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27-05-2021	Final Report	26-Aug-2013 - 25-Aug-2016

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Final Report: Mechanically Actuated Peptide-Polymer Thin Films for Selective Capture and Release

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W911NF-13-1-0242

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER
611102

6. AUTHORS
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5d. PROJECT NUMBER

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5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAMES AND ADDRESSES
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12. DISTRIBUTION AVAILIBILITY STATEMENT

Approved for public release; distribution is unlimited.

13. SUPPLEMENTARY NOTES

The views, opinions and/or findings contained in this report are those of the author(s) and should not contrued as an official Department of the Army position, policy or decision, unless so designated by other documentation.

14. ABSTRACT

The goal of this project is to develop new methods for crosslinking biopolymers to yield biocompatible hydrogels using fast, easy to use, and mild chemistries. Over the course of the project, we developed a method to crosslink alginate and gelatin polymers using tetrazine-norbornene click chemistry. Our approach is to create two separate populations of polymer, each bearing one of the reaction partners (e.g. alginate-tetrazine, AlgT, or alginate-norbornene, AlgN), then mix the two polymer types to induce gelation. The crosslinking reaction occurs spontaneously and rapidly in the absence of any catalyst. The ratio of AlgN:AlgT can be altered to modulate

15. SUBJECT TERMS

polymers, hydrogels, click chemistry

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a. REPORT	b. ABSTRACT	c. THIS PAGE			Neel Joshi
UU	UU	UU	UU		19b. TELEPHONE NUMBER 617-373-2824

RPPR Final Report
as of 01-Jun-2021

Agency Code: 21XD

Proposal Number: 63846LS **Agreement Number: W911NF-13-1-0242**
INVESTIGATOR(S):

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DUNS Number: 047006379 EIN: 042103580
Report Date: 25-Mar-2016 Date Received: 27-May-2021
Final Report for Period Beginning 26-Aug-2013 and Ending 25-Aug-2016
Title: Mechanically Actuated Peptide-Polymer Thin Films for Selective Capture and Release
Begin Performance Period: 26-Aug-2013 **End Performance Period:** 25-Aug-2016
Report Term: 0-Other
Submitted By: Neel Joshi Email: ne.joshi@northeastern.edu
Phone: (617) 373-2824
Distribution Statement: 1-Approved for public release; distribution is unlimited.

STEM Degrees: 1 **STEM Participants:** 1

Major Goals: Aim 1: Synthesize hydrogel thin films with inherent affinity for tagged proteins supported by a stretchable substrate.
We will develop synthetic procedures to fabricate two-component hydrogels with novel composition, consisting of a branched poly(ethylene glycol) polymer and an antibody-mimetic peptide (aptide). The aptide will be chosen such that it exhibits conformationally specific binding to its target. The hydrogel will be covalently crosslinked to the surface of a stretchable polymeric membrane. We will elucidate the physical characteristics of the hydrogel, including its mechanical properties, capacity to swell, and its molecular structure.

Aim 2: Evaluate the ability of peptide-polymer hydrogel thin films to specifically capture tagged proteins.
The affinity of the soluble modified aptides for their binding targets (His-tagged proteins) will be measured with surface plasmon resonance (SPR) studies. After incorporating the aptides into the substrate-supported hydrogel, their ability to bind to a His-tagged fluorescent protein will be probed using a plate-based assay. Target binding will be investigated in the presence of un-tagged proteins to demonstrate specificity.

Aim 3: Explore mechanically-actuated release of tagged proteins from the hydrogel surface.
Triggered release of the His-tagged protein from the hydrogel will be investigated using a commercially available apparatus for mechanical membrane actuation. The apparatus is capable of applying equibiaxial tension to the stretchable membrane in controlled regimens (static, cyclic, etc.). Fluorescence and ELISA assays will be used to quantify released protein as a function of hydrogel strain and time.

