

One last paper from the Tibbitt group MSE 493

Prof. Tiffany Abitbol

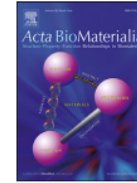
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Full length article

Designed modular protein hydrogels for biofabrication

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ABSTRACT

Designing proteins that fold and assemble over different length scales provides a way to tailor the mechanical properties and biological performance of hydrogels. In this study, we designed modular proteins that self-assemble into fibrillar networks and, as a result, form hydrogel materials with novel properties. We incorporated distinct functionalities by connecting separate self-assembling (A block) and cell-binding (B block) domains into single macromolecules. The number of self-assembling domains affects the rigidity of the fibers and the final storage modulus G' of the materials. The mechanical properties of the hydrogels could be tuned over a broad range ($G' = 0.1 - 10$ kPa), making them suitable for the cultivation and differentiation of multiple cell types, including cortical neurons and human mesenchymal stem cells. Moreover, we confirmed the bioavailability of cell attachment domains in the hydrogels that can be further tailored for specific cell types or other biological applications. Finally, we demonstrate the versatility of the designed proteins for application in biofabrication as 3D scaffolds that support cell growth and guide their function.

Statement of significance

Designed proteins that enable the decoupling of biophysical and biochemical properties within the final material could enable modular biomaterial engineering. In this context, we present a designed modular protein platform that integrates self-assembling domains (A blocks) and cell-binding domains (B blocks) within a single biopolymer. The linking of assembly domains and cell-binding domains this way provided independent tuning of mechanical properties and inclusion of biofunctional domains. We demonstrate the use of this platform for biofabrication, including neural cell culture and 3D printing of scaffolds for mesenchymal stem cell culture and differentiation. Overall, this work highlights how informed design of biopolymer sequences can enable the modular design of protein-based hydrogels with independently tunable biophysical and biochemical properties.

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- New paper
- Focus still on ECM, or hydrogels for cell growth
- Catchy title: “*modular protein hydrogels*” ...implies a flexibility in the design of the hydrogel...
“*biofabrication*” ...implies biologically engineered

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Subject areas

Biomaterials, Bioengineering, Biochemistry

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Publishing timeline

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Time to first decision ⓘ

95 days

Review time ⓘ

117 days

Submission to acceptance ⓘ

6 days

Acceptance to publication ⓘ

Abstract (break down)

Rationale: Designing proteins that fold and assemble over different length scales provides a way to tailor the mechanical properties and biological performance of hydrogels

What did they do?

- Designed modular proteins that self-assemble into fibrillar networks
- Incorporated distinct functionalities by connecting separate **self-assembling (A block)** and **cell-binding (B block)** domains into single macromolecules
- The number of self-assembling domains affects the rigidity of the fibers and the final storage modulus G' of the materials
- The mechanical properties of the hydrogels could be tuned over a broad range ($G' = 0.1 - 10$ kPa), making them suitable for the cultivation and differentiation of multiple cell types, including cortical neurons and human mesenchymal stem cells
- Confirmed the bioavailability of cell attachment domains in the hydrogels
- Demonstrated the versatility of the designed proteins for biofabrication of 3D scaffolds that support cell growth and guide their function

Significance (from statement)

Biophysical = self-assembling Block A
Biochemical = cell binding Block B

- “Designed proteins that enable ***the decoupling of biophysical and biochemical*** properties within the final material could enable ***modular biomaterial engineering***. In this context, we present a designed modular protein platform that integrates self-assembling domains (A blocks) and cell-binding domains (B blocks) within a single biopolymer... Overall, this work highlights ***how informed design of biopolymer sequences can enable the modular design of protein-based hydrogels with independently tunable biophysical and biochemical properties.***”

Some highlights from the intro (zoom out)

- Hydrogels are increasingly recognized for their use in cell growth for ex-situ models (reviews to back this up)
- Protein and peptide hydrogels offer a broad possibility for modular design of high molecular weight polymers that form hydrogels
- Protein and peptide hydrogels usually arise from the formation of fibrillar assemblies, like collagen networks in the ECM, or amyloid fiber networks (beta-sheet stacking)
- Several types of hydrogel forming peptide domains can be combined into a single macromolecule using recombinant technologies; allowing the integration of self-assembling motifs with bioactive motifs in a mix and match way

Some highlights from the intro (mid zoom)

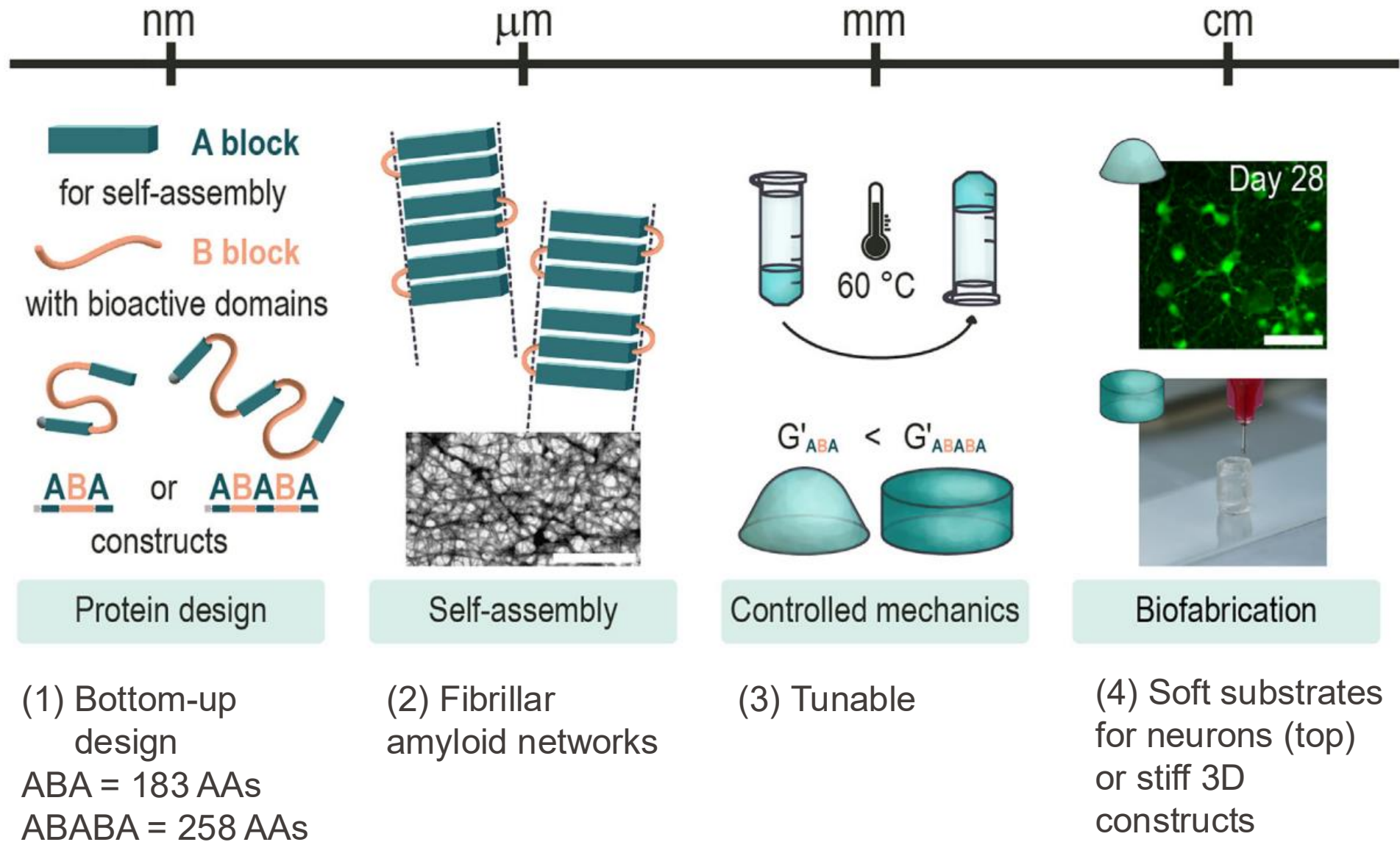
- Recombinant proteins that assemble into tertiary amyloid structures that form hydrogels can function as a *modular biomaterial platform that can be engineered at different scales* and *biological functionality can be integrated within the protein backbone*, exposed along or within the protein fibers

Some highlights from the intro (zoom in)

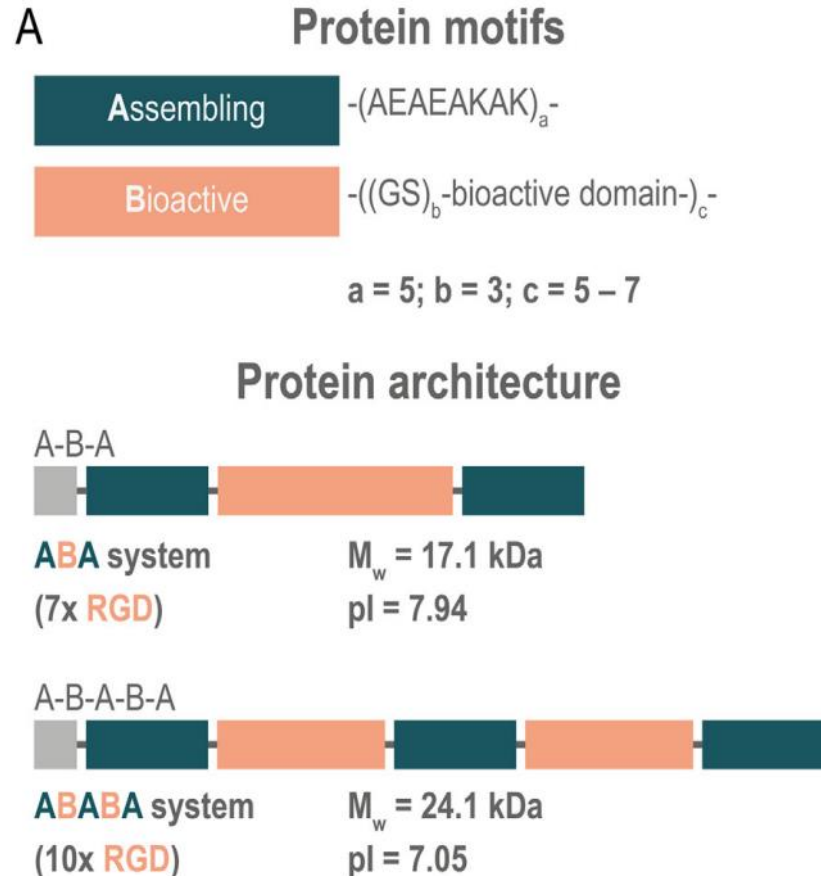
What's in this paper:

