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How crosslinking Mechanisms of Methacrylated Gellan Gum Hydrogels Alter Macrophage Phenotype

Zhuqing Li
*Iowa State University, zhuqingl@iastate.edu*

Kaitlin M. Bratlie
*Iowa State University, kbratlie@iastate.edu*

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How crosslinking Mechanisms of Methacrylated Gellan Gum Hydrogels Alter Macrophage Phenotype

Zhuqing Li and Kaitlin M. Bratlie

1 Department of Materials Science & Engineering, Iowa State University, Ames, Iowa 50011, USA
2 Department of Chemical & Biological Engineering, Iowa State University, Ames, Iowa 50011. USA
3 Division of Materials Sciences & Engineering, Ames National Laboratory, Ames, Iowa 50011, USA

CORRESPONDING AUTHOR FOOTNOTE

*To whom correspondence should be addressed: Tel: 515-294-7304, Fax: 515-294-5444, E-mail: kbratlie@iastate.edu
Abstract

In tissue engineering scaffolds, macrophages play a critical role in determining the host response to implanted biomaterials. Macrophage phenotype is dynamic throughout the host response, and a balance of phenotypes is essential for timely progression from injury to proper wound healing. Therefore, it is important to predict how materials will modulate the response of macrophages. In this study, we investigated the effect of methacrylated gellan gum (GG) hydrogels on macrophage phenotype and proliferation with the ultimate goal of improving rational design of biomedical implants. Naïve, along with classically and alternatively activated RAW 264.7 macrophages were seeded on methacrylated gellan gum hydrogels that were fabricated with different thiol-ene ratios and crosslinking mechanisms. Live/dead assays showed that all hydrogels supported cell attachment and proliferation. Stiffer substrates enhanced anti-inflammatory production of nitrites from both naïve and classically activated macrophages compared to the softer substrates. Moreover, arginine and CD206 expression – markers for alternatively activated macrophages – were inhibited by higher thiol content. Introducing ionic crosslinks using calcium did not influence the proliferation or polarization for any of the three macrophage phenotypes. Our results suggest that the macrophage phenotype shift from M1 to M2 is controlled by the different crosslinking mechanisms, physical properties, and the chemistry of methacrylated gellan gum hydrogels.

Key words: Gellan gum, methacrylation, macrophage polarization, hydrogels, biomaterials
1. Introduction

Tissue engineering scaffolds are designed to simulate the properties of specific tissues, have tunable mechanical strength, support cell growth, activate macrophages into a balanced phenotype, and incorporate growth factors to improve acceptance of the scaffold into the host.\textsuperscript{1,2,3} The physical and chemical properties of these substrates are key factors in controlling cellular responses. Ultimately, their \textit{in vivo} fate depends on the outcome of this interaction. Hydrogels are similar to native extracellular matrix and can augment the wound healing process.\textsuperscript{1,4–10} Naturally occurring polysaccharides are hydrophilic, biocompatible, have low cytotoxicity, and can be readily modified.\textsuperscript{1,2,10–12}

Gellan gum (GG) is a naturally derived polysaccharide and is composed of a tetrasaccharide repeating unit of one L-rhamnose, one D-glucuronic acid, and two D-glucoses.\textsuperscript{13} GG-based hydrogels have been studied previously for application in cartilage tissue engineering due to its biocompatibility and tunability.\textsuperscript{13–19} A variety of cells, such as, human articular chondrocytes,\textsuperscript{14} human adipose stem cells,\textsuperscript{19} lung fibroblast cells, and human intervertebral disc cells\textsuperscript{16} have shown strong attachment and proliferation on GG-based hydrogel, implying GG-based hydrogel are relatively stable both \textit{in vitro} and \textit{in vivo}. In our previous study, we analyzed the effects of thiol-ene photoclick chemistry on methacrylated modified GG via chain, step, and mix mode crosslinking mechanisms. We found that the elastic modulus of these hydrogels can be altered by three different crosslinking mechanisms as well as balancing the ratios of the thiol and vinyl groups.\textsuperscript{20} This tunability of the substrate enables us to examine how macrophage phenotype changes in response to elastic modulus.

