Pharmacological Regulation of Protein-Polymer Hydrogel Stiffness

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Abstract

Pharmacological-based Regulation of Protein-Polymer Hydrogel Stiffness

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The extracellular matrix (ECM) exhibits constant physiochemical changes throughout all biological processes, including organ development, maintenance of tissue homeostasis, and disease progression/ healing. User-programmable biomaterials afford exciting opportunities to study such dynamic processes in vitro, offering a means to probe biological fates in response to biochemical and biophysical changes in the ECM. Herein, we introduce a protein-polymer hydrogel biomaterial whose stiffness can be pharmacologically regulated with conventional antibiotics, providing a powerful first route to stimulate synthetic tissue

changes *in vivo*. Specifically, a coumermycin-mediated homodimerization of DNA gyrase subunit B (GyrB) tethered within the gel enables user-modulated physical crosslinking and a rheological increase in hydrogel mechanics. These unique platforms will prove useful in elucidating the effects of ECM-presented mechanical signals on cell function.

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Chapter 1. INTRODUCTION

The extracellular matrix (ECM) exists in all tissues and organs, serving as an important non-cellular component that supports cellular structure. ECM also directs cell fate and function, including migration, proliferation, and differentiation, through initiating crucial biophysical and biochemical signals within complex biomolecular interactions[1]. Such cues would draw out biochemical responses to regulate gene transcription and therefore change cell shape and cytoskeletal dynamics to govern cell growth as well as cell-cell communication[2,3]. Human mesenchymal stem cells (MSCs) direct their lineage-specific differentiation by varying the substrate elasticity[4]. Another example showed that ECM stiffness modulates cell proliferation through stimulated cyclin D1-dependent G1 cell cycle progression[3]. These observations demonstrate ECM-presented biophysical signals, especially material stiffness, play a critical role in regulating cell fate.

Despite this existing knowledge, understanding the specific role of ECM on cell function remains a challenge to achieve *in vivo*. The topology and composition of ECM is not only unique to each tissue, but the ECM structure is also highly dynamic[1]. For example, bone tissue has an average larger elastic modulus – a common measurement for stiffness – than brain tissue so that it can provide structural protection for organs[5]. Therefore, it is desirable to develop cell culture platforms where individual parameters can be systematically tuned, that is the dynamic

biochemical and biophysical properties offer user-defined controls to be manipulated in space and time. Researchers have turned to simplified biomaterial-based platforms for further understanding and hydrogels have proven to be a particularly attractive class of materials. Critical characteristics of hydrogels, including their high-water content, tissue-like elasticity, and facile transport of nutrients and waste, recapitulate critical aspects of the native ECM. In addition, cytocompatible hydrogel formulations can be employed to support cell encapsulation[6]. The initial stiffness and degradability of hydrogels can be simply modulated through a variety of chemical approaches, making them an ideal candidate to mimic ECM and be applied in complex cell culture environments.

Many strategies exist to form hydrogels with different static mechanics; however, far fewer exist to modify mechanical properties on demand in ways that recapitulate many developmental processes, homeostasis, and disease progression. Efforts to probe the biophysical responses in ECM that can either soften or stiffen over time are needed. Stimuli-responsive cell culture platforms have reported to be a powerful tool to recapitulate *in vivo* cellular environments owing to their capabilities to respond external stimuli with temporal and spatial precision[7]. Several techniques to date have demonstrated that cell fate can be controlled through stiffness medication with light[8–10], voltage[11], pH[12], and temperature[13].

While several strategies have been developed to understand fundamental biological processes, controlling biomaterial stiffness *in vivo* remain undeveloped. Towards this, our

group sought to create pharmacologically stiffening poly(ethylene glycol) (PEG)-protein-based hydrogels, whereby crosslinks could be added or destroyed in response to externally administered small molecules. The selected fusion protein was DNA gyrase subunit B (GyrB)[14]. The commercially available antibiotic coumermycin was obtained to dimerize GyrB protein since it does not have deleterious effect on humans. Intravenously administered antibiotic could be used to trigger gel material stiffening *in vivo*[15,16].

To enable antibiotic-mediated gel stiffening, hydrogels based on PEG were formed through a bioorthogonal strain-promoted azide-alkyne cycloaddition (SPAAC) between a 4-arm PEG bicyclononyne and a linear PEG diazide. Gels were modified with varying amounts of an azide-tagged GyrB, site-specifically modified at its C-terminus through a sortase-mediated transpeptidation reaction[17,18]. Gel stiffness was compared in the presence and absence of coumermycin-assisted protein dimerization at varying crosslinking percentages. Results successfully demonstrate antibiotic-mediated stiffening of conventional PEG hydrogels. This material offers an exciting new route to regulate biological fate *in vivo* through dynamic gel stiffening.

