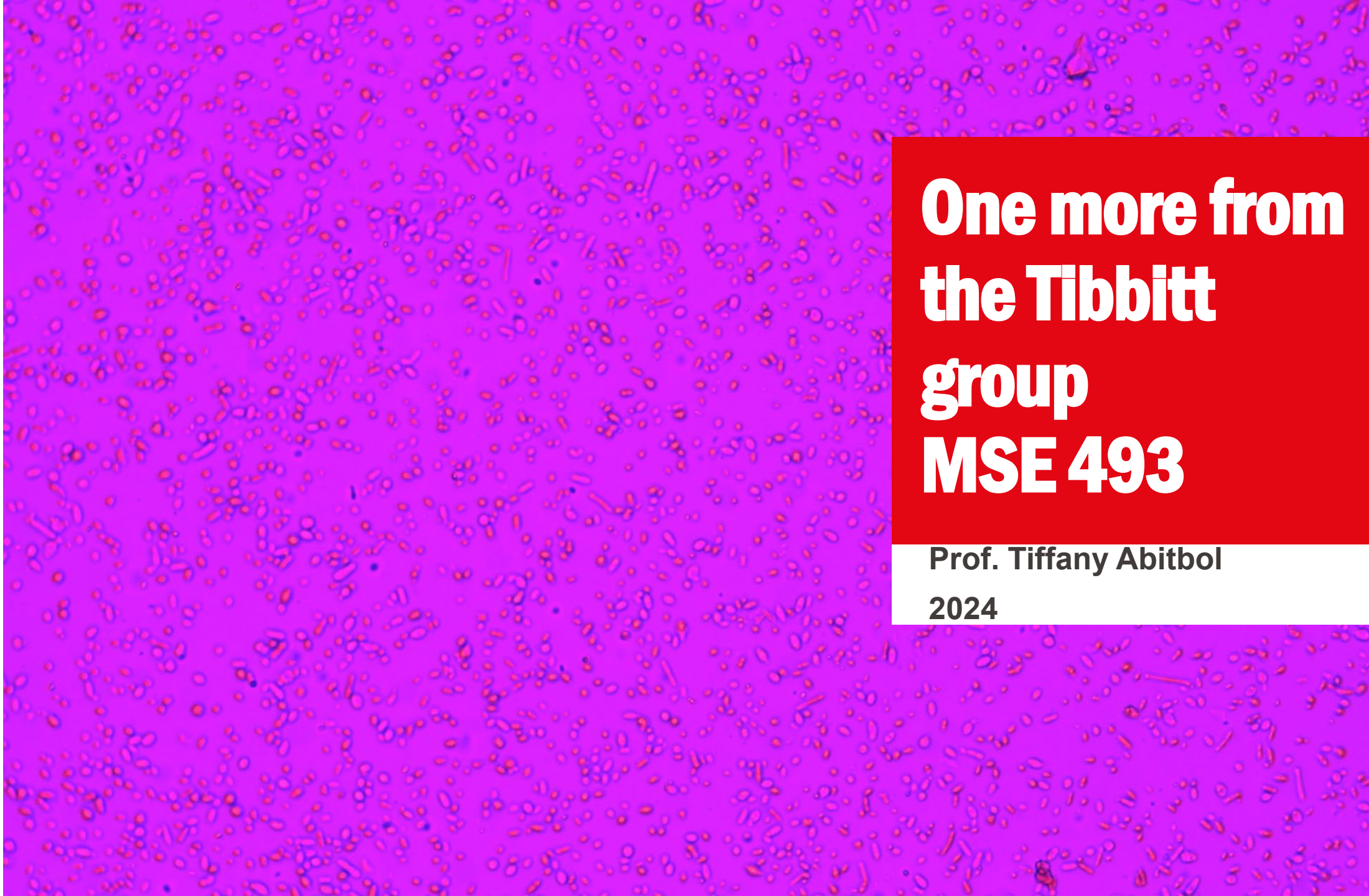

One more from the Tibbitt group MSE 493

Prof. Tiffany Abitbol

2024

Acta Biomaterialia 177 (2024) 107–117

Contents lists available at ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actbio

Full length article

Designed modular protein hydrogels for biofabrication

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ARTICLE INFO

Article history:
Received 3 October 2023
Revised 1 February 2024
Accepted 13 February 2024
Available online 19 February 2024

Keywords:
Biofabrication
Protein design
Biomaterials
Protein hydrogels

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.

- 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

Acta Biomaterialia

| Supports open access

Subject areas

Biomaterials, Bioengineering, Biochemistry

Impact

16.8
CiteScore

9.4
Impact Factor

Article publishing charge

$3210
Article publishing charge for open access

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Publishing timeline

4 days
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
- Demonstrate the versatility of the designed proteins for application in biofabrication as 3D scaffolds that support cell growth and guide their function

Significance (from statement)

- “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 off a broad possibility for modular design of high molecular weight polymers that form hydrogels
- 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
- 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)

Some highlights from the intro (mid zoom)

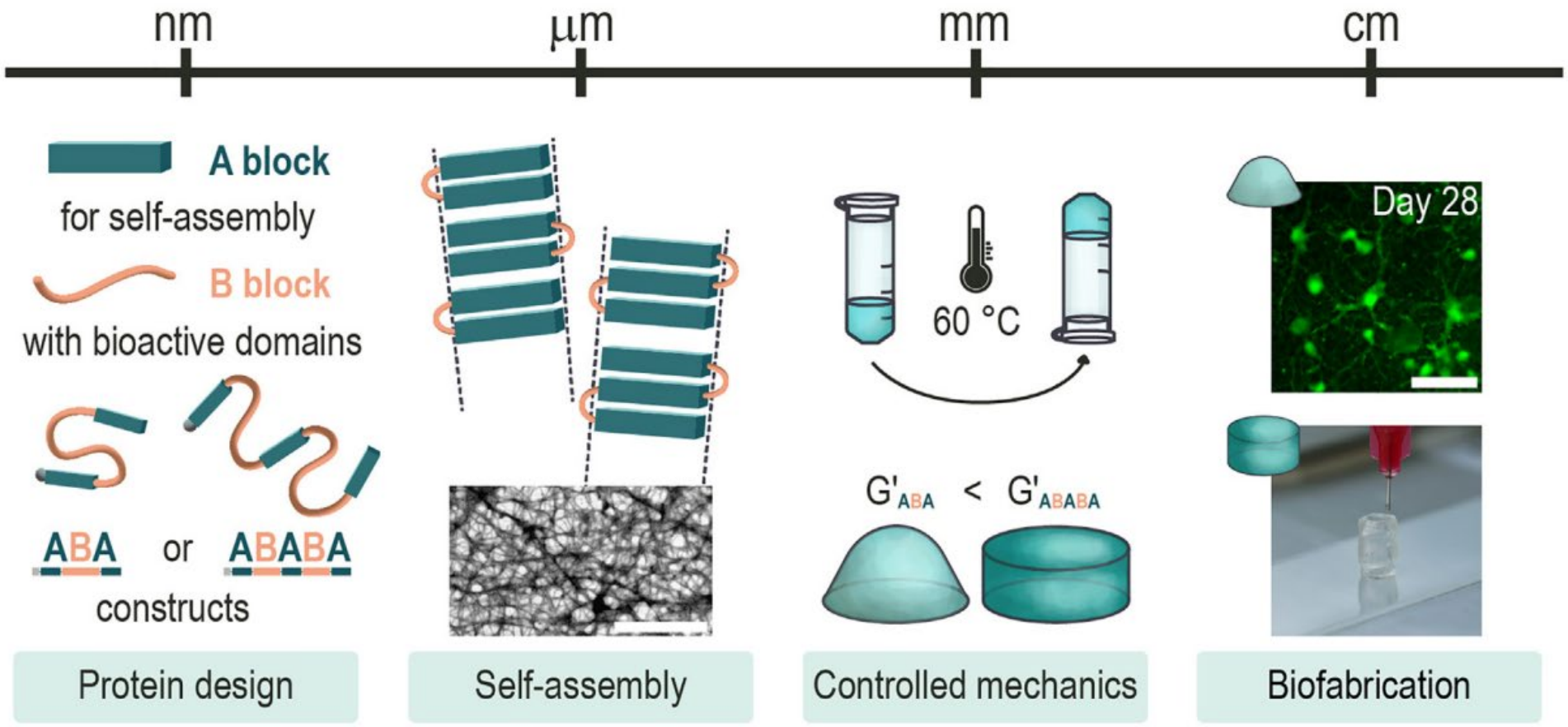
- Recombinant proteins that assemble into tertiary amyloid structures that form hydrogels can function as a ***modular biomaterial platform that can be engineered at different scales*** and ***biological functionality can be integrated within the protein backbone***, exposed along or within the protein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

Graphical abstract (Fig. 1)



