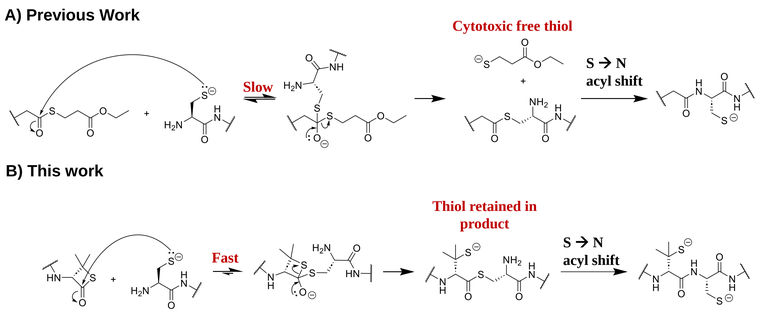
# Synthesis of Bio-Orthogonal Hydrogels via Penicillamine β-Thiolactone-Mediated Native Chemical Ligation

**Kaelin Marshall**[a], Matt Currier[a], TranTroung[a], Dylan Sager[a], Linqing Li[b], Nate Oldenhuis[a]
*University of New Hampshire, [a]Department of Chemistry, [b]Department of Chemical Engineering and Bioengineering*
Kaelin.Marshall@unh.edu

## Introduction

Although monolayer cell cultures have been the basis for in vitro studies, there has been interest in developing 3D cell culture mediums that better mimic the *in vivo* cellular environment. It is demonstrated that 3D scaffolds for cell culture provide more predictive and relevant data for future animal studies.[1] Covalently cross-linked hydrogels are typically preferred due to their stability and ability to simulate the environment of most soft tissues.[1,2] Modifications to the cross-linking strategies for hydrogel cell cultures must be robust, nontoxic, and possess favorable kinetics. Native chemical ligation (NCL) is a promising strategy, though this method has previously few applications in cell encapsulation, as the alkyl thioester of ethyl 3-mercaptopropionate-succinic acid (EMPSA) results in slow kinetics and a cytotoxic free thiol byproduct.[3,4]




**Scheme 1.** *Comparison of previous work with traditional NCL and this work. a. The reaction scheme of 4-arm PEG-EMPSA with 4-arm PEG N-terminal cysteine (cys) revealing slow kinetics and free thiol byproduct. b. Our proposed 4-arm PEG-β-thiolactone (PBET) with 4-arm PEG-cys possesses enhanced kinetics through the β-thiolactone ring opening while retaining the thiol leaving group in the PEG backbone.*

Here we introduce NCL modifications to explore eliminating cytotoxic byproducts and increase the reaction rate by using a β-thiolactone. The inclusion of the β-thiolactone results in retention of the thiol within the gel, as well as enhanced kinetics due to the thermodynamic favorability of the ring opening and amide formation. We achieved this by functionalizing 4-arm PEG with a cyclized thioester derived from the amino acid penicillamine: penicillamine-β-thiolactone (PBET).

## Experimental Design

We produced an improved PEG hydrogel system with β-thiolactone moieties, thus affording gelation in < 2 min while avoiding cytotoxic free thiols.

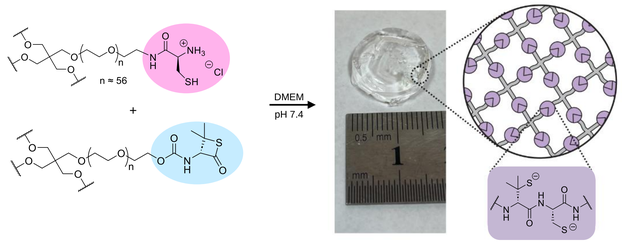
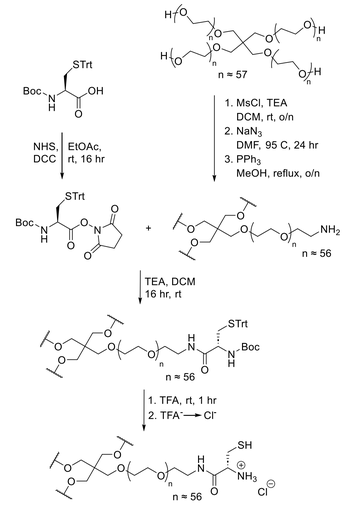