Chapter 2. EXPERIMENTAL METHODS

2.1 GyrB Sequences and Cloning Design

The GyrB gene with two restriction enzyme sites (i.e., NdeI and XhoI) at each end was obtained in the form of gBlocks through Integrated DNA Technologies (IDT). Both the GyrB gene and the STEPL plasmid template underwent restriction digest using NdeI and XhoI restriction enzymes and were then ligated together by applying T4 DNA Ligase[18,19]. The newly constructed plasmid was transformed into Rubidium Chloride (RbCl) competent Top 10 E. *Coli* and plated onto agar plates (5 g Yeast Extract, 10 g NaCl, 10 g Tryptone, 15 g Agar, 1 L dH_2O) containing carbenicillin (100 μg/mL). Colonies were formed on the plates and selected through antibiotic resistance. Plasmid mini preparation was then performed to extract and purify the plasmids. The sequence was confirmed by sending the resulting product to GENEWIZ for verifying correct mutation.

2.2 Synthesis of Azido-peptide (H-GGGGDDK(N_3)-NH_2)

To obtain GyrB protein with functional azide group through sortase-mediated reaction, peptides with an azide [H-GGGGDDK(N_3)-NH_2] were synthesized using solid-phase methodologies (Figure 2.1). The Rink amide resin-bound peptide Boc-GGGDDDK(Mtt)-NH_2 was first produced using Fmoc solid-phase peptide synthesis (SPPS, 0.25 mmol scale) performed with assistance of a CEM Liberty 1[20]. The resin underwent multiple washes with

dimethylformamide (DMF, 3x) and dichloromethane (DCM, 3x) before Mtt cleavage [2 min, 15 mL, 97:2:1 DCM:Triisopropylsilane (TIS):Trifluoroacetic Acid (TFA), 9x]. The resin was washed (DCM, 3x; DMF, 3x) prior to treatment (1 hr) with 4-azidobutanoic acid (4x, 1 mmol, 129 mg) by HATU coupling (3.95x, 0.988 mmol, 188 mg) and N,N-Diisopropylethylamine (DIEA, 8x, 2 mmol, 174 µL) in minimal DMF. Resin was washed (DMF, 3x; DCM, 3x) prior to peptide cleavage/deprotection (95:5 TFA:H_2O, 20 mL, 2 hr) and precipitation (diethyl ether, 180 mL, 0 °C, 2x). The crude peptide was purified through RP-HPLC using a 55-minute gradient from 5- 100% acetonitrile:H_2O. The collected product was lyophilized into white solids (62 mg, 0.087 mmol, 34% overall yield)[20]. Purified protein was confirmed via MALDI-TOF: calculated for $C_{26}H_{43}N_{12}O_{12}$ $[M + {}^1H]^+$, 715.31; observed 715.07 (Figure 2.2).

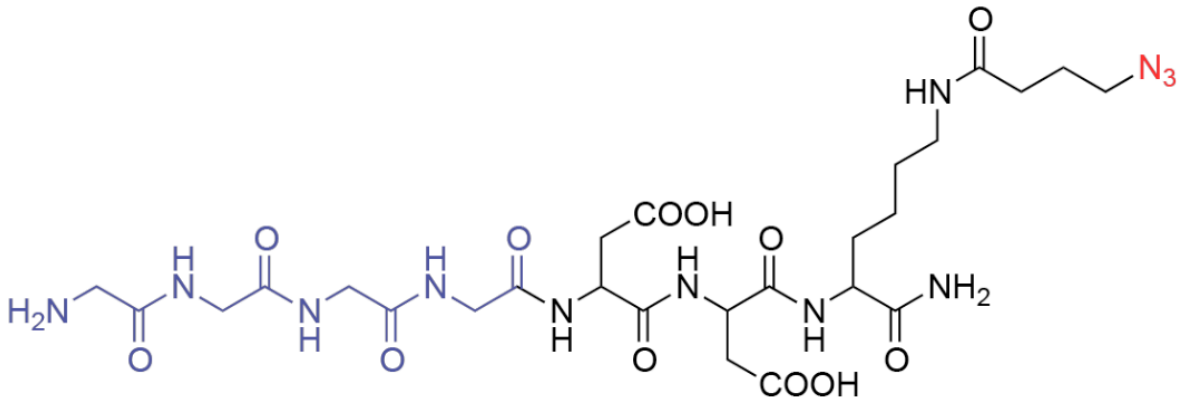

Figure 2.1 The chemical structure of azido-peptide [H-GGGGDDK(N_3)-NH_2]