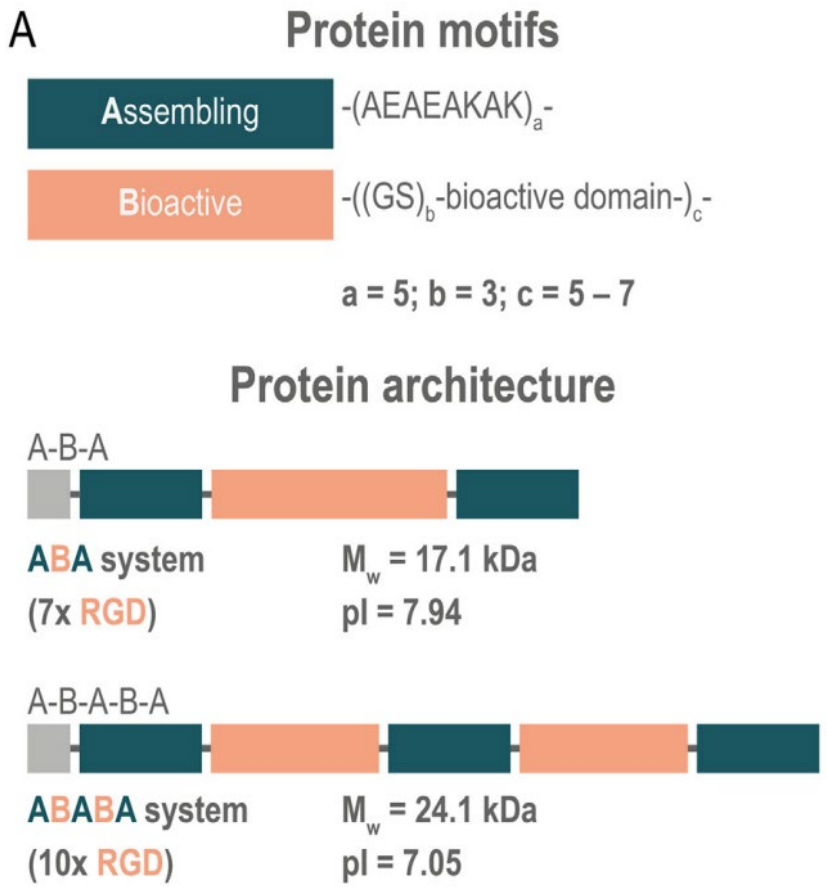
(1) Bottom up design
ABA = 183 AAs
ABABA = 258 AAs

(2) Fibrillar amyloid networks

(3) Tunable

(4) Soft substrates for neurons (top) or stiff 3D constructs

Bottom up design of modular protein

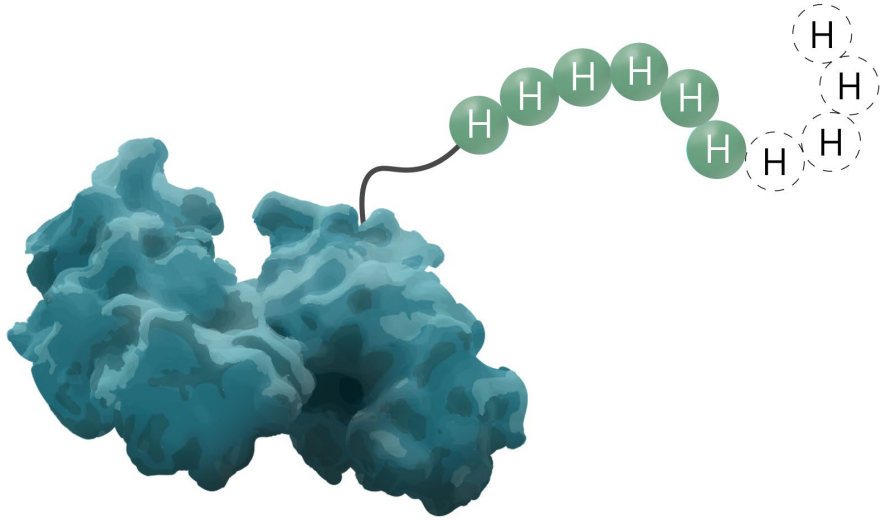


- A block and B block
- A block is 2.5 repeats of EAK-16 peptide (A – alanine, E – glutamic acid, K – lysine)
- Why? Has been shown to form stable beta sheets that can assemble in to hydrogels

- 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)

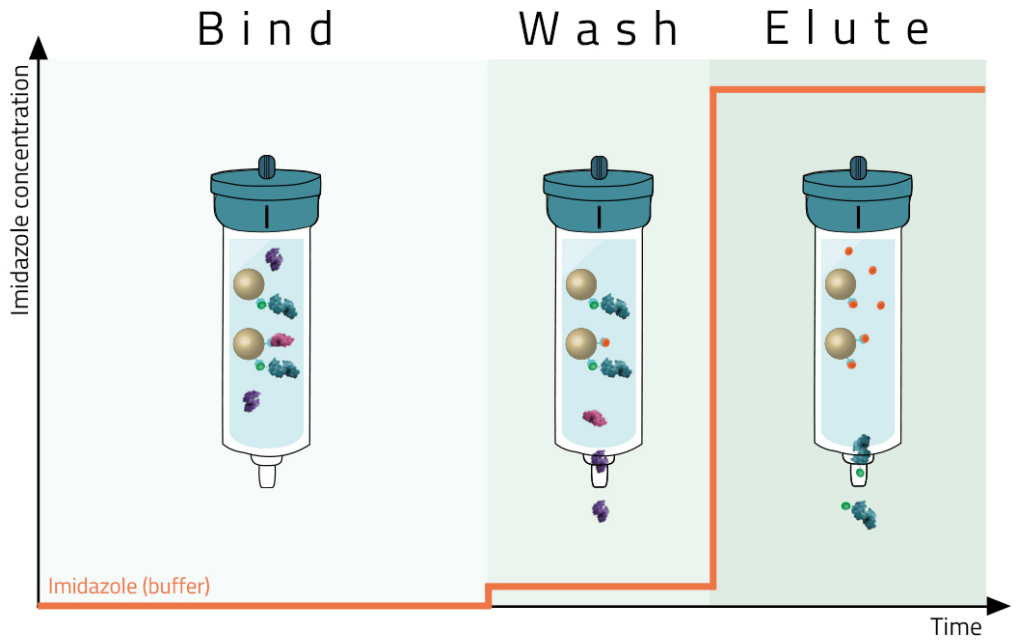
His-tag protein purification



- *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

His-tag protein purification

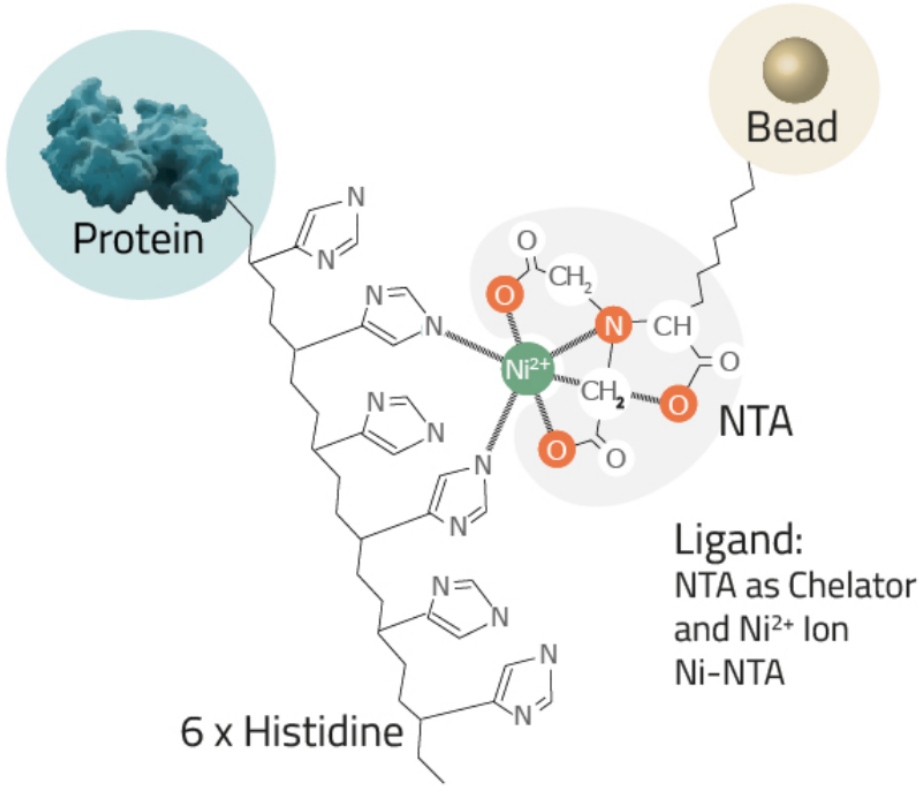


- *Cell lysate passed through column consisting of immobilized metal ions (Ni^{2+})*
- *His-tag proteins **bind** to column, while others pass through*
- *Column is **washed** to release non-specifically bound proteins*
- *His-tag protein is **eluted**, for example by competitive elution with imidazole*

His-tag

His-tag protein purification

Chelator complex structure and metal ions compared:



