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Review

# Chemical and biological engineering strategies to make and modify next-generation hydrogel biomaterials

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## SUMMARY

**There is a growing interest in the development of technologies to probe and direct *in vitro* cellular function for fundamental organoid and stem cell biology, functional tissue and metabolic engineering, and biotherapeutic formulation. Recapitulating many critical aspects of the native cellular niche, hydrogel biomaterials have proved to be a defining platform technology in this space, catapulting biological investigation from traditional two-dimensional (2D) culture into the 3D world. Seeking to better emulate the dynamic heterogeneity characteristic of all living tissues, global efforts over the last several years have centered on upgrading hydrogel design from relatively simple and static architectures into stimuli-responsive and spatiotemporally evolvable niches. Toward this end, advances from traditionally disparate fields, including bioorthogonal click chemistry, chemoenzymatic synthesis, and DNA nanotechnology, have been co-opted and integrated to construct 4D-tunable systems that undergo preprogrammed functional changes in response to user-defined inputs. In this review, we highlight how advances in synthetic, semisynthetic, and bio-based chemistries have played a critical role in the triggered creation and customization of next-generation hydrogel biomaterials. We also chart how these advances stand to energize the translational pipeline of hydrogels from bench to market and close with an outlook on outstanding opportunities and challenges that lay ahead.**


## PROGRESS AND POTENTIAL

Advanced hydrogel biomaterials have enabled near-limitless opportunities in tissue engineering, synthetic biology, therapeutic design and delivery, and advanced information storage. Innovation underlying these novel constructs stems from creative integration of cutting-edge efforts from traditionally disparate disciplines including click chemistry, enzymatic semisynthesis, and DNA nanotechnology. As these material tools mature beyond permissive and statically uniform 3D scaffolds to user-customized and exogenously controlled 4D environments, a systematic attempt to define past achievements and chart future frontiers is essential.

## INTRODUCTION

Historically, cellular biology has been interrogated in the context of two-dimensional (2D) cell culture milieux, comprised primarily of aphysiologically stiff substrates (e.g., glass, polystyrene dishes). While potentially revealing, such environments fail to mimic many essential aspects of the native cellular niche (e.g., dimensionality, viscoelasticity). Now intuitively recognized, many insights garnered through such experiments translate poorly, if at all, when moved to downstream *in vivo* studies. A second generation of investigation sought to leapfrog these shortcomings by exploiting hydrogels—water-swollen polymeric networks—for 3D cell encapsulation.[1,2] Although these early efforts permitted extended cell culture in uniform materials with bulk characteristics closer to tissue than tissue-culture plastic, such constructs were static, monocellular, and isotropic. Recognizing that the tissue microenvironment is dynamically heterogeneous in its physicochemical composition, cellular makeup, and structured architecture, the third and current generation of cell culture platforms has focused on biomaterials whose properties can be customized on demand, often in both 3D space and time (i.e., 4D), across a variety

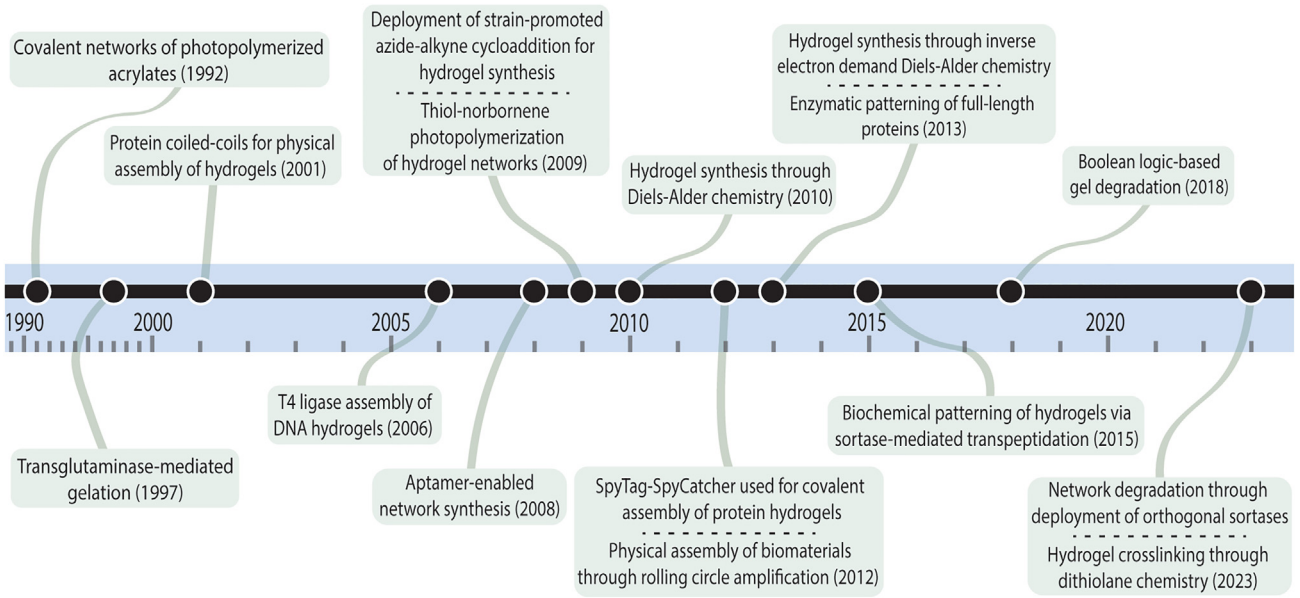


**Figure 1. Key milestones in the deployment of new chemistries for hydrogel biomaterial synthesis**

Key milestones marking the first instance of the deployment of a particular chemistry for the synthesis of a hydrogel biomaterial.

of scales.[3] Toward this goal, materials scientists, chemists, and biologists have harnessed and driven diverse chemical and biological advances to engineer exquisitely modifiable and stimuli-responsive hydrogel biomaterials (Figure 1).

