Real examples MSE 493

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Mechanically Tunable, Compostable, Healable and Scalable Engineered Living Materials

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Check for updates

Avinash Manjula-Basavanna [1,2,3], Anna M. Duraj-Thatte [2] & Neel S. Joshi [1]

Advanced design strategies are essential to realize the full potential of engineered living materials, including their biodegradability, manufacturability, sustainability, and ability to tailor functional properties. Toward these goals, we present mechanically engineered living material with compostability, healability, and scalability – a material that integrates these features in the form of a stretchable plastic that is simultaneously flushable, compostable, and exhibits the characteristics of paper. This plastic/paper-like material is produced in scalable quantities (0.5–1 g L^{-1}), directly from cultured bacterial biomass (40%) containing engineered curli protein nanofibers. The elongation at break (1–160%) and Young's modulus (6-450 MPa) is tuned to more than two orders of magnitude. By genetically encoded covalent crosslinking of curli nanofibers, we increase the Young's modulus by two times. The designed engineered living materials biodegrade completely in 15–75 days, while its mechanical properties are comparable to petrochemical plastics and thus may find use as compostable materials for primary packaging.

- 2 corresponding authors: Prof. Joshi and Dr. Majula-Basavanna, Research Scientist at MIT
- Anna Duraj-Thatte, Assistant Professor at Virginia Polytechnic
- No students?
- Impact factor = 15.7

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Anna Duraj-Thatte
Neel Joshi

Author contributions

A.M.-B. conceived the project and performed all the experiments and analysis. A.M.-B. and A.M.D.-T. cloned all the curli variants. A.M.-B., and N.S.J. wrote and edited the manuscript. All authors discussed and commented on the manuscript.

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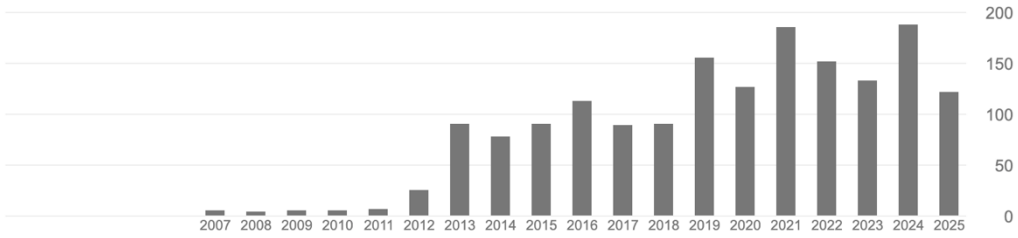

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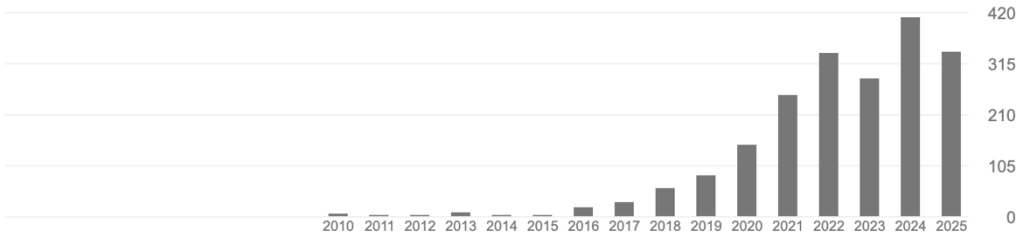

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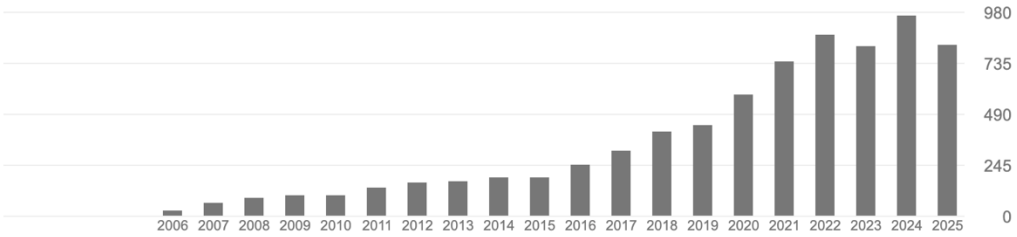

Dream team…

Abstract

Advanced design strategies are essential to realize the full potential of **engineered living materials**, including their biodegradability, manufacturability, sustainability, and ability to tailor functional properties. Toward these goals, we present mechanically engineered living material with compostability, healability, and scalability – a material that integrates these features in the form of a stretchable plastic that is simultaneously flushable, compostable, and exhibits the characteristics of paper. This **plastic/paper-like material** is produced in scalable quantities (0.5–1gL−1), directly from cultured bacterial biomass (40%) containing engineered **curli protein nanofibers**. The elongation at break (1–160%) and Young's modulus (6-450 MPa) is tuned to more than two orders of magnitude. By **genetically encoded covalent crosslinking of curli nanofibers**, we increase the Young's modulus by two times. The designed engineered living materials biodegrade completely in 15–75 days, while its mechanical properties are comparable to petrochemical plastics and thus may find use as compostable materials for **primary packaging**.

- Plastic/paper like (those are different, right?)
- Curli protein nanofibers
- Genetically encoded covalent crosslinking for improved tensile properties
- Suggested as a primary packaging material
- **MECHS**, which stands for Mechanically Engineered Living Material with Compostability, Healability, and Scalability

Previous work on curli by Joshi group

nature COMMUNICATIONS

ARTICLE

Received 17 Mar 2014 | Accepted 8 Aug 2014 | Published 17 Sep 2014

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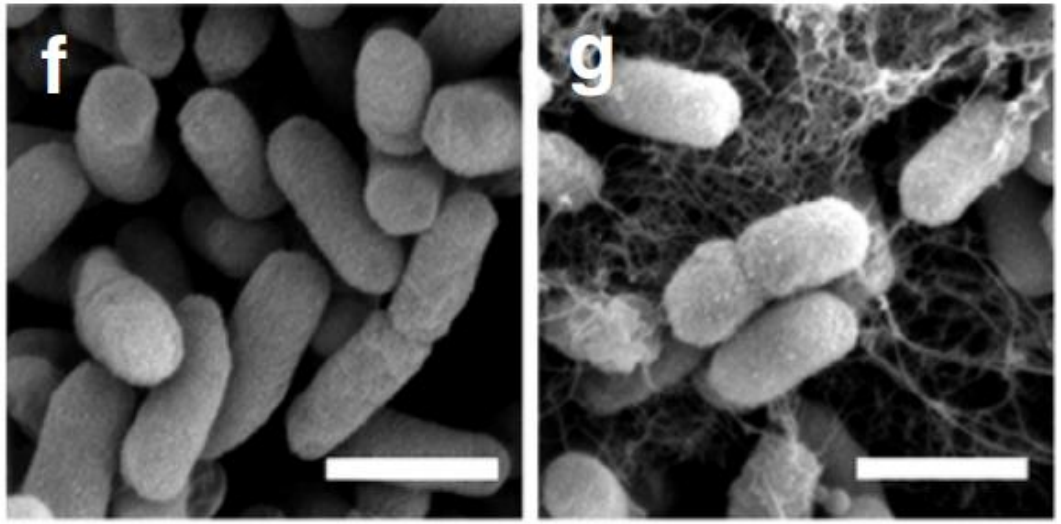
Programmable biofilm-based materials from engineered curli nanofibres

Peter Q. Nguyen[1,2], Zsofia Botyanszki[2,3], Pei Kun R. Tay[1,2] & Neel S. Joshi[1,2]

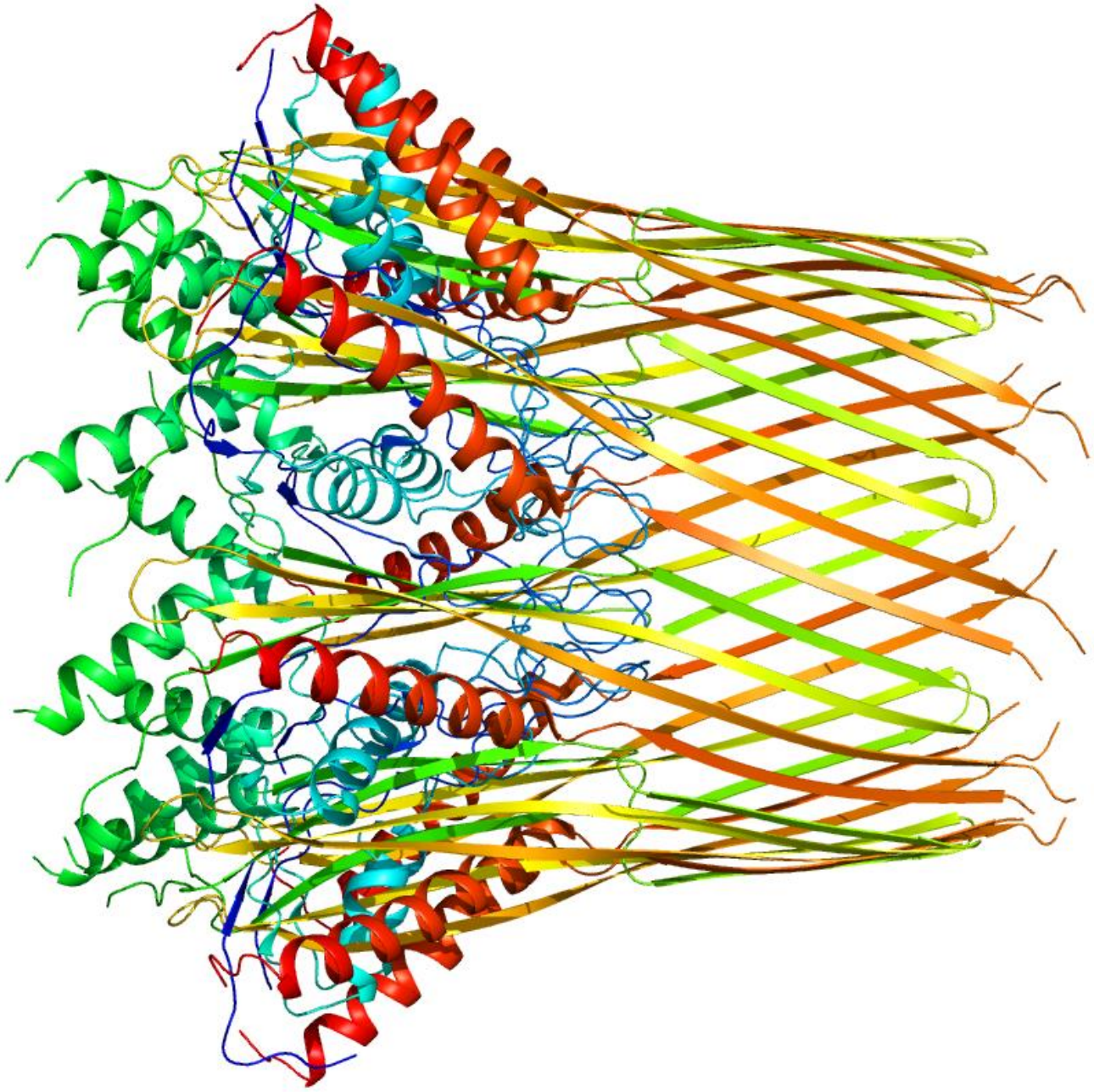
OK, what's a curli fiber?