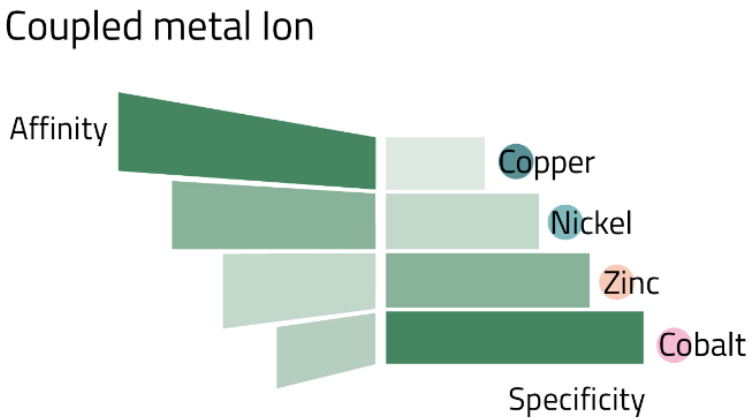
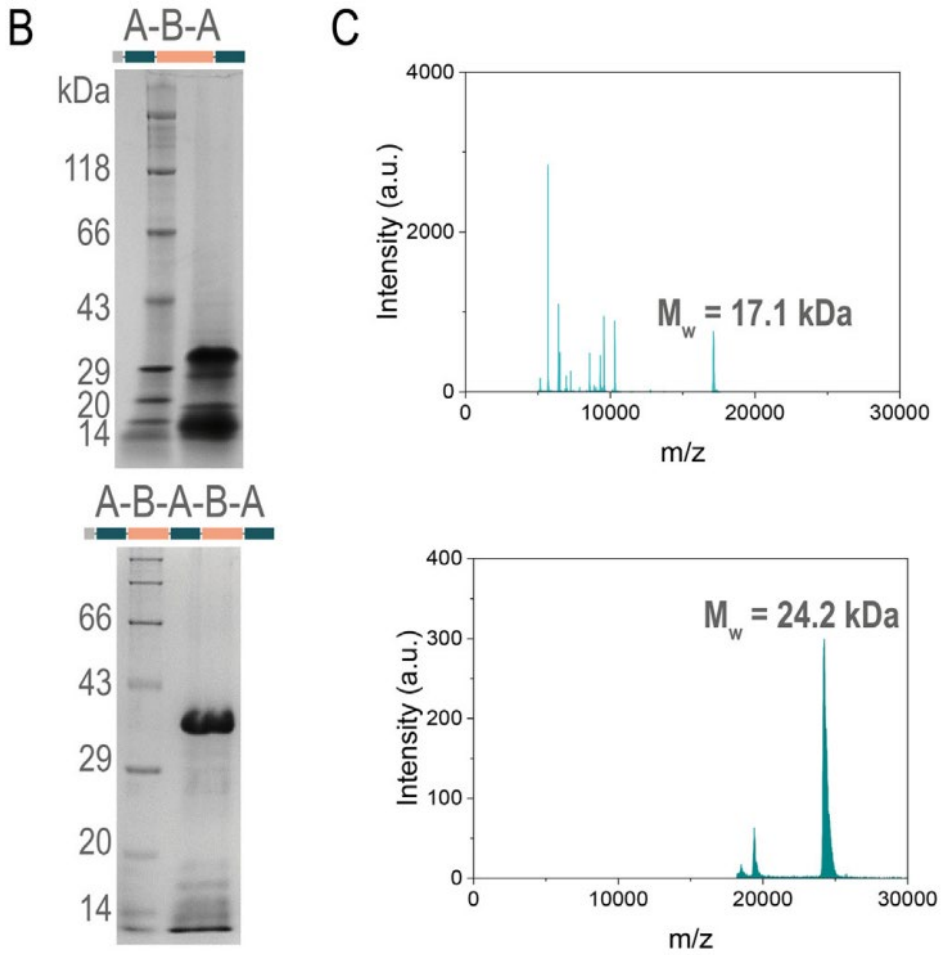


Figure 3 (Structure): Schematic depiction of a chelator complex consisting of NTA and Ni^{2+}. This nickel ion binds two imidazole rings of a His-tag that has been added to a protein. Figure 4 (Diagram): Overview of the most commonly used metal ions for His-tag protein purification. The chosen metal ion is always a trade-off between affinity and specificity.

His-tag

Protein characterization

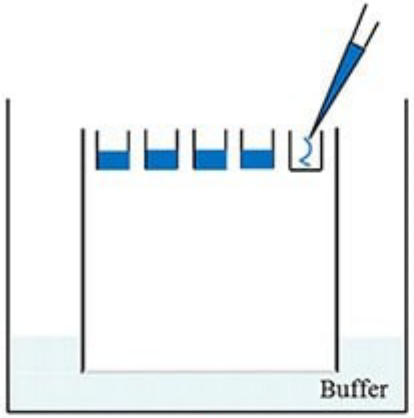


Molecular weight characterization by:

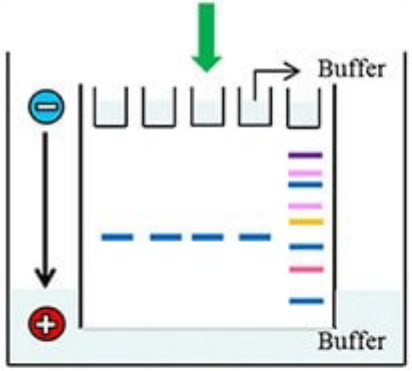
B: sulfate-polyacrylamide gel electrophoresis (SDS-PAGE); crude protein

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

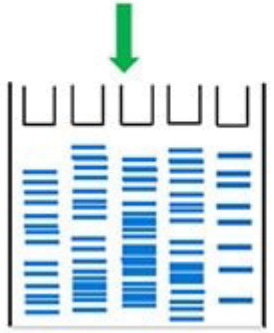
SDS-PAGE basics



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

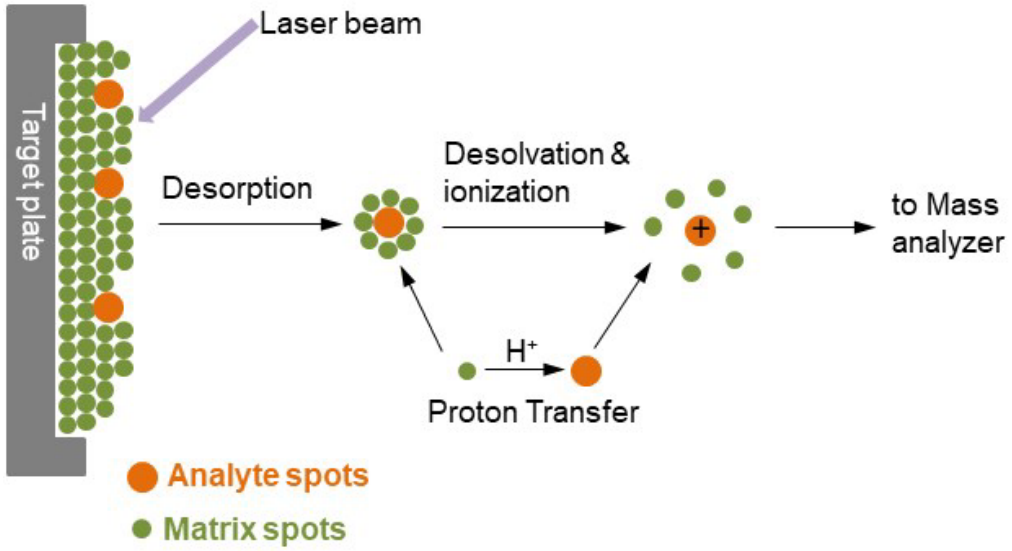
Separates proteins primarily based on their molecular weight, as SDS imparts a uniform negative charge to the denatured proteins, causing them to migrate towards the positive electrode at rates inversely proportional to their size

- 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)

SDS-PAGE

MALDI-TOF

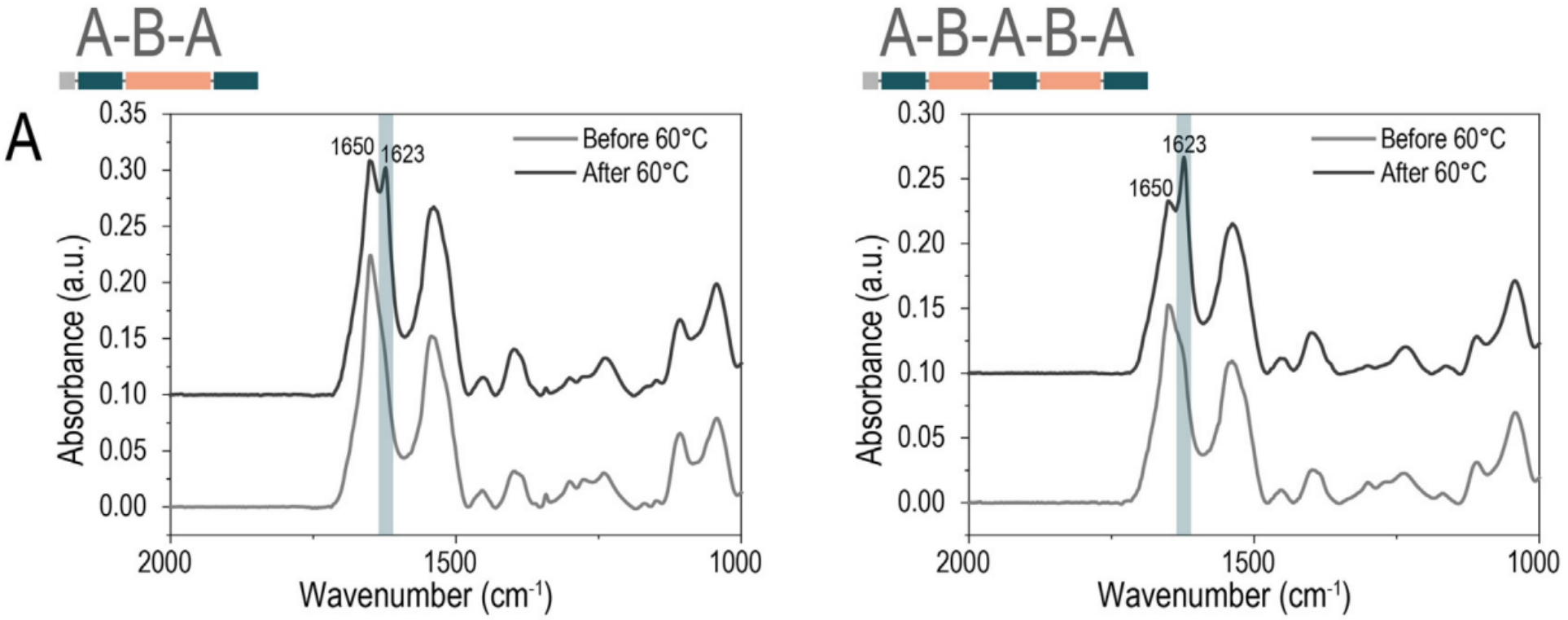
Mass spectrometry is an analytical technique in which samples are ionized into charged molecules and ratio of their mass-to-charge (m/z) can be measured. In MALDI-TOF, the ion source is matrix-assisted laser desorption/ionization (MALDI), and the mass analyzer is time-of-flight (TOF) analyzer.