Shortly after a biomaterial is implanted into a host, a cascade of inflammatory reactions will be triggered, which may lead to implant rejection.\textsuperscript{3,11} The ability to modulate the inflammatory
response could be beneficial to efficient rational design of tissue engineering scaffolds. Macrophages act as a principal mediator of inflammatory responses to either injury or implantation, and play a key role in the immune system due to their plasticity and heterogeneous phenotypes. Macrophages are thought to exist on a spectrum of phenotypes. At one extreme side of the spectrum is termed classically activated M1 macrophages and is achieved through exposure to lipopolysaccharide (LPS) or interferon (IFN)-γ. Macrophages activated through the M1 pathway differentiate into a pro-inflammatory phenotype. M1 macrophages can release important cytotoxic molecules, produce pro-inflammatory cytokines such as high levels of interleukin (IL)-12, IL-23, inducible nitric oxide synthase (iNOS), and other toxic reactive oxygen intermediates. These macrophages release pro-inflammatory cytokines to destroy microorganisms at the wound site and recruit other inflammatory cells to the injured site; however, their uncontrolled response can cause damage to native tissue. Macrophages activated through IL-4, IL-10, or IL-13 are known as alternatively activated M2 macrophages, existing on the other extreme side of the spectrum. M2 macrophages release anti-inflammatory cytokines such as IL-10 and transforming growth factor (TGF)-β. Moreover, they produce arginase, an enzyme that converts L-arginine to urea. M2 macrophages are favored for balancing pro- and anti-inflammatory responses during chronic inflammation. M2 macrophages promote wound healing, angiogenesis, and tissue regeneration; however, prolonged activation of M2 macrophages can be problematic and can lead to a fibrotic response. Macrophage phenotype is dynamic throughout the host response and a balance of phenotypes is essential for timely progression from injury to proper wound healing. Extreme macrophage phenotypes do not exist in vivo and would not be beneficial to tissue regeneration, but controlled expression of M1-like or M2-like phenotypes are useful in proper healing responses. It is essential to select appropriate biomaterials based on their
chemistry and mechanical properties as both have an impact on macrophage phenotype.\textsuperscript{10,31,32} Macrophage phenotype in response to GG hydrogels crosslinked through thiol-ene chemistry is poorly understood. Here, we investigated how the crosslinking mechanism of methacrylated GG hydrogels, along with the ratio of reactants, impacts the polarization and proliferation of different macrophage phenotypes to better identify materials parameters that influence these transitions with the ultimate goal of improving rational design of biomedical implants.

2. Materials and Methods

2.1. Materials

Gellan gum was purchased from Spectrum Chemical Mfg. Corp. (New Brunswick, NJ, USA). Methacrylate anhydride (MA) was supplied by Bean Town Chemical (Hudson, NH, USA). Dithiothreitol (DTT) was purchased from VWR Chemical (Batavia, IL, USA). Sigma-Aldrich (St. Louis, MO, USA) supplied the 2-hydroxy-4’-(2-hydroxyethoxy)-2-methylpropioiphenone (Irgacure 2959). Fresh deionized (DI) water (Milli-Q Nanopure, Thermo Scientific, Waltham, MA, USA) was used throughout this study.

2.2 Synthesis of hydrogels

The hydrogels used in this study, along with their compressive moduli, swelling ratios, and degradation in the presence of lysozyme have been previously described.\textsuperscript{20} In this study, we used GG modified with $2.0 \pm 0.11$ methacrylate groups per repeating unit.\textsuperscript{20} Methacrylated GG’s formulations are summarized in Table 1.\textsuperscript{20} Methacrylated GG (15 mg or wt-% 1%) was dissolved in 15 mL complete medium (CM, Dulbecco’s modified Eagle’s medium (Mediatech, Inc., Manassas, VA, USA) supplemented with 10% fetal bovine serum (Mediatech), 100 U/mL penicillin (Fisher, Pittsburgh, PA, USA), and 100 µg/mL streptomycin (Fisher Scientific)) at 55°C. Irgacure 2959 (1.5 mg or wt-% 0.1) was added to the solution. To this solution, either 0, 7.5, or 15
μL of 10 wt-% DTT and either 0 or 3 μL of 0.5 M CaCl₂ were added to yield a final Ca²⁺ concentration of 1 mM. The mixed mode and chain growth gels were degassed for 20 min. To 48 well plates (KSE Scientific, Durham, NC, USA), 200 μL of each hydrogel precursor solution was added. Hydrogels were then crosslinked under UV light (15 W, 365 nm, UVP, Upland, CA) for 10 min.

2.3 Water Contact Angle (WCA)

Water contact angles were measured directly by dropping DI water onto hydrogel coated glass slides. The glass slides were coated with a 0.05% PLL solution and incubated at 37°C for 1h. The coating creates a positively charged surface. Then, 50 μL of negatively charged hydrogel solution was coated on the PLL coated glass slides and crosslinked under UV irradiation. DI (5 μL) was dropped on hydrogel samples and imaged using a digital camera (Canon EOS Rebel T3i, Canon, Melville, NY, USA) Three replicates were collected for each sample before the WCAs were analyzed using Image J (NIH, Bethesda, MD, USA) software.

2.4 Cell culture

RAW 264.7 cells (ATCC, Manassas, VA, USA) were cultured in CM at 37°C in 5% CO₂. Every three to five days, the cells were passaged using a cell scraper to detach cells and subcultured at $6.7 \times 10^3$ to $2.7 \times 10^4$ cells/cm².