Beyond spatial and/or temporal initiation of gelation, systems can be endowed with defined macroarchitecture, shape memory, reversible mechanics/viscoelasticity, and anisotropic biochemical signaling, all of which enable spatiotemporal control of cell fate and behavior. Clearly, this encoded dynamism holds immense potential for different fields within biomedicine.[4,5] Importantly for biomaterial scientists and engineers, variable use cases call for dramatically different sets of properties. Within the same application space, developing a hydrogel matrix that seeks to capture bone tissue morphogenesis will require a resultant set of physicochemical properties that is qualitatively different than that geared for kidney or lung tissue engineering. Furthermore, moving our lens beyond tissue engineering proper, developing hydrogel matrices as therapeutic depots will also call for a substantially different set of design parameters; within this niche itself, whether a vehicle harbors synthetic drugs or living cells is another critical factor to consider.

As it stands, there is no shortage of problems to address, each requiring a bottom-up solution that is exquisitely tailored to meet it. (Bio)chemical advances—synthetic, semisynthetic, or biological in nature—are at the forefront of these exciting developments in biomaterials science. Through this review, we will discuss the most common, most promising, and recently emerging reaction schemes to make and modify hydrogel biomaterials—including both those that proceed spontaneously and those that can be exogenously triggered—from polymeric, small-molecule, protein, DNA, and other biomolecular precursors. In drafting this review, we found that charting the development of hydrogel biomaterial science and engineering was best tackled through the lens of understanding network crosslinking. By taking that as our starting point, we segmented the field into three main areas. Specifically, we looked at synthetic organic networks and define those as hydrogels that are crosslinked through

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routine organic-type reactions. We also ventured beyond the fume hood and elaborated upon protein- and peptide-enabled chemistries that are themselves gaining much more traction as viable crosslinking strategies. Lastly, we expanded upon DNA-enabled methods and discuss how these have also gone from an academic curiosity into a full-fledged engineering platform, both for hydrogels and other materials. We then frame this discussion within the context of translational promise for these platform technologies and expand upon the challenges faced by hydrogel biomaterials en route to the clinic. It is our hope that this work will encourage researchers to probe new questions enabled by truly next-generation biomaterials and impress the notion that different chemical and biochemical platforms can be tuned and optimized to very particular applications at hand.

A key challenge in providing a comprehensive review and perspective of the field lies in its variety. Given the breadth of the hydrogel biomaterials space and the numerous strategies that have been developed for the creation and post-synthetic modification of networks, we categorize past efforts based on the nature of the assembly (i.e., covalent vs. non-covalent crosslinking) and the extent of user control that it affords (i.e., spontaneous vs. triggerable modulation). In so doing, we hope to systematically delineate the relative advantages and disadvantages of each platform in as mutually exclusive and collectively exhaustive a method as possible.

## CHEMICAL SYNTHESIS OF HYDROGEL BIOMATERIALS

Biomaterials science has greatly benefitted from the co-opting of chemistries initially deployed for different applications and then re-purposed for materials development. These manifold reaction platforms have proved to be largely agnostic with respect to the nature of the underlying material, enabling assembly of networks from a wide variety of starting reactants. Broadly, hydrogel materials can be synthetic, naturally derived, or a hybrid of the two. Synthetic hydrogels are made up of polymer chains that are synthetic themselves, including poly(ethylene glycol) (PEG), poly(vinyl alcohol) (PVA), and poly(2-hydroxyethyl methacrylate) (PHEMA). Lacking in biological epitopes in their base form, such materials are bioinert at best, a feature that may or may not be advantageous depending on the use case being considered. Regardless, synthetic networks are readily modifiable, providing a "blank slate" upon which to engineer biological functionality.[6] Alternatively, naturally derived networks consist of biopolymers in the form of polysaccharides (e.g., hyaluronic acid, chitosan, dextran), proteins (e.g., fibrin, collagen, silk fibroin), or DNA. Innately endowed with biocompatibility, naturally derived networks can be sourced directly or recombinantly synthesized.[7] Such off-the-shelf networks (e.g., Matrigel, Geltrex) promote desirable cellular functions including adhesion, spreading, and dynamic matrix remodeling. However, batch-to-batch variability presents obstacles to widespread and reproducible deployment. An alternative strategy to address this pitfall is to produce these networks in a pristine sequence-specific manner, such as is enabled through recombinant protein expression or DNA assembly via enzymatic, solid-phase, or biological replication-based methods.

Beyond the underlying material, an important design consideration—the focus of this review—is the reaction platform through which the hydrogel is formed, particularly whether the network is held intact through covalent or non-covalent bonds. Furthermore, some material-forming chemistries proceed spontaneously, leading to gelation upon simple mixing of the constituent macromers in solution. Other platforms undergo triggered formation, in that they typically require the action of an

extraneous stimulus (e.g., temperature, pH, light) or a combination thereof to initiate the underlying gelation chemistry.

## Spontaneous hydrogel formation

Given their simplicity and relative ease of onboarding, hydrogel formation chemistries that proceed spontaneously have become a cornerstone of recent biomaterials research. Classically, these platforms lead to network formation directly upon mixing of the constituent macromers in solution, proving most useful for applications such as cell or biomolecule encapsulation into scaffolds for tissue engineering and drug delivery.