- Bottom-up design of a modular protein platform that forms fibrillar hydrogels
- Modular protein based on self-assembling (A block) and cell-binding block (B Block)
- ABA and ABABA (2 constructs are explored)
- A block based on amphiphilic sequences that form amyloids
- The number of A and B blocks control self-assembly, fiber formation, and rheology of the hydrogels
- Hydrogels suitable for culture of different cell types and for bioprinting
- Suggest that platform can be further tailored for specific applications

Graphical abstract (Fig. 1)

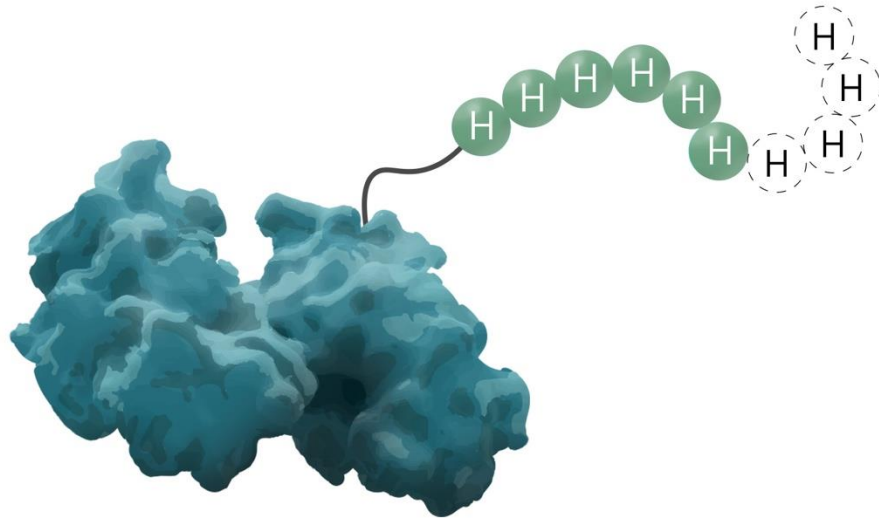


Bottom-up design of modular protein



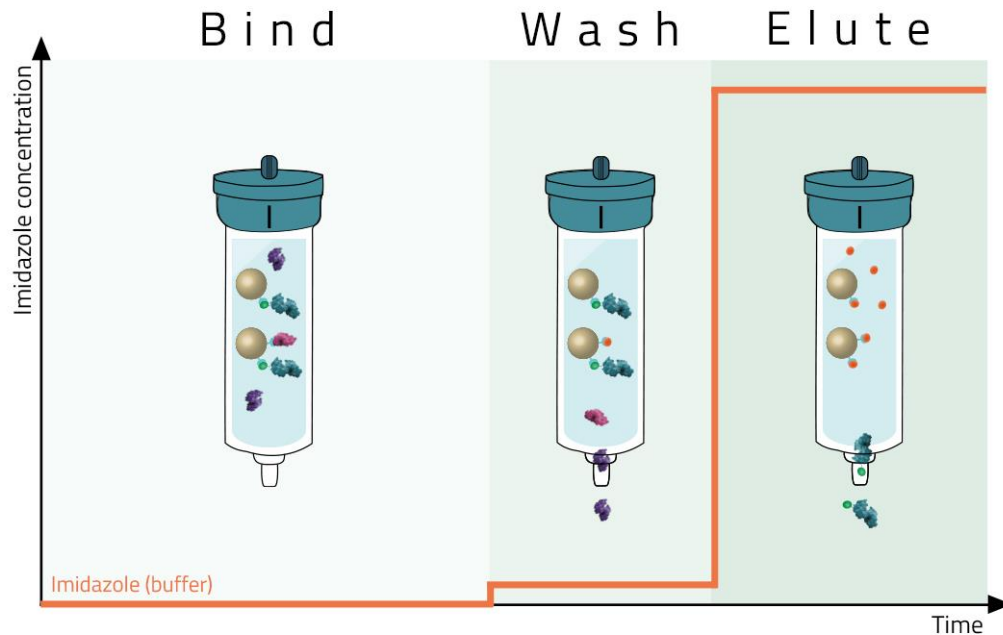
- A block and B block
- A block is 2.5 repeats of EAK-16 peptide (A – alanine, E – glutamic acid, K – lysine)
- Why? Has been shown to form stable beta sheets that can assemble into hydrogels
- B block has flexible, hydrophilic linkers based on glycine (G) and serine (S)
- Linker domain can be attached to bioactive domain
- Bioactive RGD (R – arginine, G – glycine, D – aspartic acid) for cell attachment
- Bioactive is part of linker to separate it from block A and to increase its accessibility in the resulting hydrogel
- Grey block is the N-terminal hexahistidine tag used for purification (Ni-NTA column)

His-tag protein purification



- *Commonly used tool for protein purification*
- *Underlying principle is the affinity between metal ions and the imidazole ring of histidine*
- *Protein design: DNA sequence coding for 6 consecutive his residues is added to the N-terminus of protein's gene (recombinant DNA technology)*
- *Protein expression: Gene is introduced into suitable host for expression*
- *Cell lysis: cells lysed to release protein in cell lysate*

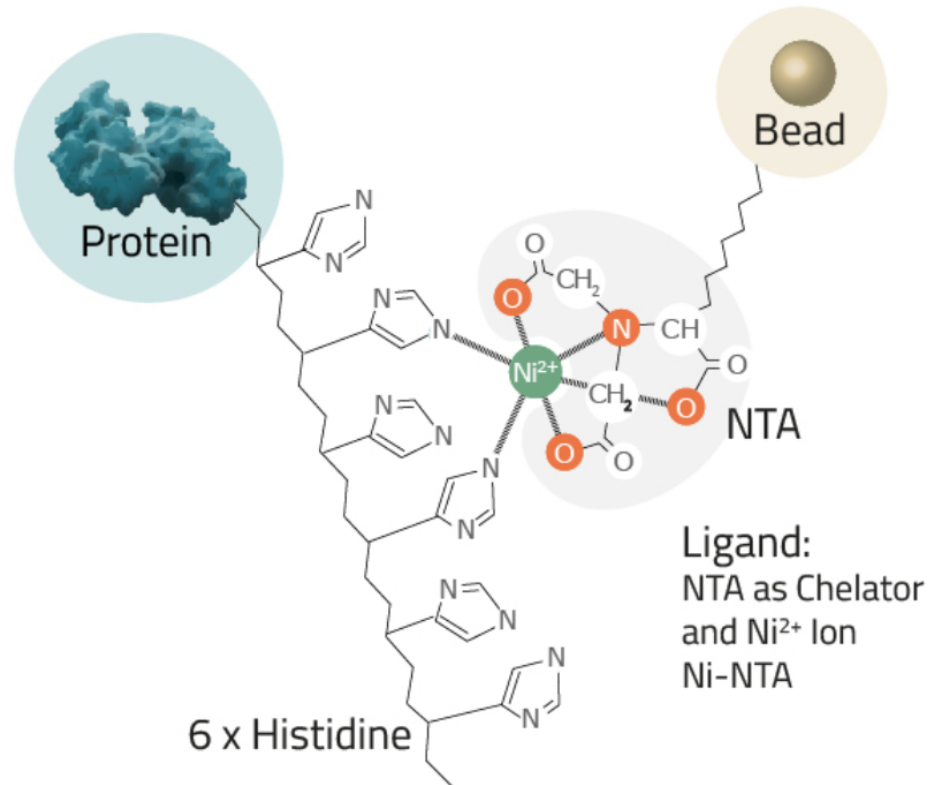
His-tag protein purification



- Cell lysate passed through column consisting of immobilized metal ions (Ni^{2+})
- His-tag proteins **bind** to column, while others pass through
- Column is **washed** to release non-specifically bound proteins
- His-tag protein is **eluted**, for example by competitive elution with imidazole

His-tag protein purification

Chelator complex structure and metal ions compared:



Coupled metal Ion

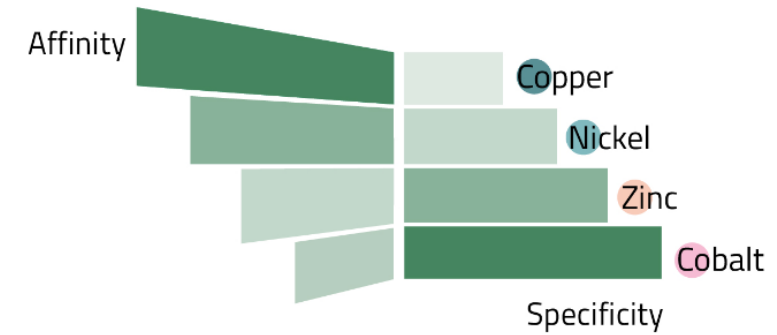
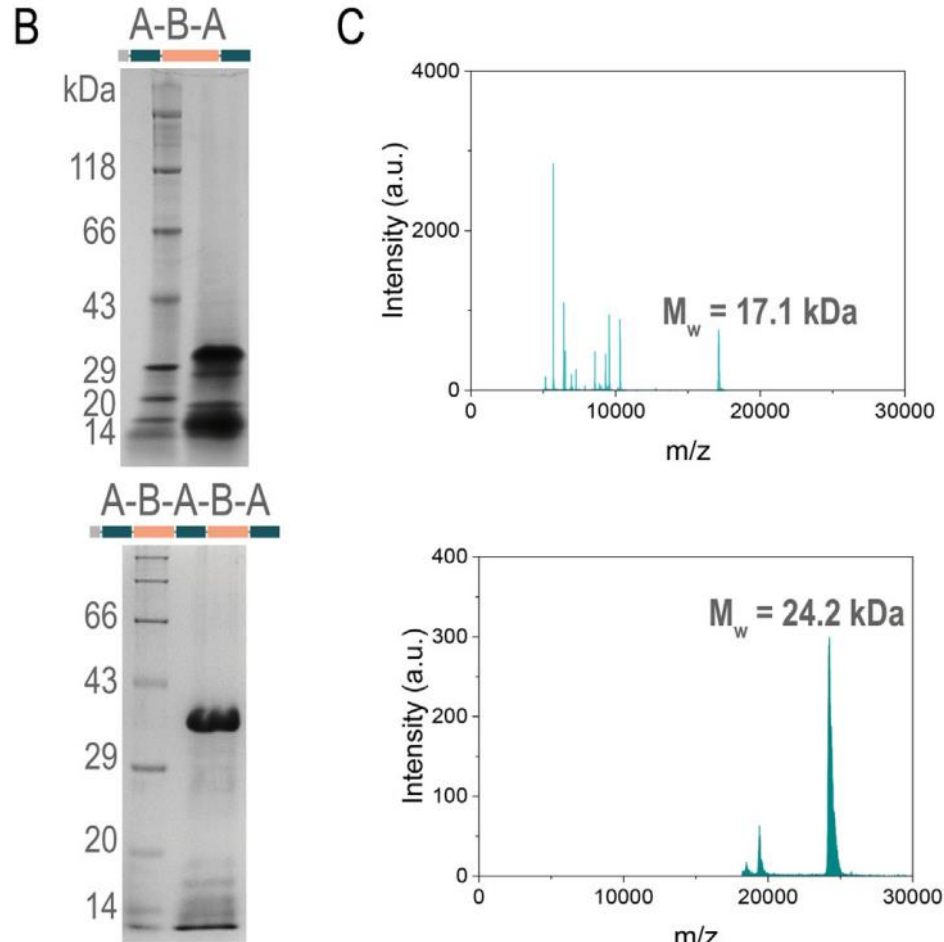


Figure 3 (Structure): Schematic depiction of a chelator complex consisting of NTA and Ni²⁺. This nickel ion binds two imidazole rings of a His-tag that has been added to a protein. Figure 4 (Diagram): Overview of the most commonly used metal ions for His-tag protein purification. The chosen metal ion is always a trade-off between affinity and specificity.

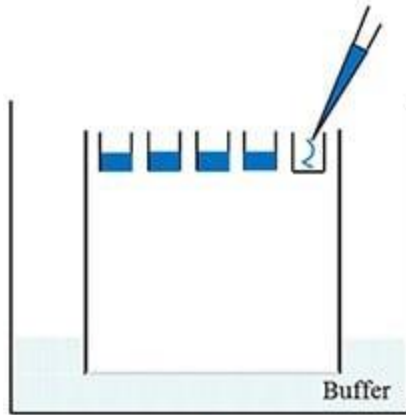
Protein characterization



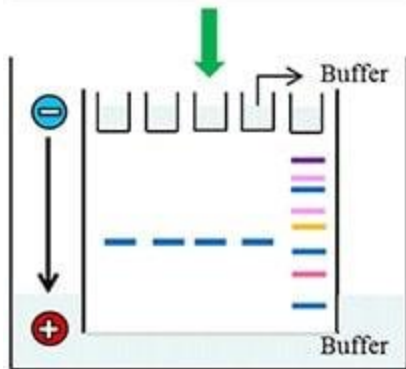
Molecular weight characterization by:

B: sulfate-polyacrylamide gel electrophoresis (SDS-PAGE); crude protein

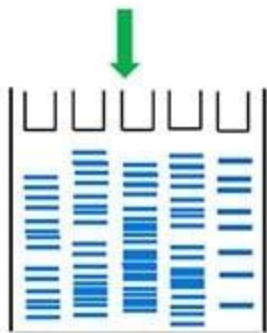
C: matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-MS); purified protein



Protein samples and marker loaded in vertical SDS-PAGE system



Direction of migration of samples in vertical SDS-PAGE system

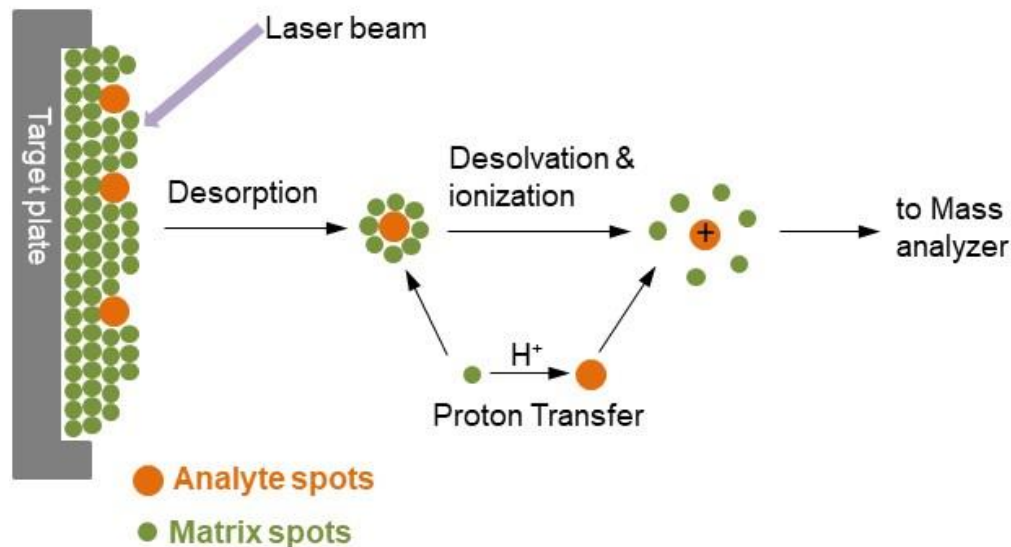


SDS-PAGE gel after Coomassie blue staining

Separates proteins primarily based on their molecular weight, as SDS imparts a uniform negative charge to the denatured proteins, causing them to migrate towards the positive electrode at rates inversely proportional to their size

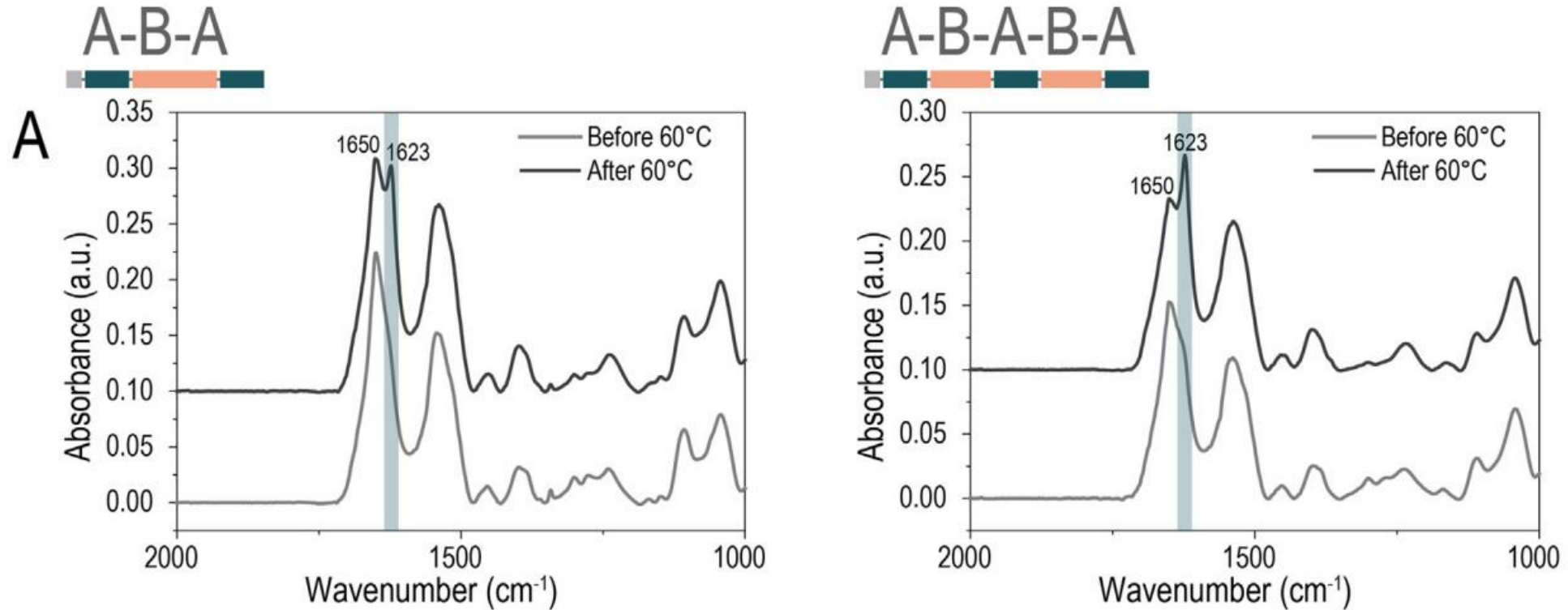
- Prepare gel (stacking and resolving)
- Denature proteins
- Load denatured proteins and molecular weight marker
- Apply current
- Run until dye front reaches bottom
- Remove gel
- Stain (e.g., Coomassie Blue)

Mass spectrometry is an analytical technique in which samples are ionized into charged molecules and ratio of their mass-to-charge (m/z) can be measured. In MALDI-TOF, the ion source is matrix-assisted laser desorption/ionization (MALDI), and the mass analyzer is time-of-flight (TOF) analyzer.