Accomplishments:
See attachment for Scientific Progress and Accomplishments

Training Opportunities: This grant supported the training of one PhD student, one masters student, and one undergraduate student over its entire duration. The graduate student (Rajiv Desai) was jointly advised by the PI (Neel Joshi), and David Mooney (Harvard SEAS). Rajiv defended his doctoral thesis ("Click Functionalized Polymeric Biomaterials for Tissue Engineering") and obtained his PhD in 2016. The masters student, Thuur van Onzen, was a visiting student funded by his home institution, Eindhoven University (Netherlands). Work on the project funded by this grant was the basis of Thuur's masters thesis, which he successfully defended in 2014. The undergraduate was David Liu (Harvard). All three students were mentored directly by the PI through regular 1-on-1 meetings and presentations at group meetings. Trainees supported by this grant also presented work at national conferences, such as the ACS National Convention (Spring 2013, New Orleans) and Materials Research Society National Convention (Fall 2015, Boston).

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Results Dissemination: The results from this project have been disseminated at conferences and invited talks, as summarize below. They have also been disseminated through publications, as listed in the “Products” section of this report.

Conferences
- ACS National Convention (Spring 2013) – Presenter: Rajiv Desai
- MRS Annual Fall Conference (Fall 2015) – Presenter: Rajiv Desai
- Gordon Research Conference, Bio-Inspired Materials (Summer 2012) – Presenter: Neel Joshi
- International Conference of Young Researchers on Advanced Materials (Singapore, 2012) – Presenter: Neel Joshi
- ACS National Convention (Fall 2013) – Presenter: Neel Joshi
- Biomedical Engineering Society Annual Conference (Tampa, 2015) – Presenter: Neel Joshi
- MRS Annual Fall Conference (Fall 2016) – Presenter: Neel Joshi
-

Invited talks
- Topics in Bioengineering (Fall 2013, Harvard BioE Seminar) – Presenter: Neel Joshi
- Emory University (Fall 2014, Chemistry Department Seminar) – Presenter: Neel Joshi
- Nanotechnology in Medicine Conference (Spring 2014, Harvard Medical School) – Presenter: Neel Joshi
- Northwestern University (Fall 2016, Intl Institute for Nanotech) – Presenter: Neel Joshi
- University of Chicago (Fall 2016, Institute for Molecular Engineering) – Presenter: Neel Joshi

Honors and Awards: Nothing to Report

Protocol Activity Status:

Technology Transfer: Patent filed:

"Click-crosslinked hydrogels and methods of use" (US10821208B2) - ARO funding acknowledged.

PARTICIPANTS:

Participant Type: Faculty
Participant: Neel Joshi
Person Months Worked: **Funding Support:**
Project Contribution:
National Academy Member:

Participant Type: Graduate Student (research assistant)
Participant: Rajiv Desai
Person Months Worked: **Funding Support:**
Project Contribution:
National Academy Member:

ARTICLES:

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as of 01-Jun-2021

Publication Type: Journal Article Peer Reviewed: Y **Publication Status:** 1-Published
Journal: Biomaterials
Publication Identifier Type: Publication Identifier:
Volume: 5.0E+001 Issue: 0 First Page #: 30
Date Submitted: Date Published:
Publication Location:
Article Title: Versatile click alginate hydrogels crosslinked via tetrazine–norbornene chemistry
Authors:
Keywords: alginate, click chemistry, cell adhesion, cell encapsulation, tissue engineering
Abstract: Alginate hydrogels are well-characterized, biologically inert materials that are used in many biomedical applications for the delivery of drugs, proteins, and cells. Unfortunately, canonical covalently crosslinked alginate hydrogels are formed using chemical strategies that can be biologically harmful due to their lack of chemoselectivity. In this work we introduce tetrazine and norbornene groups to alginate polymer chains and subsequently form covalently crosslinked click alginate hydrogels capable of encapsulating cells without damaging them. The rapid, bioorthogonal, and specific click reaction is irreversible and allows for easy incorporation of cells with high post-encapsulation viability. The swelling and mechanical properties of the click alginate hydrogel can be tuned via the total polymer concentration and the stoichiometric ratio of the complementary click functional groups. The click alginate hydrogel can be modified after gelation to display cell adhesion peptides for 2D ce

Acknowledged Federal Support:

Publication Type: Journal Article Peer Reviewed: Y **Publication Status:** 1-Published
Journal: ChemMedChem
Publication Identifier Type: Publication Identifier:
Volume: 1.0E+001 Issue: 4.0E+000 First Page #: 617
Date Submitted: Date Published:
Publication Location:
Article Title: In Vivo Targeting through Click Chemistry
Authors:
Keywords: bioorthogonal chemistry · click chemistry · drug delivery · drug targeting · gels
Abstract: Targeting small molecules to diseased tissues as therapy or di- agnosis is a significant challenge in drug delivery. Drug-eluting devices implanted during invasive surgery allow the controlled presentation of drugs at the disease site, but cannot be modi- fied once the surgery is complete. We demonstrate that bioor- thogonal click chemistry can be used to target circulating small molecules to hydrogels resident intramuscularly in dis- eased tissues. We also demonstrate that small molecules can be repeatedly targeted to the diseased area over the course of at least one month. Finally, two bioorthogonal reactions were used to segregate two small molecules injected as a mixture to two separate locations in a mouse disease model. These re- sults demonstrate that click chemistry can be used for pharma- cological drug delivery, and this concept is expected to have applications in refilling drug depots in cancer therapy, wound healing, and drug-eluting vascular grafts and stents.

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Publication Type: Journal Article Peer Reviewed: Y **Publication Status:** 1-Published
Journal: Advanced Healthcare Materials
Publication Identifier Type: DOI Publication Identifier: 10.1002/adhm.201500757
Volume: 5 Issue: 5 First Page #: 541
Date Submitted: 6/14/17 12:00AM Date Published: 3/1/16 5:00AM
Publication Location:
Article Title: Click-Crosslinked Injectable Gelatin Hydrogels
Authors: Sandeep T. Koshy, Rajiv M. Desai, Pascal Joly, Jianyu Li, Rishi K. Bagrodia, Sarah A. Lewin, Neel S. Jo
Keywords: click chemistry; degradable; gelatin; hydrogels; injectable
Abstract: Injectable gelatin hydrogels formed with bioorthogonal click chemistry (ClickGel) are cell-responsive ECM mimics for in vitro and in vivo biomaterials applications. Gelatin polymers with pendant norbornene (GelN) or tetrazine (GelT) groups can quickly and spontaneously crosslink upon mixing, allowing for high viability of encapsulated cells, establishment of 3D elongated cell morphologies, and biodegradation when injected in vivo.
Distribution Statement: 3-Distribution authorized to U.S. Government Agencies and their contractors
Acknowledged Federal Support: **Y**

CONFERENCE PAPERS:

Publication Type: Conference Paper or Presentation **Publication Status:** 1-Published
Conference Name: Materials Research Society Annual Spring Meeting
Date Received: 14-Jun-2017 Conference Date: 14-Apr-2015 Date Published:
Conference Location: San Francisco
Paper Title: Versatile click alginate hydrogels
Authors: Rajiv Desai, Sandeep T. Koshy, David J. Mooney, Neel S. Joshi
Acknowledged Federal Support: **Y**

DISSERTATIONS:

Publication Type: Thesis or Dissertation
Institution: Harvard University
Date Received: 14-Jun-2017 Completion Date: 1/1/16 6:52PM
Title: Click Functionalized Polymeric Biomaterials for Tissue Engineering
Authors: Rajiv Desai
Acknowledged Federal Support: **N**

INVENTIONS:

Intellectual Property Type: Invention
Invention Title: Click-Crosslinked Hydrogels and Methods of Use
Description:
Inventors:
Employer Name:
Employer Address:
Confirmatory Instrument:
Intellectual Property Type: Invention
Invention Title: Refillable Drug Delivery Devices and Methods of Use Thereof
Description:
Inventors:
Employer Name:

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as of 01-Jun-2021

Employer Address:
Confirmatory Instrument:

PATENTS:

Intellectual Property Type: Patent Date Received:
Patent Title: Click-Crosslinked Hydrogels and Methods of Use
Patent Abstract:
Patent Number:
Patent Country:
Application Date: Application Status:
Date Issued:

Intellectual Property Type: Patent Date Received:
Patent Title: Refillable Drug Delivery Devices and Methods of Use Thereof
Patent Abstract:
Patent Number:
Patent Country:
Application Date: Application Status:
Date Issued:

Partners

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I certify that the information in the report is complete and accurate:
Signature: Neel Joshi
Signature Date: 5/27/21 9:51AM

