratios in the composition and solution thermal transition temperature were the parameters investigated for their effect on macromer cytocompatibility. A second set of studies examines the cytocompatibility of the macromers after the addition of a reactive (meth)acrylate group over time and for different compositions and concentrations. Although these macromers can be physically gelled and chemically crosslinked, all experiments were performed with the non-crosslinked macromers to evaluate toxicity of the reactive groups and macromer composition, mainly hydrophilic-hydrophobic balance.

Cytocompatibility of unmodified thermogelling macromers

Macromer molecular weight is a factor that influences the compatibility of hydrogels with cells. Low-molecular weight oligomers are often associated with toxicity issues in hydrogels [1]. Generally, hydrogels for cell encapsulation are fabricated with macromers of molecular weights above 3000 Da [15], as they otherwise may enter the cell. No adverse effects of thermogelling macromers with the same composition but various molecular weights, ranging from 1750 to 2830 Da, were observed on cell viability. Cell viability was over 80% for most formulations, both in the leachables as well as in the direct contact assay.

As for the macromer composition, it had been shown [11] that increasing acrylamide contents correlates with higher transition temperatures of the macromer solutions (Table 1). Therefore, whether the decreasing cytocompatibility observed with increasing acrylamide in the formulation was an effect of LCST rather than composition had to be evaluated. Macromers with transition temperatures higher than the incubation temperature stay in solution or show minimal gel formation as opposed to those with transition temperatures close to physiological temperature which gel rapidly at 37 °C. The experimental data showed a decrease in cell viability for the macromer solutions with transition temperatures close to physiological. Interestingly, a macromer solution with a transition temperature of 43.5 °C, which did not gel after 24-h incubation at 37 °C, allowed for cell viability of over 70%, whereas solutions with transition temperatures significantly lower than physiological temperature showed similar cell survival values. The differences in the relative amphiphilicity may explain these observations. The lower critical phase separation phenomenon is imparted by hydrophobic interactions

between macromolecular chains [16]. For macromers with an LCST below the incubation temperature of 37°C, associations between polymer chains were more pronounced, leading to gel formation. On the other hand, an increase in the hydrophilicity of the macromers facilitated polymer-water interactions, not allowing gel formation at this temperature. This hydrophilic structure, as suggested by the viability data, caused minimal interactions with the cells. The macromers with transition temperatures around physiological temperature exhibited such a hydrophilic/hydrophobic balance at 37°C that might have caused increasing interactions with the cell membrane and could explain the lower cell viability values observed.

Another possible source of toxicity evaluated, solution osmolality, does not seem to be a contributing factor in these studies. Cells would be first exposed to the macromer solution until the gelation takes place and later to the supernatant solution after gel syneresis. Even though high osmotic stress has been shown to suppress growth [17] and induce apoptosis [18] in mammalian cells, the osmolality of solutions was within the range tolerable by cells as suggested the dextran osmolality-cell viability curve (Figure 6). This is in accordance with the observations of Zhu et al.[19], where viable density of CHO cells showed an 8% decrease for a solution osmolality of 460-500 mOsm/kg.

Cytocompatibility of modified thermogelling macromers

Some of the macromer solutions were too viscous for sterilization by filtration. Therefore, the macromers were dried in a vacuum oven and sterilized under UV irradiation for 3 h. The presence of the olefinic protons was confirmed by ¹H-NMR, indicating that after UV irradiation the structure of the (meth)acrylated macromers was preserved (data not shown). The macromers selected for (meth)acrylation were chosen based on their amphiphilic character and lower critical solution properties, and their molecular composition prior to modification was found to be cytocompatible. The parameters tested in this study were concentration, time, degree of modification and modification type (acrylation or methacrylation) on their effect on cytocompatibility. It is valuable to obtain correlations between cell viability, concentration and chemical structure of the macromers as a function of time if the hydrogel is used for cell encapsulation, as cells will be in direct contact with the macromer chains until the crosslinking of the hydrogel is completed [13].