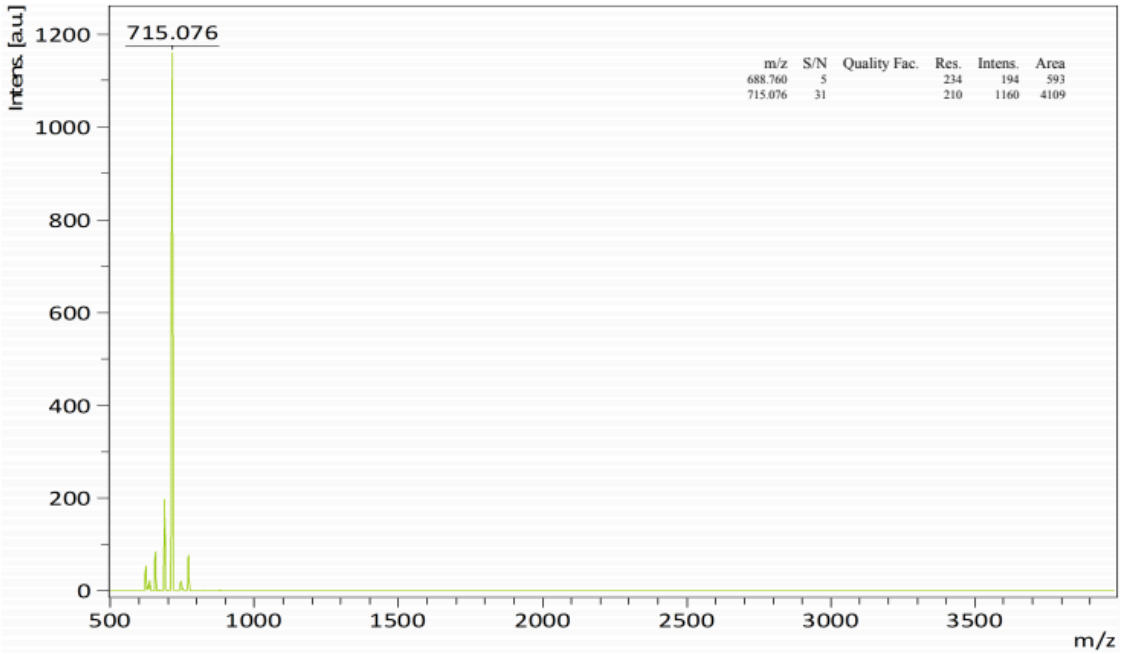

Figure 2.2 Characterization of synthesized azido-peptide [H-GGGGDDK(N_3)-NH_2]. Our synthesized peptide was characterized through MALDI-TOF mass spectrometry. The observed mass (715.07 Da) matched the expected value (715.31 Da).

2.3 GyrB Protein Synthesis

Confirmed STEPL plasmids with correct GyrB sequences were transformed into chemically competent BL21 cells for protein expression. The cells were plated onto agar plates (5 g Yeast Extract, 10 g NaCl, 10 g Tryptone, 15 g Agar, 1 L dH_2O) containing carbenicillin (100 μg/mL). Successful colonies were selected and grown in large batches of lysogeny broth (LB, 10 g Tryptone, 5 g yeast extract, 10 g NaCl, 1 L dH_2O) at 37 °C incubator with 200 rpm stirring condition. The culture growth was monitored through optical density (OD) measurements using a NanoDrop spectrophotometer (λ = 600 nm). When the value of OD

reached 0.6, isopropyl beta-D-1-thiogalactopyranoside (IPTG, 0.5 mM) was added into the culture to express the protein. The induced culture was placed at 18 °C, 200 rpm stirring condition overnight for at least 16 hours and then centrifuged. Bacteria cell pellets were obtained and resuspended in STEPL lysis buffer (20 mM Tris, 50 mM NaCl, 10 mM imidazole) using sonication. The sonicated mixture was then centrifuged at 5000 rpm for 20 minutes. The supernatant was added into a column containing 12 mL of Ni-NTA resin. The column was put on a rocking machine at 4 °C for an hour and then washed with 20 mL STEPL washing buffer (20 mM Tris, 50 mM NaCl, 20 mM imidazole) for at least 5 times. The conjugation buffer (20 mM Tris, 50 mM NaCl, 100 μM $CaCl_2$) with 20 molar excess of desired azido-peptide were added into the column to undergo Sortase-Mediated ligation at 37 °C and 200 rpm conditions for 4 hours. The protein was eluted by adding STEPL elution buffer (20 mM Tris, 50 mM NaCl, 250 mM imidazole) and the elution fractions were collected and dialyzed against STEPL buffer with snakeskin tubing (10,000 MWCO) at 4 °C. Eventually, the protein solution was concentrated applying an Amicon spin column (10 kDa molecular weight cut-off) by centrifuging at 5000 rpm for 30 minutes. GyrB protein expression and purification was repeated to generate quantities required for hydrogel formation.

