Quantifying Crosslinking Density of Photopolymerized Hydrogels with NMR

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Purpose: Photopolymerizable, protein-based biomaterials such as gelatin methacryloyl (gelMA) have been extensively developed for 3D cell-culture. These materials have been underutilized in architecturally demanding applications, such as 3D printing, due to a lack of control over mechanical stability [1]. Mechanical stability, defined here as a material's resistance to solvation and shear stress after polymerization, is dependent primarily on the density of crosslinks (DoC) formed during polymerization. Therefore, a method to quantify DoC is needed. To further enable the use of photocrosslinkable hydrogels in tissue engineering and regenerative medicine, we have developed a method to measure the extent of crosslinking of photocured soft polymers with NMR.

Methods: Hydrogel precursor solution was prepared by dissolving gelMA with a degree of functionalization of 80% (Sigma), photointiator (LAP, Sigma), and sodium trimethylsilyl-propanesulfonate (DSS) in D_2O to final concentrations of 10% m/v, 3.4 mM, and 15 mM, respectively. Precursor was polymerized by exposure to collimated 405 nm light at 50 mW/cm^2 for 30 s. Digestion solution was prepared by dissolving collagenase D (Sigma) and $CaCl_2$ in D_2O to final concentrations of 0.7 mg/mL and 5 mM, respectively. The pH was adjusted to 7.4 using phosphate buffer. A piece of polymerized gelMA was digested in excess digestion solution at 37°C, mixed for 2 days. Solution ^{1}H NMR spectra were obtained in 5-mm tubes at 25°C and 14.1 T using a standard Bruker Avance III 600-MHz spectrometer. Solid-state magic angle spinning (MAS) ^{1}H NMR was performed on a Varian NMRS 500 MHz spectrometer with a magic angle spinning probe at 25°C, 6000 Hz. Mestrenova (v14.0.0) was used to process and analyze all spectra. Spectra in Fig. 1b-d were scaled so that all DSS methyl peaks have the same intensity. Linewidths of all DSS peaks in Fig.1b-f and Fig. 1g were the same. DoC was calculated from spectra in 1g by measuring the peak integrals of exposed samples from 5.75-5.90 ppm, converting to mmols of methacryloylation (-MA) by comparison to the DSS peak, then subtracting from the degree of functionalization (DoF), which was determined by making the same measurement of an unexposed sample.

Results: First, liquid and MAS ^{1}H NMR spectra were acquired for uncured and photo-cured gelMA samples. The peaks from 5.4-5.9 ppm correspond to the vinyl protons of methacryloyl groups [2], which are consumed during polymerization. The number of crosslinks was therefore expected to be proportional to the decrease in those peaks' integrals. We observed that resolution decreased with curing time due to solidification of the sample with both liquid ^{1}H NMR (Fig. 1b-d) and MAS ^{1}H NMR (Fig. 1e), consistent with prior studies [3,4]. This prevents accurate quantification. When cured gelMA was digested with collagenase, the resulting solution ^{1}H NMR spectra was baseline resolved (Fig. 1f). A sample containing only collagenase was analyzed to identify peaks attributable to the enzyme (data not shown, * in Fig 1f). To determine if digestion affected the DoF measurement, we integrated the peak range of un-crosslinked gelMA before and after digestion and found no significant difference (data not shown). Next we tested the digestion method to measure DoC by quantifying DoF of uncured and cured gelMA. The peak integral (5.75-5.90 ppm) decreased substantially after 30-s gelation (Fig. 1g). DoF decreased from 0.306 to 0.041 mmol -MA/ g gelMA, indicating a DoC of 0.266 mmol -MA consumed / g gelMA.

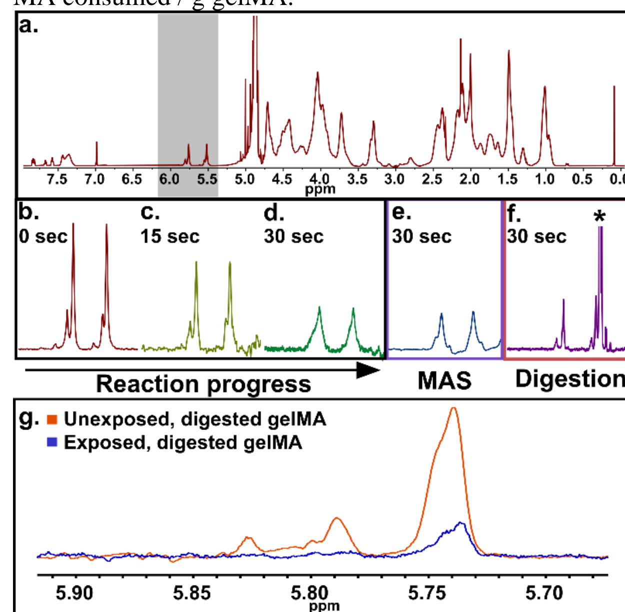

Figure 1. GelMA crosslinking density measured by ^{1}H NMR. (a) Full ^{1}H NMR spectrum of uncured gelMA. Shaded region indicates methacryloyl peaks. (b-d) Solution ^{1}H NMR peaks corresponding to shaded region for undigested gelMA samples exposed for various times. (n=2). (e) MAS ^{1}H NMR spectrum of undigested, exposed gelMA (n=1). (f) Solution ^{1}H NMR spectra of enzymatically digested, cured gelMA. *indicates peak contributed by collagenase (n=2). (g) Solution ^{1}H NMR spectra of gelMA samples exposed for 0 or 30 seconds, then digested with collagenase.

Conclusions: Comparing the three NMR methods, it is clear that collagenase digestion yielded higher resolution than solution ^{1}H NMR and MAS ^{1}H NMR, and was sufficient for quantifying DoC. This approach is convenient compared to other approaches such as lyophilization. Future work will investigate the relationship between the DoC and mechanical properties of cured gel. DoC will be a useful quality control metric for cured polymers and may improve reproducibility between studies.

References: [1] Tigner, T.J. Biomacromol. 2019; 21,2: 454-463. [2] Claaßen, C. Biomacromol. 2018; 19,1: 42-52. [3] Van Vlierberghe, S., Appl. Spectrosc. 2010; 64,10: 1176-1180. [4] Shestakova, P., Chem. Eur. J. 2011; 17,52: 14867-1487