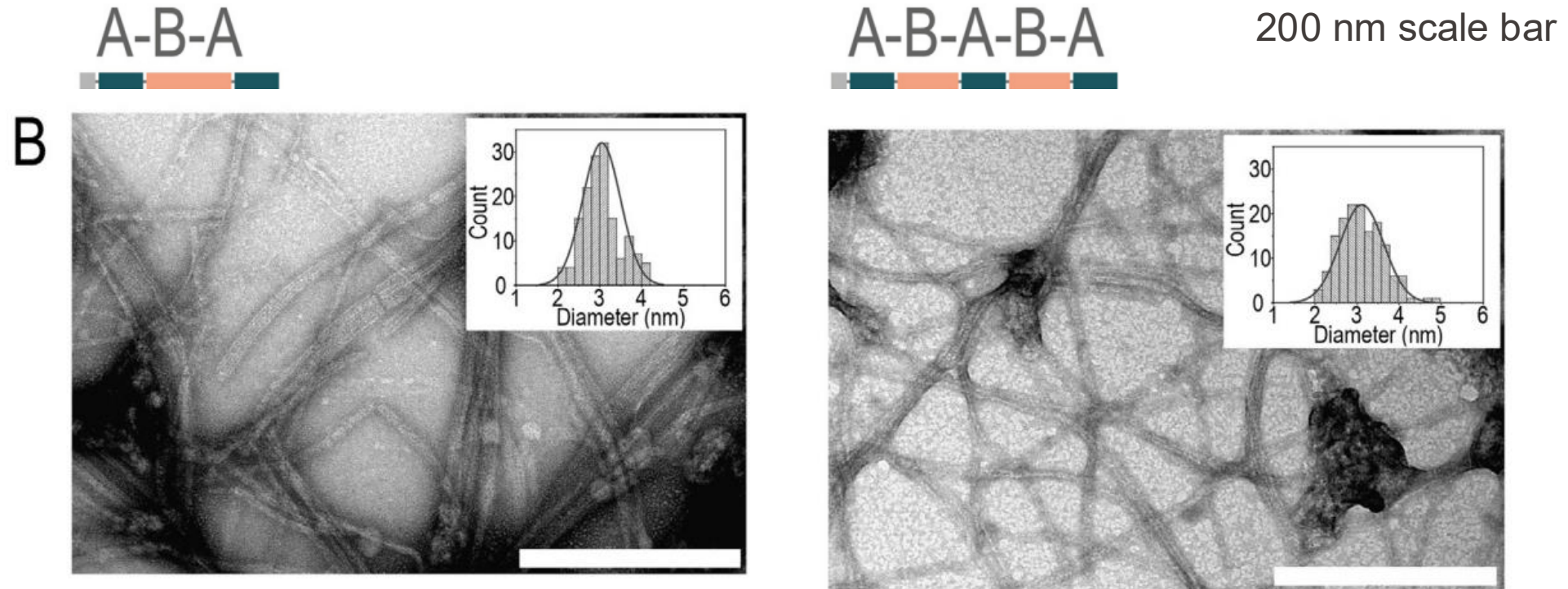
- Analyte embedded in matrix on target (conducting metal)
- After laser pulse, spot heats up and becomes vibrationally excited
- Matrix molecules ablate from surface, carrying analyte into gas phase
- During ablation, analyze is ionized, usually protonated
- TOF analyzer: ions travels through a flight tube, hitting detector at end of tube, m/z ratio based on time of flight, smaller ions faster than larger

ATR-FTIR for fibril structure (1 mg/ml)



- Self-assembly triggered by heat (1h, 60 °C)
- Increase in intensity of amide I (1623 cm^{-1}) after heating indicative of fibrillar beta-sheet structures
- Peak associated with disorder (1650 cm^{-1}) decreased with heating, but still remained – partial self-assembly?

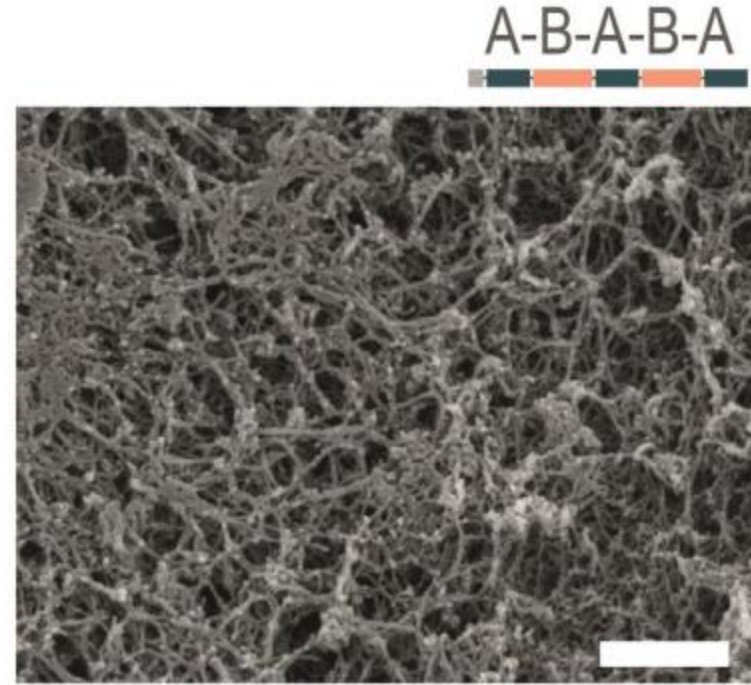
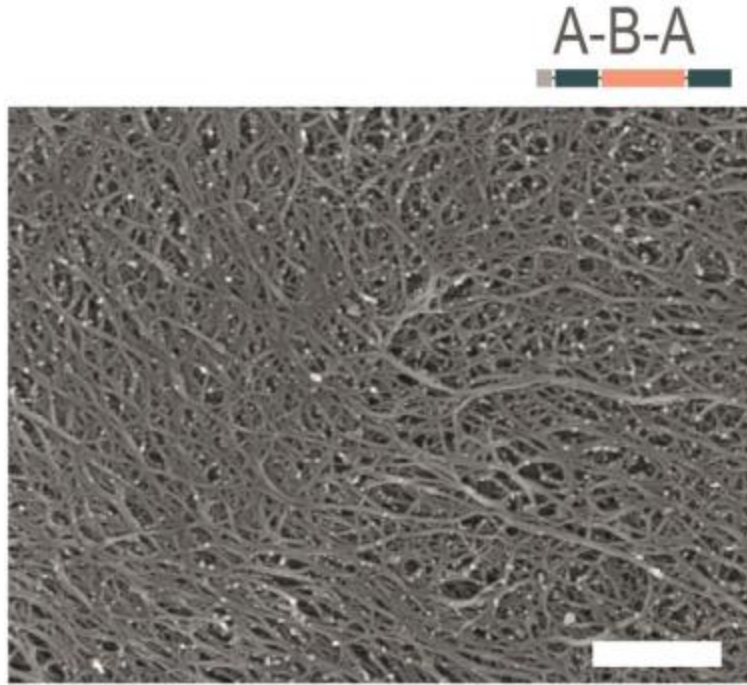
TEM for fibril structure (1 mg/ml)



- Narrowest fiber in ABA = 3.2 ± 0.5 nm
- Narrowest fiber in ABABA = 3.5 ± 0.7 nm
- Attributed to the packing pattern of the proteins perpendicular to the fiber axis

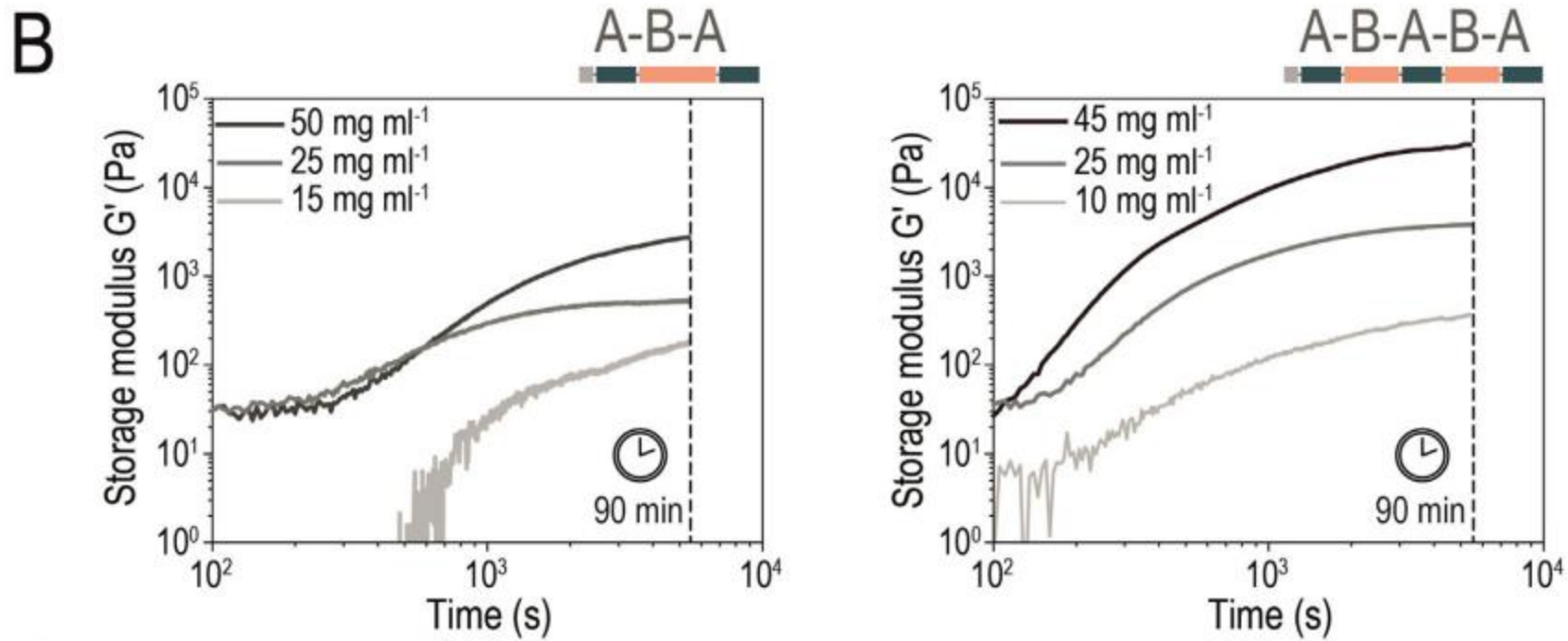
SEM for hydrogel structure (>100 mg/ml)