2.5 Cell proliferation

In order to examine cell proliferation of RAW 264.7 cells in contact with the hydrogels, a live/dead assay was performed. To the 48-well plates described above, cells were seeded at 1.25 $10^5$ cells/cm² on each of the GG hydrogels. CM (250 μL) was added to each well. Macrophages were activated with 25 ng/mL IL-4 (denoted M(IL-4) cells, eBioscience Inc., San Diego, CA, USA) or 500 ng/mL LPS (M(LPS), Sigma-Aldrich) and the plate was incubated for 48 h. Naïve
cells (M(0)) were also included. Live and dead controls were obtained by culturing cells directly on the tissue culture plastic. The plate was incubated for 48 h, after which the dead control supernatant was aspirated and replaced with 70% ethanol and incubated for 20 min. After this incubation period, all wells were aspirated and 150 µL of working solution (2 µM calcein AM (AnaSpec, Fremont, CA, USA) and 7.5 µM 7-aminoactinomycin D (Tonbo Biosciences, San Diego, CA, USA) in phosphate buffered saline (PBS, diluted from 10x solution, pH 7.4, Fisher)) was added to the wells. The plates were incubated for 30 – 40 min at 37°C in 5% CO$_2$. Live and dead cells were quantified using an excitation/emission of 485/590 nm and 528/645 nm, respectively, using a plate reader (BioTek Synergy HT Multidetection Microplate Rreader BioTek, Winooski, VT, USA). The percentage of live cells was calculated using equation 1.

$$\% \text{ Live Cells} = \frac{F(530)_{\text{sam}} - F(530)_{\text{min}}}{F(530)_{\text{Max}} - F(530)_{\text{min}}} \times 100\%$$ (1)

RAW 264.7 cells were stained using the above live/dead assay were also imaged with an EVOS® FLoid® Imaging Station (Life Technologies, Grand Island, NY, USA) using the red (excitation/emission 586/646 nm) and the green channels (482/532 nm).

2.6 Urea assay

Arginase activity, an M(IL-4) marker, was measured through the conversion of L-arginine into urea. After 48 h incubation as described above, the supernatant was collected for later use and the cells were washed with 400 µL PBS. The plates were placed on ice for at least 10 min with 100 µL of cell lysis buffer (150 µL protease inhibitor cocktail (Amresco, Solon, OH, USA) and 15 µL Triton X-100 (Acros Organics, Elgin, IL, USA) diluted to 15 mL with DI water) in each well. The lysate solution (25 µL) was transferred from each well to a 96 well plate (Argos Technologies, Elgin, IL, USA) along with 25 µL of 10 mM MnCl$_2$ (Fisher) and 50 mM Tris solution (Fisher). The plates were incubated for 10 min at 55°C. After this incubation, 50 µL of 1
Arginine (pH 9.7) was added to each well and the plates were incubated at 37°C for 20 h. A
colorimetric response was obtained by adding 200 µL of solution 1 (1.2 g o-phthalaldehyde (Alfa
Aesar, Ward Hill, MA, USA), 1 L H2O, and 500 µL HCl (Fisher)) and solution 2 (0.6 g N-
(naphthyl) ethylenediamine dihydrochloride (Acros Organics), 5 g boric acid (Fisher), 800 mL
H2O, and 111 mL sulfuric acid (Fisher) diluted to 1 L in H2O) at a 1:2 ratio. The plates were read
at 520 nm with a reference at 630 nm.

2.7 Griess assay

Nitrite production, an M(LPS) phenotype marker, was determined using the supernatant
collected from the urea assay. A nitrite standard curve was generated using serial dilutions of 100
µM NaNO2 with a volume of 150 µL in a 96 well plate. After transferring 150 µL of samples into
the remaining wells, 130 µL DI water and 20 µL Griess reagent (Acros Organics) were added to
each well and incubated for 20 min. The plates were read at 448 nm with a 690 nm reference.

2.8 Immunocytochemistry

RAW 264.7 cells were fluorescently labeled using a previously described protocol. Briefly, 100 µL hydrogel was crosslinked on clean, poly-L-lysine (PLL) coated glass coverslips
and were placed in 35 × 10 mm Petri dishes (Corning, Tewksbury, MA, USA). Cells were seeded
at 1×10^6 cells/cm² and activated as M(LPS), M(IL-4), or M(0) as described above. The cells were
incubated for 48 h. The cells were stained for CD11c (ab33483, Abcam, Cambridge, MA, USA)
and CD206 (ab64693, Abcam). The nuclei were stained using DAPI (4',6-diamindino-2-
phenylindole, U0031, Abnova, Walnut, CA, USA). These coverslips were imaged with an EVOS®
FLoid® Imaging Station (Life Technologies) using the red (586/646 nm), blue (390/446 nm), and
green channels (482/532 nm).