- 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

MALDI-TOF

ATR-FTIR for 2° 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?

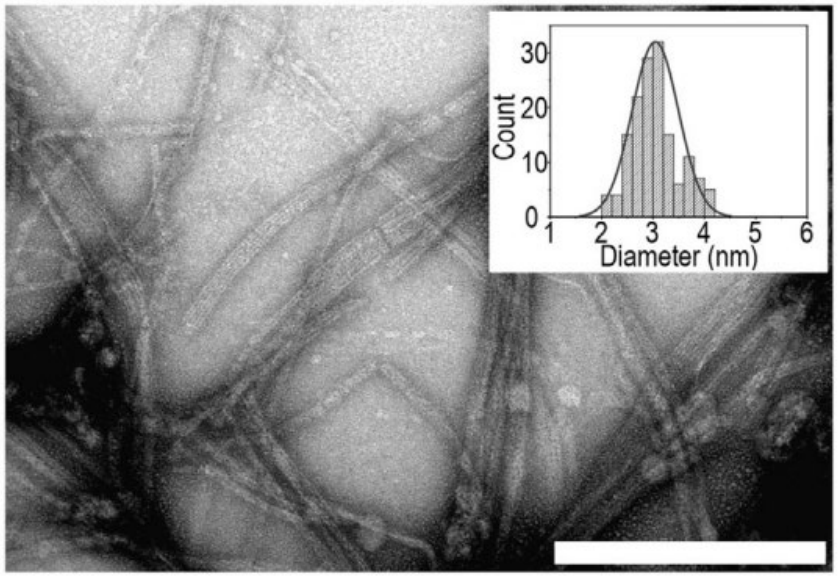
TEM for fibril structure (1 mg/ml)

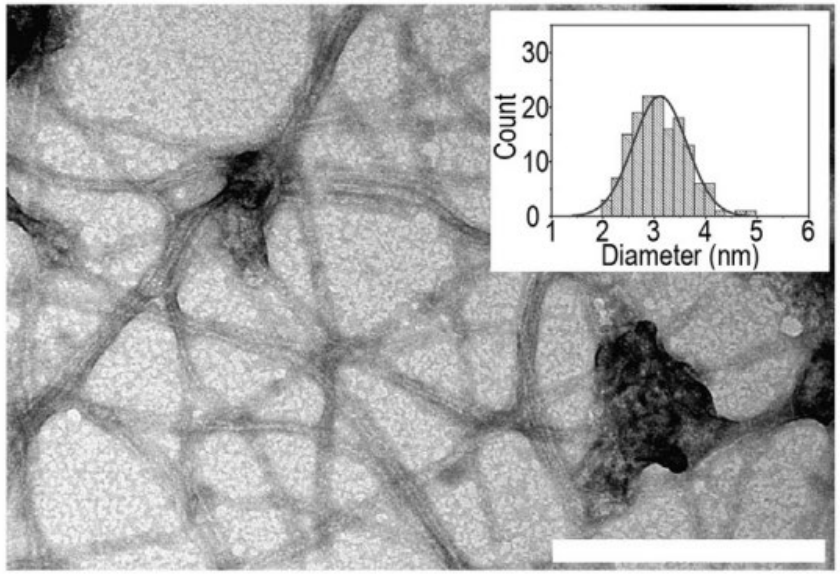
A-B-A

A-B-A-B-A

200 nm scale bar

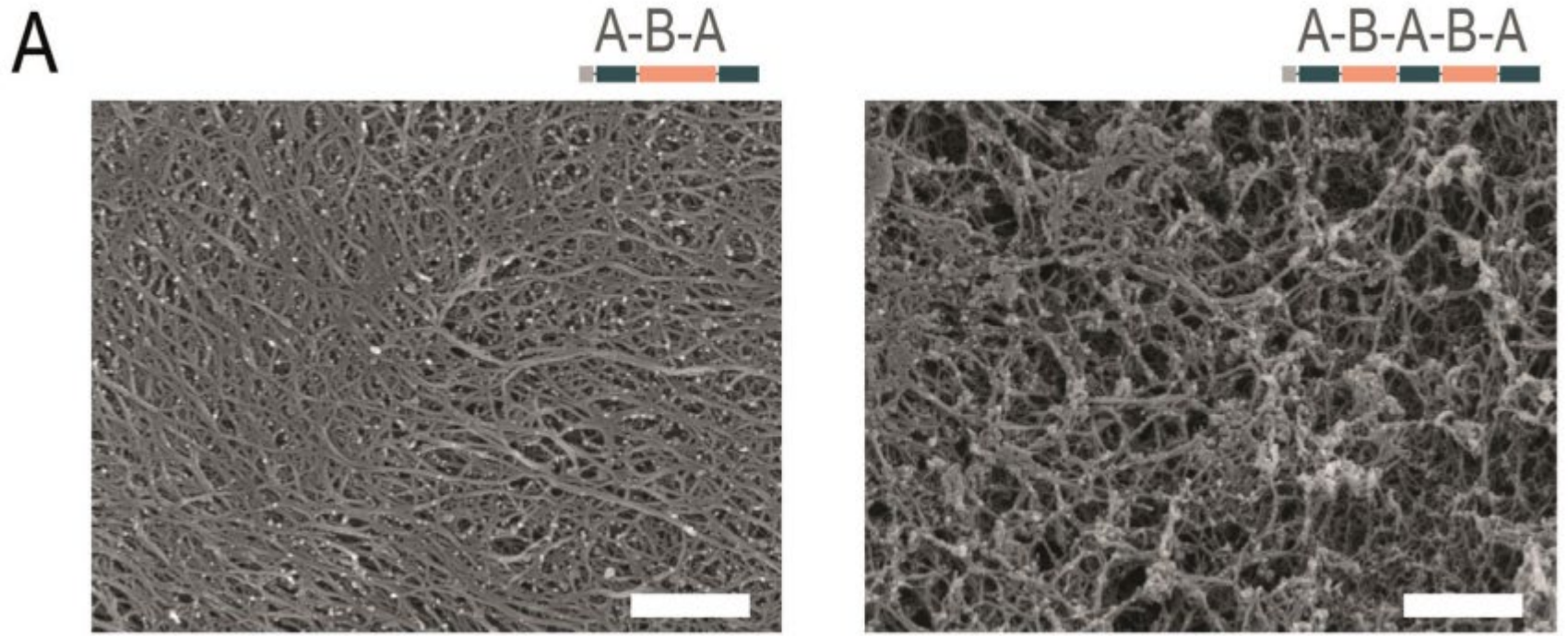
B





- 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

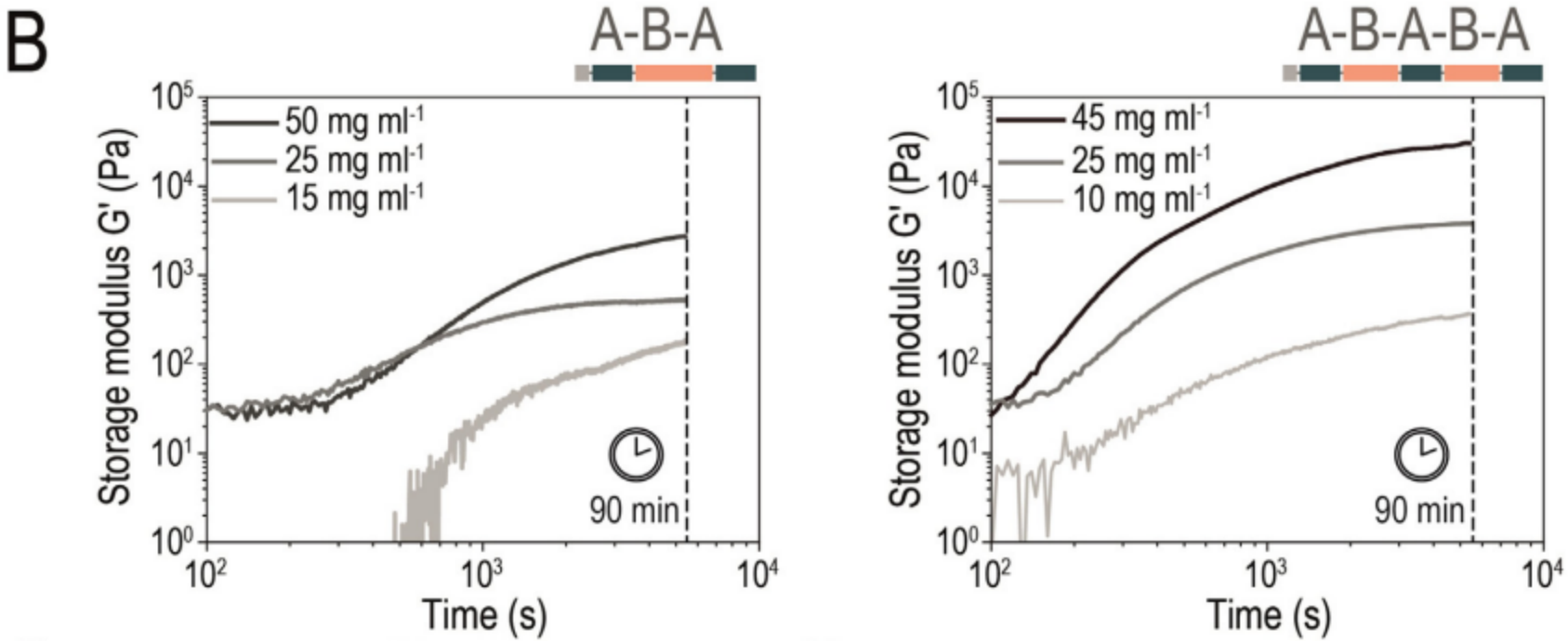
SEM for hydrogel structure (>100 mg/ml)