A



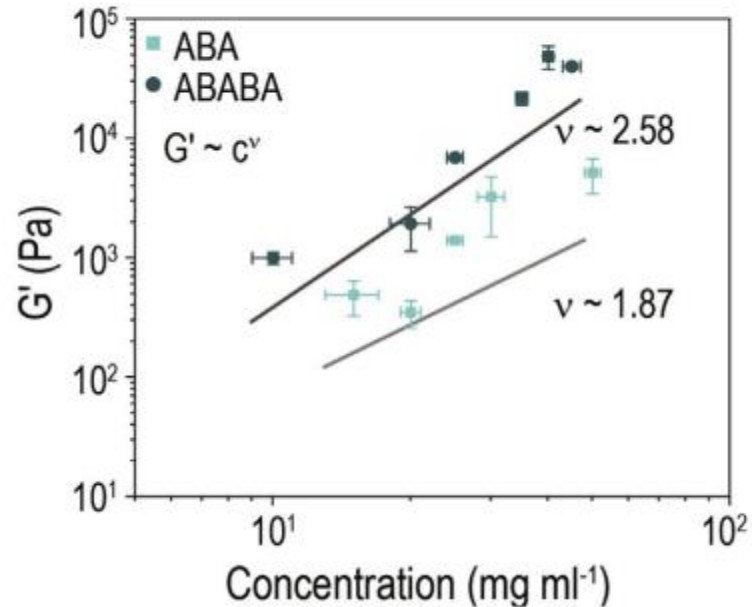
1 μm scale bar

- ABA is less branched?
- 120 mg/ml for ABA
- 105 mg/ml for ABABA



- Increase in G' observed within the first 5 min of heating at 60 °C

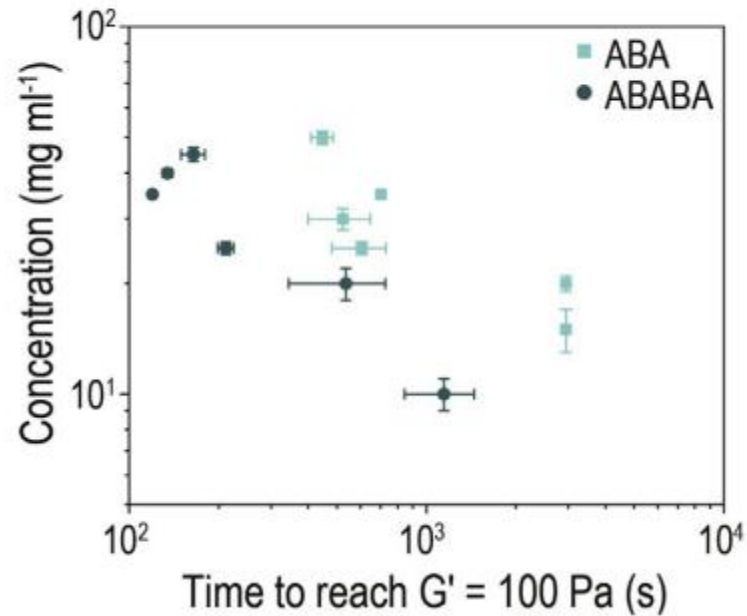
C



- For all concentrations, G' 90 min after gelation was initiated were higher for ABABA than ABA
- G' scaled with protein concentration and followed a power law
- Scaling parameter within the range found for fibrillar networks
- Scaling for ABABA ($\nu \leq 2.5$) indicated rigid fibrils compared to semiflexible fibrils for ABA ($\nu \leq 2.2$)

G' values after 90 min at 60 °C for different concentrations

D

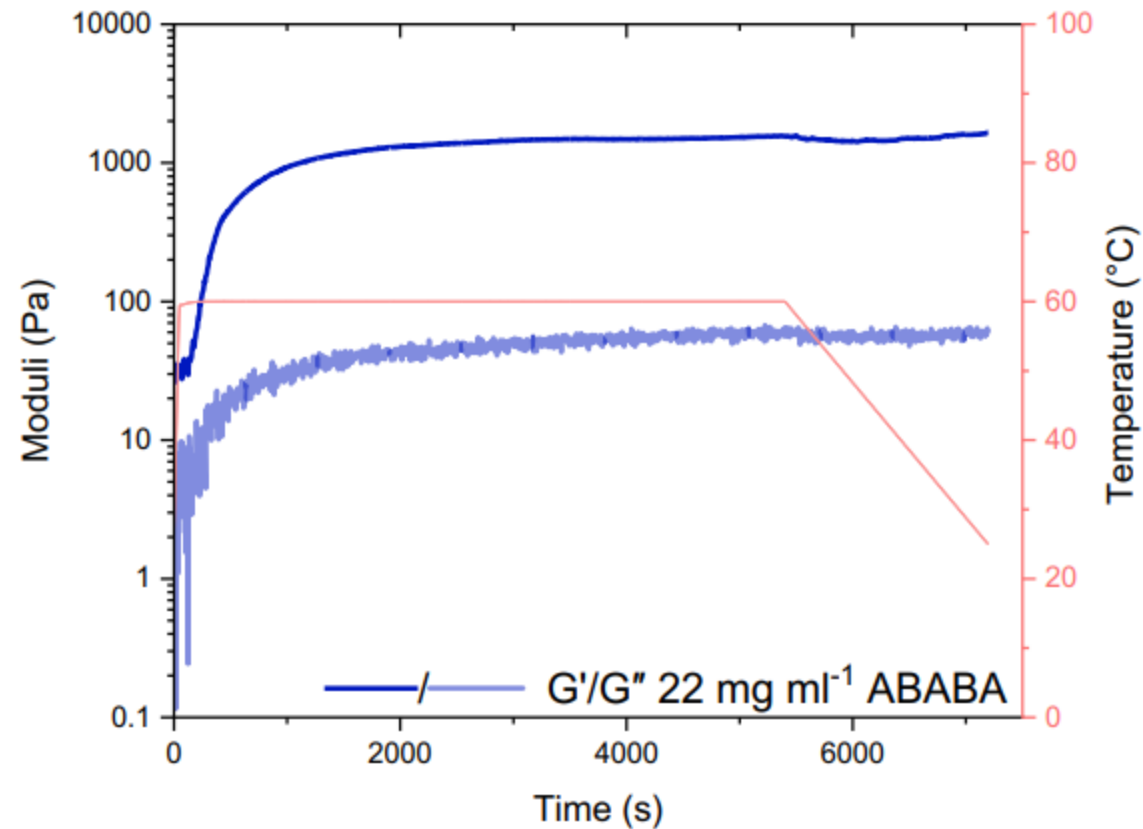


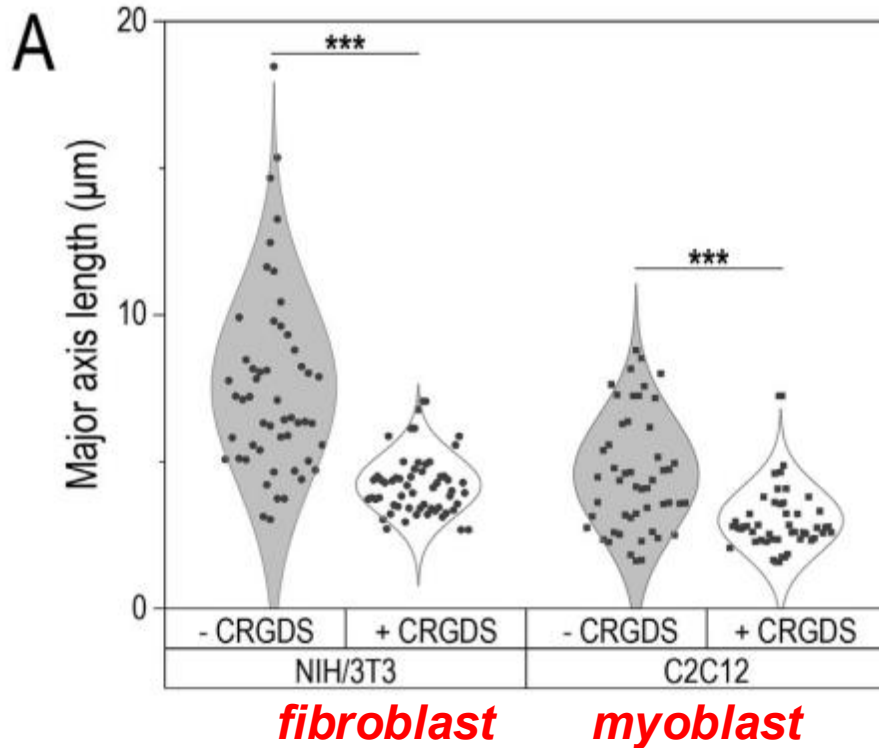
- Time required for $G' = 100$ Pa was considered “gel initiation point”
- Based on this ABABA formed earlier than ABA

Time required to reach $G' = 100$ Pa for protein solutions of different concentrations.