Abstract
The goal of this project is to develop new methods for crosslinking biopolymers to yield biocompatible hydrogels using fast, easy to use, and mild chemistries. Over the course of the project, we developed a method to crosslink alginate and gelatin polymers using tetrazine-norbornene click chemistry. Our approach is to create two separate populations of polymer, each bearing one of the reaction partners (e.g. alginate-tetrazine, AlgT, or alginate-norbornene, AlgN), then mix the two polymer types to induce gelation. The crosslinking reaction occurs spontaneously and rapidly in the absence of any catalyst. The ratio of AlgN:AlgT can be altered to modulate mechanical properties and availability of pendant functional groups after crosslinking for further functionalization (for example, with cell adhesive peptides). We have demonstrated hydrogels made in this manner can support cell growth and proliferation in three-dimensional culture. Furthermore, unlike other covalent crosslinking chemistries, this approach does not cross-react with any biological functional groups, enabling the encapsulation of cells and other biological cargo in situ without deleterious effects. We have also extended the methodology to apply to gelatin-based polymers. Overall, this represents a new method to create a range of hydrogels that are compatible with in vivo injection using fast, convenient, and reliable chemistry.

Final Report

1) Foreword

The goals of this project have shifted considerably since the project began. The details of this shift are summarized here for context. The original goal of the project was to create mechanically actuated hydrogels for drug delivery (Fig. 1). These systems were inspired by

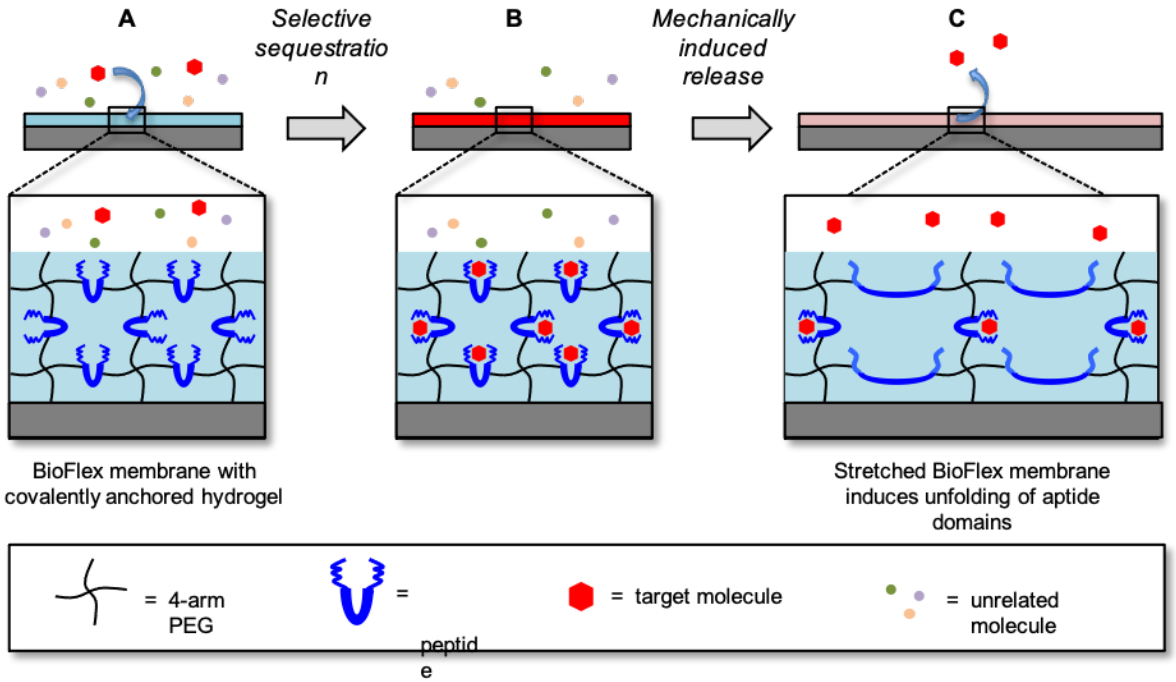

Figure 1: Mechano-sensitive hydrogels for selective sequestration and release (original proposal Aims). **A**) The hydrogel will contain antibody-mimetic peptides (aptides) integrated into its structure as a crosslinking unit. **B**) The inherent affinity of the aptide for His-tagged proteins will allow it to selectively sequester a target from a complex mixture. **C**) Mechanical tension applied to the hydrogel film will unfold the integrated aptide domains, diminishing their affinity for the target and releasing it.