2.9 Dose-dependent DTT study
LPS and IL-4 polarized RAW 264.7 cells were incubated with DTT (0.01 – 0.06 mg/mL) in a dose-dependent manner in CM at 37°C in 5% CO₂ for 48 h. Urea production was measured as described above.

2.10 Statistics and data analysis

Statistical analysis was performed using JMP statistical software (Cary, NC, USA). Statistical significance of the mean comparisons was determined by a two-way ANOVA. Pair-wise comparisons were analyzed with Tukey’s honest significance difference test. Differences were considered statistically significant for p < 0.05.

3. Results

3.1 Cell proliferation

Hydrogels used for tissue engineering scaffolds must be biocompatible to allow cell survival and proliferation. Cell proliferation of M(0), M(LPS), and M(IL-4) macrophages seeded on GG hydrogels were quantified and visualized by live/dead fluorescence staining (Figure 1). Proliferation of the cells on the gels was normalized to those cultured on tissue culture plastic. No dead cells were observed on any of the hydrogels. Chain growth hydrogels, fabricated through free radical polymerization of methacrylate groups in the absence of thiol (Table 1), showed the lowest cell proliferation (12 ± 1 to 21± 2%) for all three macrophage phenotypes. The proliferation of M(0) macrophages was lower on step growth hydrogels, crosslinked via thiol-ene chemistry, compared to those on the mixed mode gels, a combination of step and chain growth mechanisms. It is also possible that M(0) cells did not adhere as well to the step growth hydrogels compared to the other substrates when the cells were seeded. Step growth hydrogels are labeled as not degassed in the presence of thiol, while mixed mode gels were degassed with thiol. M(LPS) proliferation was higher than either M(0) or M(IL-4) macrophages seeded on equimolar step growth hydrogels.
M(IL-4) seeded on equimolar thiol-ene ratio gels showed no statistical difference in the presence of calcium. However, proliferation significantly increased on mixed mode hydrogels formed through the half-molar thiol-ene ratio compared to their step growth hydrogel counterparts. Proliferation of the three macrophage phenotypes were significantly higher on the step growth hydrogels formed using the equimolar thiol-ene ratio than those formed using a half molar thiol-ene ratio. Mixed mode hydrogels exhibit a significant higher proliferation for M(0) compared to corresponding step growth hydrogels. Additional crosslinking using calcium in step growth gels enhanced M(0) proliferation. M(LPS) and M(IL-4) were not influenced by ionic crosslinking for any of the crosslinking mechanisms. Live/dead fluorescent images of M(0), M(LPS), and M(IL-4) were used to visualize cell attachment (Figure 1B and Figure S1). Cells were generally rounded and grew in clusters. These results qualitatively agreed with Figure 1A.

3.2 Arginase/iNOS

Polarization of RAW 264.7 macrophages (M(0), M(LPS), and M(IL-4)) was evaluated by examining the urea:nitrite ratio. We indirectly measured arginase activity – a measure of M2 polarization – using a urea assay (Figure 2A). Nitrite production – a measure of M1 polarization – was quantified through a Griess reagent assay (Figure 2B). Arginine is converted to urea and nitrite by arginase and iNOS, respectively. To better elucidate the results we obtained here, urea:nitrite was applied as a functional readout of M1 and M2 macrophage polarization because both enzymes compete for arginine as a precursor (Figure 2C).

M(LPS) cells, when cultured on GG hydrogels, shifted to a more inflammatory phenotype on softer materials. The compressive moduli of the hydrogels were previously determined by measuring the slope of linear region of the stress-strain curve. The step growth mechanism yields a lower compressive stiffness. In Figures 2A and B, hydrogels crosslinked via step growth
mechanism had at least 50% higher nitrite production and at least 50% lower urea production when compared to their corresponding mixed mode hydrogel counterparts. Urea production increased with decreasing hydrogel stiffness and nitrite production increased with increasing stiffness (Figures 3A and B). The plots of the compressive moduli of hydrogels versus urea (R=0.89) and nitrite (R= -0.85) produced by M(LPS) cells on hydrogels are represented in Figure 3A and 3B, respectively. M(LPS) cells cultured on the stiffest hydrogels (~16.5 kPa) – those crosslinked via chain growth mechanism – had the highest urea:nitrite ratio (87.8 ± 7.45 to 96.9 ± 4.25 mg/µmol). The urea:nitrite ratio was positively correlated with the compressive moduli (R = 0.81, Figure 3C). The softest gels – those formed via step growth mechanism with equimolar thiol-ene ratio – yielded the lowest urea: nitrite ratio (9.98 ± 1.22 to 14.0 ± 2.56 mg/µmol). Maintaining consistent calcium concentration and thiol-ene ratio, mixed mode gels yielded a higher urea:nitrite ratio compared to those formed through step growth mechanism. Mixed mode hydrogels with equimolar thiol-ene ratio significantly increased the urea:nitrite ratio by ~20 mg/µmol when compared with the corresponding step growth crosslinked gels. Furthermore, when maintaining consistent crosslinking mechanism, gels formed via half-molar thiol-ene ratio showed a higher urea:nitrite ratio compared to those formed by the equimolar thiol-ene ratio. Higher amounts of thiol present in mixed mode hydrogels reduced the urea:nitrite ratio by ~30 mg/µmol when compared with the corresponding hydrogel crosslinked via half-molar thiol-ene.