Gel maintained after cooling

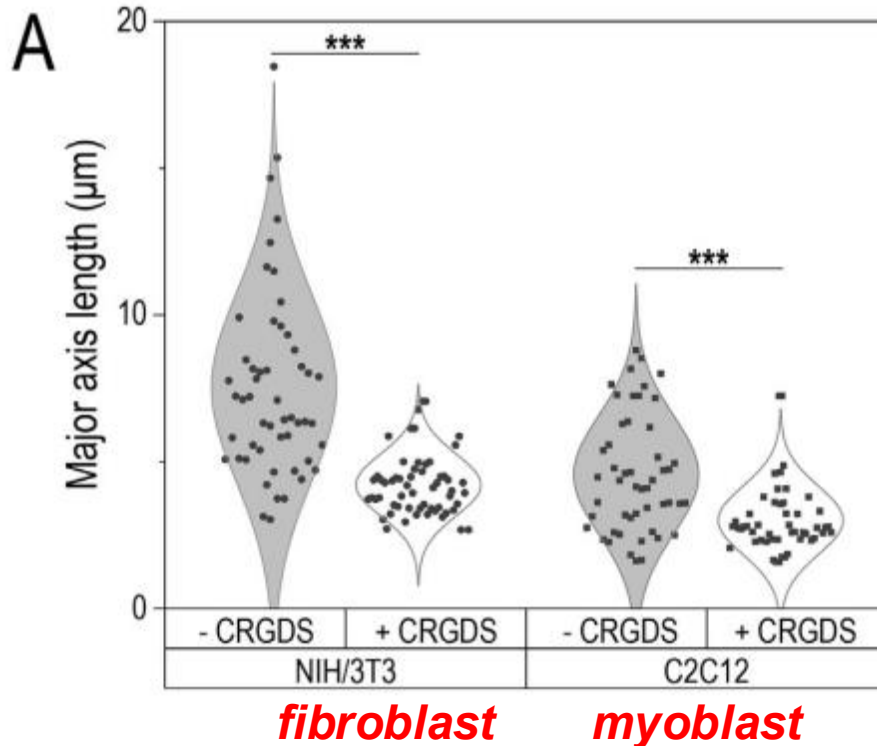
Figure S11. Time sweep during 22 mg mL^{-1} ABABA sample gelation (at $T = 60 \text{ }^\circ\text{C}$, $t = 90 \text{ min}$) followed by a cooling cycle (rate $\sim 1 \text{ }^\circ\text{C min}^{-1}$) to $25 \text{ }^\circ\text{C}$.





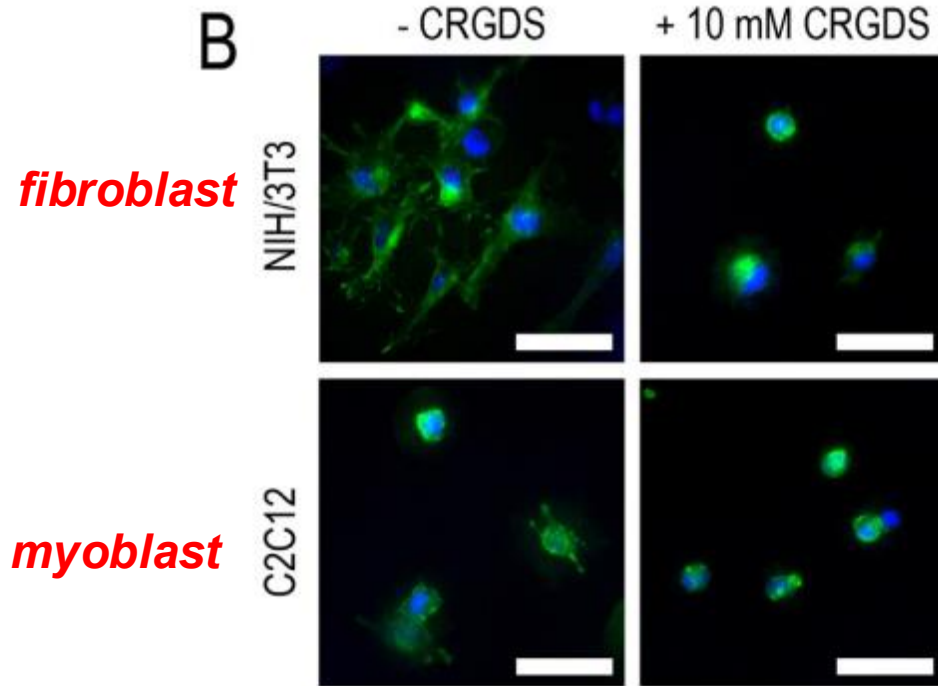
Investigation of cell attachment and spreading in 15 mg/ml ABABA hydrogel, with and without 10 mM CRGDS

- **What's the experiment?**
- Hydrogel has a bioactive RGD part that can bind to integrin on the cell surface; if binding occurs, the cell can attach and elongate on the hydrogel
- As a control, to soluble CRGDS peptide is added (+CRDGS condition), to competitively block RGD-integrin binding with hydrogel. In this case, the cells cannot adhere to the hydrogel and remain rounded (permissive/non-adhesive environment)
- Myoblast (muscle fibers) & fibroblast (ECM proteins) – differ in integrin expression (fibroblasts rely on RGD-integrin binding, myoblasts less so, so should be less impacted by blocking RGD)



Investigation of cell attachment and spreading in 15 mg/ml ABABA hydrogel, with and without 10 mM CRGDS

- For fibroblasts, with CRGDS, the mean major axis cell length decreased from $7.5 \pm 3 \mu\text{m}$ to $4.2 \pm 1 \mu\text{m}$ (reduction in cell elongation) – “indicates that RGD specific integrins are needed for cells to adhere and spread on our modular protein substrates”
- For myoblasts, same result = cell elongation was significantly reduced with competition from soluble CRGDS
- Overall spreading lower for myoblasts, hypothesized to be related to integrins primarily binding to collagen and laminin in muscle ECM



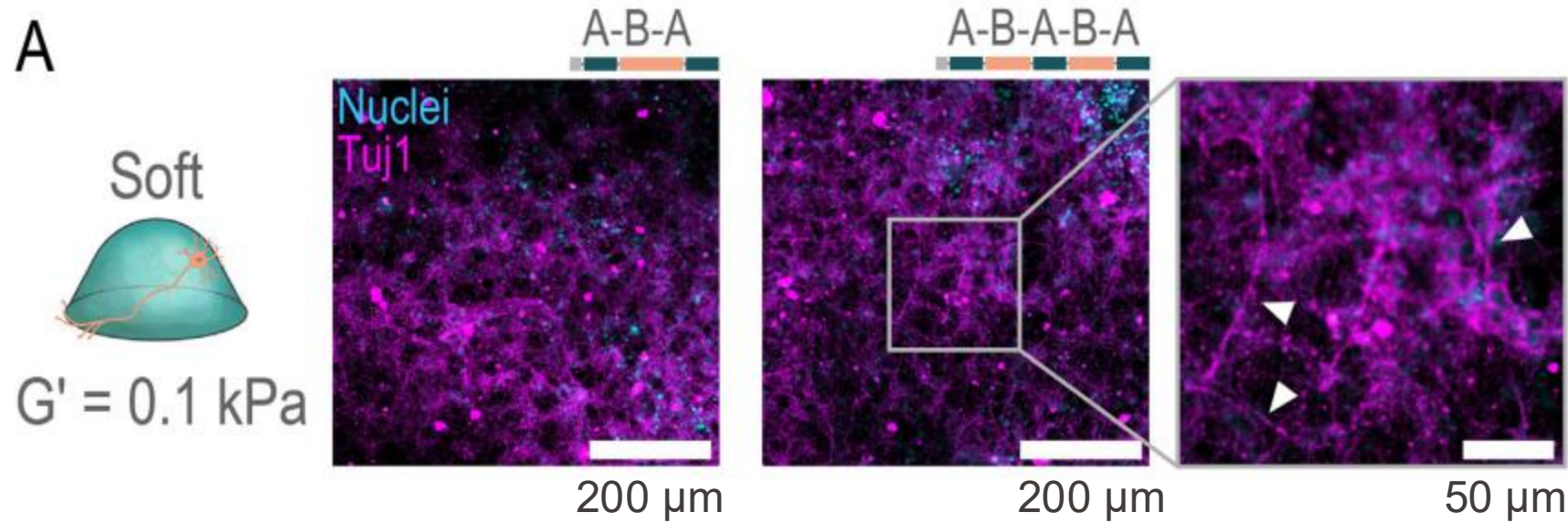
Investigation of cell attachment
and spreading in ABABA gel

- Visualization of the same phenomenon
- Better elongation/adhesion in absence of CRGDS, which otherwise will compete with RGD integrin binding sites on cell surfaces
- *Moving forward with biofabrication part now that they have established that their design enables tunable mechanics and has exposed cell adhesion sites after self-assembly...*