natural ECM protein, which undergo predictable conformational changes in response to tensile stresses, and thereby switch between two states with differing affinity for affinity for a target molecule. We wanted to create synthetic systems that mimicked this behavior by using folded protein/peptide domains as crosslinkers in a macroscopic hydrogel network. Accordingly, mechanical force applied to the bulk structure would impart force on the protein crosslinkers, thereby causing them to unfold. The protein crosslinkers would be designed such that they exhibited a conformationally specific binding ability so that mechanically induced unfolding would cause them to release a bound target.

During the early phases of the project, when we were investigating different polymer and protein systems with which to fabricate the mechanically actuated hydrogels, we found that existing crosslinking chemistries were too slow, low yielding, and inconsistent to result in macroscopic hydrogels with reproducible properties. Furthermore, the amounts of protein required to fabricate hydrogels reliably was prohibitively expensive. In order to address the first problem, we developed a new crosslinking strategy based on tetrazine-norbornene click chemistry. However, despite our best efforts we were not able to synthesize protein crosslinked hydrogels in sufficient quantities to measure their mechanical properties and binding/release characteristics.

After consulting with our program officer, we decided to shift the goals of the project to focus on further developing the click chemistry crosslinking strategy, as we found this to be both novel and highly effective. During the project period, we demonstrated the use of the click chemistry for fabricating alginate and gelatin hydrogels, and investigated their use in cell encapsulation studies. We feel we have been quite successful in this endeavor, with three published papers in high-impact journals and filed one patent.[1–3] Our progress in this area is detailed below.

2) Statement of the problem to be studied

Hydrogels are used widely for a range of applications from drug delivery to tissue engineering because of their high hydration that mimics biological tissues and their compatibility in vivo. Several such applications rely on the ability to encapsulate biological cargo (i.e. cells, biomolecules, or small molecules) and deliver them in vivo. Despite this main function of hydrogels, there remains a need for better chemistries for crosslinking their constituent polymers. Most covalent crosslinking chemistries that are used conventionally cross-react with functional groups found on cells, decreasing cell viability and leading to unpredictable biological effects. A crosslinking chemistry that is fast, easy to use, and bio-orthogonal is needed. A more detailed description of the deficiencies in existing crosslinking chemistries, with respect to specific polymers, can be found in our attached publications.

3) Summary of most important results

Our results are described in detail in the attached publications. A brief summary is provided here. We have demonstrated that tetrazine-norbornene click chemistry is a highly effective method for creating hydrogel constructs with encapsulated cells. We first demonstrated this approach with both alginate and gelatin, but in principle, it should be compatible with any polymer that has functionalizable side chains. The overall strategy is to modify the polymer backbone with the tetrazine (T) or norbornene (N) functional groups to create two different polymer species (Fig. 2). All but one of the components for these reactions are commercially

available. The tetrazine-bearing molecule can be synthesized in one step from commercially available precursors and isolated in gram quantities per reaction. This combination should make our approach easily accessible to other researchers, even without synthetic chemistry expertise. The N and T polymers are individually stable and can be stored in dry form on the shelf for months or perhaps longer. When combined, the N and T functional groups react spontaneously without the need for any catalyst or energy input to form a crosslinked hydrogel network. The ratio between the N and T polymers can be varied to modulate the mechanical and swelling properties of the gels, with plateau moduli ranging from 1-20 kPa for alginate gels and 0.2-5 kPa for gelatin gels (Fig. 3).