- Protein nanofibers produced by some bacteria, like *E. coli*, as main protein part of biofilm
- 4-7 nm in diameter, highly entangled networks
- Curli are formed by the extracellular self-assembly of CsgA, a small secreted 13 kDa protein
- A type of amyloid fiber



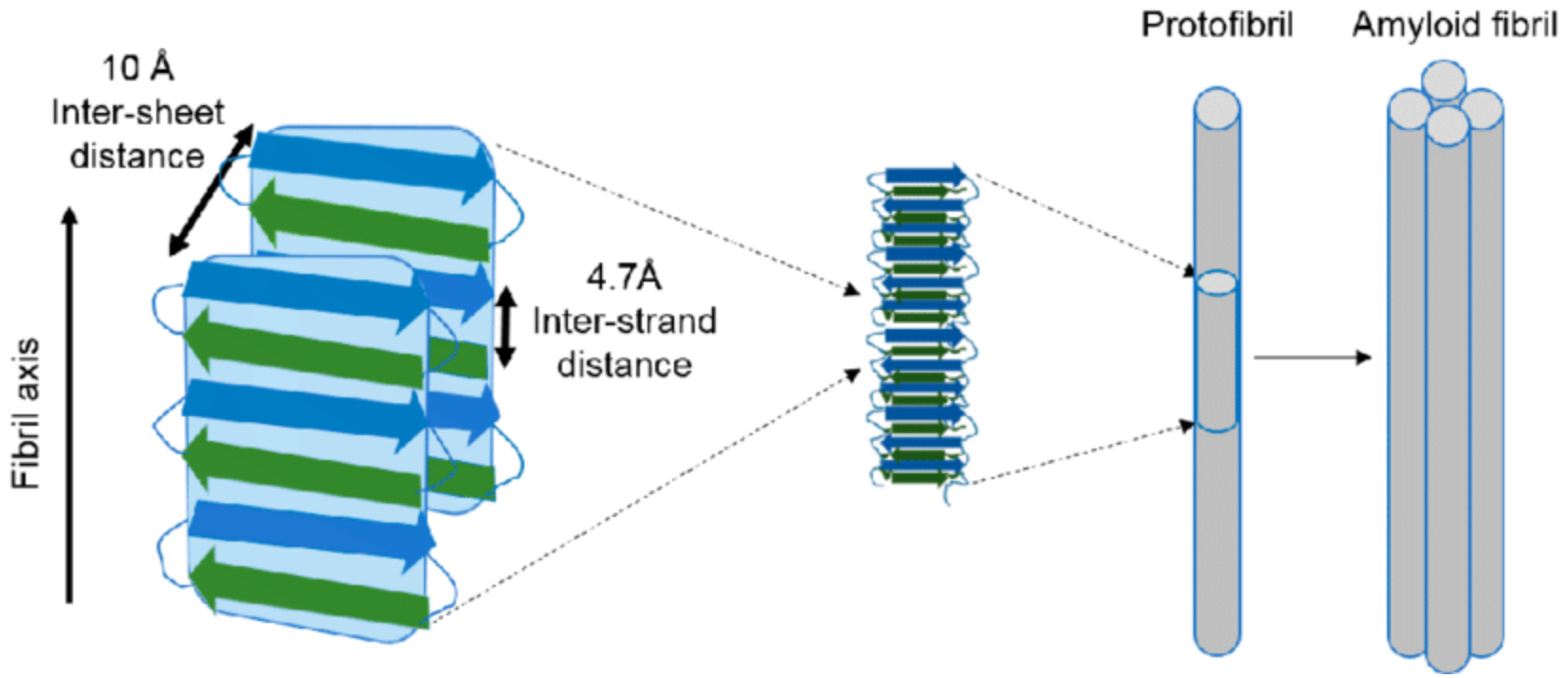
1 µm scale bars
(f) *E. coli* variant with no nanofiber (no CsgA)
(g) *E. coli* variant with nanofibers (wild-type CsgA)

https://doi.org/10.1038/ncomms5945



Crystallographic structure of the enterobacteria Curli protein structures

https://en.wikipedia.org/wiki/Curli



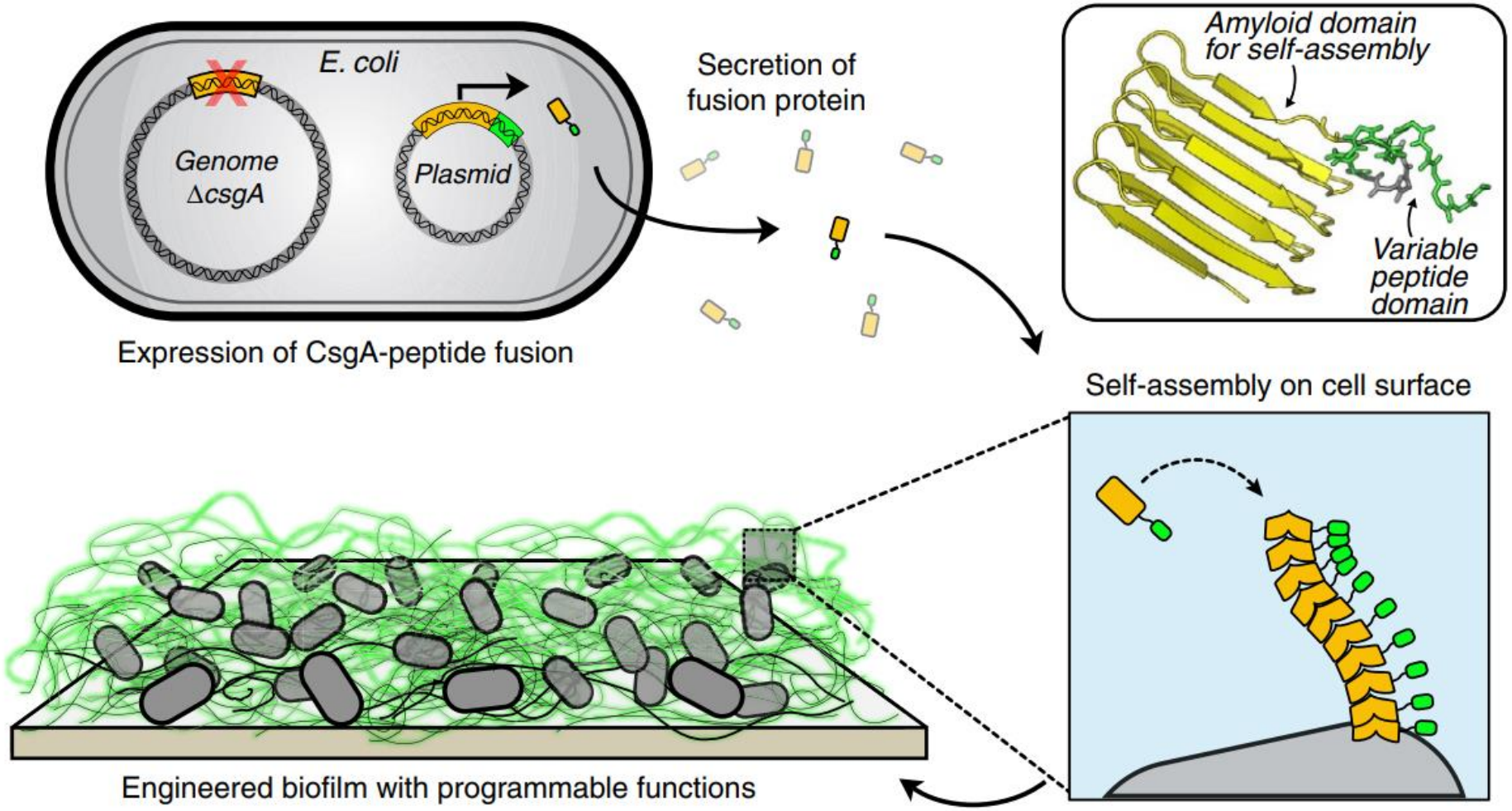
- β-sheets are perpendicular to the fibril axis
- H-bonds between β-sheets run parallel to fibril axis
- Cross β-sheet structure contributes to high strength and stability

Why Curli nanofibers?

- “Ideal platform” for materials engineering: Why?
- Programmability – composed from the self-assembly of one protein, providing an entry point to genetic engineering (recall: 1 gene, 1 protein)
- Robust –withstands harsh environments – heat, solvents, pH, detergents, denaturants, high strength
- Abundant – up to 10-40% of biofilm

https://doi.org/10.1038/ncomms5945

Previous work: Curli nanofibers with non-natural functions



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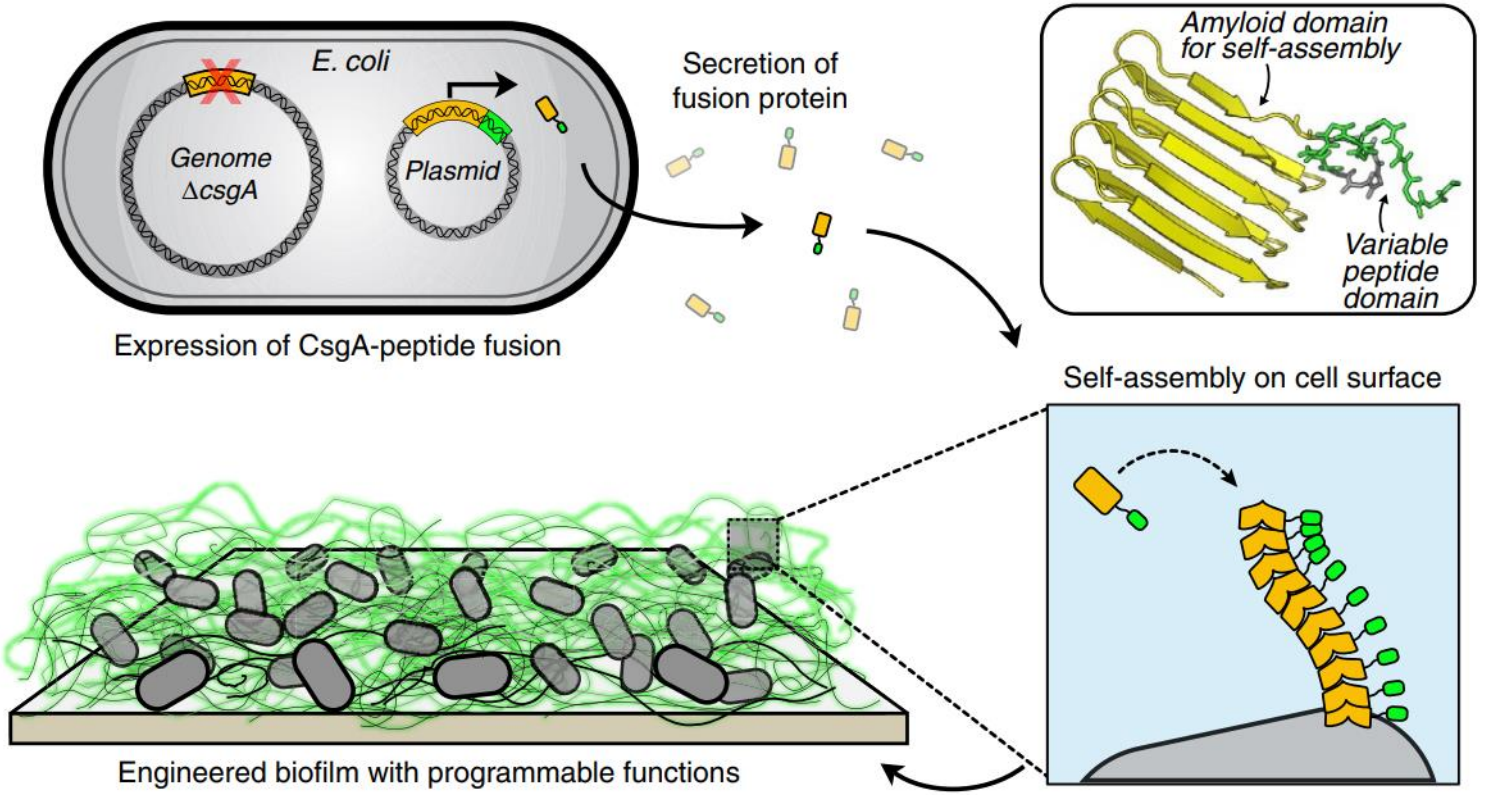
Biofilm-Integrated Nanofiber Display (BIND) system



- DcsgA cells express and secrete the fusion protein
- Fusion protein consist of the CsgA (orange) and the functional peptide domain (green)
- Secreted fusion protein self-assembles on cell surfaces, making up a biofilm, that is programmed with non-natural functions

https://doi.org/10.1038/ncomms5945

Previous work: "AquaPlastic"

ARTICLES
https://doi.org/10.1038/s41589-021-00773-y
nature chemical biology
Check for updates