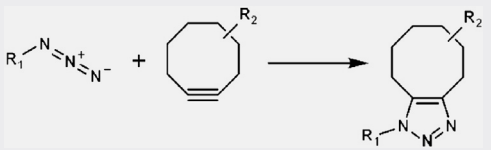
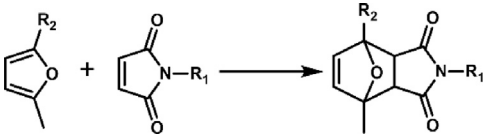
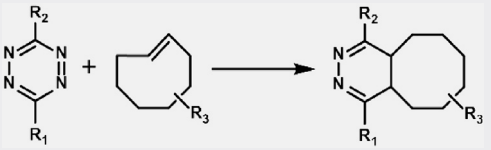
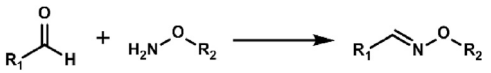
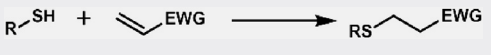
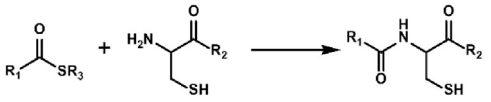
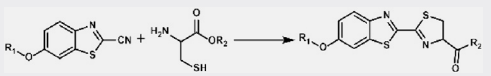
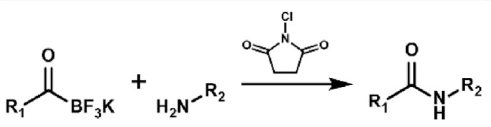
### *Spontaneous gel formation via covalent reaction*

*Synthetic organic-based.* Owing to their high degree of tunability, crosslinking schemes that exploit synthetic organic reactions have propelled the field of hydrogel biomaterials forward to a largely unparalleled degree (Table 1). In fact, through rational modification of the starting macromers and employed gelation chemistry, reaction kinetics, network microarchitecture, and material mechanics/viscoelasticity can be exquisitely controlled. Since these chemistries have been profiled extensively in previous reviews,[8,9] we limit our survey to only the most broadly used and recently developed platforms.

Step-growth polymerizations have become the most prominent synthetic crosslinking chemistries, whereby complementary reactive groups react specifically in a one-to-one manner. Directly contrasting most chain-growth chemistries in which reaction is propagated through active chemical radicals, such step-growth chemistries generally proceed in a radical-free manner and lead to a more uniform hydrogel microstructure (Figure 2). Not only does this hold immediate implications for the creation of more structurally sound therapeutic depots or extracellular matrix (ECM) mimics, it also translates directly to adjacent avenues such as bioprinting where ultimate network integrity is predicated on the platform chemistry deployed.[10] Lastly, many step-growth chemistries can be considered "click,"[11] a designation that is limited to reactions that are specific, high yielding, generate non-toxic products, and proceed in aqueous or otherwise benign solvents.

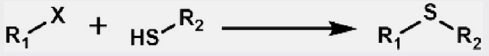
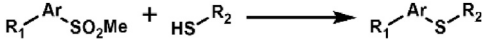
Strain-promoted azide-alkyne cycloaddition (SPAAC) represents a now-classic example of a click platform that has been co-opted successfully for the generation of hydrogel networks. Developed by the Bertozzi group as a non-toxic alternative to the conventional copper-catalyzed cycloaddition (CuAAC),[12] ring-induced strain encoded within the alkyne moiety drives the reaction forward under physiological conditions and without the metal catalyst. Additionally, modifications to the strained-alkyne structure can directly modulate resultant kinetics and thereby accommodate use cases with different gelation time requirements. Owing to its limited cross reaction with chemical moieties on natural biomolecules, the platform found use in and heralded an era of "bioorthogonal click chemistry."[13] Given its potential, it was subsequently co-opted for the cytocompatible synthesis of hydrogel biomaterials. Toward that end, DeForest and Anseth were the first to employ it for the synthesis of cell-laden hydrogels, using multi-armed PEG chains end-functionalized with azide groups and peptide chains capped with DIFO3 cyclooctyne moieties.[14] SPAAC is now broadly used for hydrogel synthesis for a range of applications; for instance, the Haag group harnessed this platform for engineering PEG-based networks crosslinked with acid-labile benzacetal groups, thereby enabling cell capture and subsequent triggered release over a cytocompatible pH range.[15] Additionally, SPAAC-based PEG networks have also shown promise as injectable

**Table 1. Comparison of Chemical Platforms for Hydrogel Synthesis**

| Chemistry | Mechanism | Kinetics | Bio-compatible | Synthetic Tractability | Orthogonality | Amenability to 4D Control | Reversibility | Other notes |
|---|---|---|---|---|---|---|---|---|
| SPAAC |  | (+)(+) | (+)(+) | Moderate | Fair; may cross-react with thiols | Fair | Irreversible | Strained alkynes prone to dimerization |
| Diels-Alder |  | (+) | (+)(+) | Moderate | Fair; may cross-react with thiols | No | Reversible | Highly acid-sensitive until recent reagent optimization |
| Inverse-Electron Demand Diels-Alder |  | (+)(+) | (+)(+) | Moderate | Good | Fair | Irreversible | Highly acid-sensitive until recent reagent optimization |
| Oxime Ligation |  | (+)(+) | (-) | Good | Good | Yes | Reversible | Base-catalyzed reaction until recent iterations |
| Thiol-Michael Addition |  | (+)(+) | (+)(+) | Good | Poor; cross-reacts with thiols | Yes | Reversible | Driving force for the reaction is dictated by the choice of vinyl group. |
| Native Chemical Ligation |  | (-) | (-) | Moderate | Poor; cross-reacts with thiols | No | Reversible | Oxo-ester counterpart of this platform obviates the redox-dependency of the platform |
| CBT-Cys |  | (+)(+) | (+)(+) | Moderate | Poor; cross-reacts with thiols | No | Irreversible | Recently engineered to be redox-activatable |
| KAT Ligation |  | (+)(+) | (+)(+) | Good | Good | No | Reversible | Kinetics are strongly pH-dependent; reaction is much slower at physiological pH levels |