1 µm scale bar

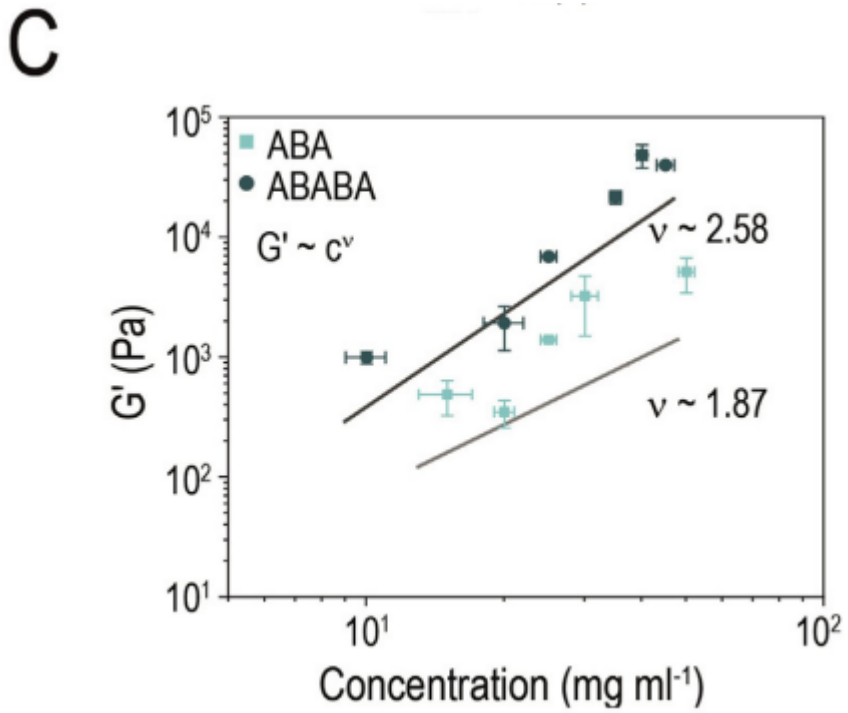
- ABA is less branched?
- 120 mg/ml for ABA
- 105 mg/ml for ABABA

Gelation kinetics to study self-assembly



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

Self-assembly by rheology

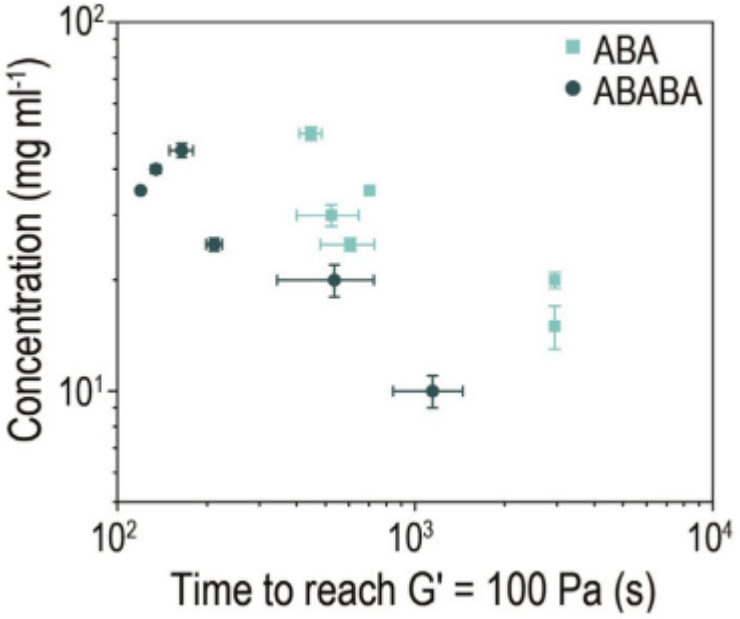


G’ values after 90 min at 60 °C for different concentrations

- 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 ($\nu \leq 2.2$)

Self-assembly by rheology

D

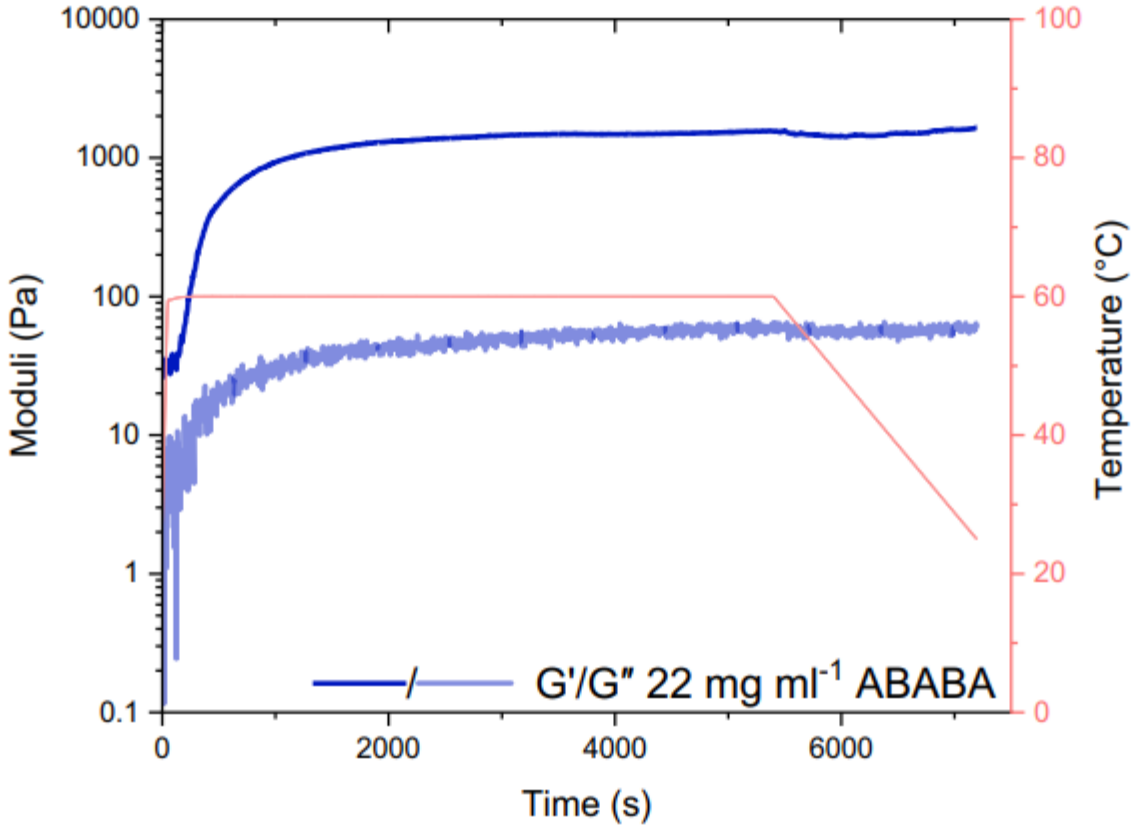


- Time required for G’ = 100 Pa was considered “gel initiation point”
- Based on this ABABA formed earlier than ABA

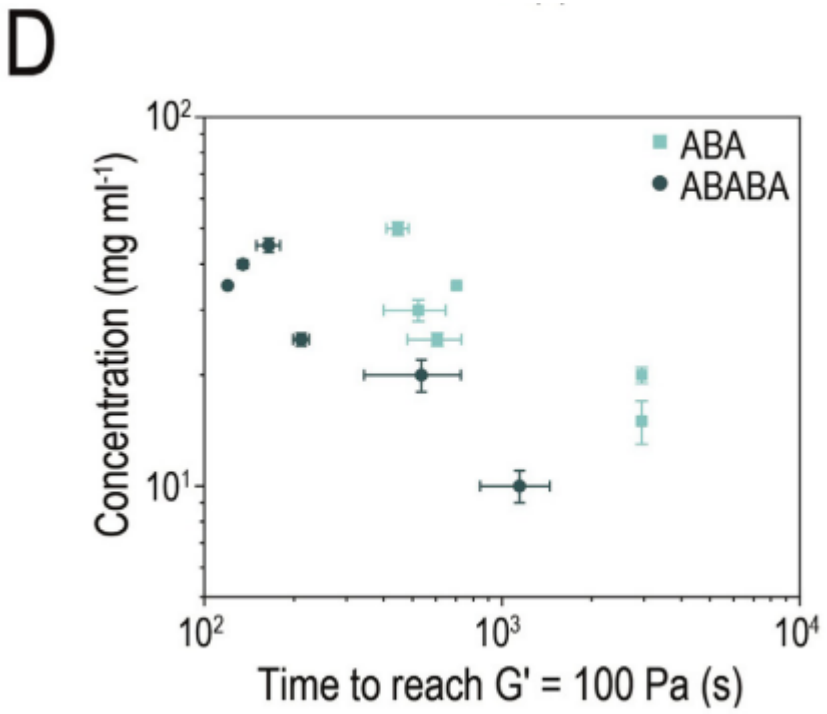
Time required to reach G’ = 100 Pa for protein solutions of different concentrations.