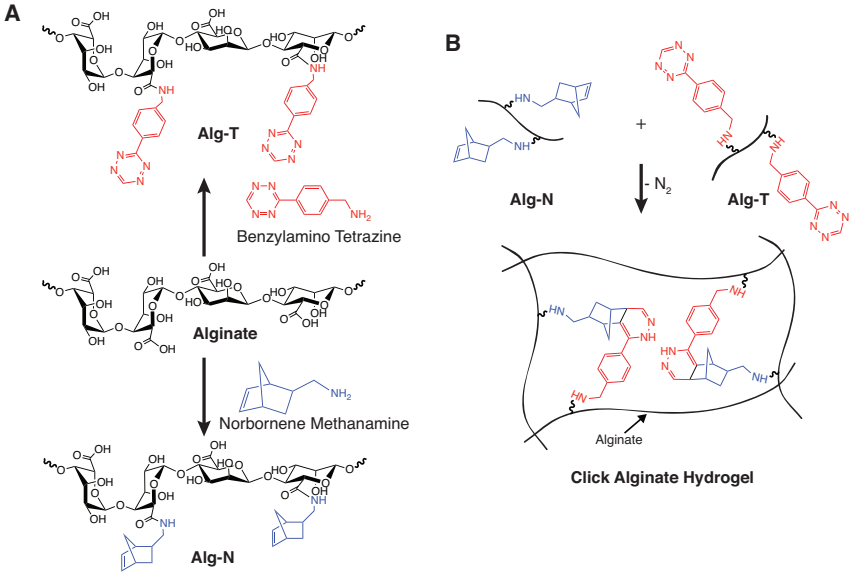

Figure 2: Fabrication of click alginate hydrogels. Schematic of click alginate polymer synthesis. Aqueous carbodiimide chemistry is used to modify alginate backbone car- boxylic acids with tetrazine or norbornene, resulting in Alg-T or Alg-N polymers respectively (A). Alg-T and Alg-N polymers are mixed together to create a covalently crosslinked click alginate hydrogel network, with the loss of N_2 (B).

Hydrogels fabricated using this method are highly biocompatible. Since the crosslinking reaction does not go to 100% completion, there remain pendant N and T groups that can be further functionalized. For example, we used photocatalyzed thiol-ene chemistry to append cell binding epitopes to preformed alginate hydrogels, which promoted cell spreading and adhesion. Cells that are encapsulated in the gels remain viable and metabolically active for several days and proliferate. The gelation kinetics of the hydrogels makes them convenient to use for encapsulation and injection, since they can be combined and handled for several minutes before they become too viscous for injection or mixing. Click-alginate gels injected subcutaneously into mice remain intact for at least 3 months without exhibiting any degradation or inducing significant inflammation. This may make them useful for cell implantation studies where long-term survival is needed in concert with isolation from the surrounding environment, as might be the case for islet cell implantation in diabetic therapies. In contrast, calcium crosslinked alginate gels degraded rapidly in vivo. Click-gelatin gels are biocompatible and exhibit cell spreading, adhesion, and viability characteristics (Fig. 4). However, since gelatin contains protease cleavable sequences, cells encapsulated in these gels can degrade and remodel their environment. This makes Click-gelatin gels potentially useful for tissue engineering applications where in vivo scaffold degradation must be precisely tuned.

Overall, the chemistry we have developed is highly practical and effective way to encapsulate cells and other biological cargo. Several groups who work with these polymers have approached about collaborations due to the inadequacies in the gel systems they are currently using.[4] We are pursuing other applications of these polymers, related to three-dimensional printing to create physiologically relevant tissue constructs like muscle, bone, and vascular systems.