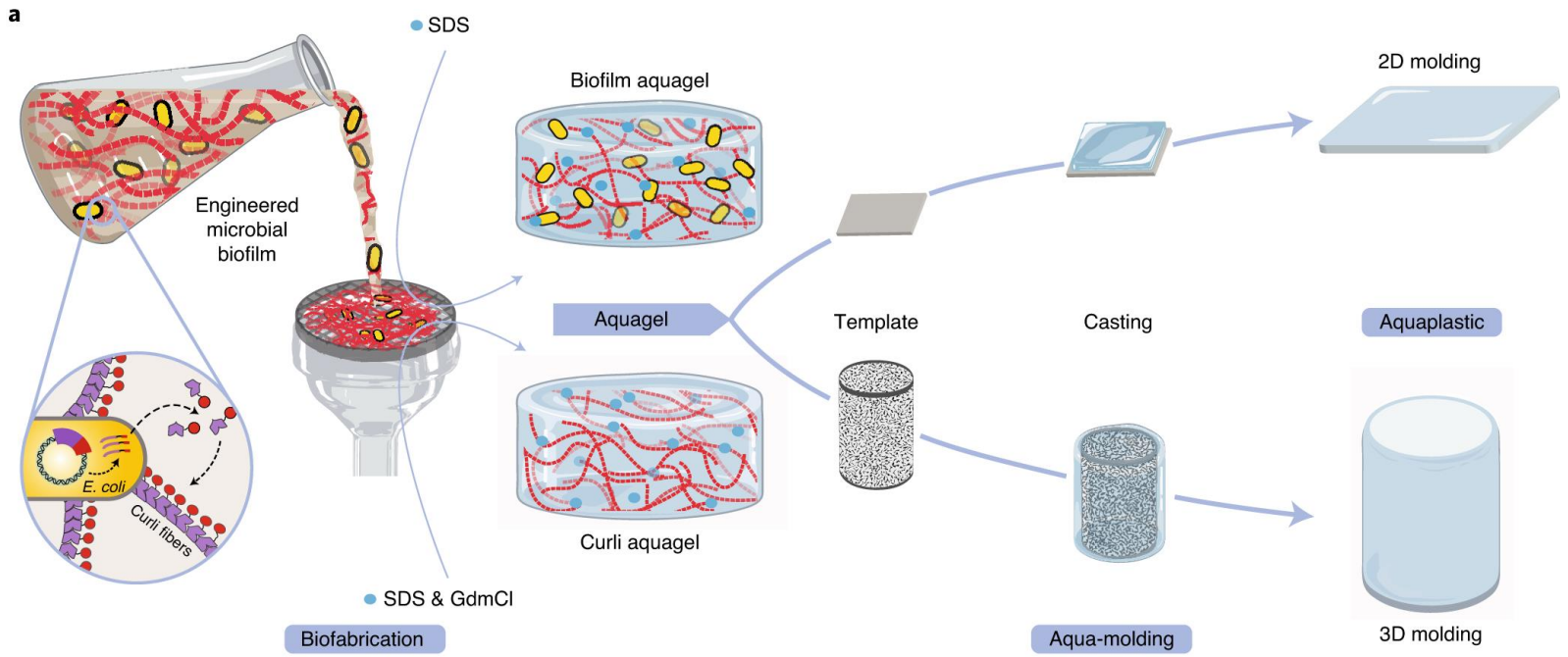
Water-processable, biodegradable and coatable aquaplastic from engineered biofilms

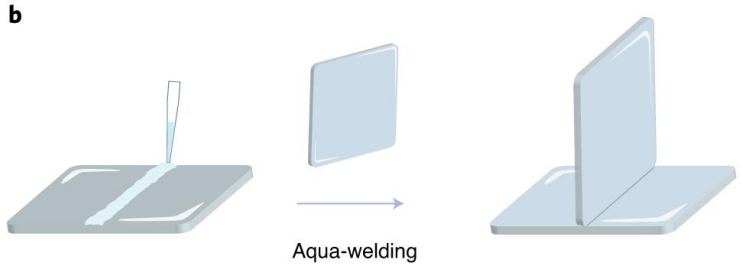
Anna M. Duraj-Thatte[1,2,3,9], Avinash Manjula-Basavanna[2,3,9], Noémie-Manuelle Dorval Courchesne[2,4,9], Giorgia I. Cannici[2,5], Antoni Sánchez-Ferrer[6], Benjamin P. Frank[7], Leonie van't Hag[6], Sarah K. Cotts[8], D. Howard Fairbrother[7], Raffaele Mezzenga[6] and Neel S. Joshi[1,2,3] ✉

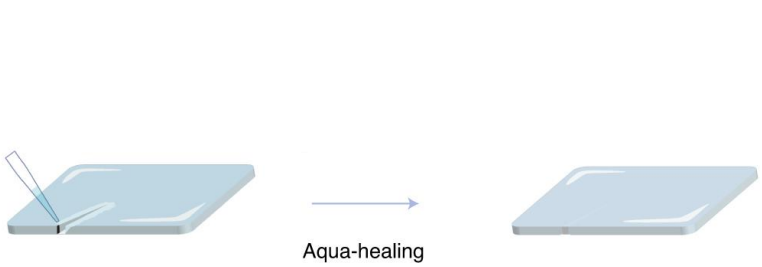
Petrochemical-based plastics have not only contaminated all parts of the globe, but are also causing potentially irreversible damage to our ecosystem because of their non-biodegradability. As bioplastics are limited in number, there is an urgent need to design and develop more biodegradable alternatives to mitigate the plastic menace. In this regard, we report aquaplastic, a new class of microbial biofilm-based biodegradable bioplastic that is water-processable, robust, templatable and coatable. Here, *Escherichia coli* was genetically engineered to produce protein-based hydrogels, which are cast and dried under ambient conditions to produce aquaplastic, which can withstand strong acid/base and organic solvents. In addition, aquaplastic can be healed and welded to form three-dimensional architectures using water. The combination of straightforward microbial fabrication, water processability and biodegradability makes aquaplastic a unique material worthy of further exploration for packaging and coating applications.

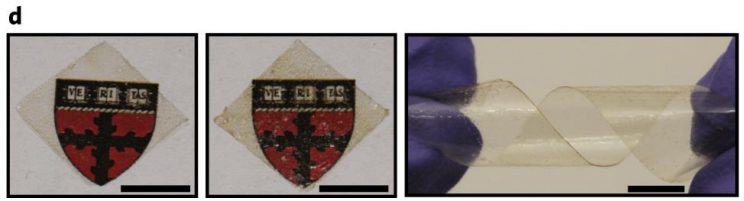
- 2021
- Same 3 authors
- Swiss contribution

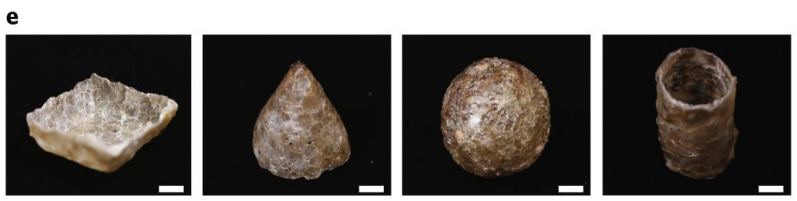
https://doi.org/10.1038/s41589-021-00773-y











Maybe a focus of another class?

https://doi.org/10.1038/s41589-021-00773-y

MECHS to address AquaPlastic shortcomings

- AquaPlastic is composed is recombinant protein nanofibers produced by *E. Coli*
- Young's modulus ca. 1 GPa and ultimate tensile strength ca. 25 MPa
- Resistant to chemicals (acid, base, organic solvents)
- Coatable
- But:
- Brittle and not scalable
- To address: MECHS combines "whole cellular biomass and engineered extracellular matrix protein nanofibers that enables tuning of their mechanical properties"

Biofrabrication of MECHS

- 2 main ingredients: whole *E. coli* cells + engineered recombinant curli nanofibers
- Use PQN4 in which chromosomal curli genes (*csgBAC*, *csgDEFG*) have been deleted
- PQN4 was transformed with pET21d plasmid vector encoding a ***synthetic curli operon***, *csgBACEFG*, containing all genes needed for *CsgA* production, secretion, and extracellular assembly (not new in this work)
- In this work – the biology part and the materials part come from a bacteria!

Gene transcription recap from last week

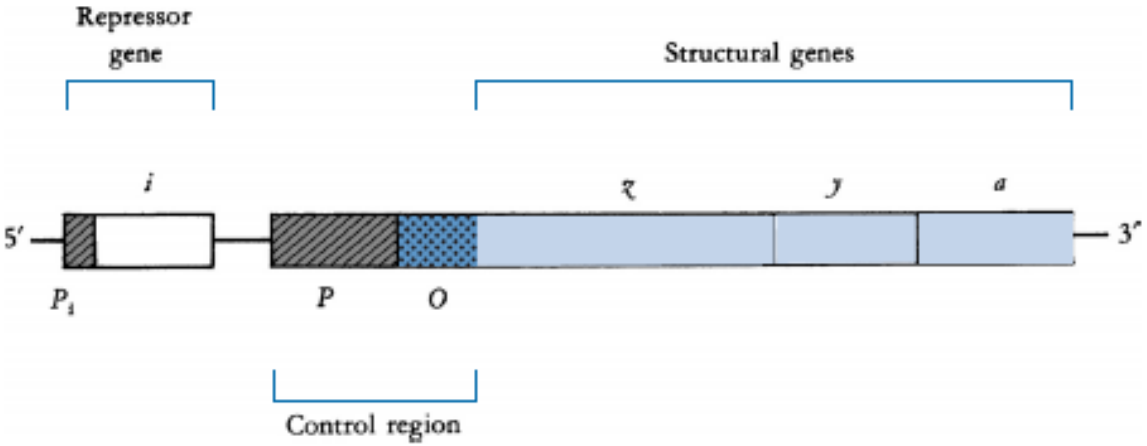
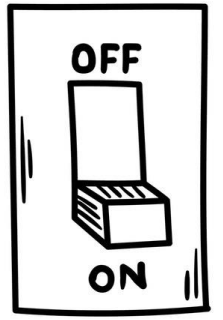


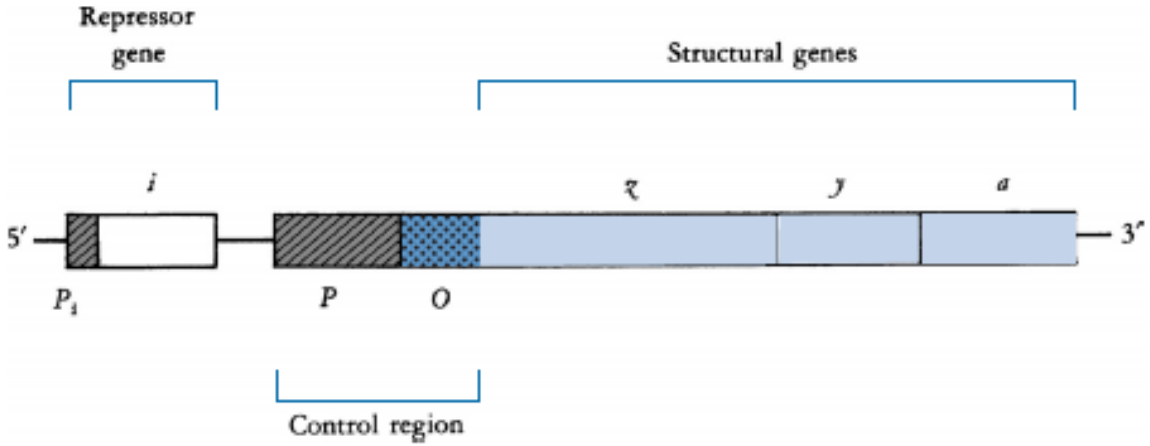
Fig. 2.7 The *lac* operon. The structural genes *lacZ*, *lacY*, and *lacA* (noted as *z*, *y*, and *a*) encode ß-galactosidase, galactoside permease, and a transacetylase, respectively. The cluster is controlled by a promoter (*P*) and an operator region (*O*). The operator is the binding site for the repressor protein, encoded by the *lacI* gene (*i*). The repressor gene lies outside the operon itself and is controlled by its own promoter, P_i.