(*Continued on next page*)

**Table 1. *Continued***

| Chemistry | Mechanism | Kinetics | Bio-compatible | Synthetic Tractability | Orthogonality | Amenability to 4D Control | Reversibility | Other notes |
|---|---|---|---|---|---|---|---|---|
| Thiol-Halide |  | (+)(+) | (+) | Good | Poor; cross-reacts with thiols | No | Irreversible | Halide groups are easily synthetically tractable. |
| Thiol-Methylsulfone |  | (+) | (+)(+) | Moderate | Poor; cross-reacts with thiols | No | Irreversible | Moderate and tunable platform kinetics render it a suitable intermediate between different thiol-Michael chemistries. |

Widely used synthetic assembly schemes are compared based on their kinetic profile, biocompatibility, tractability, orthogonality, amenability to spatiotemporal control, and reversibility.

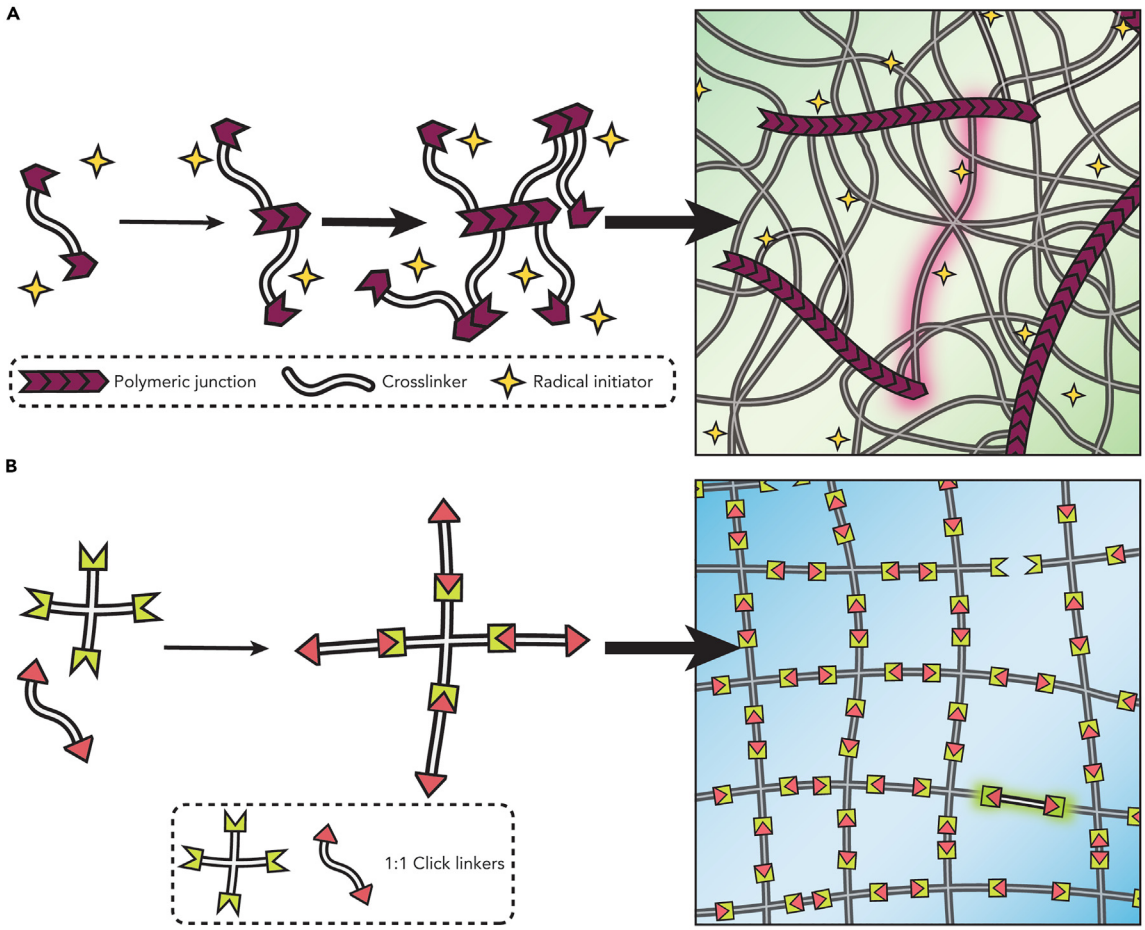



**Figure 2. Chain-growth vs. step-growth chemistries for hydrogel network synthesis**

(A) Radical-initiated chain-growth crosslinking results in a molecularly undefined and spatially heterogeneous network.

(B) Click chemistry-mediated step-growth networks spontaneously form more homogeneous and well-defined mesh structures, typically without the need for initiators.

embolic agents; for example, the Zhong group synthesized routine PEG-based matrices and successfully injected these in the auricular central artery of rabbits, enabling fast-flow blockage without the need for invasive surgical interventions.[16] Promisingly, these gels demonstrated good cytocompatibility and degraded over the span of 2 days, a timeframe that can be shortened or extended based on ultimate crosslinker design.