2.4 GryB Protein Hydrogel Formation through SPAAC

GyrB protein hydrogel was formed by strain-promoted azide-alkyne cycloaddition (SPAAC). Synthesized GyrB protein with an azide group can bind with PEG-tetraBCN, which has a strained alkyne bond as part of its ring structure. The synthesis of linear PEG diazide and PEG-tetraBCN were based off previously published protocols reported by our group[20]. The coumermycin was dissolved in dimethyl sulfoxide (DMSO) for stock (50 mg/mL). The hydrogels with the total volume of 30 μL were formed by adding linear PEG diazide (4.64 mM, M_n~ 3.4 kDa), azide-modified GyrB protein (2.73 mM), Coumermycin (1.40 mM) and PEG-tetraBCN (3 mM, M_n~ 20 kDa) in this sequential order. While the concentration of each materials was modified to test the stiffness changes of hydrogel, the molar ratio of PEG-tetraBCN, azide-modified GyrB protein, and Coumermycin remained 1:4:2.

2.5 GyrB Protein Hydrogel Rheological Studies

Rheological experiments were performed by applying GyrB protein hydrogels on an Anton Paar rheometer. The hydrogels were formed on the rheometer by mixing PEG-tetraBCN (20 kDa) either in concentrations of 2, 3, and 4 mM with a linear PEG-diazide (3.09 – 7.01 mM), the azide-modified GyrB protein (0 – 2.72 mM) and Coumermycin (0 – 1.40 mM) between parallel plates (8 mm diameter). The hydrogels with the same constitution but without Coumermycin were also applied for rheological studies to compare the stiffness differences

between hydrogels with and without the pharmacological substances. A total volume of 30 μL was used to form hydrogels within 0.5 cm thickness between the plates. The storage and loss moduli (G' and G" respectively) were measured at constant strain (1%) and frequency (1 rad/s). G' and G" were recorded for 1 hour when hydrogels were formed on the rheometer.

Chapter 3. RESULT AND DISSCUSSION

Enlightened by the Weber et al. work that developed PEG-based pharmacologically tunable vaccine depot[16], our group successfully created stimuli-responsible hydrogels by incorporating GyrB protein and coumermycin crosslinkers as switches in PEG-based block polymers. We perform step-growth polymerization through strain-promoted azide-alkyne cycloaddition (SPAAC). A bicyclononyne structure on PEG polymers reacts with an azido functionality to rapidly form a triazole linkage under cytocompatible and physiological conditions. The bioorthogonal reaction enables protein species to be installed in network uniformly without concerning the loss of activity due to specific site mutagenesis. The SPAAC reaction connect a four-armed poly(ethylene glycol) tetra-bicyclononyne (PEG tetraBCN, Molecular weight ~ 20 kDa) with a linear PEG diazide (N_3-PEG-N_3, Molecular weight ~ 3.5 kDa), or a GyrB protein C-terminally modified with a single azide (GyrB-N_3). Addition of aminocoumarin antibiotic coumermycin promotes GyrB homodimerization. Therefore, the biomechanical property of this biohybrid material can be precisely controlled by adjusting its content, the ratio of the azide-modified components and the coumermycin antibiotic.

GyrB proteins with a reactive, bioorthogonal azide group at the end were obtained through sortase-mediated reaction. This enzyme-based bioconjugation technique utilize Sortase A (SrtA) from *Staphylococcus aureus*. SrtA is a calcium-assisted transpeptidase that recognizes

the LPXTG (where X represents random amino acid) peptide motif and helps bind with a targeted component once a N-terminal polyglycine peptide or protein present. Utilizing a strategy first introduced by Warden et al., we are capable of using sortase expressed protein ligation (STEPL) technique to process protein purification and conjugation with a single step[19,20]. On the C-terminus of GyrB gene sequence, it was fused with the amino acid sequence LPXTG, a flexible $(GGS)_5$ linker, SrtA and a hexahistidine (6xHis) tag. The whole protein was expressed and purified in a Ni-NTA column. Upon addition of Ca^{2+} and an azide-containing polyglycine peptide, the activated SrtA-ligated GyrB-N_3. The GyrB-N_3 was obtained while the rest of sortase chimera was cleaved off and remained on the column via 6xHis-tag. This chemoenzymatic process gives rise to creating protein-based material appending with reactive handle on its C-terminus with high labeling efficiencies[21].

To verify successful GyrB purification and modification with a functional azide group, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed with testing samples from each step during protein expression and purification (Figure 3.1). The result suggested that we obtained the desired biomolecules since a strong band near 28 kDa was present on the purified protein lane and our conjugated GyrB protein was estimated to possess 26 kDa molecular weight. On the other hand, a band showing near 55 kDa on washing and buffer elution lanes indicated the sortase chimera was separated successfully. The GyrB proteins were obtained with relatively high purity. Not satisfied with the approximation via

SDS-PAGE gel assay, mass spectroscopy was performed to precisely verify the synthesized GyrB bio-conjugated molecules. The observed mass (25.6 kDa) approximately matched the expected value (25.1 kDa) confirming that the mutant was correctly functionalized (Figure 3.2).