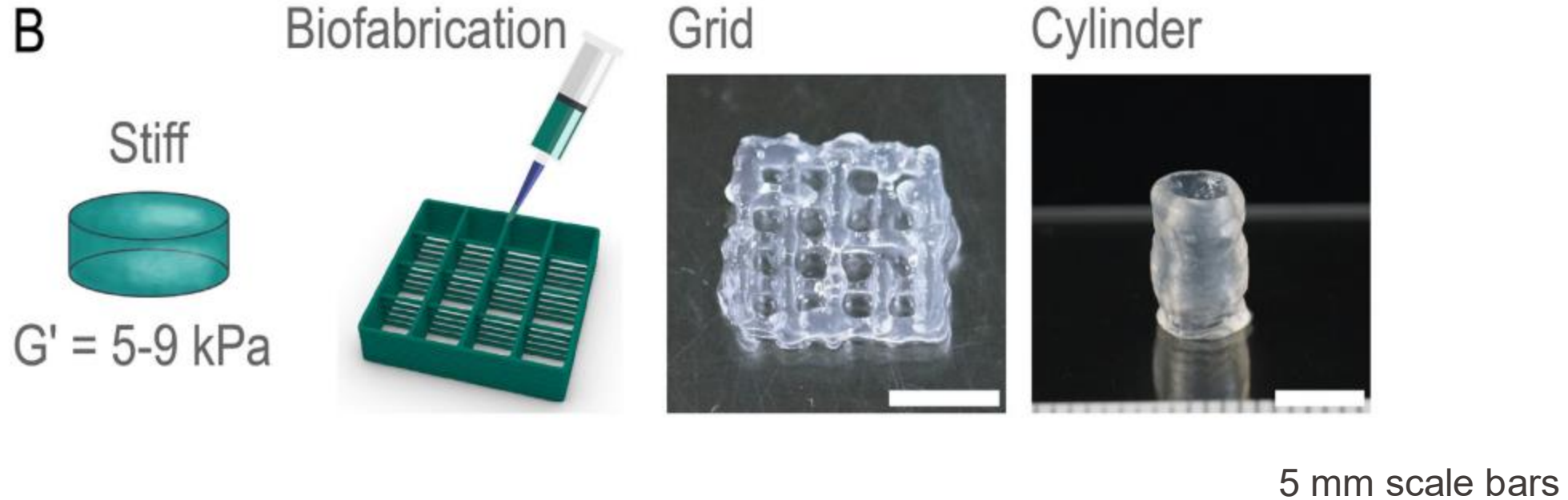
50 μ m scale bar

Biofab of soft hydrogel scaffolds

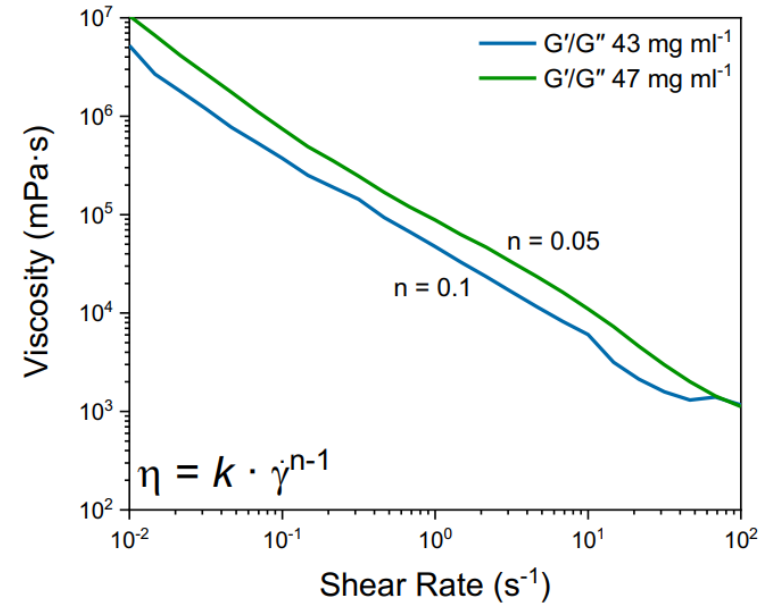
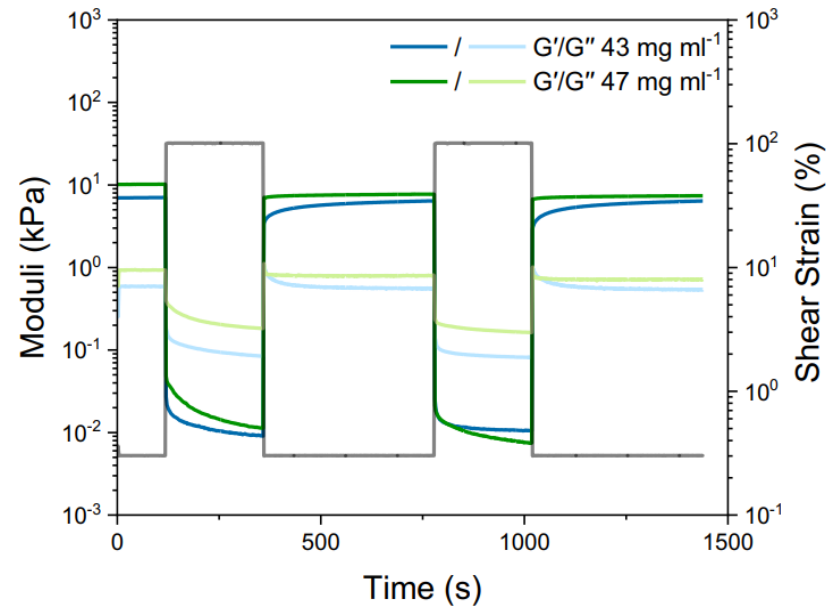
Nuclei = cyan
Neurons = magenta



- Can hydrogels support neuronal cell growth and function?
- To test, seeded primary cortical neurons on soft ABA and ABABA hydrogels (G' ca. 0.1 kPa)
- Cells attached and spread uniformly with viability up to 4 weeks
- Neurites formed after 2 weeks of culture to form an extensive network as shown by beta3-tubulin staining
- After 4 weeks, neurite networks were expansive and spanned the entire surface area of the gel
- Doesn't work when seeded on stiffer gels (Fig. S16)



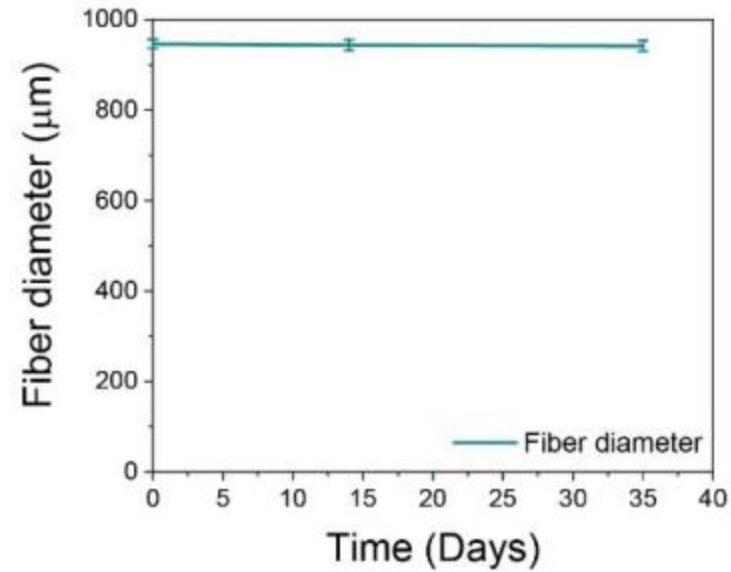
- Used 3D printing to achieve more complex scaffolds
- Multilayered grids and a self-supporting multilayer cylinder



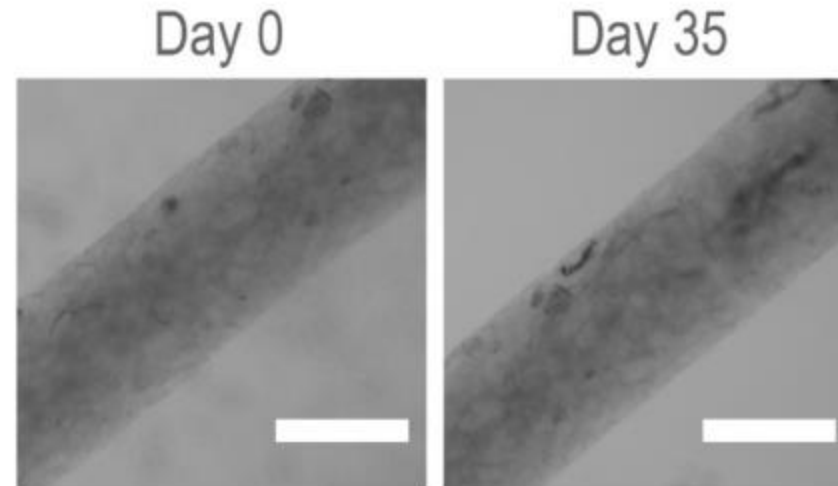
- Stiff ABABA hydrogels (45 ± 2 mg/ml, storage modulus $G' = 5-9$ kPa) showed high elastic recovery of G' after high strain intervals and shear thinning – suggesting good for 3D printing

750 μm scale bar

A

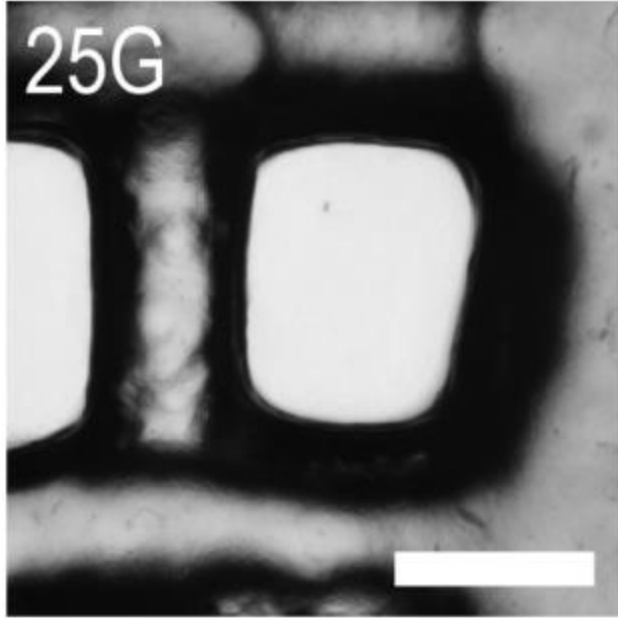


B



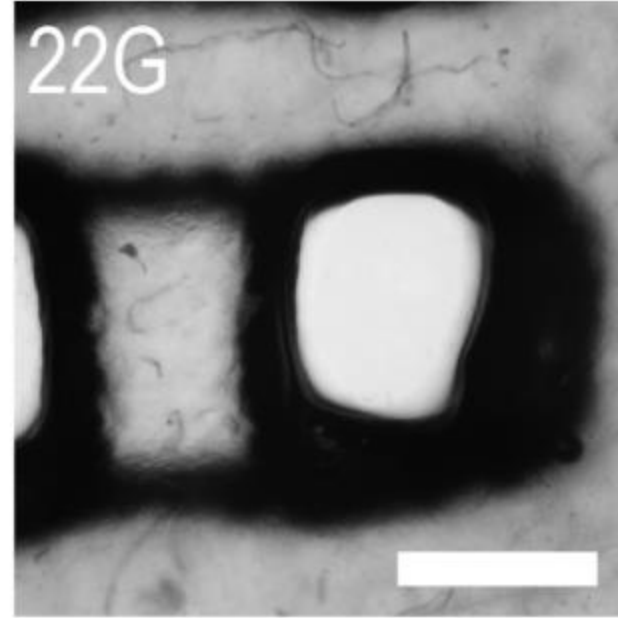
- Extruded filament in PBS, ABABA hydrogel (45 mg/ml) stable over 5 weeks, suggesting they would remain stable during long-term cell culture