DNA part:

- Promoter: binding site for RNA polymerase
- Operator: sequence where repressor proteins can bind
- Structural genes: genes that will be transcribed and translated

Gene transcription recap from last week



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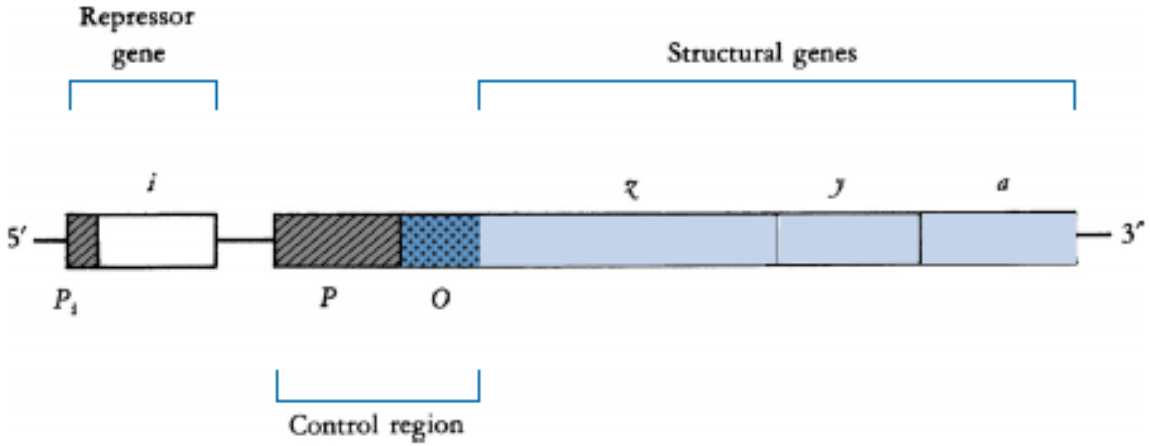
Protein part:

- Repressor (LacI) binds to operator to prevent transcription
- RNA polymerase: enzyme that transcribes DNA in o RNA, binds to promoter

Gene transcription recap from last week



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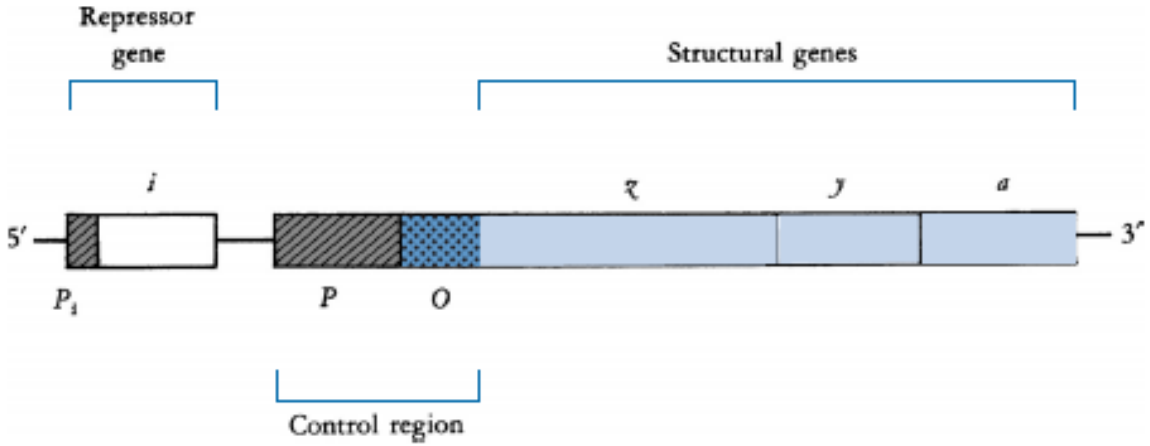
Inducer

- Lactose/IPTG – molecule that binds to the repressor (LacI) changing its shape

Gene transcription recap from last week



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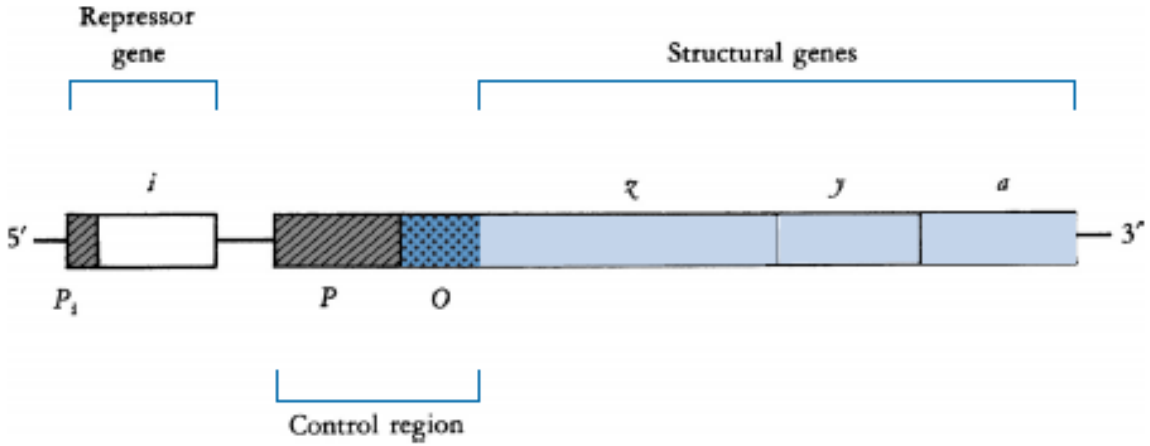
Repressed/OFF state

- LacI repressor is bound to operator
- This prevents RNA polymerase from binding to the promoter and transcribing the genes
- No gene expression occurs = no protein formed

Gene transcription recap from last week



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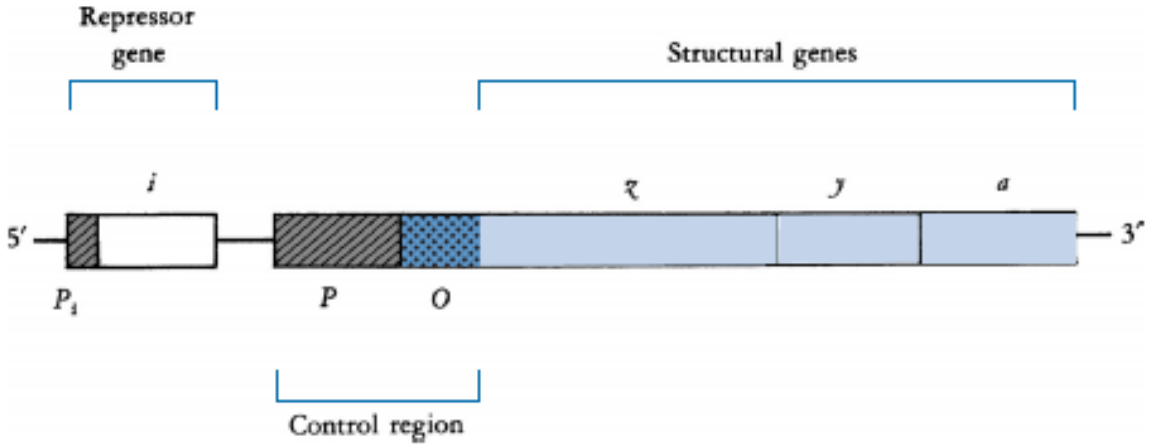
Induction

- IPTG or lactose enters cell and binds to repressor (LacI)
- Shape change in repressor causes it to release from operator = protein is formed

Gene transcription recap from last week



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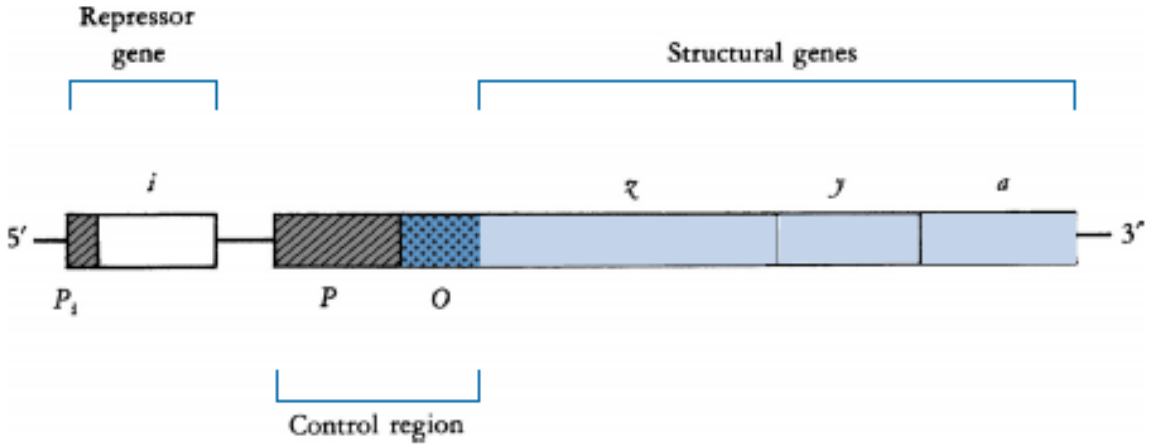
Transcription

- RNA polymerase can now bind to the promoter and start gene transcription, producing mRNA

Gene transcription recap from last week



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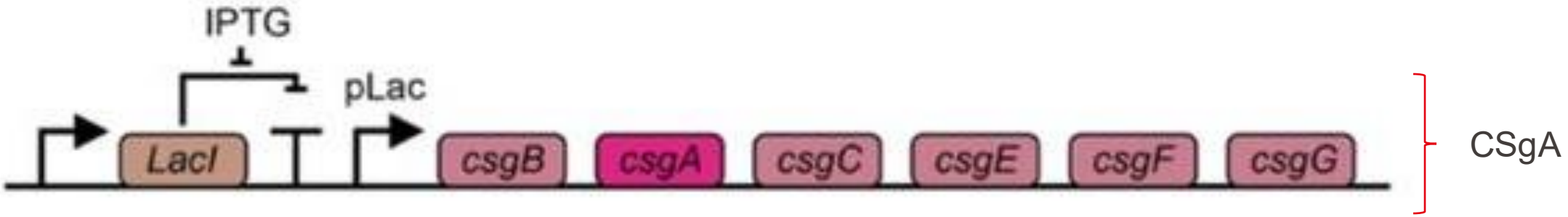
Translation

- mRNA is bound by ribosomes and translated into proteins (in nature: enzymes for lactose metabolism = laccases)

Summary: Inducers turn gene expression on/off

“ON” switch = lactose or IPTG
“OFF switch = no lactose or IPTG

Biofabrication of MECHS



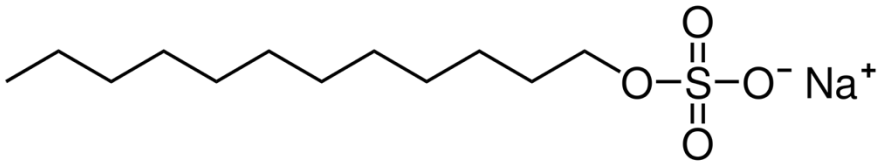
- Curli genes are place under the control of the lac promoter (pLac), which is regulated by IPTG and lac repressor (LacI)
- Without IPTG, LacI binds to the lac operator, repressing curli gene expression
- With IPTG, LacI is released from operator, allowing transcription of curli genes (induce curli gene expression on demand)

Biofabrication of MECHS – SI section

Biofabrication of MECHS: The 48 h cell culture (500 ml) of PQN4-pET21d-CsgA (CsgA) was centrifuged (5000 rpm, 10 min) to pelletize the curli biomass, which was then washed with 250 ml of deionized water by centrifuging (5000 rpm, 10 min) to remove the residual quantities of culture media. 1 g (wet pellet) of curli biofilm biomass was first dispersed in 5 ml of deionized water and subsequently added with 5 ml of 1%, 2%, 3%, 4% or 5% (w v^{-1}) of sodium dodecyl sulfate (SDS, serves as a gelator), which was then mixed on a shaker for 2 h at room temperature. The resulting gelatinous biomass was washed with 10 ml of deionized water twice by centrifuging (5000 rpm, 10 min) to remove the soluble biomolecules and the excess SDS. This SDS treated gelatinous biomass was casted and ambient dried on a silicone mold to obtain the MECHS films that were extremely brittle.

To realize the flexible films of MECHS, the 3% SDS treated gelatinous biomass of PQN4-pET21d-CsgA (CsgA) was added with 5 ml of 1%, 2%, 3%, 4%, or 5% (w v^{-1}) of glycerol (serves as a plasticizer) and mixed on a shaker for 1 h at room temperature. The glycerol treated and centrifuged (5000 rpm, 10 min) biomass was casted on a silicone mold and ambient dried to obtain the flexible MECHS films.

Similarly, to realize the Covalently Crosslinked (CL1 and CL2) films of MECHS, 5 ml of 3% SDS and 5 ml of 3% glycerol treated curli biomass was utilized. For all constructs, a minimum of ten replicates were reported.