M(IL-4) cells shifted to a more anti-inflammatory phenotype when seeded on hydrogels with half-molar or no thiol equivalents. Unlike M(LPS), M(IL-4) phenotypic shifts appeared to be materials chemistry driven, as opposed to substrate stiffness. The urea production in M(IL-4) depended on the thiol content with higher amounts of thiol reducing the measured amount of urea (Figure 2A). There were very few statistical differences in nitrite production for M(IL-4) cells for
the different crosslinking mechanisms, thiol-ene ratios, or calcium content (Figure 2B). Hydrogels formed through chain growth crosslinking had the highest urea:nitrite ratio (378 ± 27.0 to 375 ± 23.3 mg/µmol). Gels formed using equimolar thiol-ene ratios had the lowest urea production (4.27 ± 0.28 to 9.33 ± 0.64 mg/dL) and lowest urea:nitrite ratio (10.6 ± 1.6 to 18.9 ± 2.5 mg/µmol) relative to half molar thiol-ene gels. There was no statistically significant difference for the urea:nitrite levels for gels formed using equimolar thiol-ene ratios. For step growth and mixed mode gels crosslinked with half molar thiol-ene ratios, the urea:nitrite ratio increased 6 – 20 times (from 10.8 ± 2.6 to 60.5 ± 10.2 – 219 ± 0.7 mg/µmol) compared to equimolar gels. However, the urea:nitrite value for mixed mode gels formed with half-molar thiol-ene ratio was significantly lower than corresponding gels formed via step growth mechanism. To examine whether this effect could be the result of residual DTT present in the solution or partially unreacted DTT in the hydrogel, we incubated the macrophages with varying concentrations of DTT. DTT negatively impacted the amount of urea produced by the M(IL-4) cells (Figure S2). Urea production dropped from 93.8 ± 16.8 to 1.6 ± 0.20 mg/µmol when DTT concentration increased from 0.025 mg/mL to 0.04 mg/mL.

Since materials properties appear to be an important factor in macrophage polarization, we examined water contact angles (WCA) of the hydrogels to determine if hydrophobicity played a role in macrophages responses to these hydrogels. The WCAs of all the hydrogels studied here fell between 75.7 – 85.2° (Figure S3). This range was not sufficiently large to determine if hydrophobicity influenced macrophages on these gels.

There were no statistical differences for the other cell activations and assays. The measured nitrite levels were 33.9 ± 2.41 and 2.55 ± 0.35µmol for the M(LPS) and M(IL-4) controls respectively (Figure 2B). The urea production measured for controls were higher than the cells
exposed to modified hydrogels with the exception of M(0) cells (79.3 ± 5.50, 309.9 ± 31.8, 4.27 ± 0.28 mg/dL for M(LPS), M(IL-4), and M(0) cells, respectively, Figure 2A). Nitrite levels for M(0) macrophages were below the limit of detection and were excluded from Figures 2B and 2C. The measured urea:nitrite values were 20.5 ± 2.73 mg/µmol and 1.15± 0.98× 10^3 mg/µmol for the M(LPS) and M (IL-4) controls, respectively (Figure 2C).

3.3 Fluorescent imaging

Macrophage shifts in phenotypes resulting from the methacrylated GG hydrogels were identified using immunocytochemistry (ICC) fluorescent staining (Figure 4). Cells were stained for CD11c (M1 macrophages) and CD 206 (M2 macrophages). Selected images are presented in Figure 4 with additional images given in Figure S4. These images were used to support the results from urea and nitrite production assays.