Exposure to (meth)acrylated macromers was shown to have a dose-dependent effect on cell viability (Figure 7). This effect was more pronounced with higher modification and after longer exposure. The cytotoxicity of hydrogel-forming macromers is often reported in concentrations of up to 1 mg/mL in cell culture media [20,21]. We have opted to evaluate higher concentrations relevant for hydrogel fabrication. In this study, when the direct contact assay was performed, cell culture medium was added on top of the hydrogel layer after its solidification. In the course of the 24-h incubation, the hydrogel was phase-separated, which means that the addition of medium diluted down the supernatant solution. This step was however required to ensure nutrient delivery to the cells. Solutions of lower concentrations did not show significant gel formation, and no additional media were added. The cytotoxicity of the macromers showed also a time-dependent behavior (Figure 8). Acrylated macromers all showed statistically significant higher cytotoxicity at the 24 h time interval compared to 2 and 6-h exposure. The same correlation was observed for the methacrylated macromers with lower modification. Macromers with a higher degree of methacrylation showed significantly higher toxicity already after 6-h incubation compared to 2 h. This study seems to be a good indicator for determining the kinetics of the crosslinking reaction by establishing the tolerable time of exposure of cells to the (meth)acrylated macromers. It represents the worst-case scenario, since within the time frame examined, chains would have already started to crosslink with the addition of an initiator system. After crosslinking is completed, the detrimental effects of the (meth)acrylate end groups should be eliminated. The presence of reactive end groups, such as the (meth)acrylate

groups, contributes to the toxicity of materials. Increasing degrees of modification drastically reduce cell viability, as can be seen in Figure 9.

To the best of our knowledge, there are only a few studies that investigate the cytocompatibility of (meth)acrylated, uncrosslinked macromers. Crosslinking molecules such as poly(ethylene glycol) diacrylate (PEG-DA-575 Da) [13] and hydrophobic propylene fumarate diacrylate [23] were found to be very toxic on cells, whereas higher molecular weight macromers such as dextran derivatized with methacrylate- and hydroxyethyl methacrylate groups [22] and PEG-DA (molecular weight 3.4 kDa) [13] showed more cytocompatible results. Many studies have been carried out to elucidate the cytotoxic effects of acrylic and methacrylic monomers. In general, acrylic monomers have been reported to be more toxic than their methacrylic analogues [24,25]. Some researchers suggested that toxicity correlates with monomer lipophilicity, and that cytotoxic effects of (meth)acrylates are exerted through reacting with and disrupting the lipid bilayer in cell membranes [26,27]. Other researchers identified a mechanism involving the covalent binding of electrophilic reactive (meth)acrylates to cellular targets such as thiol groups on proteins through a Michael-type reaction [28,29]. In the present case, we found acrylated macromers to be more cytotoxic than methacrylated ones after 2-h incubation, but after 24 hours, this phenomenon seemed to have reversed. Figure 10 shows the fraction of live cells at 2 and 24 hours as a function of transition temperature of the macromer solutions. Our previous studies [11] showed that the hydrogel stability at 37°C is proportional to the LCST of the macromer solutions, and hydrogels fabricated of macromers with low LCST tend to phase-separate and precipitate faster. This offers an explanation for the higher toxicity observed for methacrylates as compared to acrylates with the same degree of modification after 24-h incubation. At 2 hours, the gels are still stable, but the precipitation over the 24-h time interval happens sooner for macromers with lower transition temperatures, as in the case of methacrylates. This effect prolongs the period in which non-gelled, (meth)acrylated, reactive macromer chains interact directly with structures of the cells, such as membranes, as opposed to chains that are fully assembled into a gel, thus enhancing their toxic action over 24 hours. The results suggest that when (meth)acrylates form a gel, their cytotoxic effects are diminished. Macromer toxicity seems to be associated with their soluble form, or in this specific case, precipitated, and not when the macromolecular chains are assembled in a physical network.