Brittle MECHS (SDS gelator)

- 48h culture
- Centrifuge to pelletize **curli biomass**
- Wash with water
- “Gelation” – 1 g (wet) + 5 ml water + 5 ml SDS (1,2,3, 4, or 5% w/v)
- Shake for 2h at RT
- Wash with water by centrifugation to remove “biomolecules” and excess SDS
- Cast on silicone molds

Flexible MECHS (glycerol plasticizer)

- 3% SDS treated biomass + 5 ml glycerol (1, 2, 3, 4, or 5% w/v)
- Shake for 1 h at RT
- Centrifuge to wash
- Cast on silicone molds

Note: Casting directly from whole biomass without filtration763 or extensive washing

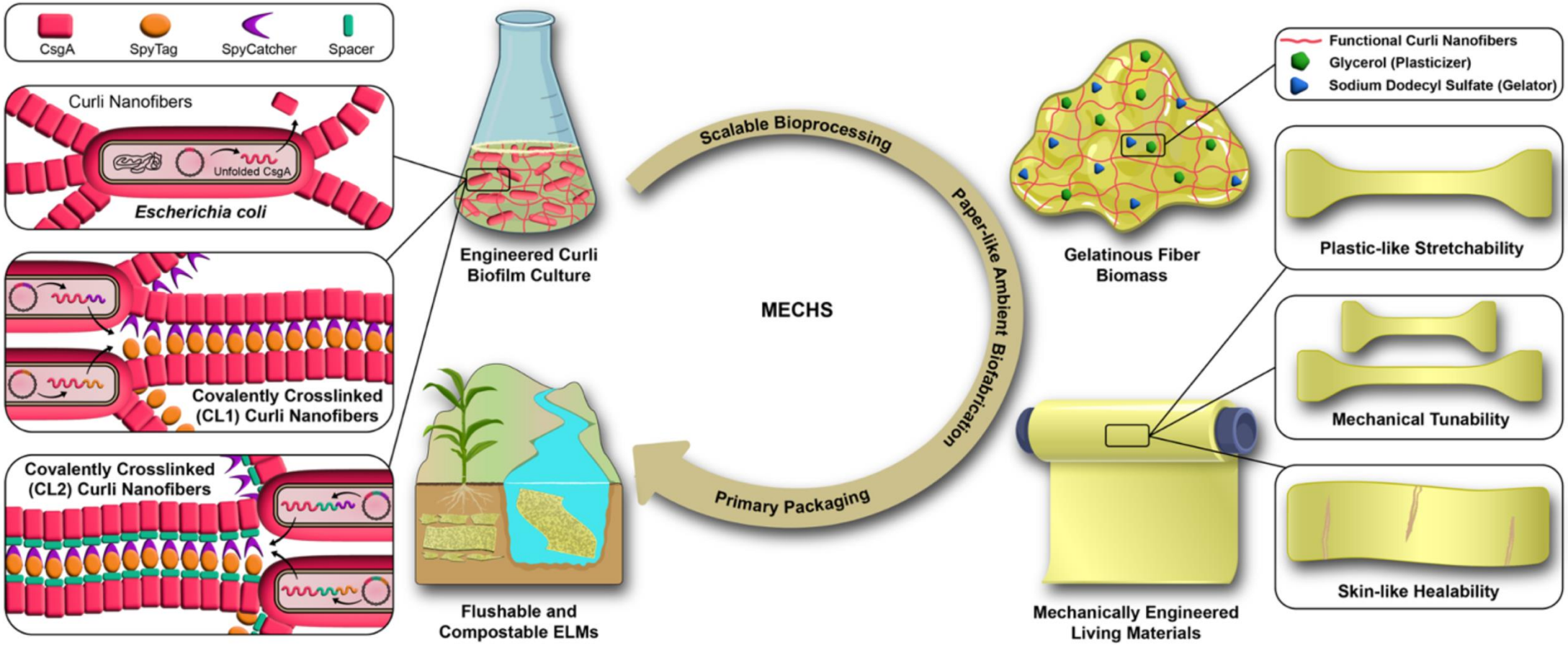
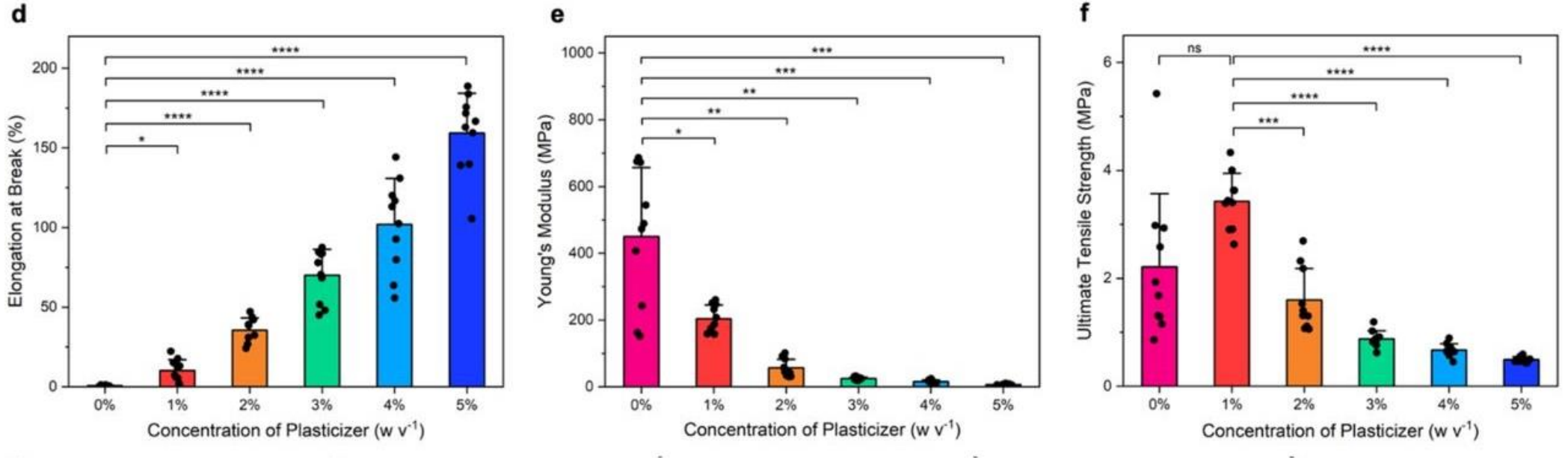


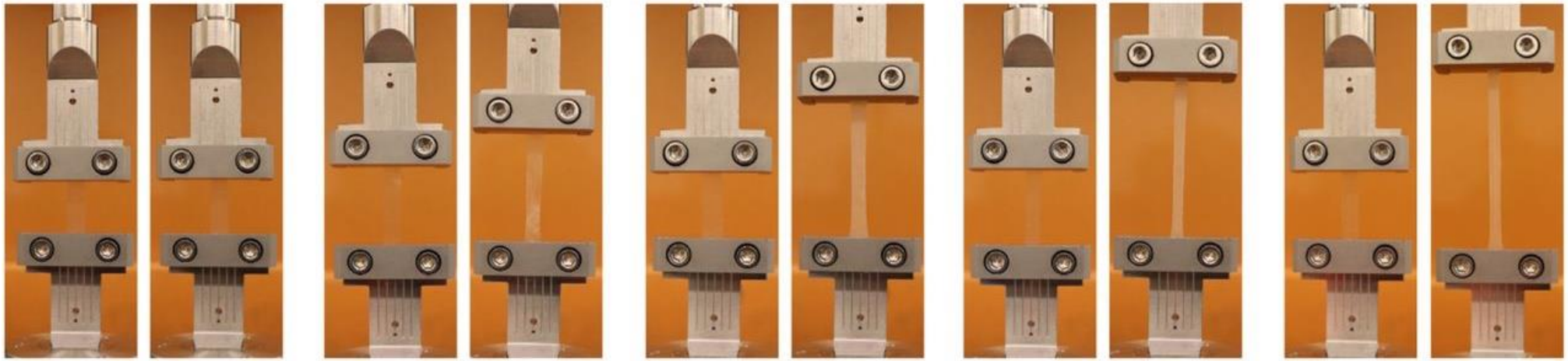
Figure 1: Schematic summary of Mechanically Engineered Living Materials with Compostability, Healability and Scalability (MECHS). Native and functional curli nanofibers were produced from engineered *Escherichia coli* and the treated biomass was dried ambiently to biofabricate MECHS films in a scalable manner. MECHS films exhibit plastic-like stretchability, mechanical tunability and skin-like healability. Parts of the schematics were adapted from BioRender.com.

Genetic constructs

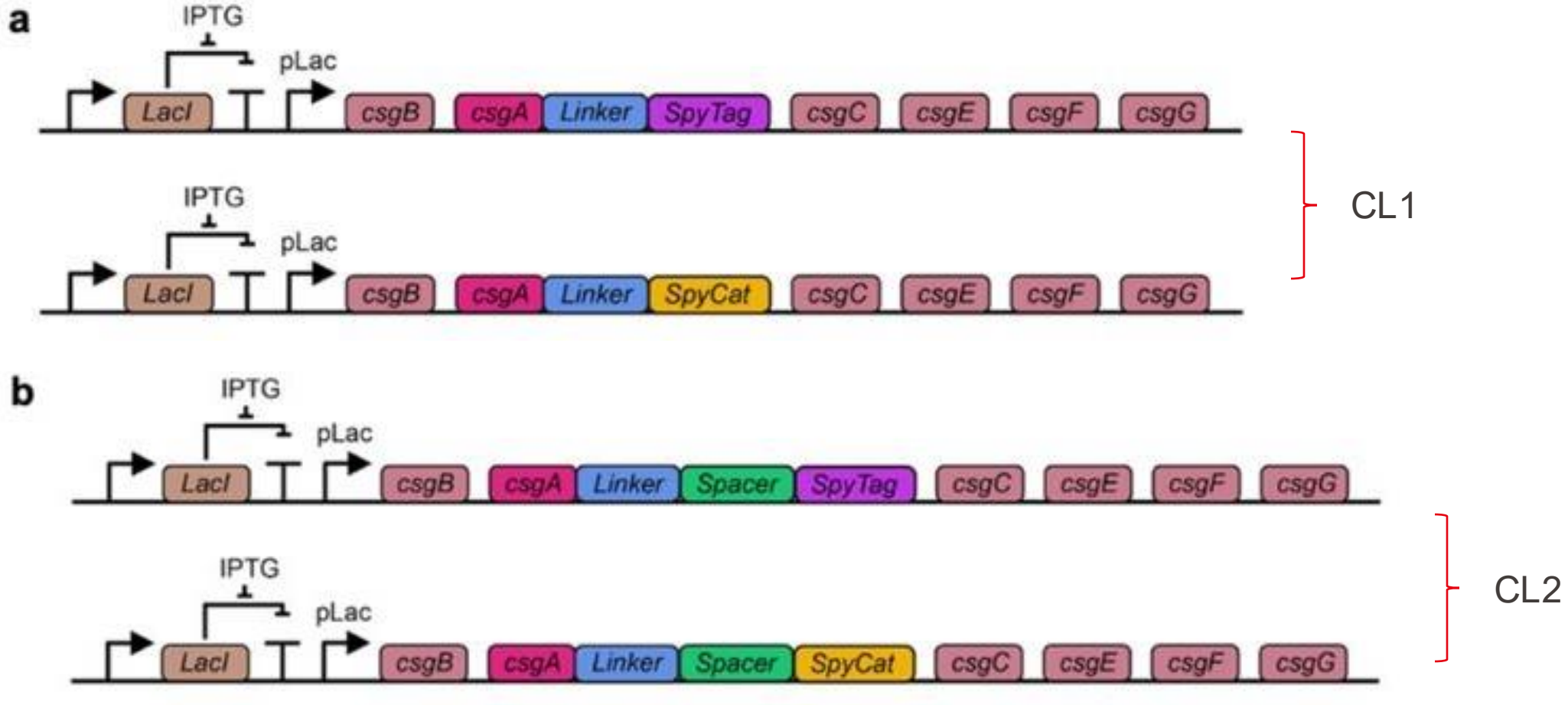
Peptide/Protein	Amino Acid Sequence	Length
CsgA	GVVPQYGGGGNHGGGGNNSGPNSELNIYQYGGGNSALALQTDARNSDLTITQH GGGNGADVGQGSDDSSIDLTQRGFGNSATLDQWNGKNSEMTVKQFGGGNGAA VDQTASNSSVNVTQVGFGNNATAHQY	131
Linker	GGSGSSGSGGSGGGSGSSGSGGSGGGSGSSGSGGSG	36
SpyTag	RGVPHIVMVDAYKRYK	16
SpyCatcher	VTTLSGLSGEQGPSGDMTTEEDSATHIKFSKRDEDGRELAGATMELRDSSGKTIS TWISDGHVKDFYLYPGKYTFVETAAPDGYEVATPIEFTVNEDGQVTVDGEATEGDAHT	113
Spacer	KVLILACLVALALARETIESLSSSEESITEYKQKVEKVKHEDQQQGEDEHQDKIYPS FQPQPLIYPFVEPIPYGFLPQNILPLAQPAVVLPVPQPEIMEVPKAKDTVYTKGRVM PVLKSPTIPFFDPQIPKLTDLENLHLPLPLLQPLMQQVPQPIPQTLALPPQPLWSVP QPKVLPIPQQVVPYPQRAVPVQALLLNQELLLNPTHQIYPVTQPLAPVHNPISV	225

Effect of plasticizer on curli biomass (no crosslinking – CsgA)





More genetic engineering to further tailor mechanical properties (based on BIND)