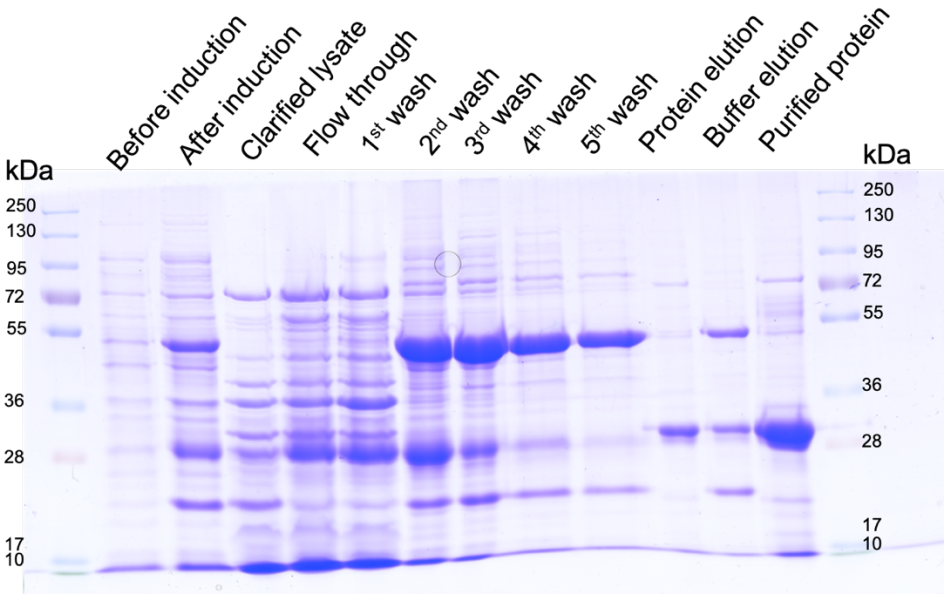

Figure 3.1. SDS-PAGE gel assay for GyrB protein expression and purification. SDS-PAGE gel assay was performed after protein expression and purification to approximately clarify or desired GyrB protein and check its purity. Samples from each steps of synthesis process were collected. GyrB (molecular weight ~26 kDa) were confirmed to be synthesized successfully due to the strong band shown near 28 kDa on the purified protein lane.