**Figure 1.** *Hydrogel cross-linking via β-thiolactone-mediated NCL. The hydrogel backbone is composed of 4-arm PEG-PBET and 4-arm PEG-cys, with the thiol byproduct retained in the network.*

## Synthesis & Properties




**Scheme 2.** *Synthesis of 4-arm PEG-cys from 4-arm PEG (10kDa) and Boc/Trityl-protected cys. 4-arm PEG-PBET was afforded through carbamate formation.*

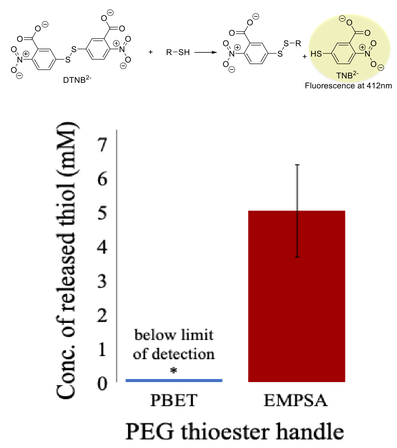



**Figure 2.** *Ellman's assay for 5 wt% gels verify no detectable free thiol in PBET gels compared to 5 mM in EMSPA gels. Elevated free thiol levels induce protein misfolding disrupt glutathione metabolism.*

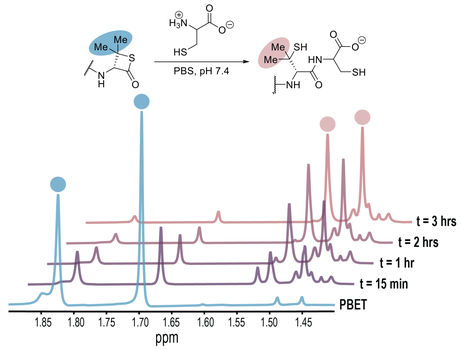



**Figure 3.** *$^1$H NMR spectrum confirms PEG-PBET NCL with cys. After NCL ring opening, PBET methyl peaks shift from 1.83 and 1.70 ppm to 1.53 and 1.48 ppm, respectively. Conditions: 2 mM PEG-PBET, 32 mM cys in PBS (pH 7.4).*

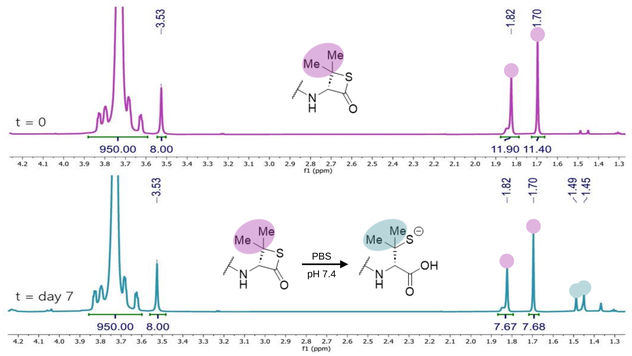
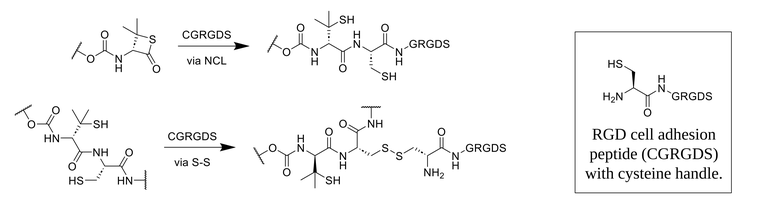