Gel maintained after cooling

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



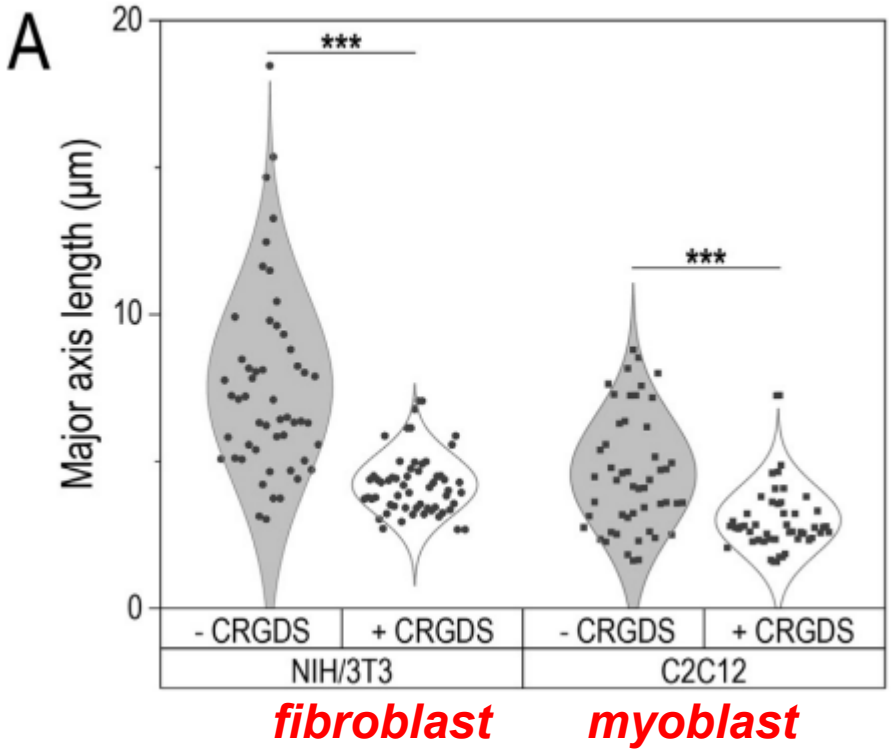
Self-assembly by rheology



- 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.

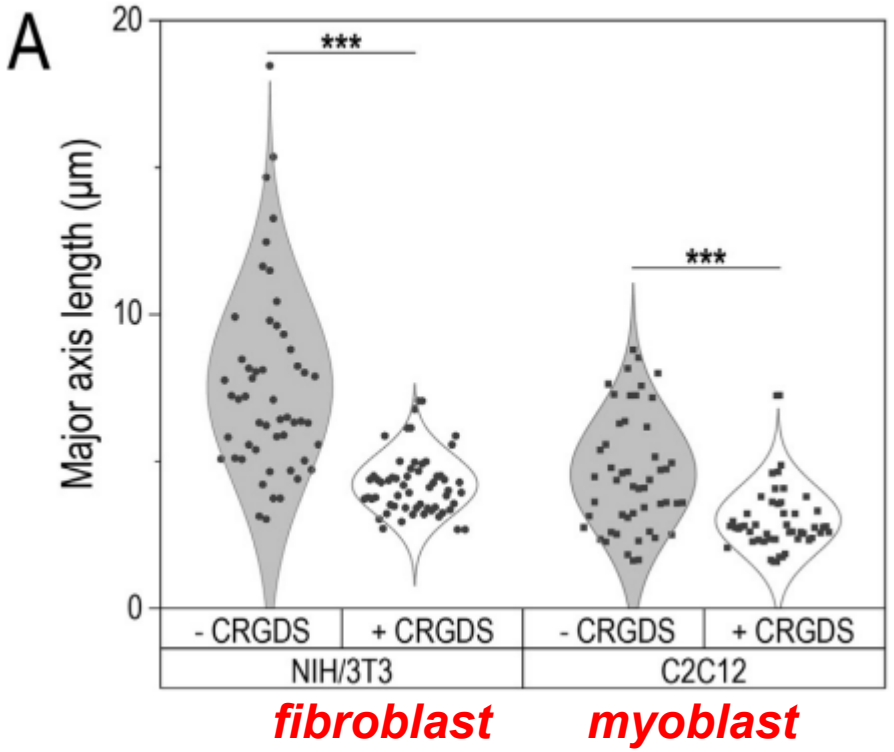
Cell attachment on protein hydrogels



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

- Design has cell attachment peptides (RGD) decoupled from network formation domains
- To ensure RGD specific attachment, added soluble CRGDS peptide to cell culture medium to competitively bind to RGD-integrins on cell surfaces
- Specificity was tested by comparing fibroblasts ad myoblasts, which differ in integrin expression

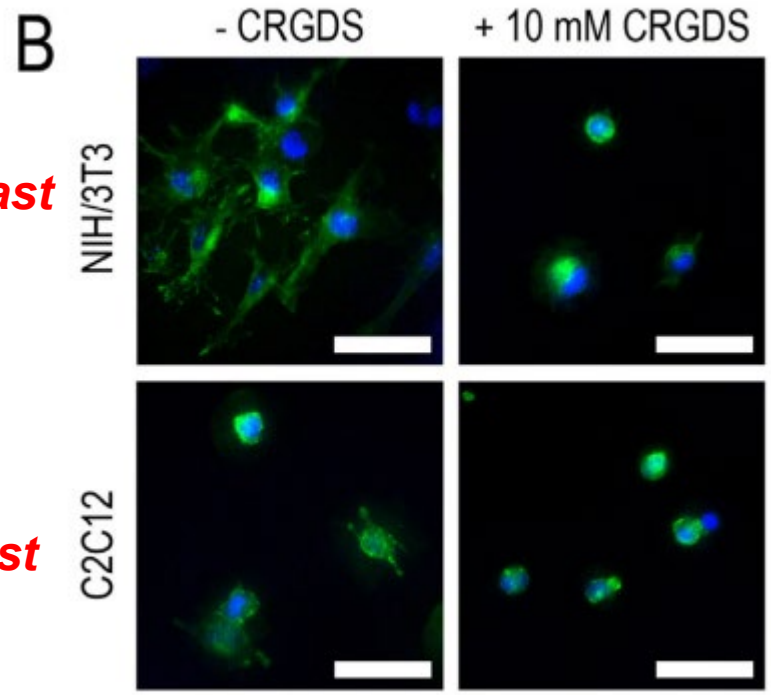
Cell attachment on protein hydrogels



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 ± 3 µm to 4.2 ± 1 µm (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

Cell attachment on protein hydrogels



fibroblast

myoblast

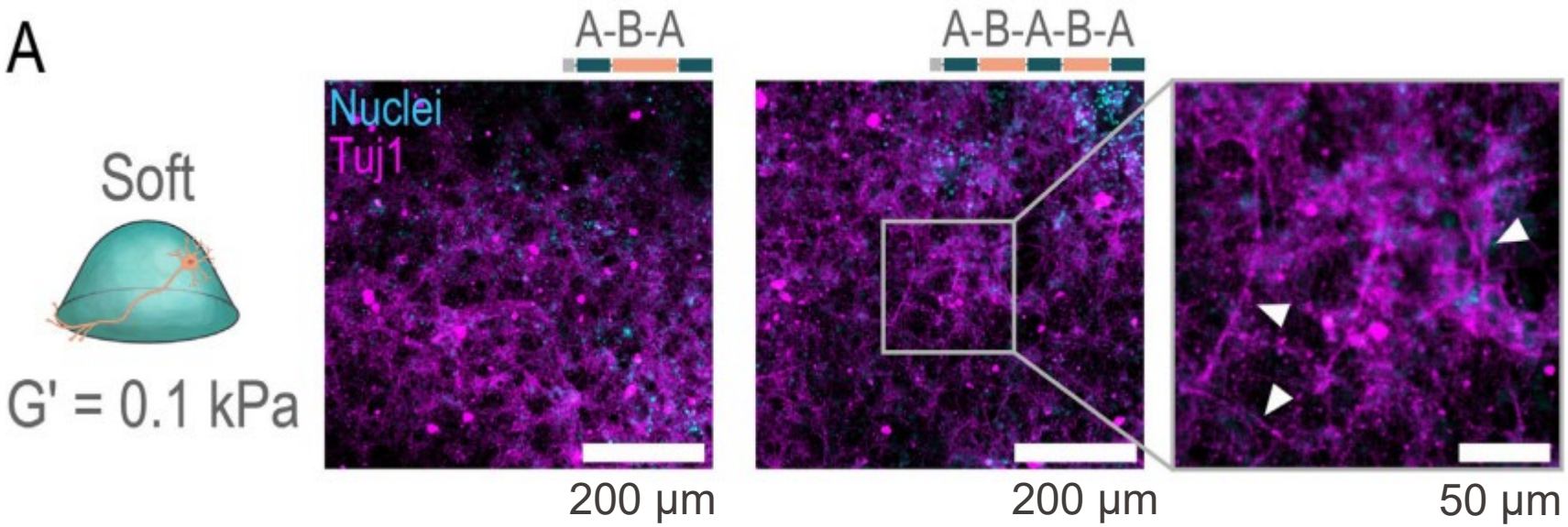
Investigation of cell attachment and spreading in ABABA gel