Similar to SPAAC, Diels-Alder (DA) is another widely used chemistry for cell encapsulation, but with additional avenues for modification afforded through its innate reversibility. DA platforms employ two starting groups—diene- and dienophile-modified macromers—that undergo a 1:1 cycloaddition upon mixing. Broad application of the chemistry was initially limited because of its reliance on acidic conditions to drive the DA reaction forward. Specifically, the first report by the Shoichet group that showcased this chemistry necessitated the reaction of furan- and maleimide-end-functionalized precursors at a pH of 5.5 to enable gelation at reasonable timescales, precluding their use in cell encapsulation.[17] However, by replacing the furan diene with a more electron-rich methylfuran, the group successfully side-stepped such acid dependence to enable cytocompatible network assembly at a pH

level of 7.4 on the order of minutes, and were able to encapsulate five different cancer cell lines, highlighting the portability of the system.[18] Since these foundational reports, the relative ease of onboarding this platform from a synthesis standpoint, coupled with its aforementioned reversibility, have made it a staple in different biomaterial labs looking to recreate near-native cellular environments[19] or develop new therapeutic delivery modalities.[20,21]

The inverse-electron-demand Diels-Alder (IEDDA) reaction has been gaining significant traction as a bioorthogonal crosslinking chemistry since the publication of foundational reports deploying it for *in vivo* cellular labeling[22] and endogenous epitope targeting.[23] First utilized for biomaterial formation by the Anseth group as a synthetically more tractable[24] platform than SPAAC,[25] its reaction involves a tetrazine and an appropriate dienophile group (e.g., norbornene, *trans*-cyclooctene). Specifically, the first proof of concept for this chemistry in a hydrogel context relied on the reaction of PEG macromers end-functionalized with a benzylamino tetrazine with a dinorbornene synthetic peptide, which showed that the approach is highly suitable for network formation, cellular encapsulation, and potential post-synthetic patterning. Stemming from its promising and tunable kinetics, which are readily modulated by rationally introduced modifications to either the tetrazine or the dienophile, the chemistry is rapidly gaining widespread use as a workhorse reaction in manifold chemical biology applications (reviewed elsewhere[26,27]) and as a viable hydrogel crosslinking strategy. Emblematic of the promise of the latter, the Vega group systematically investigated different ratios and multiple substitutions of the starting tetrazine or norbornene end-functionalized groups and probed for downstream effects in their obtained hyaluronic acid networks. Their study showed direct and straightforward encoding of ultimate hydrogel mechanical properties through easily adjustable starting macromer concentrations, relative stoichiometry, and degree of substitution with photopatternable methacrylic anhydride moieties.[28] The tetrazine-norbornene chemistry was also employed for the creation of hydrolytically degradable alginate hydrogels, which was achieved through oxidation of the polymer backbone prior to gel crosslinking.[29] By controlling this initial extent of oxidation (e.g., unoxidized vs. 5% oxidized), mechanical properties and degradation timescales could be exquisitely controlled by the user.

Oxime ligation[30] is another commonly employed platform for the synthesis of hydrogel biomaterials. It involves the reaction of a hydroxylamine and a carbonyl (e.g., aldehyde, ketone), forming an oxime linkage and a water byproduct. Similar to DA, oxime ligation can be reversed with pH, making it suitable for the creation of dynamically switchable matrices. While early examples of this strategy were hampered by slow gelation times (on the order of hours) at physiological pH,[31] the Becker group found that tuning pH and the aniline catalyst concentration can yield gelation in seconds.[32] This newfound tunability enabled pristine control over network mesh size and modulus based on selected gelation parameters.[33] There are now multiple successful reports of oxime-crosslinked hydrogels, such as for the creation of hyaluronic acid (HA)-based vitreous substitutes[34] and the culture of tumor spheroids.[35]

Thiol-involved reactions also represent a very common route toward hydrogel biomaterial synthesis. In fact, thiols can react with a number of different chemical groups and are found natively on proteins (i.e., cysteine amino acids), making thiol-based reactions uniquely suited for applications interfacing materials and polypeptides.[36] The most prominent example of such "thiol-X" reactions is the thiol-ene platform,[37] which involves the reaction of a thiol group with an alkene. Classically,

the thiol-ene reaction is radical-mediated via photochemical or thermal initiation. While triggering gelation is important given the ensuant spatiotemporal control (a topic discussed at length in a subsequent section), propagating free radicals can be cytotoxic and non-specifically reactive, making such strategies much less attractive for applications that require interfacing with living cells. A powerful workaround is the adoption of a thiol-ene reaction that harnesses the reaction of a thiol with more electron-deficient alkenes (e.g., maleimides, vinyl-sulfones). Referred to as thiol-Michael additions,[38] these reactions are typically base- or nucleophile-catalyzed and progress through anion propagation rather than a free radical. Given these relative advantages, in addition to their powerful kinetics and resultant product stability, such reactions have been widely used for the creation of biomaterials for post-surgical implants,[39] stem cell encapsulation and maintenance,[40] lineage specification,[41] among others.

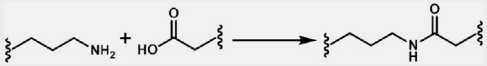
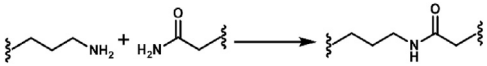
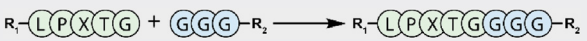
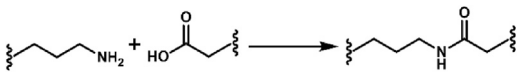
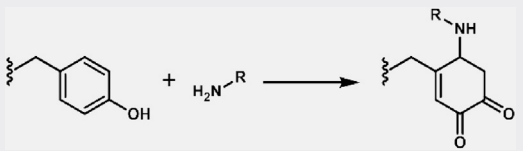
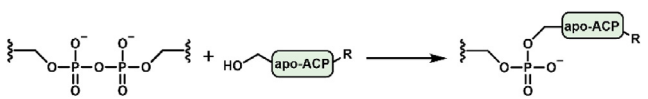
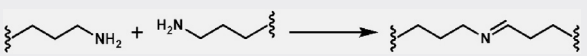
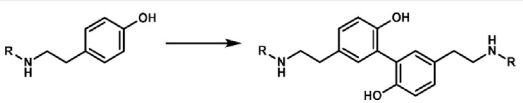
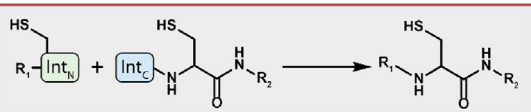
Less employed but still worthy of note as a synthetic crosslinking platform is "native chemical ligation,"[42] which enabled the Messersmith group to generate PEG-based gels from multi-armed macromers functionalized with either an N-terminal cysteine or a thioester,[43] with follow-up work establishing the system's suitability for pancreatic islet cell encapsulation.[44] A subsequent report by the group sought to eliminate the crosslinking chemistry's dependence on reducing conditions, leading to an oxo-ester-mediated reaction rather than the more common thioester.[45]