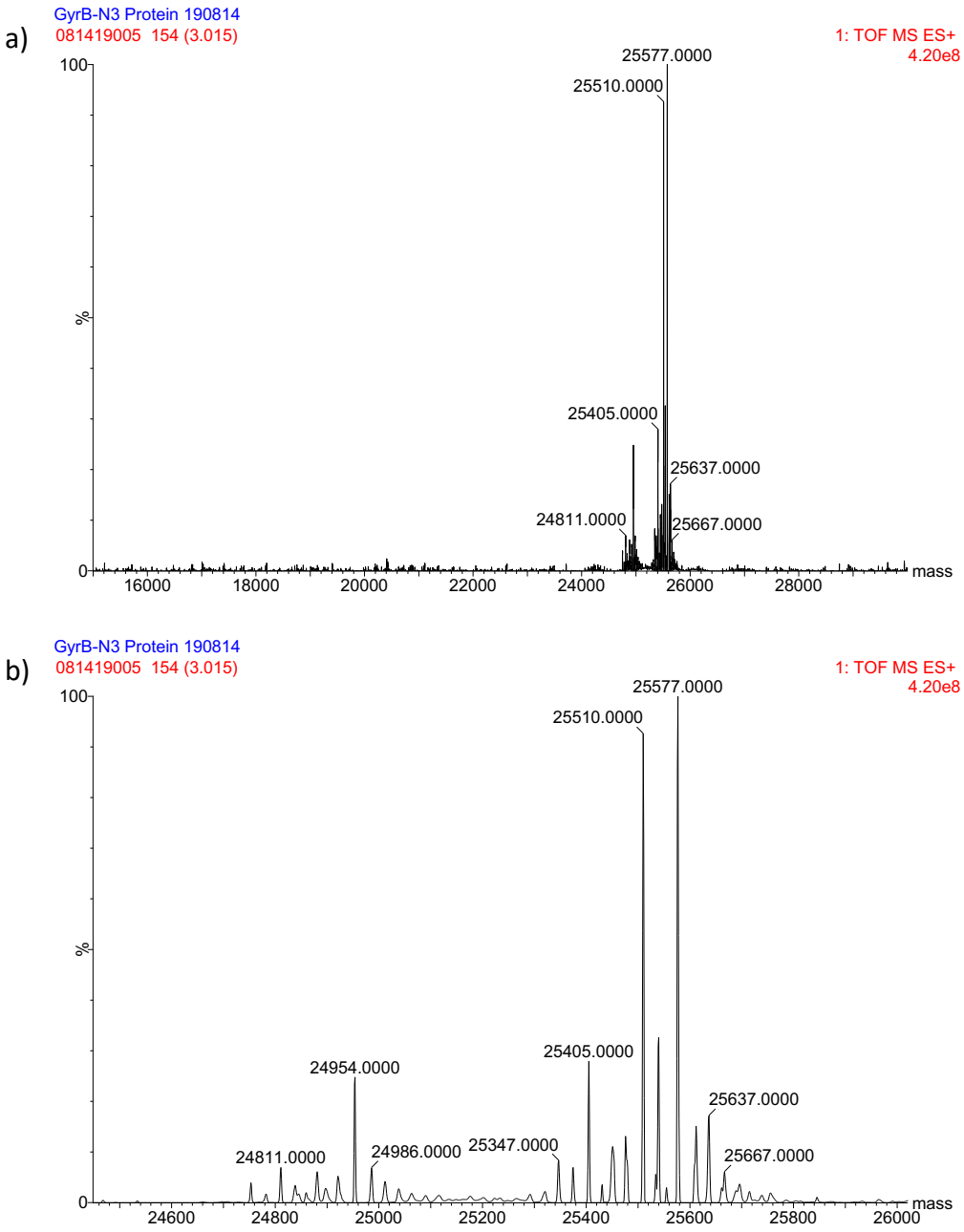

Figure 3.2. Mass spectroscopy of GyrB protein. GyrB fusion protein with an azide group functional end was accurately verified by mass spectrometry. The modified GyrB proteins have molecular weight around 25.6 kDa. a) Zoomed out mass spectrometric analysis of GyrB proteins. b) Zoomed in mass spectrometric analysis of GyrB proteins.

Antibiotic-responsive hydrogels were created through the SPAAC reaction by combining a 4-armed PEG bicyclononyne, a linear PEG diazide, and the azide-tagged GyrB protein in phosphate-buffered saline (pH = 7.4). The purified GyrB-N_3 proteins can be successfully produced at 5-6 mg per liter of 2xYT growth medium. In order to maximize the GyrB and coumermycin crosslinks in our hydrogel system, the gel is formed by mixing PEG-tetraBCN (3mM), linear PEG-diazide (4.63 mM) and the modified GyrB protein (2.73 mM), resulting in filling GyrB proteins up to 22% of the crosslinking structure. We examined the stiffness differences by recording the storage modulus (G') and loss modulus (G") of hydrogels in the presence or absence of coumermycin. The storage modulus of our antibiotic-mediated gels correlated to our hypothesis that gels with the coumermycin tend to be stiffer than the ones without coumermycin (Figure 3.3). We confirmed coumermycin dimerize the GyrB proteins and render the hydrogels with stiffer structure. It is confirmed that the stiffness of our designed hydrogel systems can be adjusted by the addition of antibiotics.

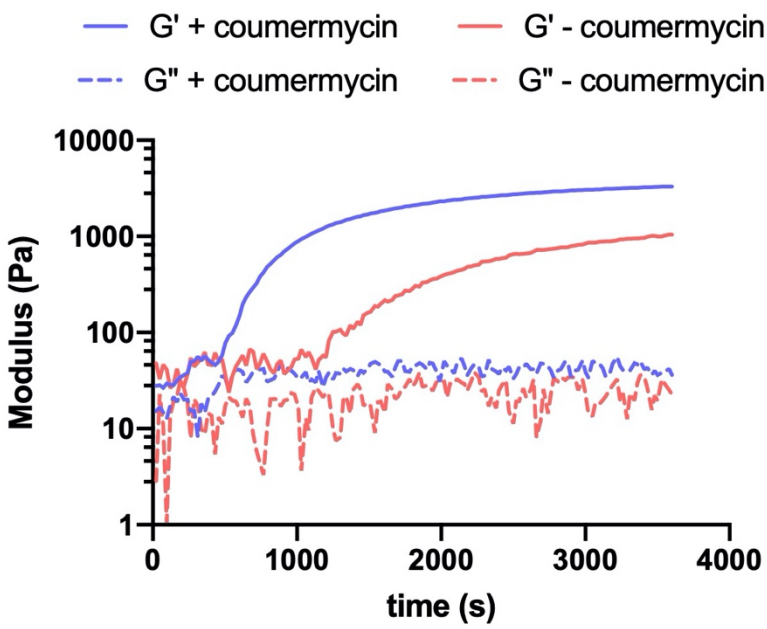

Figure 3.3. Storage (G') and loss (G") modulus of antibiotic-responsive hydrogels. The hydrogel was formed by combining PEG-tetraBCN (3 mM), linear PEG-diazide (4.63 mM) and the modified GyrB protein (2.73 mM) in the presence and absence of coumermycin. When coumermycin was applied, the hydrogel system has 22% of crosslink formed by the antibiotic dimerization with GyrB protein. The diagram showed that the hydrogels treated with coumermycin have higher G' indicating they possess a relative stiffer structure. Our biomaterials stiffness was proven to be user-defined by the addition of pharmacological substances.