**Figure 4.** *$^1$H NMR spectrum shows slight hydrolysis of PEG-PBET, with 33% degradation at day 7. Conditions: 2 mM PEG-PBET in PBS (pH 7.4). Hydrolysis of PBET causes methyl peaks to shift from 1.82 and 1.70 ppm to 1.49 and 1.45 ppm, respectively.*

## Cell Adhesion




***Scheme 3.*** *Modes of RGD attachment through NCL reaction with free PEG-PBET or through disulfide bridging.*

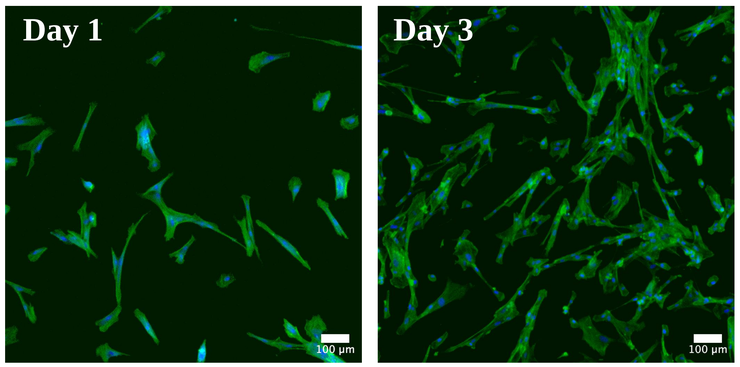
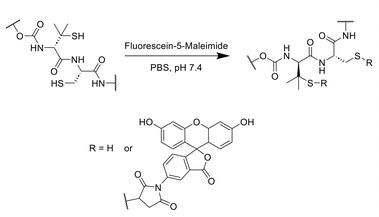



**Figure 5.** *2D cell studies with human dermal fibroblasts (HDFs). Laser scanning confocal microscopy (LSCM) of HDF cells shows cell proliferation and adhesion to RGD attachment motifs. Day 1 and 3, 5% wt., 10k, 4 mM RGD. Stained with DAPI (DNA, blue) amd Phalloidin 488 (actin, green).*

## Post-Gelation Modifications

Post-gelation modifications of our hydrogels expand their application and tunability. Just like S-S bridging can incorporate CGRGDS motifs, the incorporated thiols can serve as functional handles for modifications via thiol-Michael addition (S-MA) with acrylates, acrylamides, and maleimides (Mal).




**Scheme 4.** *Attachment of fluorescein-5-mal to thiols in the formed hydrogel. The Mal moiety undergoes (S-MA) at the PEG-cys and PEG-PBET junctions, covalently attaching the fluorophore to the gel backbone.*

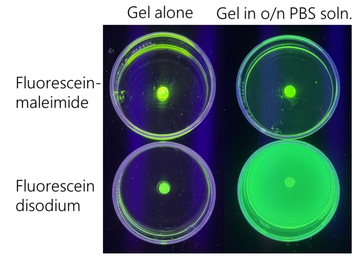



**Figure 6.** *PEG-PBET/cys gels treated with fluorescein +/− maleimide. Minimal diffusion when soaked in buffer shows covalent attachment via S-MA (+Mal), compared to the −Mal control. Conditions: 10% wt, 10 mM fluorescein (+/− Mal), PBS (pH 7.4).*

## Acknowledgments

Thank you to Wynter Pavia, the Oldenhuis Group, UNH Chemistry Department, UIC, UNH CIBBR, and our funding sources: NIH P20GM113131, NSF IIA, NSF1757371, NSF CAREER 2340569, UNH Core Pilot.

## References

1. Cacciamali, A., et al. *Front. Physiol.* **2022**, *13*, 836480.
2. Echalier, C.,et al., E. S. Mater. Today Commun. 2019, 20, 100536.
3. Hu, B.H., et al., *Biomacromolecules* **2009**, *10* (8), 2194–2200.
4. Jung, J.P., et al., *Biomacromolecules* **2013**, *14* (9), 3102–3111.