Still more synthetic organic-based reaction schemes are being developed for spontaneous hydrogel formation, promisingly including the luciferin-inspired cyanobenzothiazole-cysteine (CBT-Cys) reaction,[46] potassium acyltrifluoroborate (KAT) ligation,[47] and alternative thiol-X reactions (e.g., thiol-halide,[48] thiol-epoxy; [49] thiol-methylsulfone[50]).

Summarily, multiple click-type platforms have now been developed for the covalent synthesis of gels. Since each has comparative advantages with regard to different metrics such as kinetics, reversibility, and temperature-dependence, the decision to deploy one or the other should ultimately be made on a use case basis.

*Protein-based.* Protein-enabled chemistries harness platforms evolved by nature to genetically encode recognition and reactivity (Table 2). While protein-based interactions are predominantly non-covalent, there exist an increasing number of schemes enabling covalent network formation, most generally based on split-fragment reconstitution.[51] A quintessential example is the SpyTag-SpyCatcher system, developed by the Howarth lab through splitting the fibronectin-binding domain of *Streptococcus pyogenes* prior to rational fragment engineering.[52] Reconstitution is highly energetically favorable upon splitting; protein-protein ligation occurs rapidly through the formation of an isopeptide bond between the lysine 31 on the SpyCatcher protein and aspartate 230 on a short SpyTag peptide. In their seminal work, the Arnold and Tirrell groups exploited SpyTag/SpyCatcher repeating motifs interspaced by telechelic elastin-like polypeptides (ELPs) to engineer fully protein-based covalently assembled networks.[53] Subsequent work by Li employed the self-same strategy to synthesize protein networks based on globular domains (GB) rather than the telechelic elastin, showcasing the versatility of fragment reconstitution-based approaches with respect to different forms of protein folds.[54] Moreover, this system was also co-opted by the Niemeyer group to assemble flow biocatalysis hydrogel units; after expressing the two tetrameric enzymes *Lactobacillus brevis* alcohol dehydrogenase (LbADH) and glucose-1-dehydrogenase (GDH) as genetic

**Table 2. Comparison of Protein-Enabled Approaches for Hydrogel Synthesis**

| Platform | Mechanism | Kinetics | Reversibility | Other notes |
|---|---|---|---|---|
| **Fragment Reconstitution** | | | | |
| SpyTag – SpyCatcher Ligation |  | (+)(+) | Irreversible | Newer evolved SpyTag and SpyCatcher variants (e.g., 002 and 003 versions) exhibit enhanced kinetic profiles |
| SnoopTag – SnoopCatcher Ligation |  | (+) | Irreversible | Challenging to bring the SnoopTag – SnoopCatcher ligation to full completion |
| GB Tag Ligation |  | Fair | Reversible | GN and GC fragments may each homo-dimerize, limiting reaction efficiency. |
| **Enzymatic Crosslinking** | | | | |
| Sortase-mediated |  | (+)(+) | Reversible | The existence of evolved sortases can enable different mode of reaction fully orthogonally. |
| Transglutaminase-mediated |  | (+) | Irreversible | Reaction kinetics are ultimately dictated by the choice of peptide recognition sequence. |
| Tyrosinase-mediated |  | (+)(+) | Irreversible | Anecdotally reported to result in mechanically weak hydrogel networks. |
| Horseradish Peroxidase-mediated |  | (+) | Irreversible | Concerns remain over the cytotoxicity of $H_2O_2$ and the immunological response caused by trace amounts of enzyme. |
| Lysyl Oxidase-mediated |  | Fair | Irreversible | LOx is found endogenously, enabling straightforward *in situ* crosslinking if required. |
| Phosphopantetheinyl transferase-mediated |  | Poor | Irreversible | Requires Coenzyme A as cofactor for crosslinking. |
| **Other Approaches** | | | | |
| Intein Trans-Splicing |  | (+)(+) | Irreversible | Requires reducing conditions for splicing and crosslinking to occur; concerns also remain over the toxicity of splice products. |

Widely used protein-enabled assembly schemes are compared based on their kinetic profile and reversibility. Additional notes regarding the platform are provided when appropriate.