Conclusions

This study demonstrated the good cytocompatibility properties of thermally responsive, chemically crosslinkable macromers. It was found that unmodified macromers with number-average molecular weights in the range of approximately 1500 to 3000 Da with varying molecular compositions allowed for high cell viability over a 24-h time interval. The chemical modification with an acrylate or methacrylate group enables the chemical crosslinking of the macromers. We investigated the effect of the (meth)acrylate derivatization on the toxicity of uncrosslinked macromers and determined that a 6-h time frame for crosslinking completion would allow for viable cell encapsulation. Increased degrees of modification were shown to decrease the thermal transition temperature as well as the cytocompatibility of the macromers. Formulations with the parameters established in this study will be used towards the fabrication of injectable, thermoresponsive, *in situ* forming hydrogel systems for cell and drug delivery.

Acknowledgments

This work was supported by a grant from the National Institutes of Health (R01-DE017441) (A.G.M.). M.C.H. also acknowledges financial support from the Deutsche Forschungsgemeinschaft (German Research Foundation) (HA4444-1/1). J.D.K. acknowledges support by the National Institutes of Health under Ruth L. Kirschstein National Research Service Award (T32-GM008362) from the National Institute of General Medical Sciences. J.D.K. is a student in the Baylor College of Medicine Medical Scientist Training Program and was supported by the National Institute of

General Medical Sciences (T32 GM07330). We would also like to acknowledge Ryan McGuire for his help with the osmolality measurements and Anita Saraf for assisting with the fluorescent microscopy.

References

1. Peppas NA, Bures P, Leobandung W, Ichikawa H. Hydrogels in pharmaceutical formulations. *Eur J Pharm Biopharm* 2000;50:27–46. [PubMed: 10840191]
2. Bryant SJ, Anseth KS. The effects of scaffold thickness on tissue engineered cartilage in photocrosslinked poly(ethylene oxide) hydrogels. *Biomaterials* 2001;22:619–26. [PubMed: 11219727]
3. Kretlow JD, Klouda L, Mikos AG. Injectable matrices and scaffolds for drug delivery in tissue engineering. *Adv Drug Deliv Rev* 2007;59:263–73. [PubMed: 17507111]
4. Temenoff JS, Mikos AG. Injectable biodegradable materials for orthopedic tissue engineering. *Biomaterials* 2000;21:2405–12. [PubMed: 11055288]
5. Temenoff JS, Park H, Jabbari E, Conway DE, Sheffield TL, Ambrose CG, et al. Thermally cross-linked oligo(poly(ethylene glycol) fumarate) hydrogels support osteogenic differentiation of encapsulated marrow stromal cells in vitro. *Biomacromolecules* 2004;5:5–10. [PubMed: 14715001]
6. Klouda L, Mikos AG. Thermoresponsive hydrogels in biomedical applications. *Eur J Pharm Biopharm* 2008;68:34–45. [PubMed: 17881200]
7. Cellesi F, Tirelli N, Hubbell JA. Towards a fully-synthetic substitute of alginate: development of a new process using thermal gelation and chemical cross-linking. *Biomaterials* 2004;25:5115–24. [PubMed: 15109835]
8. Lee BH, West B, McLemore R, Pauken C, Vernon BL. In-situ injectable physically and chemically gelling NIPAAm-based copolymer system for embolization. *Biomacromolecules* 2006;7:2059–64. [PubMed: 16768434]
9. Robb SA, Lee BH, McLemore R, Vernon BL. Simultaneously physically and chemically gelling polymer system utilizing a poly(NIPAAm-co-cysteamine)-based copolymer. *Biomacromolecules* 2007;8:2294–300. [PubMed: 17567067]
10. Vermonden T, Fedorovich NE, van Geemen D, Alblas J, van Nostrum CF, et al. Photopolymerized thermosensitive hydrogels: synthesis, degradation, and cytocompatibility. *Biomacromolecules* 2008;9:919–26. [PubMed: 18288801]
11. Hacker MC, Klouda L, Ma BB, Kretlow JD, Mikos AG. Synthesis and characterization of injectable, thermally and chemically gelable, amphiphilic poly(N-isopropylacrylamide)-based macromers. *Biomacromolecules* 2008;9:1558–70. [PubMed: 18481893]
12. Betz MW, Modi PC, Caccamese JF, Coletti DP, Sauk JJ, Fisher JP. Cyclic acetal hydrogel system for bone marrow stromal cell encapsulation and osteodifferentiation. *J Biomed Mater Res A* 2008;86:662–70. [PubMed: 18022839]
13. Shin H, Temenoff JS, Mikos AG. In vitro cytotoxicity of unsaturated oligo[poly(ethylene glycol) fumarate] macromers and their cross-linked hydrogels. *Biomacromolecules* 2003;4:552–60. [PubMed: 12741769]
14. Temenoff JS, Shin H, Conway DE, Engel PS, Mikos AG. In vitro cytotoxicity of redox radical initiators for cross-linking of oligo(poly(ethylene glycol) fumarate) macromers. *Biomacromolecules* 2003;4:1605–13. [PubMed: 14606886]
15. Nicodemus GD, Bryant SJ. Cell encapsulation in biodegradable hydrogels for tissue engineering applications. *Tissue Eng Part B Rev* 2008;14:149–65. [PubMed: 18498217]
16. Southall NT, Dill KA, Haymet ADJ. A View of the Hydrophobic Effect. *Journal of Physical Chemistry B* 2002;106:521–33.
17. Kim NS, Lee GM. Response of recombinant Chinese hamster ovary cells to hyperosmotic pressure: effect of Bcl-2 overexpression. *J Biotechnol* 2002;95:237–48. [PubMed: 12007864]
18. Mockridge JW, Benton EC, Andreeva LV, Latchman DS, Marber MS, Heads RJ. IGF-1 regulates cardiac fibroblast apoptosis induced by osmotic stress. *Biochem Biophys Res Commun* 2000;273:322–7. [PubMed: 10873605]