"genetically grafting to CsgA via a linker to obtain CsgA-SpyTag and CsgA-SpyCatcher"

Approaching infinite affinity through engineering of peptide–protein interaction

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Much of life's complexity depends upon contacts between proteins with precise affinity and specificity. The successful application of engineered proteins often depends on high-stability binding to their target. In recent years, various approaches have enabled proteins to form irreversible covalent interactions with protein targets. However, the rate of such reactions is a major limitation to their use. Infinite affinity refers to the ideal where such covalent interaction occurs at the diffusion limit. Prototypes of infinite affinity pairs have been achieved using nonnatural reactive groups. After library-based evolution and rational design, here we establish a peptide–protein pair composed of the regular 20 amino acids that link together through an amide bond at a rate approaching the diffusion limit. Reaction occurs in a few minutes with both partners at low nanomolar concentration. Stopped flow fluorimetry illuminated the conformational dynamics involved in docking and reaction. Hydrogen–deuterium exchange mass spectrometry gave insight into the conformational flexibility of this split protein and the process of enhancing its reaction rate. We applied this reactive pair for specific labeling of a plasma membrane target in 1 min on live mammalian cells. Sensitive and specific detection was also confirmed by Western blot in a range of model organisms. The peptide–protein pair allowed reconstitution of a critical mechanotransmitter in the cytosol of mammalian cells, restoring cell adhesion and migration. This simple genetic encoding for rapid irreversible reaction should provide diverse opportunities to enhance protein function by rapid detection, stable anchoring, and multiplexing of protein functionality.

protein engineering | synthetic biology | mechanobiology | cytoskeleton | nanobiotechnology

interaction (9). Alternative studies have taken this route for covalent ligation via posttranslational chemical modification or unnatural amino acid incorporation (10–12). Nonetheless, such activated complexes require substantial manipulation. Wide application depends upon moving toward infinite affinity for protein–protein interactions where both partners only contain the natural 20 amino acids. This requires a challenging balance between reactivity and specificity.

Here, building on unusual chemistry from Gram-positive bacteria (13), evolution, and computational design, we have established a genetically encoded interaction between a protein and a peptide tag that forms a spontaneous amide bond with close to

Significance

Interactions between proteins normally depend on a range of noncovalent contacts. Under challenging conditions, such as with mechanical force or over long time periods, noncovalent interactions break. Unbreakable protein–protein interactions, linked by covalent bonding, provide many opportunities for robust connection of molecular building blocks, including for biomaterials, enzymes, and vaccines. When evaluating unbreakable interactions, it is important to consider whether reaction happens quickly even at low concentrations. Here we establish a genetically encoded peptide that reacts with its genetically encoded protein partner with a speed close to the limit set by diffusion. We apply a range of biophysical methods to understand the dynamics required for this interaction, demonstrating applicability to rapid and specific detection in a range of species.

- 2019 PNAS paper
- Unbreakable protein-protein interactions
- Genetically encoded peptide that reacts with a genetically encoded protein partner

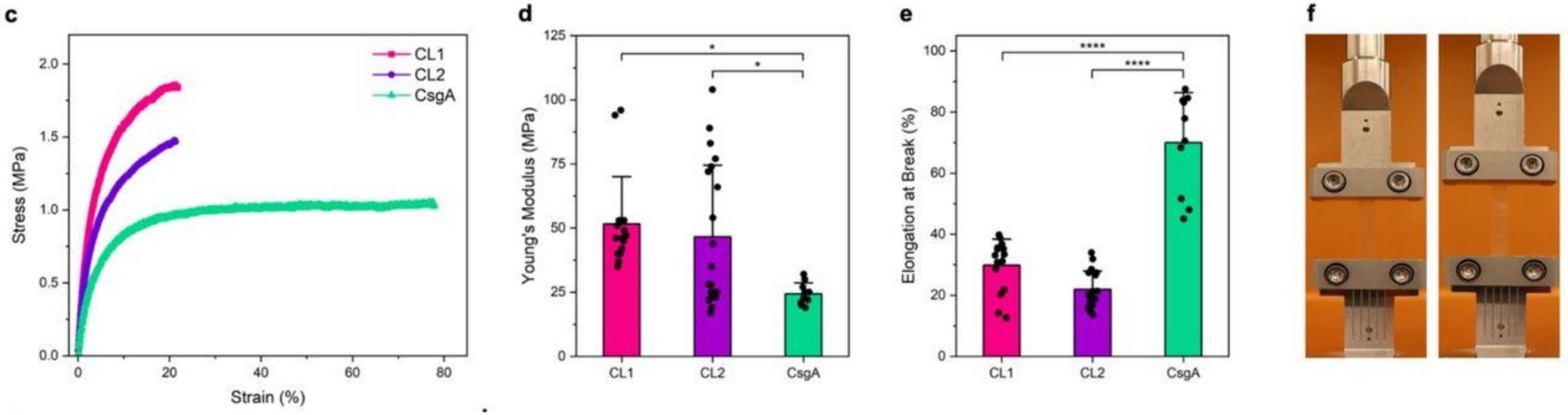
https://doi.org/10.1073/pnas.1909653116

More genetic engineering to further tailor mechanical properties (based on BIND and SpyTag/SpyCatcher)

SpyTag/Spy Catcher

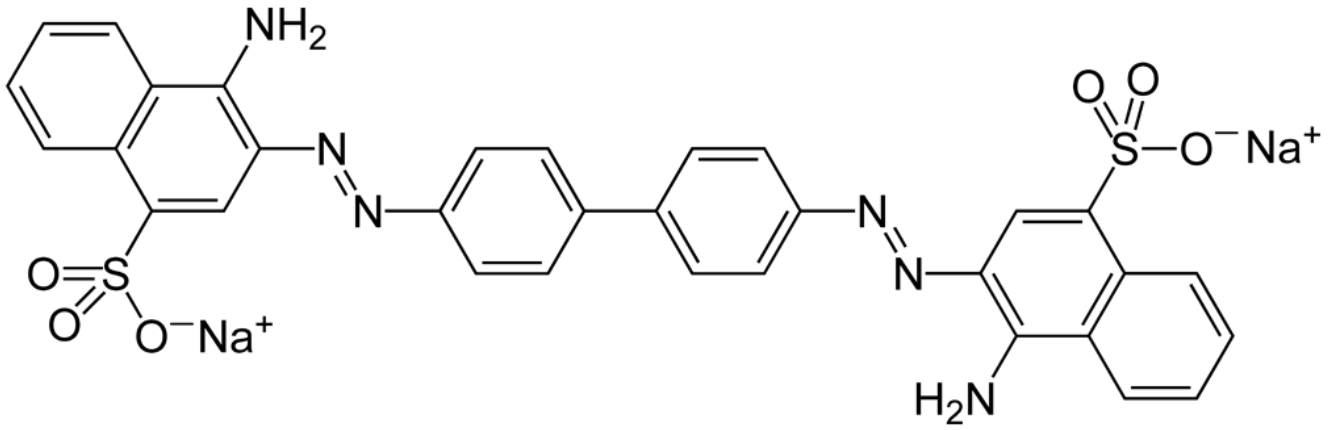
- Spontaneous reaction between lysine in SpyCatcher and aspartic acid in SpyTag to give an isopeptide bond
- Does not require activating groups
- Highly specific even in complex biological media
- Two CsgA constructs (CsgA-SpyTag & CsgA-Spycatcher) were expressed from separate plasmids in co-culture and the resulting curli biomass was used to make MECHS films, called CL1
- Analogous experiments with a large spacer (disordered protein domain of 225 amino acids, called CL2 – *why do you think they tried this experiment?*

Mechanical properties of crosslinked curli biomass, with (CL2) and without a spacer (CL1), 3% plasticizer



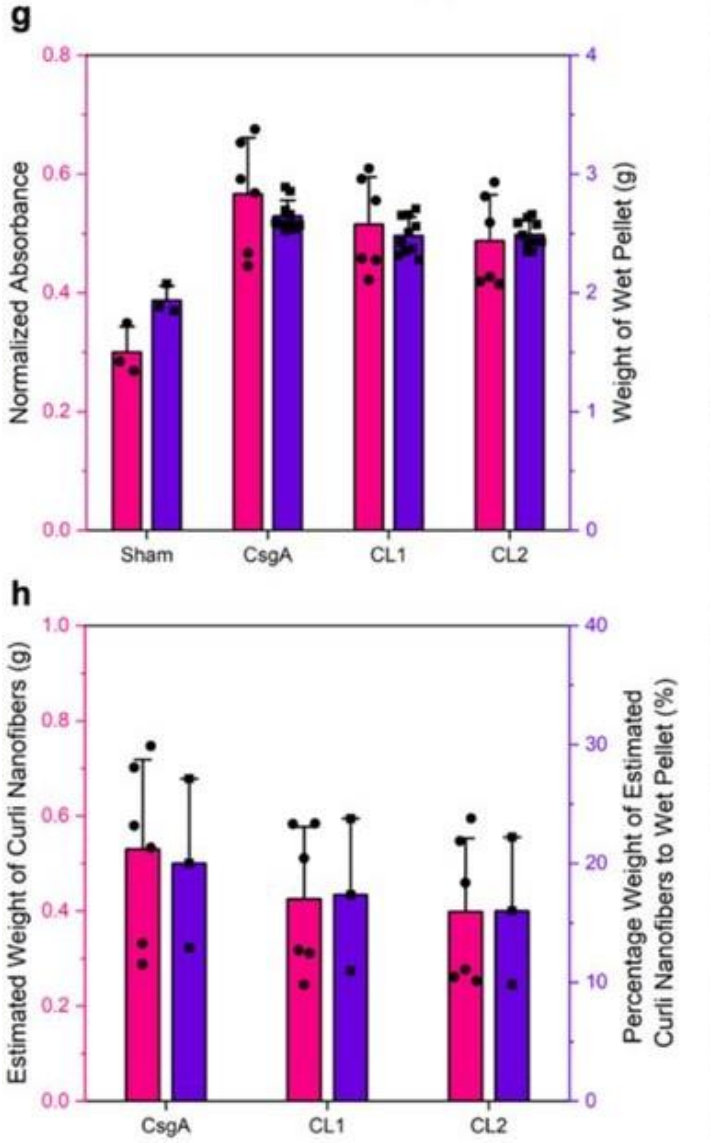
- Crosslinked films are stiffer
- Spacer does not seem to add much
- Speculate that an even bigger spacer might reduce the stiffness and enhance extensibility

Congo red assay



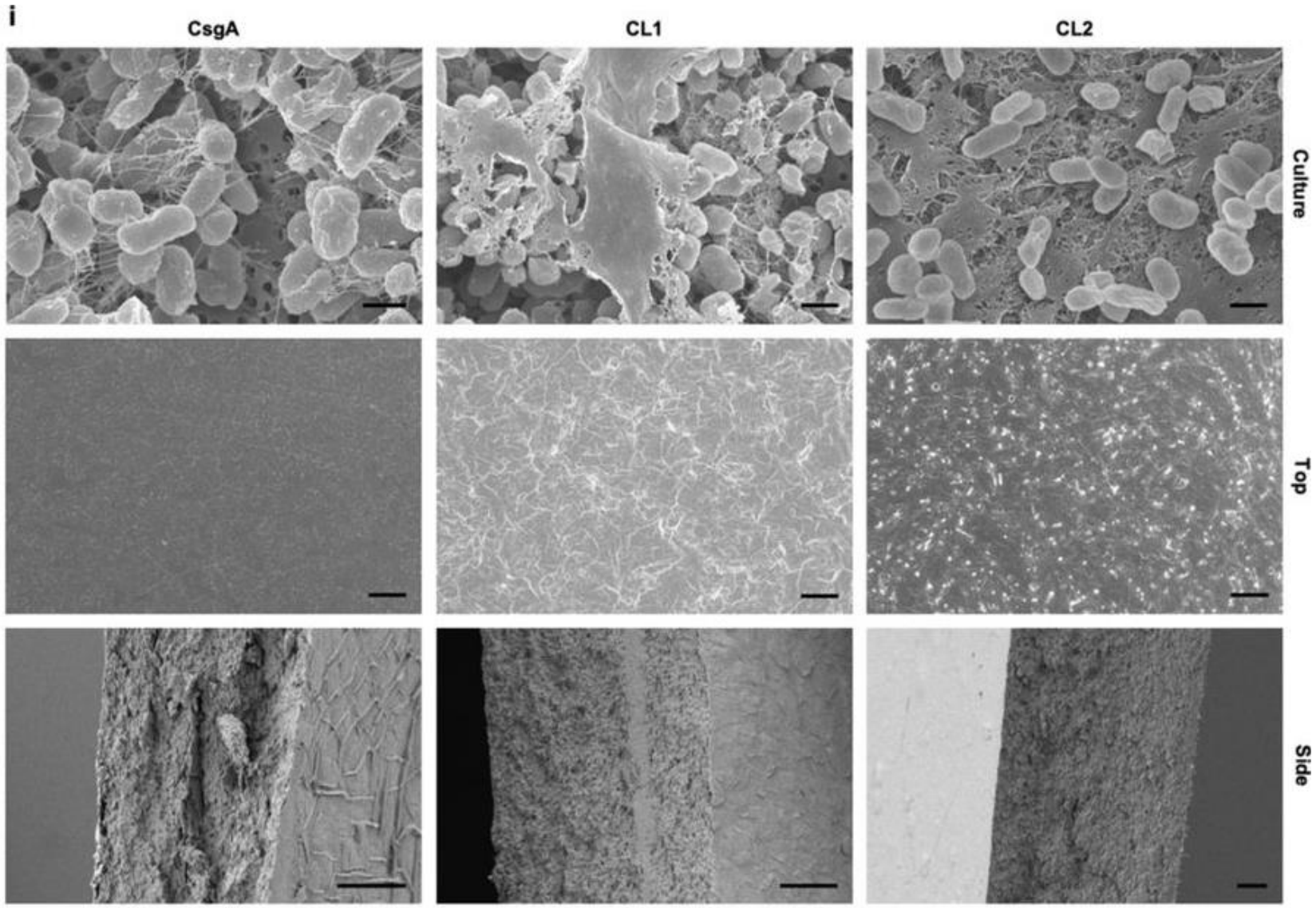
- Dye can be used to detect amyloids
- Align parallel to β-sheet axis
- Upon binding, absorbance shifts from 490 nm to 540 nm
- Not entirely specific to amyloids

