Sequential Click Reactions for Polymerizing and Functionalizing Hydrogel Biomaterials

Daniel L. Alge and Kristi S. Anseth.
Department of Chemical and Biological Engineering, the BioFrontiers Institute, and the Howard Hughes Medical Institute, University of Colorado, Boulder, CO 80309 USA.

Statement of Purpose: Synthetic hydrogels are broadly useful as biomaterials, with applications spanning drug delivery, fundamental studies of cell biology *in vitro*, and *in vivo* tissue engineering. Polymer chemistry has played an integral role in the development of synthetic hydrogels, and recent advances in click chemistry have pushed the evolution of the field by enabling the design of advanced functional biomaterials. The impact of click chemistry is exemplified by the work combining highly specific, efficient, and cytocompatible reactions (*i.e.*, bioorthogonal click reactions) with photoreactive chemistries to create biochemically dynamic 3D cellular microenvironments[1]. However, the click chemistry toolbox is rapidly expanding, and nearly a dozen different reactions falling under the "click" paradigm have now been used to polymerize and functionalize bioactive synthetic hydrogels. Combining multiple click reactions to create advanced functional hydrogel biomaterials is an emerging frontier in the field. Here, we report on the use of sequential thiol-norbornene and tetrazine-norbornene click reactions for creating protein functionalized poly(ethylene glycol) (PEG) hydrogels. This approach exploits the dual reactivity of norbornene, which reacts readily with thiols via a photoinitiated, radical mediated click reaction and also with tetrazines via an inverse electron demand Diels-Alder mechanism.

Methods: Hydrogels were photopolymerized from a PEG-norbornene macromer (4 arm, M_n ~ 20 kDa) and di-thiol enzymatically degradable peptide (KCGPQGIAGQCK), similar to what has been previously described[2], using a [SH]:[norbornene] ratio of 0.85. A bioactive protein, alkaline phosphatase (ALP), was subsequently conjugated to the unreacted norbornenes in the hydrogel using tetrazine-norbornene click chemistry. For protein conjugation, ALP was functionalized with 5-(4-(1,2,4,5-tetrazin-3-yl)-benzylamino)-5-oxopentanoic acid, which was synthesized as previously described[3], using standard bioconjugate chemistry techniques. Photopolymerized hydrogels were soaked in a 0-25 mg/ml solution of tetrazine functionalized ALP (Tz-ALP) in phosphate buffered saline (PBS) for 4 hours at 37°C. Non-functionaled ALP was used as a control. Unconjugated protein was washed out of the hydrogels by soaking in PBS overnight. To quantify both the amount and bioactivity of tethered ALP, intact hydrogels and collagenase digests of hydrogels were incubated with p-nitrophenyl phosphate (pNPP), a synthetic substrate for ALP, and the change in absorbance at 405 nm was monitored. In addition, to demonstrate the ability of ALP functionalized hydrogels to promote biomineralization, which is the physiologic role of ALP, hydrogels were incubated in cell culture media containing 10% fetal bovine serum and 20 mM β-glycerophosphate and ALP mediated calcium phosphate deposition was quantified using a commercially available calcium assay.

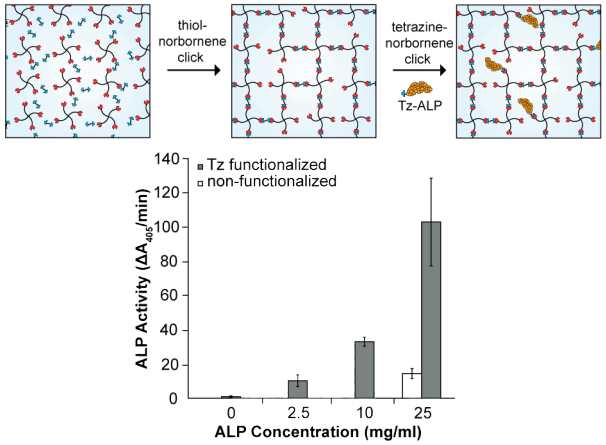

Fig. 1. Schematic illustrates hydrogel polymerization and ALP conjugation by sequential thiol-norbornene and tetrazine-norbornene click reactions. Plot shows concentration dependent ALP bioactivity, as measured by pNPP hydrolysis. Non-functionalized ALP was used as a control.

Results: PEG-peptide hydrogels were rapidly photopolymerized using thiol-norbornene click chemistry. While this approach offered excellent throughput for biomaterial fabrication, the presence of free radicals could potentially damage fragile proteins. For this reason, we used bioorthogonal tetrazine-norbornene click chemistry, which proceeds in water at physiologic temperature and pH without the need for an initiator or catalyst, for protein conjugation to unreacted norbornenes within the hydrogel network. ALP was used as a model protein, and the results showed that protein bioactivity was maintained, as the conjugated enzyme was able to hydrolyze pNPP (Fig. 1). Furthermore, the amount of tethered protein was tunable based on the concentration added to the hydrogel. However, pNPP hydrolysis was low for hydrogels incubated with non-functionalized ALP, demonstrating the efficacy of the tetrazine-norbornene click reaction. Finally, conjugated ALP promoted calcium deposition within the hydrogels, suggesting potential utility for applications in bone tissue engineering.

Conclusions: Click chemistry has emerged as a powerful tool for the design and synthesis of hydrogel biomaterials. Here, we demonstrate that sequential thiol-norbornene and tetrazine-norbornene click reactions can be used to polymerize synthetic PEG hydrogels and then conjugate proteins under mild conditions that maintain bioactivity. This approach could have broad applicability for the development of advanced functional hydrogels for applications in drug delivery and tissue engineering.

References:

1. DeForest CA, Anseth KS. Angew Chem Int Ed Engl. 2012; 51:1816-1819.
2. Fairbanks BD, et al. Adv Mater. 2009; 21:5005-5010.
3. Alge DL, et al. Tet Lett. 2013; 54:5639-5641.