ICC fluorescent staining images were consistent with our urea:nitrite analyses. CD206 and CD11c signals were influenced by surface chemistry and mechanical properties, respectively. Hydrogels formed via chain growth mechanism (Figure 4A) expressed the highest fluorescent intensity of CD206 for all three macrophage activations. When the compressive modulus decreased (Figure 4A: 16.5 kPa, Figure 4B: 7.4 kPa, Figure 4C: 16.4 kPa, Figure 4D: 17.3 kPa), the CD11c signal for M(LPS) cells was suppressed and their CD206 signal was enhanced. We observed the same trend for M(0) macrophages. There was weak CD206 presence when M(IL-4) cells were cultured on hydrogels fabricated through equimolar thiol-ene gels (Figure 4B). Higher CD206 expression was observed when thiol-ene ratio decreased to half molar (Figures 4B and C). Stiffer substrates decreased the CD11c expression for M(LPS) and M(0) macrophages; lower thiol content increased the CD206 expression for M(IL-4) macrophages. Control images for each activation (Figure S4K) demonstrated the ability of this staining procedure to identify the
macrophage phenotypes. Based on the immunofluorescent images, a semi-quantitative evaluation of the percent CD206+ cell and CD11c + cells are present in Figure 4 E and F.

4. Discussion

Modulated macrophage phenotypes are desired in progression from injury to proper wound healing and biomaterial integration. To reduce the foreign body response caused by bio-implants, a balanced of phenotypes at the host-hydrogel interface is desired.3,27,28,30,34 Many polysaccharide-based hydrogels have been extensively studied to better understand how surface chemistry and physical properties can be used to polarize macrophages to a desired phenotype.1,2,7,8,10,22,35–37 Hydrogels based on GG has been studied for several tissue engineering applications owing to its biocompatibility and flexibility for modification.13–17,38–40 However, the relationship between GG hydrogel crosslinking mechanisms and macrophage polarization has not been well studied. Here, we investigated the effect of methacrylated GG hydrogels on macrophage phenotype and proliferation with the ultimate goal of improving rational design of biomedical implants.

4.1 Macrophage attachment and morphology

Our results demonstrate that all substrates studied here support adhesion and proliferation of RAW 264.7 macrophages. Crosslinking mechanisms and thiol content modulated the growth of all macrophage phenotypes. Radicals generated by photoinitiators under UV light can induce cellular damage,41 so we seeded cells on the prepared hydrogels to better understand how crosslinking mechanisms and surface chemistry influence cell proliferation and polarization. Compared to tissue culture plastic, cell proliferation on all substrates was suppressed to a range of 5 – 40%, however, no dead cells were observed on any of the hydrogels meaning that either cell growth or cell adhesion was lower on the substrates compared to tissue culture plastic. Chain growth crosslinking caused a significant reduction in cell proliferation for all three macrophage
phenotypes. Mixed model hydrogels appeared to be more cytocompatible for M(0) compared to corresponding step growth hydrogels. Moreover, step growth crosslinked hydrogels with equimolar thiol-ene ratios had a higher number of adherent M(LPS) and M(IL-4) macrophages. No statistical difference was observed for either of these two types of macrophages on mixed mode hydrogels.

4.2 Activated macrophage response to hydrogel stiffness

A temporal phenotype balance between pro-inflammatory and wound healing macrophages is essential for successful tissue engineering applications. Cytokine secretion by macrophage phenotypes can be modulated by the surface chemistry and mechanical properties of the substrates.\textsuperscript{10,21,31,32} It has long been observed that physical mechanical cues play an important role in regulating differentiation, phenotypic change, and adhesion of many cell types.\textsuperscript{28,42–44} Previously, we have studied the physical properties for these hydrogels, along with the cytocompatibility of NIH/3T3 fibroblasts seeded on these gels.\textsuperscript{24} A positive correlation between NIH/3T3 fibroblast proliferation and substrate stiffness was observed. Here, the correlation between classically activated macrophages and substrate stiffness suggests cell differentiation was driven by the mechanical cues. M(LPS) cells seeded on softer substrates produced less urea after lysis and incubation with arginine, presumably resulting from less arginase activity or production. We also used nitrite production as a marker for the M1-like phenotype. Macrophages showed the highest nitrite production on the softest substrates under an inflammatory condition. The nitrite production was reduced ~ 50\% when the compressive modulus increased. This is consistent with our urea production data for M(LPS) cells. Stiffer substrates induced lower nitrite production and elevated urea production with a R = -0.85 and 0.89, respectively (Figure 3A and B). The stiffest substrate enhanced anti-inflammatory arginase production for naïve and M(LPS) macrophages by
at least a factor of three compared to the softest substrate. Stiffer substrates modulated the M1 phenotype towards M2-like characteristics. The effect of substrate stiffness on macrophage phenotype has been previously reported.\textsuperscript{32,27} Primary human macrophage polarization in stiffness defined 3D collagen based matrices crosslinked with either carbodiimide chemistry or functionalized with hyaluronic acid was investigated.\textsuperscript{32} Under inflammatory conditions, the carbodiimide crosslinked matrix had the highest stiffness and polarized macrophages towards an anti-inflammatory phenotype.\textsuperscript{32} In another study, LPS stimulated macrophages had a decreased expression of TNF-\( \alpha \), IL-1\( \beta \), and IL-6 when cultured on softer poly(ethylene glycol)-RGD hydrogels. The authors suggested a lower stiffness led to reduced macrophages activation and foreign body response.\textsuperscript{27} ICC images of LPS activated macrophages cultured on all GG hydrogels studied here qualitatively confirmed the results obtain in the urea and Griess assays, namely the phenotypic shift of the macrophages. This suggests that higher compressive modulus may improve biocompatibility of the hydrogels in applications where a decreased pro-inflammatory response is desired. Our results also demonstrate that the thiol ratio has no effect on the polarization of LPS activated macrophages. Previously, M(IFN-\( \gamma \)) macrophages were shown to be unaffected by the amount of thiol used to crosslink the gel, in terms of their IFN-\( \gamma \) production,\textsuperscript{45} which agrees with our results.