50 µm scale bar

- 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…*

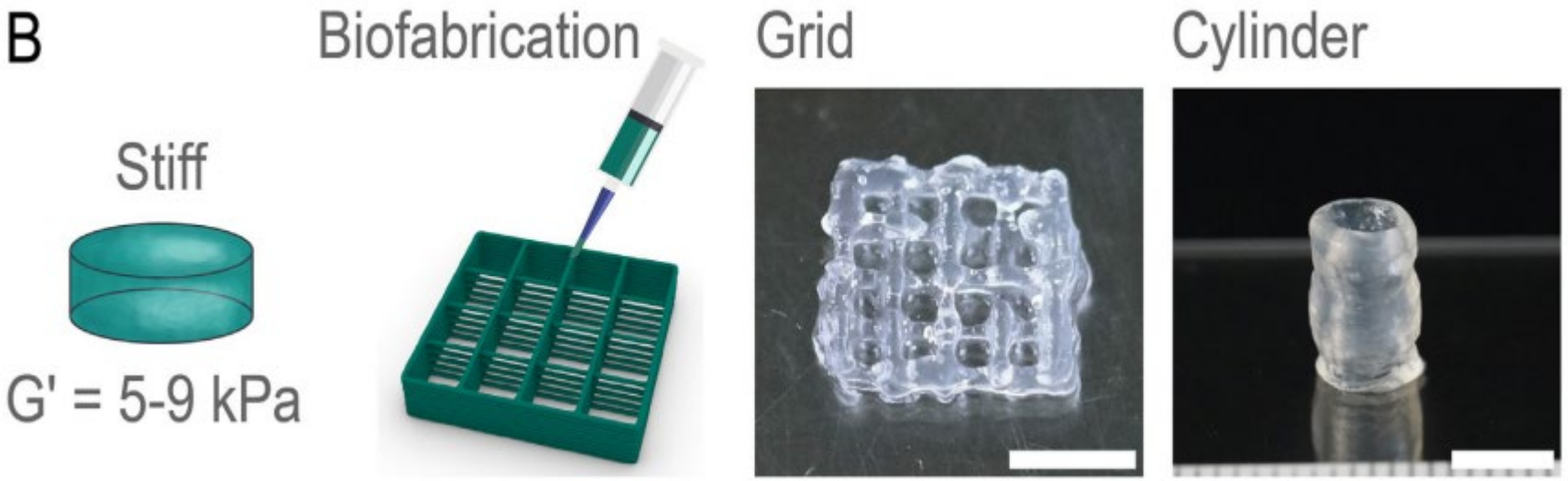
Biofab of soft hydrogel scaffolds

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)

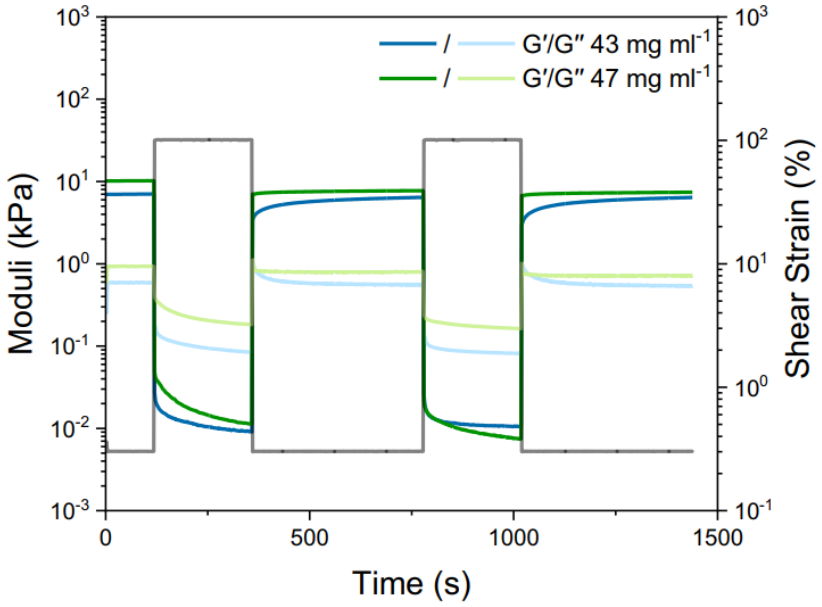
Biofab of soft hydrogel scaffolds

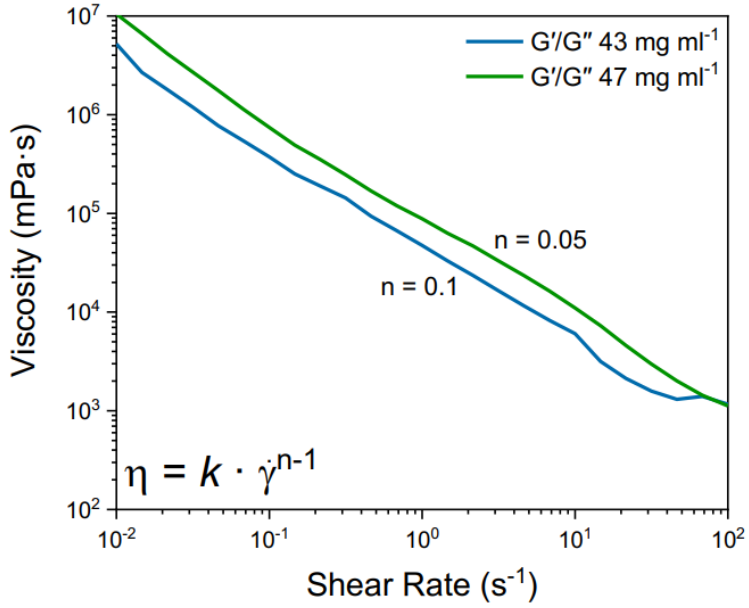


5 mm scale bars

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

Biofab of soft hydrogel scaffolds

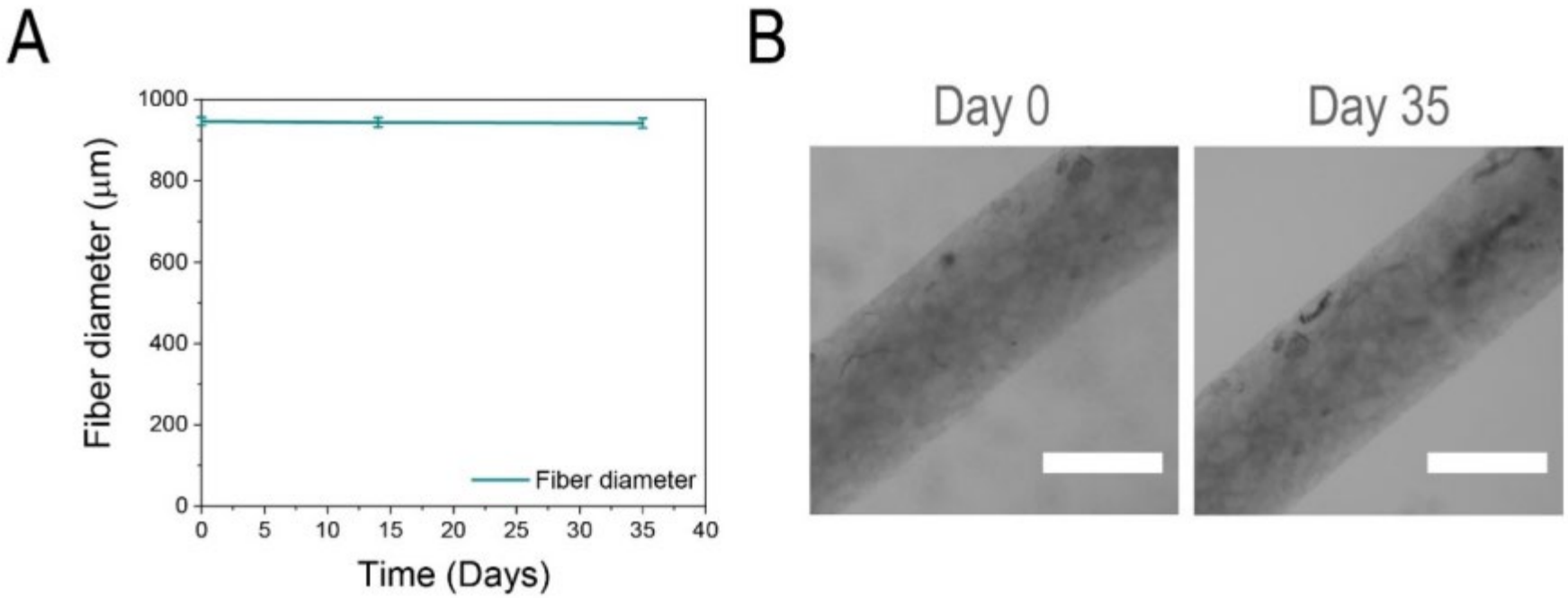




- Stiff ABABA hydrogels (45 ± 2 mg/ml, storage modulus G’ = 5-9 kPa) showed high elastic recovery of G’ after high strain intervals and shear thinning – suggesting good for 3D printing