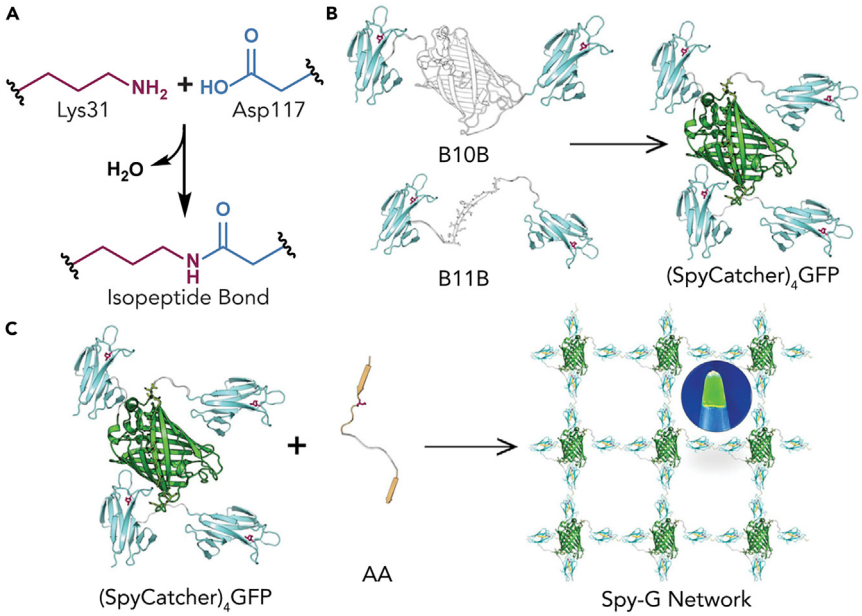



**Figure 3. Hydrogel synthesis through SpyTag-SpyCatcher ligation and split GFP reconstitution**
(A) SpyCatcher and SpyTag react spontaneously upon mixing and form an isopeptide bond between Lys31 on SpyCatcher and Asp117 on SpyTag.
(B) Star-like proteins bearing four reactive SpyCatchers physically assemble through spontaneous reconstitution of split GFP. Adapted with permission from Yang et al.[56] Copyright 2020, Elsevier.
(C) Covalent crosslinking of four-arm star-like protein macromers with difunctional SpyTag reagents yields a "Spy-G" hydrogel. Reproduced with permission from Yang et al[56] Copyright 2020, Elsevier.

fusions with either SpyTag or SpyCatcher, component mixing led to hydrogel bioreactor formation at near-quantitative yields without expensive co-factors.[55]

A related strategy uses proteins that are artificially split and maintain a high thermodynamic driving force for spontaneous reconstitution. A prominent example of this approach is demonstrated by the Sun group, who supplemented a SpyTag- and SpyCatcher-based strategy with split GFP reconstitution to create highly tunable protein-based networks, analogous to synthetic hydrogels formed via step-growth polymerization of multifunctional macromers (Figure 3).[56]

Moving forward, the main limiting factor in developing networks with added functionality lies in the availability of amenable split protein pairs. Indeed, newer splits are continuously being evolved to hold better kinetic and thermodynamic assembly profiles[57] or proceed through a reaction that is orthogonal to existing systems.[58] However, as our understanding of protein design develops,[59] we anticipate that the development of computationally generated *de novo* proteins[60] will expand the palette of genetically encoded click-type chemistries that can induce gelation, both alone and in tandem with other orthogonal pairs.

Although relatively underexplored in biomaterials science, another potentially powerful approach exploits inteins to assemble protein-based networks. Inteins consist of autocatalytic protein processing domains that assemble, link their concomitant flanking "extein" proteins, and excise themselves out without the need for an exogenous cofactor or catalyst.[61] While harnessed extensively in the protein-protein ligation and semisynthesis sphere, inteins hold exciting potential

toward the creation of entirely protein-based covalent materials. One example was demonstrated by the Chen group, who expressed trimeric CutA proteins as *Nostoc punctiforme* (Npu) intein fusions to rapidly generate pH- and temperature-stable networks for enzyme encapsulation.[62] A growing library of orthogonal and well-behaved intein pairs[63] with robust reaction profiles may enable hydrogel assembly with increasing levels of biological functionality.

*Spontaneous gel formation via non-covalent reaction*

*Synthetic organic-based.* While covalent linkage of macromolecular precursors yields stably persistent hydrogels, utilization of non-covalent reaction schemes to create hydrogel networks uniquely affords network dynamism; physically assembled gels can be formed and modified in a user-directed fashion, often including in response to applied shear.[64,65]

A widespread example of non-covalent synthetic reactions utilized for biomaterial formation is that of host-guest chemistries—a supramolecular chemistry in which structural relationships define the assembly of two species—best typified by a cyclodextrin host and a concomitant guest molecule. A notable early application of a host-guest gelation strategy was demonstrated by the Stoddard group, physically assembling a poly(acrylic acid) network through cyclodextrin/azobenzene interaction,[66] whereupon the *trans*-azobenzene isomer—but not the ultraviolet (UV) light-generated *cis* form—sets into the hydrophobic cyclodextrin cavitand. The Burdick group extensively characterized HA-based networks physically crosslinked through a cyclodextrin host and an adamantane guest (Figures 4A and 4B).[67] Variation in starting macromer concentration, chemical modifications to adamantane, as well as the host-to-guest molar ratio, enabled broad macroscopic tunability including erosion rate, shape memory, and gel stiffness. The group also exploited the effects of adamantane-cyclodextrin complexation on the affinity of HA toward encapsulated small molecules, enabling the tunable and sustained release of model small-molecule drugs.[68]

Beyond cyclodextrin host-based physical networks, a newer generation of non-covalent gels exploits curcubit[n]uril (CB[n]) as host. While not yet as widespread as cyclodextrins in pharmaceutical formulations,[70] CB[n] hosts offer wider tunability and high affinities toward specific guest molecules, wherein binding usually occurs at or near the diffusion limit.[71] The Webber group extensively characterized CB[7] host-based PEG hydrogel formulations, where the expanded host-guest affinity range afforded orders-of-magnitude changes in macroscopic properties (e.g., stress relaxation, solute release).[72] These elevated affinities have made possible the use of supramolecular chemical methods of hydrogel assembly to "home in" on a target location within the body to deliver a therapeutic payload. The Webber lab has successfully demonstrated that, provided spatial localization of a host-modified hydrogel in a specific tissue, intravenously delivered guest-functionalized therapeutics will preferentially accumulate at the host site and yield site-specific drug targeting.[73]