To understand how the crosslink percentage of fusion GyrB proteins contributes to our hydrogel system, the coverage of 0, 12, and 22% were selected. The total volume of testing hydrogels, the content of PEG-tetraBCN (3 mM) and linear PEG-diazide (4.63 mM) remained

the same while adjusting the ratio between GyrB proteins and 1x STEPL buffer. Each crosslink percentage of GyrB hydrogels were tested with the presence or absence of coumermycin. For the materials with coumermycin, the storage modulus increases when the GyrB crosslink percentages increase, owing to the fact that G' is directly related to the extent of crosslinking. More GyrB protein coverage renders our hydrogel system a stiffer structure indicating the materials to obtain better performance on storing deformation energy in an elastic manner. On the other hand, G' shown in materials without coumermycin dimerization are lower than the ones with, and they all have similar values owing to their stiffness only contributed from the crosslinks of linear PEG-diazide (Figure 3.4 a).

The same approach was applied in hydrogels with different PEG-tetraBCN concentration, which are 4 mM and 2 mM respectively. This is to identify the role of PEG-tetraBCN in our hydrogel system. Similar results obtained indicating the stiffness increase when the inducible hydrogels were treated with coumermycin, while the hydrogels without coumermycin show roughly the same storage modulus as ones without GyrB crosslinks (Figure 3.4 b and c). Each G' results with 12% coverage of GyrB protein were put together for further comparison. The storage modulus was expected to increase with the increment of GyrB protein percentage for both conditions, with and without the addition of coumermycin. G' from the hydrogels with coumermycin follow the trend of our hypothesis while the ones without the addition of coumermycin do not have much differences in their values (Figure 3.4 d). This could be due to

the fact that the stiffness changes in the hydrogel system are too small to be accurately measured on the rheometer. Regardless, rheology experiments have successfully proven that the storage modulus of our novel biomaterials is generally higher in the presence of coumermycin than without. We created the first hydrogel system where intact stiffness, or the biomechanical properties in general, can be modified through the addition of pharmacological substances.