19. Zhu MM, Goyal A, Rank DL, Gupta SK, Vanden Boom T, Lee SS. Effects of elevated pCO₂ and osmolality on growth of CHO cells and production of antibody-fusion protein B1: a case study. *Biotechnol Prog* 2005;21:70–7. [PubMed: 15903242]
20. Kaihara S, Matsumura S, Fisher JP. Cellular responses to degradable cyclic acetal modified PEG hydrogels. *J Biomed Mater Res A*. 2008;10.1002/jbm.a.32149
21. Du JZ, Sun TM, Weng SQ, Chen XS, Wang J. Synthesis and characterization of photo-cross-linked hydrogels based on biodegradable polyphosphoesters and poly(ethylene glycol) copolymers. *Biomacromolecules* 2007;8:3375–81. [PubMed: 17902689]
22. De Groot CJ, Van Luyn MJ, Van Dijk-Wolthuis WN, Cadee JA, Plantinga JA, Den Otter W, et al. In vitro biocompatibility of biodegradable dextran-based hydrogels tested with human fibroblasts. *Biomaterials* 2001;22:1197–203. [PubMed: 11336291]
23. Timmer MD, Shin H, Horch RA, Ambrose CG, Mikos AG. In vitro cytotoxicity of injectable and biodegradable poly(propylene fumarate)-based networks: unreacted macromers, cross-linked networks, and degradation products. *Biomacromolecules* 2003;4:1026–33. [PubMed: 12857088]
24. Yoshii E. Cytotoxic effects of acrylates and methacrylates: relationships of monomer structures and cytotoxicity. *J Biomed Mater Res* 1997;37:517–24. [PubMed: 9407300]
25. Atsumi T, Fujisawa S, Tonosaki K. (Meth)acrylate monomer-induced cytotoxicity and intracellular Ca(2+) mobilization in human salivary gland carcinoma cells and human gingival fibroblast cells related to monomer hydrophobicity. *Biomaterials* 2006;27:5794–800. [PubMed: 16934868]
26. Fujisawa S, Atsumi T, Kadoma Y. Cytotoxicity of methyl methacrylate (MMA) and related compounds and their interaction with dipalmitoylphosphatidylcholine (DPPC) liposomes as a model for biomembranes. *Oral Dis* 2000;6:215–21. [PubMed: 10918558]
27. Fujisawa S, Kadoma Y, Komoda Y. ¹H and ¹³C NMR studies of the interaction of eugenol, phenol, and triethyleneglycol dimethacrylate with phospholipid liposomes as a model system for odontoblast membranes. *J Dent Res* 1988;67:1438–41. [PubMed: 3183163]
28. Schweikl H, Spagnuolo G, Schmalz G. Genetic and cellular toxicology of dental resin monomers. *J Dent Res* 2006;85:870–7. [PubMed: 16998124]
29. Chan K, O'Brien PJ. Structure-activity relationships for hepatocyte toxicity and electrophilic reactivity of alpha,beta-unsaturated esters, acrylates and methacrylates. *J Appl Toxicol* 2008;28:1004–15. [PubMed: 18615533]

