Head-to-Head Comparison of Thiol-Ene and Tetrazine Click Annealing Chemistry on Scaffold Properties

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Statement of Purpose: Microporous annealed particle (MAP) hydrogels are an emerging class of materials that that have been shown to result in superior regenerative outcomes *in vivo* compared to conventional hydrogels. MAP hydrogels are fabricated from microparticles, which can be assembled together through a variety of annealing chemistries. In contrast to conventional hydrogels, which are nonporous and limit cell migration and proliferation within the structure, MAP hydrogels are comprised of a network of interconnected pores that promote immediate cell migration and proliferation. While MAP scaffolds offer improved regenerative outcomes when compared to conventional hydrogels, there has been little investigation on the impact of the annealing chemistries on MAP scaffolds. This work aims to characterize and compare the impact of thiol-norbornene ("thiol-ene") and tetrazine-norbornene click chemistry on poly(ethylene glycol) (PEG)-based MAP scaffolds.

Methods: Hydrogel microparticles were fabricated by electrospraying a precursor solution with a final concentration of 10wt% of PEG-amide-norbornene (PEG-aNB; 4-arm, 5kDa or 20kDa), 10mM of lithium acylphosphinate (LAP), 1mM of cell adhesive peptide CGRGDS, and a dithiol matrix metalloproteinase (MMP)-degradable peptide crosslinker KCGPQGIWGQCK. A thiol-ene ratio of 0.75:1 was used to ensure the presence of free norbornene groups, which were subsequently used for microparticle annealing. Microparticles were characterized using light microscopy. Thiol-ene-based MAP scaffolds (ThiolMAPs) were annealed using a non-degradable PEG dithiol (3.4kDa) linker and photopolymerizing at 365nm for 5 minutes. Tetrazine click-based MAP scaffolds (TzMAPs) were annealed at 37°C for 1 hour using a PEG-di-tetrazine (4.2kDa) linker. Rheology was performed to characterize the storage moduli of the scaffolds. Samples were subjected to oscillatory shear at 1% strain and 1 rad/sec. To characterize the microporosity of the scaffolds, samples were perfused with a 5mg/mL, tetramethylrhodamine isothiocyanate-conjugated Dextran (155kDa) solution and imaged by confocal microscopy. Images with a z-depth of 200µm were obtained and used to quantify samples' porosity. Scaffold degradation was assessed by incubating scaffolds in a 0.1mg/mL collagenase B solution at 37°C. Changes in sample mass were recorded over 2 hours in 15-minute increments.

Results: The majority of the electrosprayed microparticles measured less than 350µm and 500µm for the 5kDa and 20kDa formulations, respectively (Fig. 1A), although diameters up to 700µm were observed. MAP scaffolds fabricated with 5kDa microparticles demonstrated measurable differences in stiffness, porosity, and degradability depending on the annealing chemistry used to form the overall structure. 5kDa ThiolMAPs generated lower storage moduli when compared to their TzMAP counterpart (Fig. 1B). 5kDa ThiolMAPs were more porous than 5kDa TzMAPs (Fig. 1C-D). 5kDa ThiolMAPs underwent complete degradation and 5kDa TzMAPs retained around half of their initial mass by 2 hours (Fig. 1E). Lower storage moduli were observed in 20kDa MAP scaffolds when compared to their 5kDa counterparts, but the differences between the 20kDa ThiolMAPs and TzMAPs were negligible. A similar trend was also apparent in the 20kDa scaffolds' porosity measurements. Degradation occurred at a faster rate in 20kDa-based MAP scaffolds, but degradation between 20kDa ThiolMAPs and TzMAPs were comparable to each other.

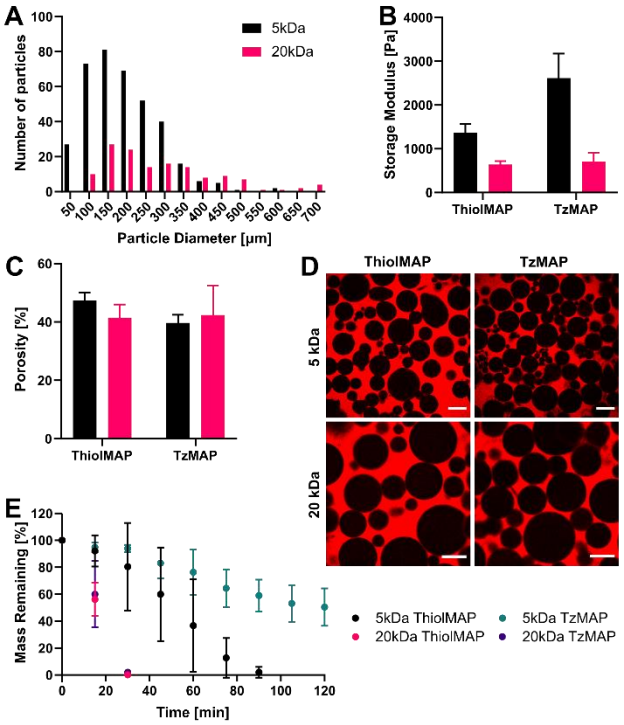

Figure 1. **A)** Size distribution of 5kDa and 20kDa microparticles. **B)** Storage modulus of ThiolMAPs and TzMAPs. **C)** Porosity measurements of MAP scaffolds. **D)** Confocal images of Dextran-perfused MAP scaffolds. Scale bar = 200µm. **E)** Accelerated degradation of 5kDa and 20kDa MAP scaffolds in a collagenase solution.

Conclusions: This head-to-head comparison of ThiolMAPs and TzMAPs indicates the emergence of distinct scaffold properties due to annealing chemistry. However, the effects were dependent on the microparticles used in the assembly process. Future studies will investigate ThiolMAP and TzMAP scaffolds' impact *in vitro*, with specific studies quantifying human mesenchymal stem cells migration through the scaffold over time.