Compositional analysis



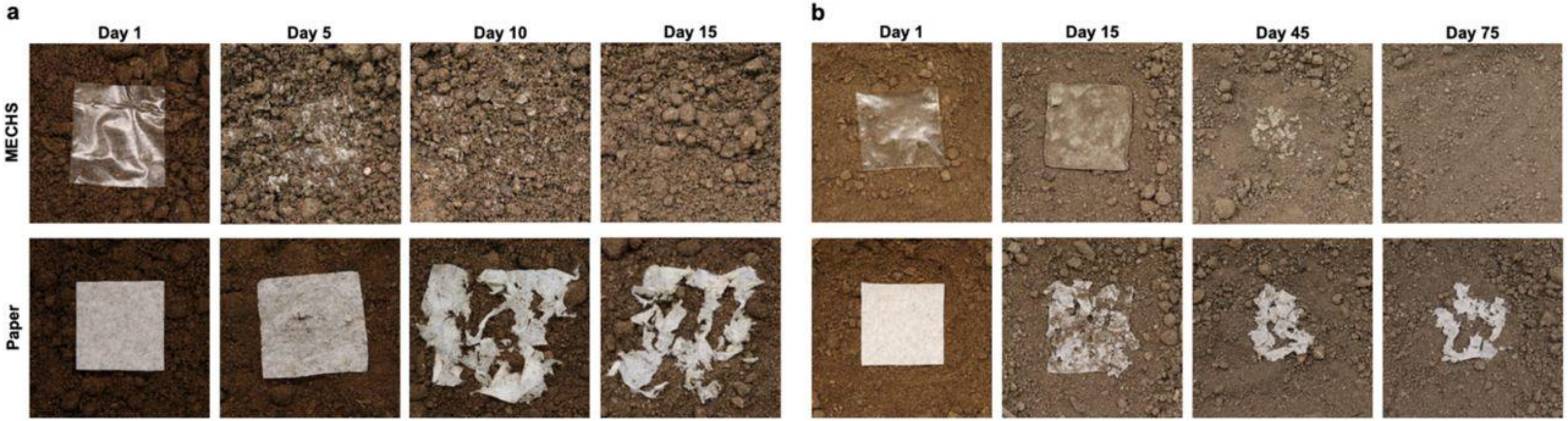
- Calibration curve with Congo Red and purified curli nanofibers
- Curli from 500 mL cultures: CsgA = 530 mg; CL1 = 431 mg; CL2 = 399 mg (calculated from Fig 3g absorbance and calibration curve, plotted in Fig 3h)
- Wet weight of whole cell pellets: CsgA = 2647 mg; CL1 = 2483 mg; CL2 = 2490 mg (Fig 3g)
- % curli nanofibers in wet whole cell pellets, 15-20% (Fig 3h)
- If you subtract wet pellet mass between curli-producing and sham construct (1936 mg), roughly get the mass of curli nanofibers from each curli-producing construct (coincidence?)

Morphological analysis (FESEM)



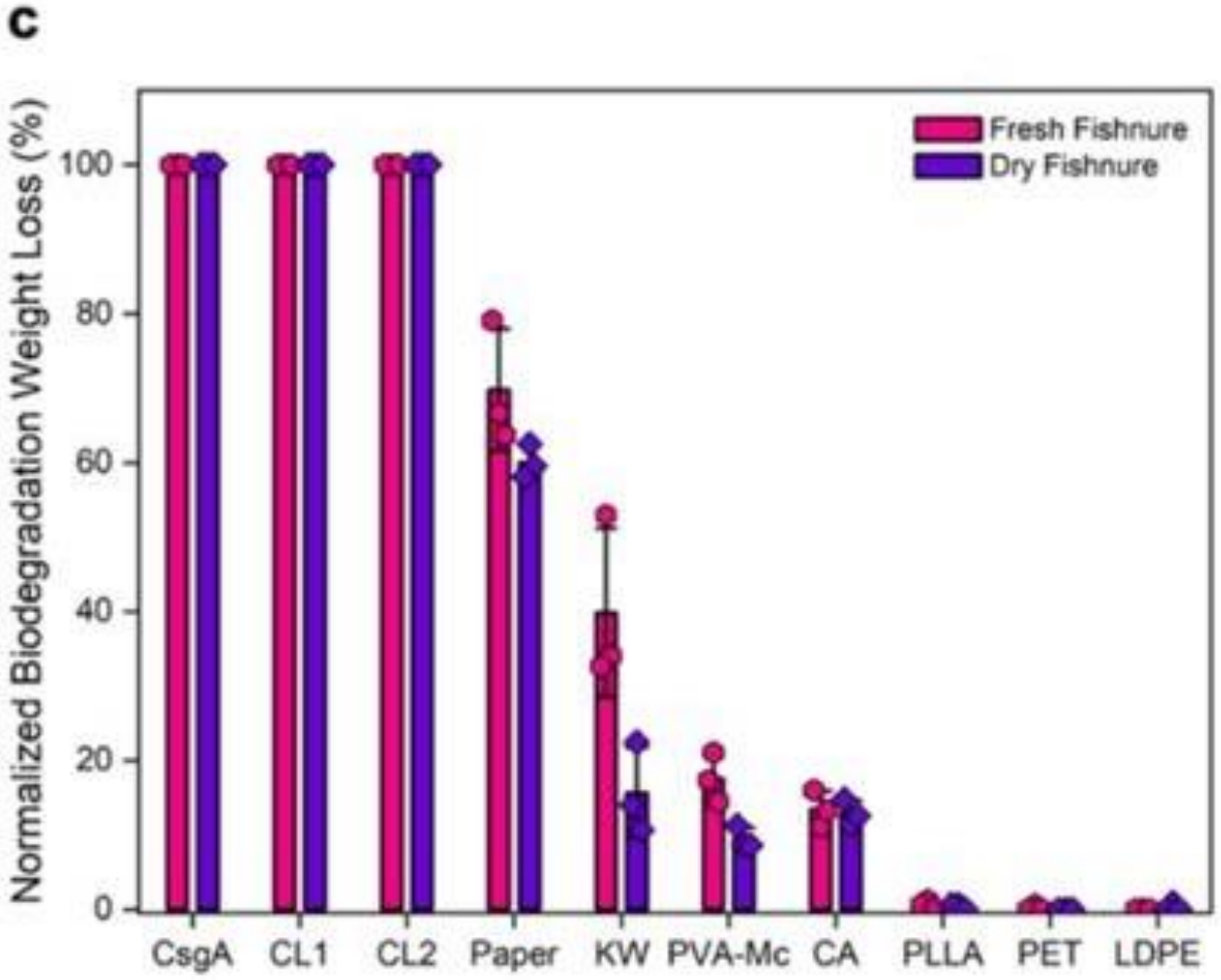
- Scale bar is 1 μm
- Top: cell cultures
- Middle: Top surface MECHS
- Bottom: Cross-section MECHS
- CL1 and CL2 look like “aggregated mats” – “presumably due to nanofiber bundling…covalent crosslinking”
- Cross-sections show dense packing

Compostability



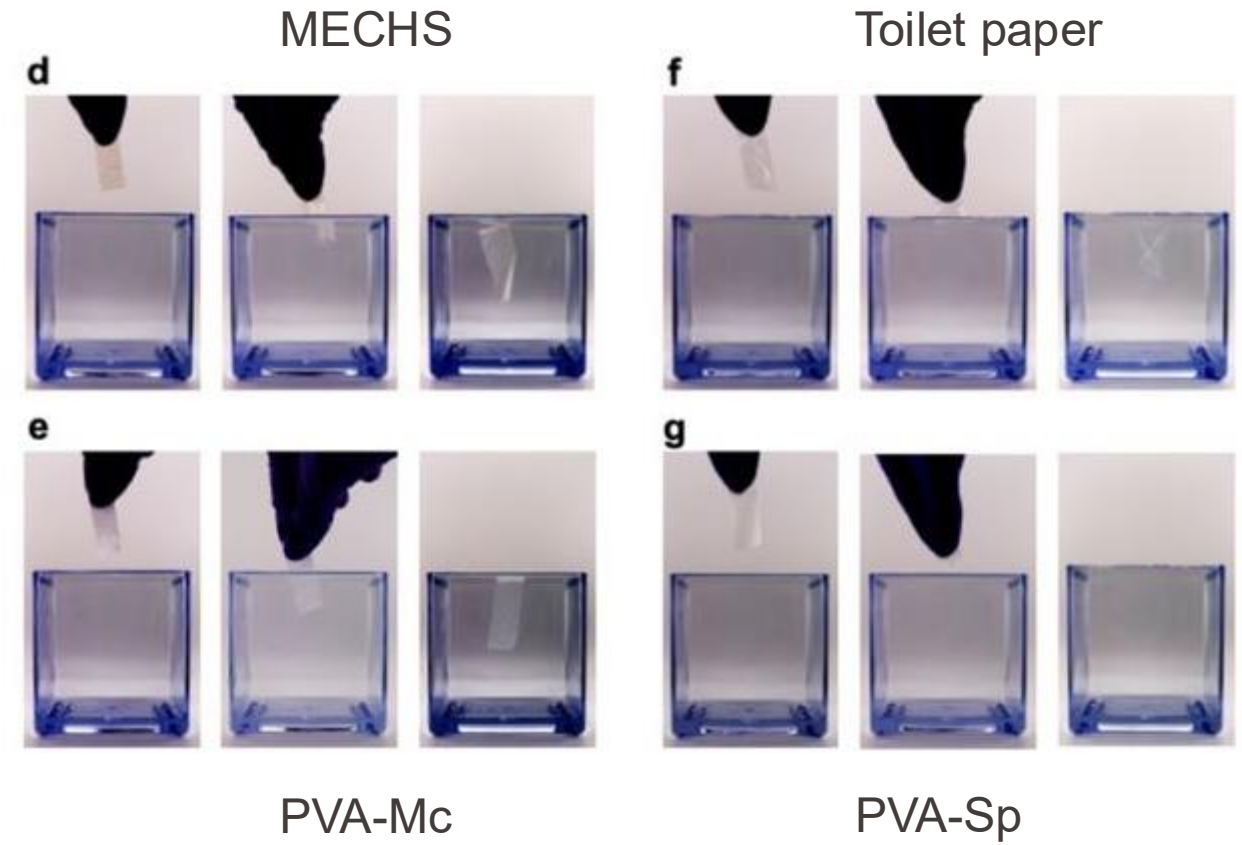
- MECHS (top) vs. toilet paper (bottom) in fresh (a) or dry (b) “fishnure”
- 5 cm by 5 cm samples