Figure 1.

Structure of thermogelling macromers (A) before and (B) after modification with the addition of a (meth)acrylate group. The asterisk indicates possible branching sites. The indices m, n, o could equal zero in some formulations. The sum of the modified (z) and unmodified (x) hydroxyl groups equals the initial hydroxyl group number prior to (meth)acrylation ($x+z=o$).

Figure 2.

Cell viability after 24-h incubation with macromers of different number-average molecular weights and polydispersity indices (PI). A) Leachables assay: incubation with hydrogel supernatant B) Direct contact assay: incubation with hydrogel. Error bars represent standard deviation; n= 3-5.

Figure 3. Cell viability after 24-h incubation with macromers of different molecular ratios of 2-hydroxyethyl acrylate (HEA) and acrylamide (AAm) in their compositions. A) Leachables assay: incubation with hydrogel supernatant B) Direct contact assay: incubation with hydrogel. Error bars represent standard deviation; n= 3-5. Characters on experimental groups indicate statistically significant differences when groups are not marked with the same letter ($p < 0.05$). For clarity purposes, in figure (A) differences are marked for undiluted group only.

Figure 4. Cell viability after 24-h incubation with macromers of different transition temperatures (T_{η}). A) Leachables assay: incubation with hydrogel supernatant B) Direct contact assay: incubation with hydrogel. Error bars represent standard deviation; $n=5$. Experimental groups denoted by different letters show statistically significant differences ($p<0.05$). For clarity purposes, in figure (A) differences are marked for undiluted group only.

Figure 5.

Fluorescence microscopy images of fibroblasts after 24-h exposure to hydrogel leachables or in direct contact with hydrogel and treatment with Live/Dead reagent. Letters denote the positive control (A), negative control (B), treatment with a formulation composed of PEDAS₁NiPAAm₂₀ in leachables assay (C) and direct contact (D), treatment with a formulation composed of PEDAS₁NiPAAm₁₆AAm₄ in polymer-conditioned media (E) and direct contact (F). Scale bars indicate 100 μ m.

Figure 6. Osmolality of dextran solutions of different concentrations and cell viability values after 24-h incubation with these solutions. Differences in cytocompatibility are not statistically significant ($p < 0.05$).

Figure 7. Cell viability after (A) 2-h, (B) 6-h and (C) 24-h incubation with various concentrations of (meth)acrylated macromers with a theoretical composition of PEDAS₁-NiPAAm₁₅-AAm_{3,5}-HEA_{1,5}. Error bars represent standard deviation for n=3-5.