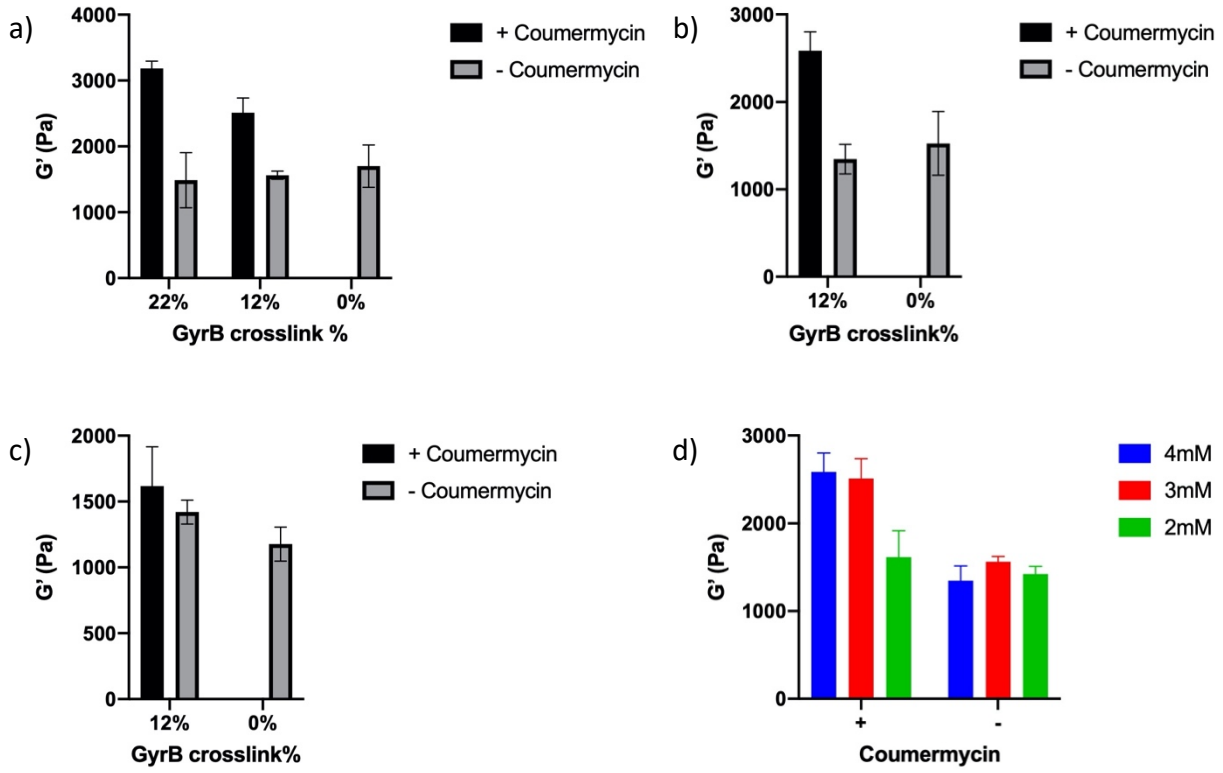

Figure 3.4. Stiffness results of hydrogels with different GyrB crosslink percentage and PEG polymer concentrations. a) Hydrogels were formed by PEG-tetraBCN (3 mM) with varying percent of GyrB crosslinks (22, 12, and 0%). b) Hydrogels were formed by PEG-tetraBCN (4 mM) with varying percent of GyrB crosslinks (12 and 0%). c) Hydrogels were

formed by PEG-tetraBCN (2 mM) with varying percent of GyrB crosslinks (12 and 0%). d) Comparison of hydrogels with 12% GyrB crosslinks but different PEG polymer concentrations.

Chapter 4. CONCLUSION

Our findings illustrate how the stiffness of biomaterials can be modified in response to the addition of common pharmacological antibiotics. Antibiotic-mediated hydrogels were synthesized through SPAAC click chemistry to incorporate PEG polymers with GyrB fusion proteins, allowing common aminocoumarin antibiotics to dimerize in between. Rheological studies of this novel materials were conducted to investigate the stiffness changes through the presence or absence of antibiotics as well as the different concentrations PEG polymers. Future work can apply other antibiotics to the materials in order to reversibly control its biomechanical property. We anticipate that this material uniquely enables regulation of material stiffness in the presence of living cells and potentially in vivo, strategies that could yield new approaches for tissue engineering and drug delivery.

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APPENDIX A

GyrB sequence was fused with LPXTG motif, GGS linked and STEPL sequence to undergo sortase expressed protein ligation for protein synthesis. The DNA sequences of the mutant were shown below reading from 5' end to 3' end. The GyrB sequences, LPXTG, GGS linker, and STEPL were marked black, blue, green, and red for recognition.

GyrB – LPXTG – GGS linker - STEPL

ATGTGCTCGAATTCTTATGACTCCTCCAGTATCAAAGTCCTGAAAGGGCTGGATGCGGTGCG
TAAGCGCCCGGGTATGTATATCGGCGACACGGATGACGGCACCGGTCTGCACCACATGGTAT
TCGAGGTGGTAGATAACGCTATCGACGAAGCGCTCGCGGGTCACTGTAAAGAAATTATCGTC
ACCATTCACGCCGATAACTCTGTCTCTGTACAGGATGACGGGCGCGGCATTCCGACCGGTAT
TCACCCGGAAGAGGGCGTATCGGCGGCGGAAGTGATCATGACCGTTCTGCACGCAGGCGGTA
AATTTGACGATAACTCCTATAAAGTGTCCGGCGGTCTGCACGGCGTTGGTGTTTCGGTAGTA
AACGCCCTGTCGCAAAAACTGGAGCTGGTTATCCAGCGCGAGGGTAAAATTCACCGTCAGAT
CTACGAACACGGTGTACCGCAGGCCCCGCTGGCGGTTACCGGCGAGACTGAAAAAACCGGCA
CCATGGTGCGTTTCTGGCCCAGCCTCGAAACCTTCACCAATGTGACCGAGTTCGAATATGAA
ATTCTGGCGAAACGTCTGCGTGAGTTGTCGTTCCTCAACTCCGGCGTTTCCATTCGTCTGCG
CGACAAGCGCGACGGCAAAGAAGACCACTTCCACTATGAAGGCCTCGAGCTGCCGGAAACCG
GTGGTGGTAGTGGTGGCTCTGGCGGTTCTGGTGGCAGTGGCGGTAGCCAAGCTAAACCTCAA
ATTCCGAAAGATAAATCAAAAGTGGCAGGCTATATTGAAATTCCAGATGCTGATATTAAAGA
ACCAGTATATCCAGGACCAGCAACACCTGAACAATTAAATAGAGGTGTAAGCTTTGCAGAAG
AAAATGAATCACTAGATGATCAAAATATTTCAATTGCAGGACACACTTTCATTGACCGTCCG
AACTATCAATTTACAAATCTTAAAGCAGCCAAAAAAGGTAGTATGGTGTACTTTAAAGTTGG
TAATGAAACACGTAAGTATAAAATGACAAGTATAAGAGATGTTAAGCCAACAGATGTAGAAG
TTCTAGATGAACAAAAAGGTAAAGATAAACAATTAACATTAATTACTTGTGATGATTACAAT
GAAAAGACAGGCGTTTGGGAAAAACGTAAAATCTTTGTAGCTACAGAAGTCAAA