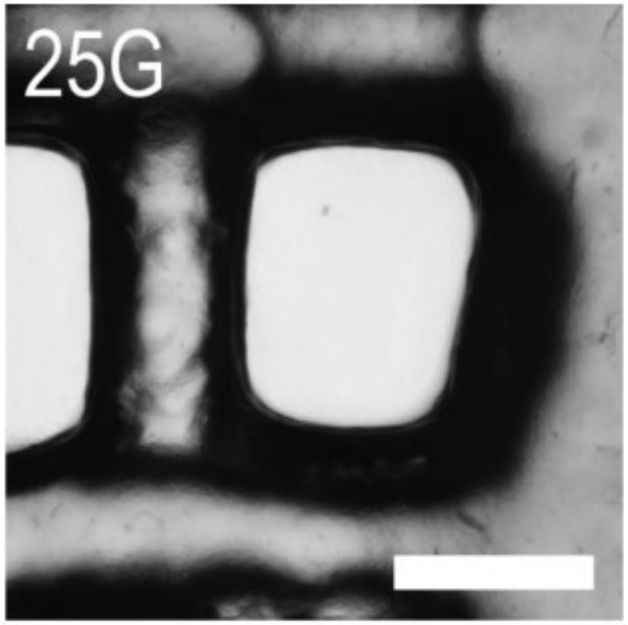
Biofab of soft hydrogel scaffolds

750 µm scale bar



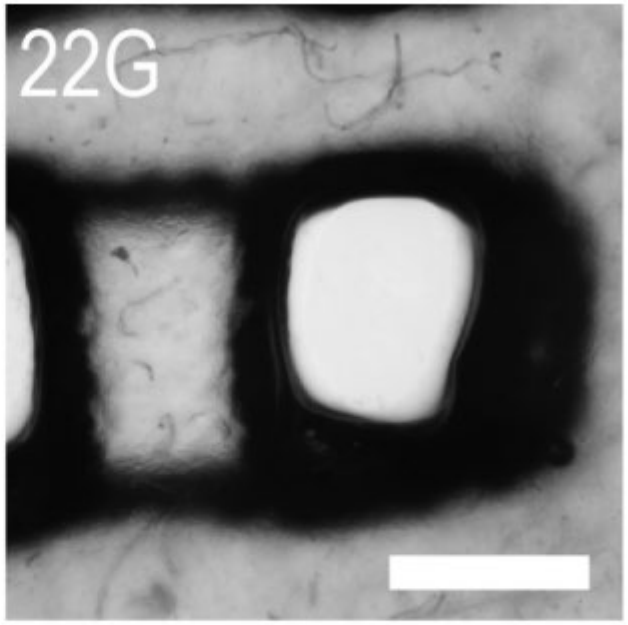
- Extruded filament In PBS, ABABA hydrogel (45 mg/ml) stable over 5 weeks, suggesting they would remain stable during long-term cell culture

Biofab of soft hydrogel scaffolds



Pr = 0.9

SR = 2.6



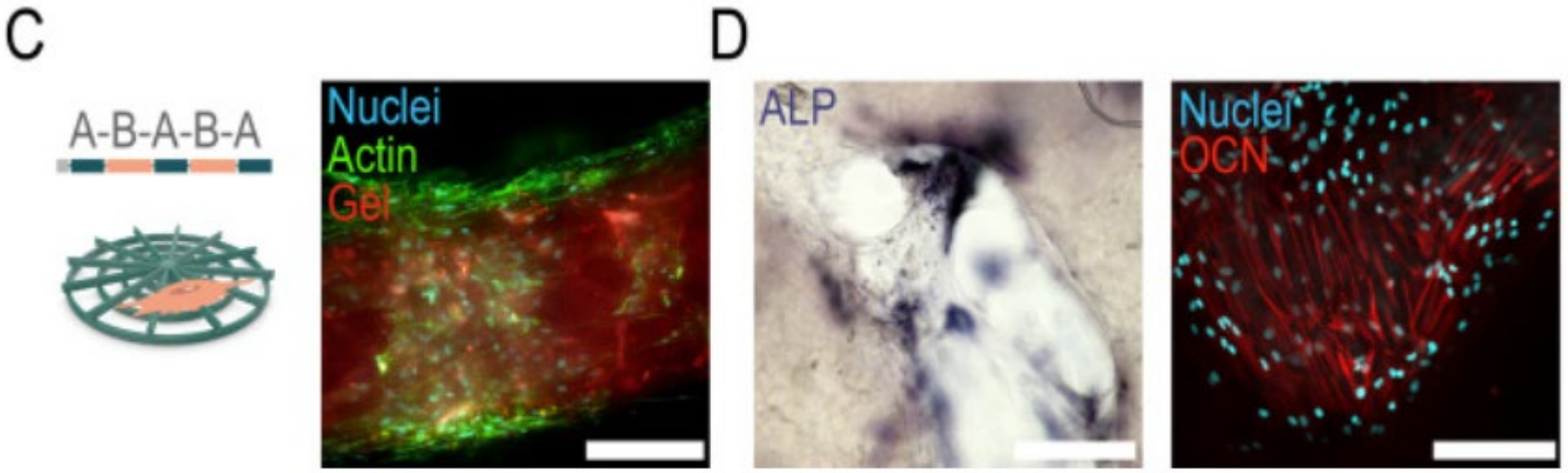
Pr = 0.9

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

Biofab of soft hydrogel scaffolds



Take away:

High modulus protein hydrogels are suitable for *digital fabrication* of biomaterials and osteogenic differentiation of hMSCs

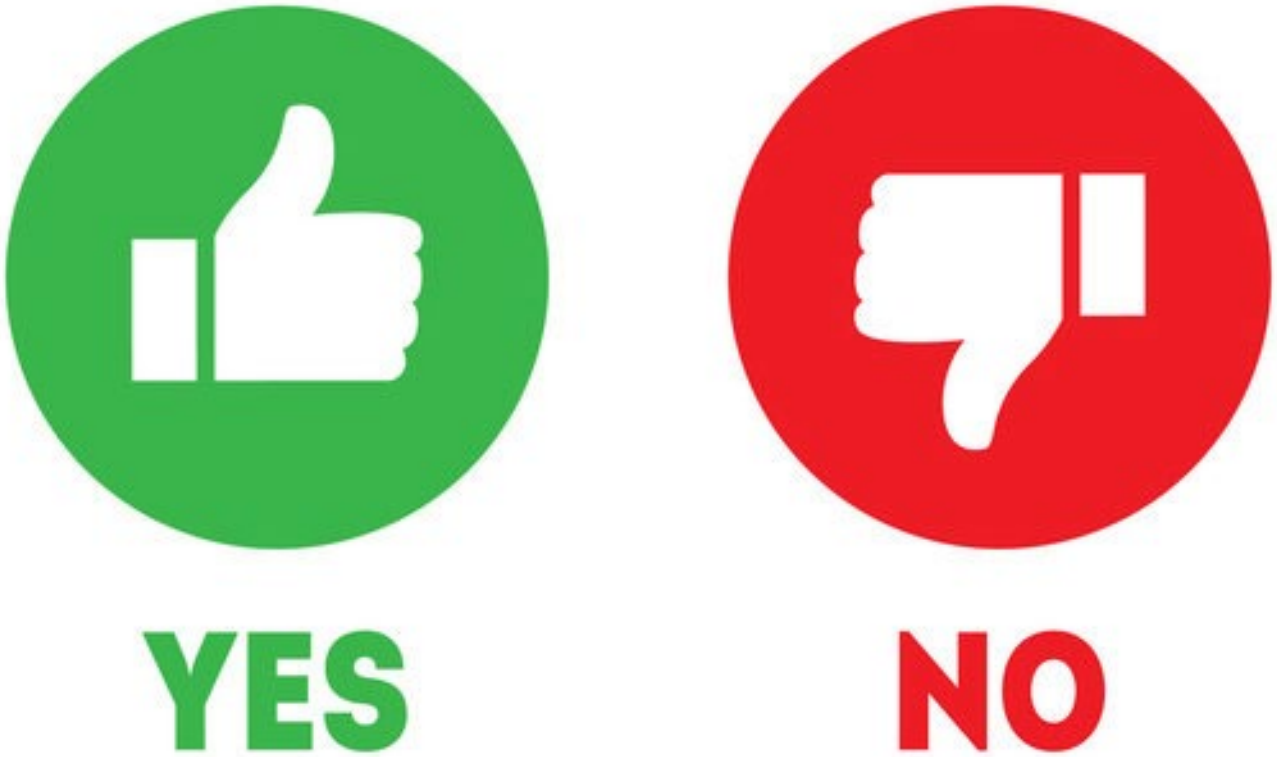
Discussion (a few points)

- Hypothesized that ABABA self-assembly would be enhanced due to more A blocks
- Unexplored consideration – entanglements during heating, which can influence gelation (suggest liquid AFM in the future)
- To replicate ECM, would be useful to have a library of fibers with different rigidity
- Possible influence of low molecular weight contaminants – further purification to improve material performance?
- Other integrin attachment domains (not just RGD) in the future, or other bioactive sites, etc., sustained release, wound healing, etc.,

Conclusions/ Take aways

- Modular protein design to form supramolecular fibrillar hydrogels
- Self-assembly behavior and mechanics depend on protein design and concentrations
- 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)



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)!