\textbf{4.3 Activated macrophage response to hydrogel surface chemistry}

In contrast to M(LPS) and M(0) cells, the polarization of M(IL-4) is altered by surface chemistry instead of substrate stiffness. Hydrogels crosslinked via equimolar thiol-ene had the lowest urea production (3 – 5 mg/dL). The next highest urea production was for those formed via half-molar thiol-ene (20 – 50 mg/dL). Finally, chain growth crosslinking induced the highest urea production (56 – 70 mg/dL). No correlation was observed between compressive modulus and urea
production (R = 0.16). Surface chemistry appears to be the driving force for phenotypic shifts for M(IL-4) cells cultured on these gels. This is in line with a previous study examining the effect of solubilized thiol on macrophages. Higher levels of DTT can inhibit IL-4 production. The results showed that gels formed with a half-molar thiol-ene ratio had significantly higher urea production than those crosslinked with equimolar ratios. We hypothesize that the degassing process created an oxygen poor environment and led to a more homogeneous polymerization. When fluorescently imaging the M(IL-4) cells, the CD206 signal was highest for chain growth hydrogels and lowest for hydrogels crosslinked via equimolar thiol-ene using either step growth or mixed mode mechanisms.

M(IL-4) cells showed a nitrite production in a range of 1.5 – 4 µM for all GG hydrogels studied here. Neither compressive modulus nor surface chemistry had an impact on nitrite production for the M(IL-4) cells. Nitrite production was below the detection limit for M(0) cells cultured on these GG substrates. There was also no observed correlation between the addition of calcium and the overall function of macrophages cultured on the materials studied here.

Recently, many reports have demonstrated that macrophage phenotype and cell behavior may be controlled with biomaterial surface chemistry. One example of modifying surface chemistry to alter macrophage phenotype is by varying surface hydrophobicity. Human monocytes cultured on hydrophobic polystyrene surfaces (WCA = 84°) exhibited an M2-like phenotype with higher levels of the anti-inflammatory cytokine IL-10. In comparison, the hydrophilic surface fabricated through O₂ plasma-etching of polystyrene (WCA = 10°) shifted the macrophage phenotype toward a pro-inflammatory response with significant increases in the expression of IL-6 and IL-1β. These studies showed that surface chemistry could impact macrophage polarization. The WCAs of all the hydrogels studied here fell in a narrow range of 75.7 – 85.2° (Figure S3),
which indicates that another property, other than hydrophobicity, is inducing these phenotypic changes. It was also found that by simply incorporating a cell adhesion ligand, arginine-glycine-aspartic acid (RGD), into the PEG hydrogel to modulate surface chemistry also leads to a decreased inflammatory response. Here, we found that the level of DTT played a key role in altering macrophage polarization. The present of thiol has previously been shown to significantly inhibit the anti-inflammatory response. Our results are in agreement with this study with urea production being lowered when M(IL-4) cells were cultured on hydrogels formed via equimolar thiol-ene ratios. These results were further confirmed by incubating LPS and IL-4 polarized RAW 264.7 cells with DTT in a dose-dependent manner (Figure S2). Here, urea production from M(IL-4) cells was significantly impacted by DTT concentration. Altogether, these findings support the postulation that the amount of thiol present in the hydrogel (likely as unreacted species) impacts the response of M(IL-4) cells.

Our findings have implications in strategies that involve GG-based hydrogels. A well understand macrophage response is beneficial for material design and improving implant outcomes. By applying different crosslinking mechanisms, a variety of substrate stiffnesses can be achieved along slight differences in their surface chemistry. This study provides evidence that GG hydrogel stiffness is one of the external cues sensed by activated macrophages. The amount of thiol also appears to influence macrophage polarization. The recognition of the predominant macrophage phenotype or the assessment of the relative balance between M1 and M2 macrophages after biomaterial implantation could lead to a prediction of implantation outcomes. Our hydrogels have the potential to be applied as dermal implants, cartilage tissue substitutes, and biologically active wound dressings.