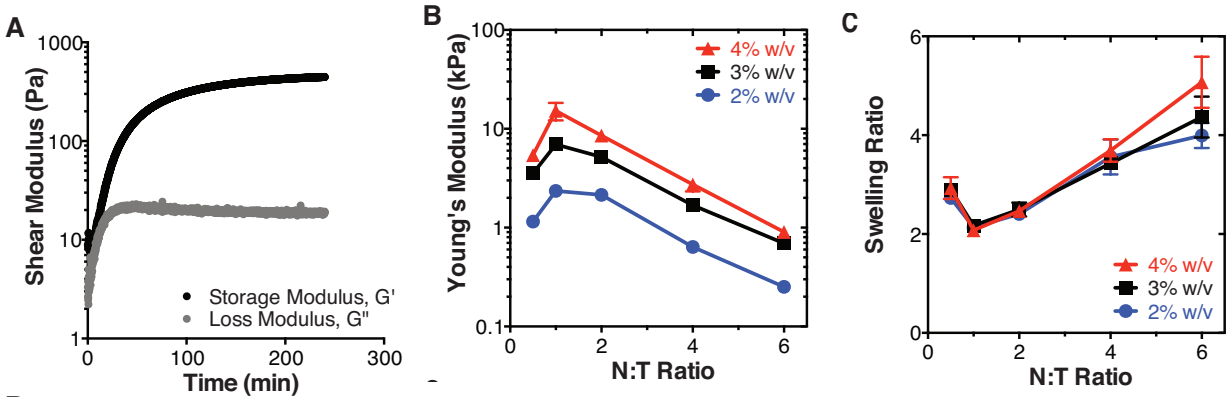

Figure 3. Click alginate hydrogel mechanical properties. Representative in situ dynamic rheometry plot at 25 C for 3% w/v click alginate at N:T 1⁄4 1, demonstrating modulus evolution with time (A). Compressive Young's modulus (B) and volumetric swelling ratios (C) for 2%, 3% and 4% w/v click alginate hydrogels at varying N:T ratio. Values represent mean and standard deviation (n 1⁄4 4).

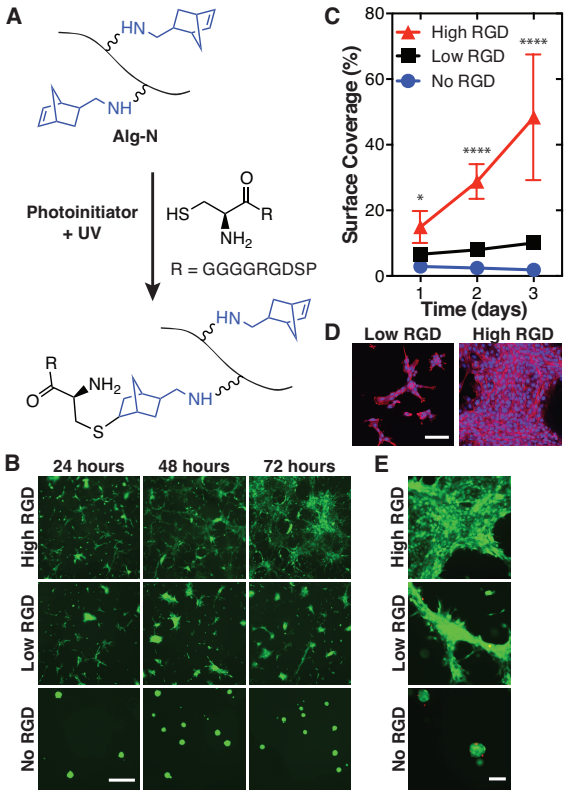

Figure 4. Cell adhesion, spreading, and proliferation on click alginate hydrogels modified with RGD peptides after synthesis. Schematic of CGGGGRGDSP peptide coupling reaction onto click alginate hydrogel surface using photoinitiated thiol-ene chemistry (A). Representative images of 3T3 fibroblast adhesion, spreading, and proliferation on click alginate hydrogels with varying RGD peptide density (scale bar 1⁄4 200 mm) (B), and quantification (Two-Way ANOVA with Turkey's post-hoc test, *p < 0.05, ****p < 0.0001 relative to No RGD control; Values represent mean and standard deviation, n 1⁄4 4e7) by endogenous EGFP expression (green) over 3 days (C). Phalloidin (red) and Hoescht 33342 (blue) staining of F- actin filaments and nuclei at 3 days for cells adherent to RGD modified click alginate hydrogels (scale bar 1⁄4 100 mm) (D). Representative fluorescent images of EGFP (green) 3T3 cells cultured on click alginate hydrogels with varying ligand density for 3 days and stained with ethidium homodimer-1 (red) (scale bar 1⁄4 100 mm) (E). The High, Low, and No RGD conditions refer to the 2 mM, 0.2 mM, and 0 mM peptide solutions used to modify the click alginate hydrogel surface.

4) Bibliography

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3. Desai, R. M., Koshy, S. T., Hilderbrand, S. A., Mooney, D. J. & Joshi, N. S. Versatile click alginate hydrogels crosslinked via tetrazine-norbornene chemistry. *Biomaterials* **50,** 30–37 (2015). doi:10.1016/j.biomaterials.2015.01.048

4. Gao, Y., Peng, K. & Mitragotri, S. Covalently Crosslinked Hydrogels via Step-Growth Reactions: Crosslinking Chemistries, Polymers, and Clinical Impact. *Adv Mater* 2006362 (2021). doi:10.1002/adma.202006362 doi:10.1002/adma.202006362