Biofab of soft hydrogel scaffolds



Pr = 0.9

SR = 2.6

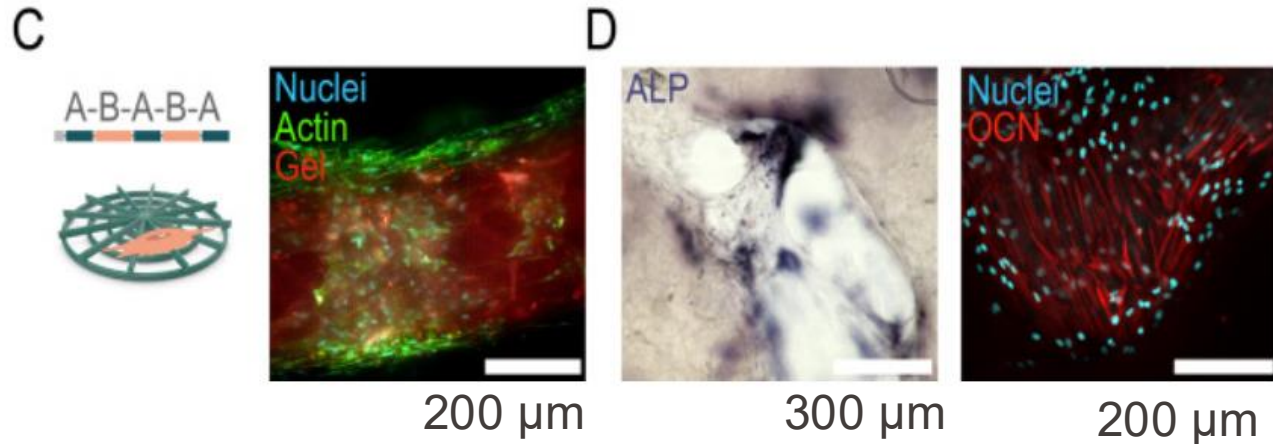


Pr = 0.9

SR = 2.5

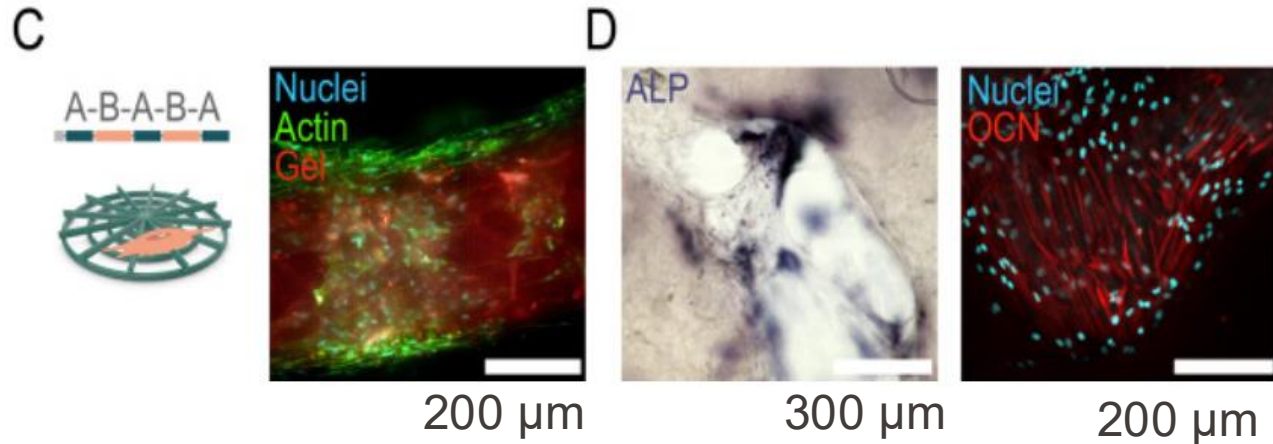
- 750 μm scale bar
- Bigger gauge, smaller nozzle diameter

- Printability evaluated by direct ink writing
- Pr = printability index
- SR = spreading ratio = filament width/nozzle width



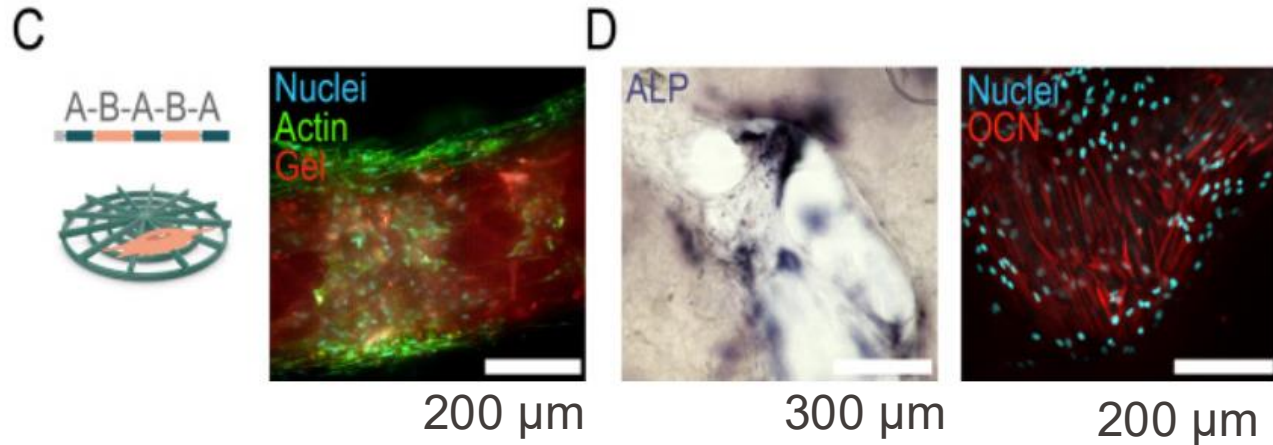
- Studied **osteogenic differentiation** of human mesenchymal stem cells (hMSCs)
- Can the stem cells turn into bone forming cells (osteoblasts) with the right biochemical and mechanical cues?

- Used stiff ABABA protein hydrogel with RGD motifs for integrin mediated adhesion
- Either used osteogenic media (contains osteo-inductive factors) or control growth medium
- Used biomarkers and staining assays to assess osteogenic differentiation, e.g., ALP (alkaline phosphatases) - an early marker for osteogenic differentiation, alizarin red staining, to detect calcium mineralization, a late marker of osteogenesis, gene expression analysis



- Studied **osteogenic differentiation** of human mesenchymal stem cells (hMSCs)
- Can the stem cells turn into bone forming cells (osteoblasts) with the right biochemical and mechanical cues?

- After 2 weeks, gel (red) was proliferated by cells (actin/green, nuclei/blue)
- In osteogenic media (BMP-2-containing media), alkaline phosphatase (ALP) activity and osteocalcin (OCN) detected in cells attached to scaffolds
- After 4 weeks, without BMP-2, some ALP activity also detected (hMSCs tend toward osteogenesis over time)



- Studied **osteogenic differentiation** of human mesenchymal stem cells (hMSCs)
- Can the stem cells turn into bone forming cells (osteoblasts) with the right biochemical and mechanical cues?

Takeaways:

- hMSCs encapsulated in the ABABA hydrogel showed **increased ALP activity** and **mineral deposition** when cultured in osteogenic medium.
- Gene expression confirmed **upregulation of osteogenic markers**, indicating that the hydrogel supported differentiation into an osteoblastic phenotype.
- The presence of RGD motifs likely facilitated **integrin-mediated signaling** that promoted this osteogenic response.
- In contrast, cells in growth medium or on non-bioactive controls did **not** show strong osteogenic activity.

Conclusions/ Take aways

- Modular protein design to form supramolecular fibrillar hydrogels
- Self-assembly behavior and mechanics depend on protein design and concentrations – control over biochemistry and material properties!
- These fibrillar hydrogels can be used as soft scaffolds for the growth of neurons and stiffer scaffolds for osteogenic differentiation of human mesenchymal stem cells
- Promising for development of cell culture platforms suitable for tissue engineering applications, controlled drug delivery, regenerative medicine

Did you like this paper? Why or why not? (a research thread from 2009-2024)



YES



NO

Overall lesson

takeaways

- Cell environment is not just a static supporting architecture that keeps cells viable
- It is dynamic and redesigned depending on what is going on in the cell
- Strategies that enable “synthetic-biologic” ECM designs, like modular proteins, for fibrillar structure and specific biological sequences, like for adhesion, are interesting
- Depending on how the modular protein is assembled (e.g., concentration, number of A or B units), you can access very different mechanical properties, leveraging that cells respond to mechanical cues that can very much influence/decide their phenotype
- Feels like many more designs should be possible via recombinant engineering to enable fiber motif+ function motif(s)!