Compostability



- Normalized biodegradation weight loss (%) after 75 days

Flushability

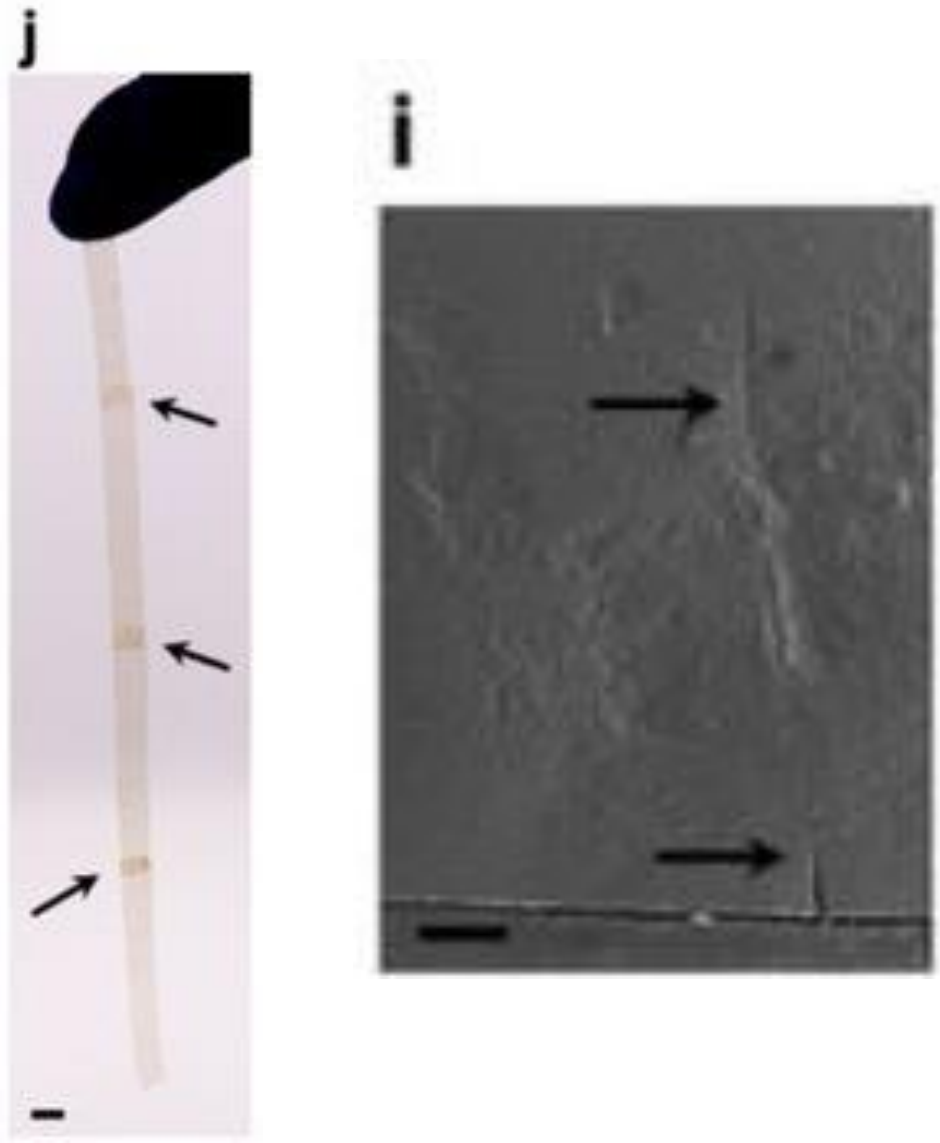


- MECHS do not dissolve completely – “likely due to network of hydrophobic curli nanofibers”
- PVA-Sp dissolves fully
- Is this flushable or not?

Biofertilizer to support plant growth?

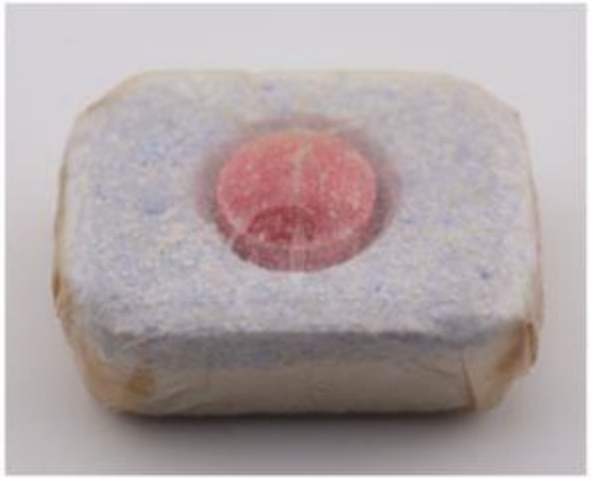
- MECHS are made of protein, therefore have a significant nitrogen content
- Photograph of black bean seedling grown in soil consisting of fishnure and MECHS
- Convincing?

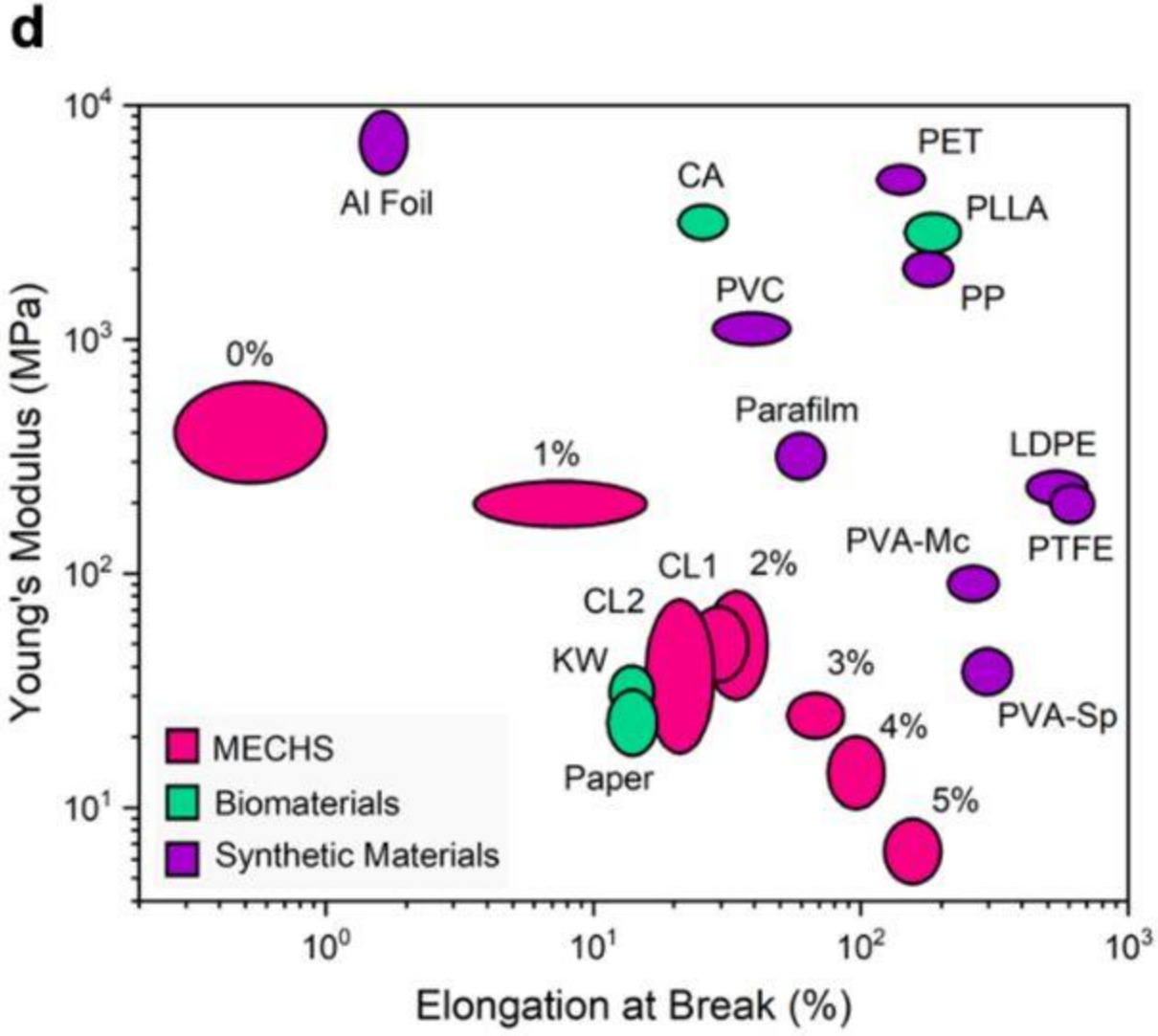
Weldable? Healable?



- Welded together by using microliters of water followed by ambient drying
- Convincing?

Prototypes and the mechanical landscape

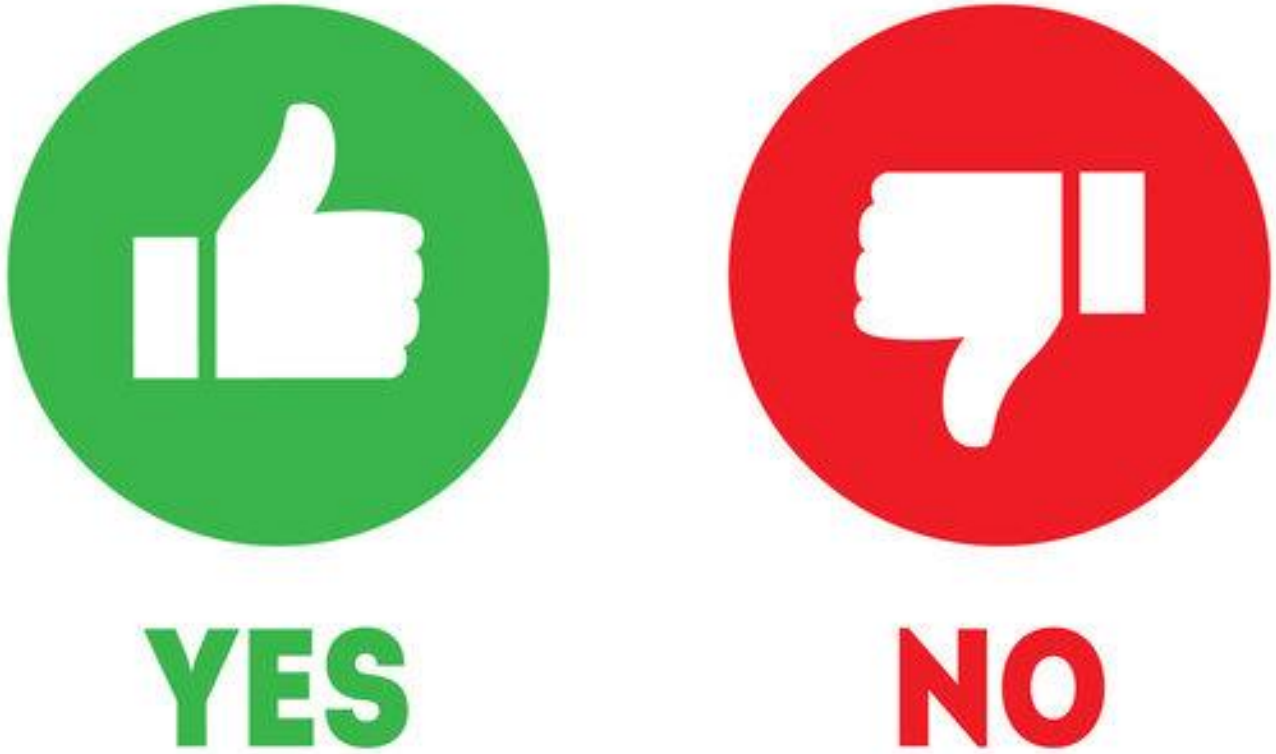




Conclusions

- Compared to AquaPlastic, MECHS are tunable (*where does the tuneabiity actually come from*?)
- MECHS properties:
  - 1-160% elongation at break, Young's modulus 6-450 MPa (paper/plastic like)
  - Transparent
  - Higher yield (increased by a factor of 10) by using whole cells, 500-1000 mg compared to 50-100 mg for AquaPlastic
  - SDS "gelator" coupled with glycerol plasticizer can reduce brittleness (*how does SDS act as a gelator?*)
  - Processed by fancy words like "aquamolding", "aquahealing", "aquawelding" (*what do you think?)*
  - Plastic/paper like properties (*ok…)*

Did you like this paper? Why or why not?



Lesson takeaways

- An even better idea of ELMs
- A real example of how you can use recombinant protein engineering to change mechanical properties (at least in theory), including the use of the lac operon
- A more exotic and modern presentation of a bioplastic (consisiting of biofilm and associated nanofibers and bacteria)
- How to contextualize a new material in terms of existing materials (important but not straightforward)