5. Conclusion
In this study, we investigated the macrophage proliferation and polarization responses to different crosslinking mechanisms of methacrylated GG hydrogels \textit{in vitro}. Our data showed that all hydrogels fabricated here support cell attachment and proliferation. Macrophage phenotypic shifts were altered by compressive modulus and surface chemistry. Stiffer hydrogels seemed to favor polarization of naïve and classical activated macrophages to a M2-like phenotype, with the thiol-ene ratio having little impact on phenotypic changes. In contrast, increasing the thiol ratio negatively caused M(IL-4) cells to shift to a more M1-like phenotype. The addition of calcium had no influence on polarization. Taken together, macrophage polarization induced by different crosslinking mechanisms showed that our materials were able to alter macrophage phenotype. This study demonstrates that phenotypic changes can be altered by different crosslinking mechanisms with tunable physical properties and surface chemistry.

Supporting Information

Live/dead staining images of M(0), M(LPS), and M(IL-4) cells; urea production on dose-dependent DTT concentration; water contact angles; immunocytochemistry fluorescent staining.

Acknowledgements

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References:


8. Vasconcelos, D. P.; Costa, M.; Amaral, I. F.; Barbosa, M. A.; Águas, A. P.; Barbosa, J. N.


Table 1. Crosslinking mechanism and compressive modulus for GG hydrogels.

Methacrylated GG hydrogels were crosslinked using chain growth, step growth, and mixed mode mechanisms in the presence and absence of calcium. The thiol concentration was also varied. The compressive modulus was measured for each hydrogel.

Figure 1. Proliferation of macrophages on gellan gum hydrogels. RAW 264.7 macrophages were seeded on hydrogels and tissue culture plastic (control). (A) Quantification of live and dead cells. Data represents the mean ± SD. n = 4. Statistical analysis through two-way ANOVA and Tukey’s HSD post-hoc test. Bars with the same letter (a-f) are not statistically different (p < 0.05). (B) Representative micrographs of live (green) and dead (red) cells cultured for 48 h. Scale bar is 100 μm.

Figure 2. Urea and Nitrite production from stimulated and naïve macrophages on gellan gum hydrogels. Absolute values for (A) urea) and (B) nitrite production for M(LPS), M(IL-4), and M(0) cells seeded on hydrogels and tissue culture plastic (control). Polarization changes of macrophages were monitored through (C) urea:nitrite. Data represents the mean ± SD. n = 4. Statistical analysis through two-way ANOVA and Tukey’s HSD post-hoc test. Bars with the same letter (a-f) are not statistically different (p < 0.05).

Figure 3. Plots of the compressive moduli of gellan gum hydrogels versus molecules produced by M(LPS) cells on gellan gum hydrogels. (A) Urea production; (B) nitrite production; (C) urea: nitrite

Figure 4. Immunocytochemistry staining of stimulated and naïve macrophages on gellan gum hydrogels. RAW 264.7 macrophages were seeded on hydrogels and polarization were altered by either physical properties or surface chemistry. (A) chain grow mechanism with no thiol and calcium; (B) Step growth mechanism hydrogel with equimolar thiol-ene ratio, no
calcium; (C) Mixed model mechanism hydrogel with half-molar thiol-ene ratio, no calcium; (D) Mixed model mechanism hydrogel with half-molar thiol-ene ratio and calcium. Cells were stained with CD206 (red, M2 marker) and CD11c (green, M1 marker) markers as well as DAPI (blue). Scale bar is 100μm. Semiquantitative analysis of percentage of (E) CD206+ and (F) CD11c+ cells determined from the ICC images.
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<table>
<thead>
<tr>
<th>Mechanism</th>
<th>SH:C=C ratio</th>
<th>Calcium concentration (1mM)</th>
<th>Degassing</th>
<th>Gellan gum concentration</th>
<th>Initiator content</th>
<th>Modulus (kPa)</th>
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<td>Chain growth</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>1%</td>
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<td>16.55 ± 1.02</td>
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<td>Chain growth</td>
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<td>7.37 ± 0.81</td>
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<td>+</td>
<td>1%</td>
<td>0.1%</td>
<td>12.43 ± 0.81</td>
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<tr>
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<td>9.94 ± 0.64</td>
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<tr>
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<tr>
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<td>+</td>
<td>1%</td>
<td>0.1%</td>
<td>17.25 ± 1.03</td>
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</table>
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175x378mm (200 x 200 DPI)
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Table of content graphic.

338x190mm (200 x 200 DPI)