Figure 8. Cell viability over time after exposure to 100 mg/mL solutions of (A) acrylated and (B) methacrylated macromers, with an unmodified composition as a control. Error bars represent standard deviation for $n=3-5$. Statistically significant differences over time within one composition group are marked with an asterisk ($p<0.05$).

Figure 9.

Cell viability after 24-h incubation with 100 mg/mL solutions of acrylated and methacrylated macromers with different degrees of modification. Modification is expressed as the fraction of hydroxyl moieties substituted by (meth)acrylate groups. Error bars represent standard deviation for $n=3-5$.

Figure 10.

Cell viability after 2 and 24-h incubation with 100 mg/mL solutions of acrylated and methacrylated macromers as a function of their thermal transition temperature. Error bars represent standard deviation for n=3-5.

Table 1

Molecular composition, molecular weight characteristics and transition temperature of unmodified thermogelling macromers^a

Molecular weight group				
Molar Composition PEDAS/NiPAAm/AAm/HEA	M _n [Da]	PI	T _g [°C]	
1/15.4/2.6/2	1750±370	3.4±0.6	31.6±0.3	
1/15.4/2.6/2	2300±140	2.9±0.3	32.7±0.8	
1/15.4/2.6/2	2440±420	3.0±0.8	32.6±0.2	
1/15.4/2.6/2	2830±170	3.6±0.5	31.5±1.2	
Composition group				
Molar Composition PEDAS/NiPAAm/AAm/HEA	Acrylic Monomer Ratios (%HEA/%AAm)	M _n [Da]	PI	T _g [°C]
1/16/0/4	20/0	3050±140	2.9±0.2	23.2±0.1
1/15.4/1/3.6	18/5	3810±260	3.0±0.3	26.5±0.1
1/15.4/2.6/2	10/13	1750±370	3.4±0.6	31.6±0.3
1/14/3/3	15/15	2700±120	2.7±0.1	33.7±0.2
1/16/4/0	0/20	1700±80	2.7±0.1	38.7±3.0
LCST group				
Molar Composition PEDAS/NiPAAm/AAm/HEA	M _n [Da]	PI	T _g [°C]	
1/20/0/0	2560±250	2.7±0.1	27.2±0.2	
1/18.5/1.5/0	2450±280	2.6±0.2	30.6±0.4	
1/16/4/0	1700±80	2.7±0.1	38.7±3.0	
1/16/4/0	1370±100 ^b	2.9±0.1 ^b	40.8±1.4	
1/14/6/0	1470±250	6.9±2.1	43.5±2.3	

^a Molecular composition describes the molar feed ratio of monomers as used for polymerization. Molecular weight characteristics were obtained by gel permeation chromatography. The transition temperature T_g was determined from rheological measurements of 10% (w/v) macromer solutions as the inflection point of the temperature-complex viscosity curve. Values represent means ± standard deviation for a sample size of n=3. Table is adapted from [11] with permission.

^b The average and range for this formulation were determined for n=2.

Table 2
Acrylated and methacrylated thermogelling macromers with varying degrees of modification and their transition temperatures ^c

Molar Composition: PEDAS/NiPAAm/AAm/HEA = 1/15/3.5/1.5		
Modification	% of hydroxyls modified	T_{DSC} [°C]
No modification	0	35.6 ± 0.5
Acrylation	8	31.2 ± 0.2
Acrylation	32	24.6 ± 0.3
Acrylation	49	20.8 ± 0.9
Methacrylation	10	24.1 ± 0.6
Methacrylation	29	15.2 ± 0.9

^cThe degree of hydroxyl group conversion was determined by ¹H-NMR. The transition temperature T_{DSC} was obtained by differential scanning calorimetry (DSC) for 10% (w/v) macromer solutions. Values represent means ± standard deviation for a sample size of n=3. Table is adapted from [11] with permission.