Hydrogels can also be physically assembled through hydrophobic-hydrophobic interactions. For example, the Okay group demonstrated the assembly of physically tough, highly stretchable, and self-healing hydrogels through copolymerization of large hydrophobic monomers (i.e., stearyl methacrylate, dodocyl acrylate) with a hydrophilic acrylamide monomer in a micellar solution of sodium dodecyl sulfate with sodium chloride (Figures 4C and 4D).[69] While the conditions necessary for network assembly (e.g., required surfactant, macromer interface hydrophobicity) are not the most suitable for bio-focused applications, the study highlighted the promise of tuning and optimizing hydrophobic-hydrophobic associations to control resultant

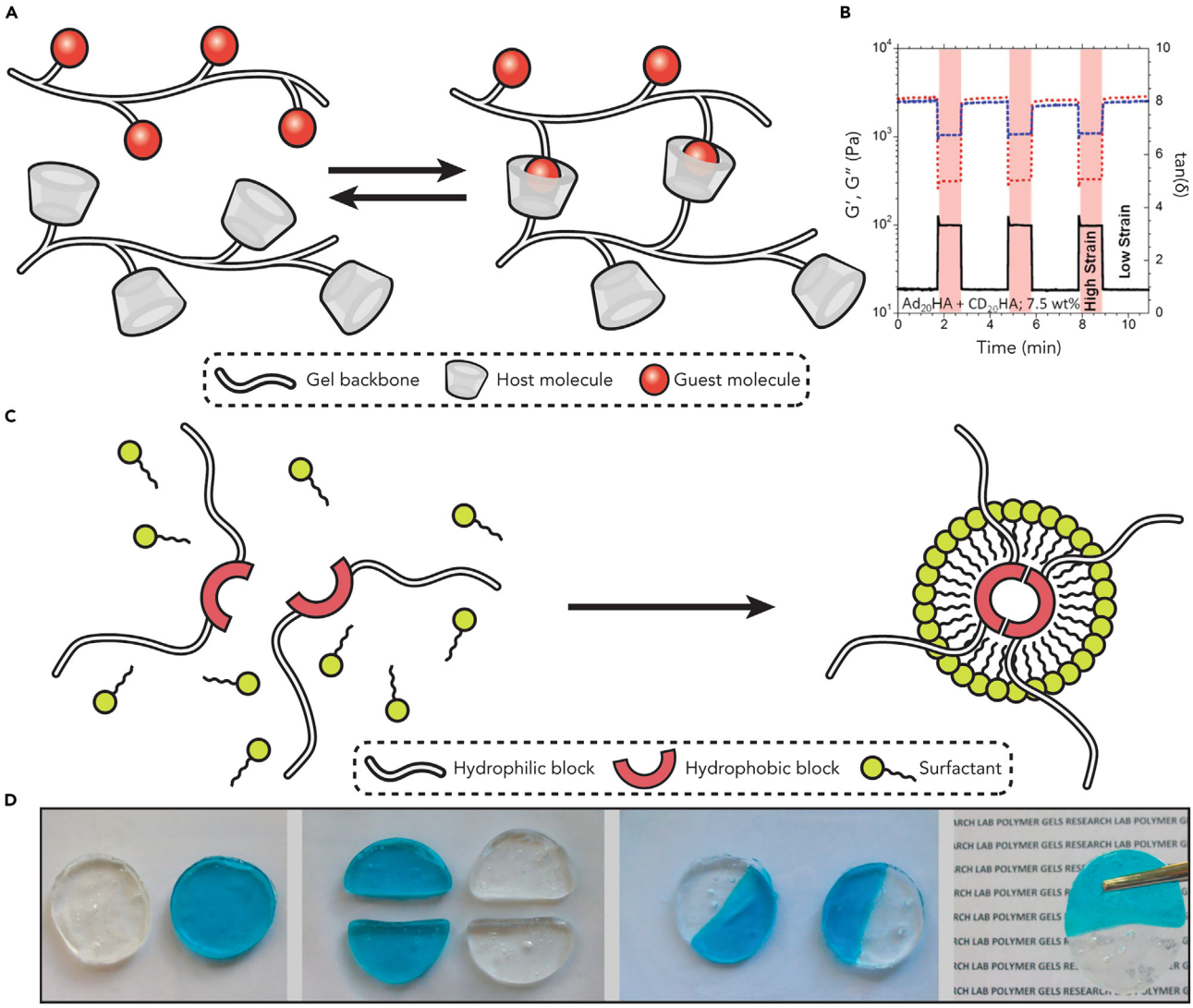



**Figure 4. Assembly of non-covalently linked hydrogel networks through host-guest chemistry and hydrophobic-hydrophobic interactions**

(A) Host-guest chemistries enable the formation of physically and reversibly crosslinked hydrogels.

(B) Demonstration of self-healing properties of a host-guest crosslinked hyaluronic acid-based hydrogel. White and red regions represent cyclic deformation at 0.5% and 250%, respectively. Storage and loss modulus are recovered after each cycle. Reproduced with permission from Rodell et al.[67] Copyright 2013, The American Chemical Society.

(C) Gel fragments formed by hydrophobic association undergo physical grafting when placed in direct contact. Reproduced with permission from Tuncaboylu et al.[69] Copyright 2011, The American Chemical Society.

macroscopic properties of physically crosslinked hydrogels. Specifically, alkyl side-chain length on the hydrophobic monomer and surfactant concentration were both found to crucially affect self-healing efficiency.[74]

Trends in non-covalent assembly schemes closely mirror those observed in chemical crosslinking methodologies, whereupon newer systems with increased breadth of tunability and applicability are continuously being developed.

*Protein-based.* Protein-enabled chemistries permitting non-covalent gelation are numerous and well characterized compared to their covalent counterparts. Many interactions proceed through biorecognition, wherein complementary polypeptide

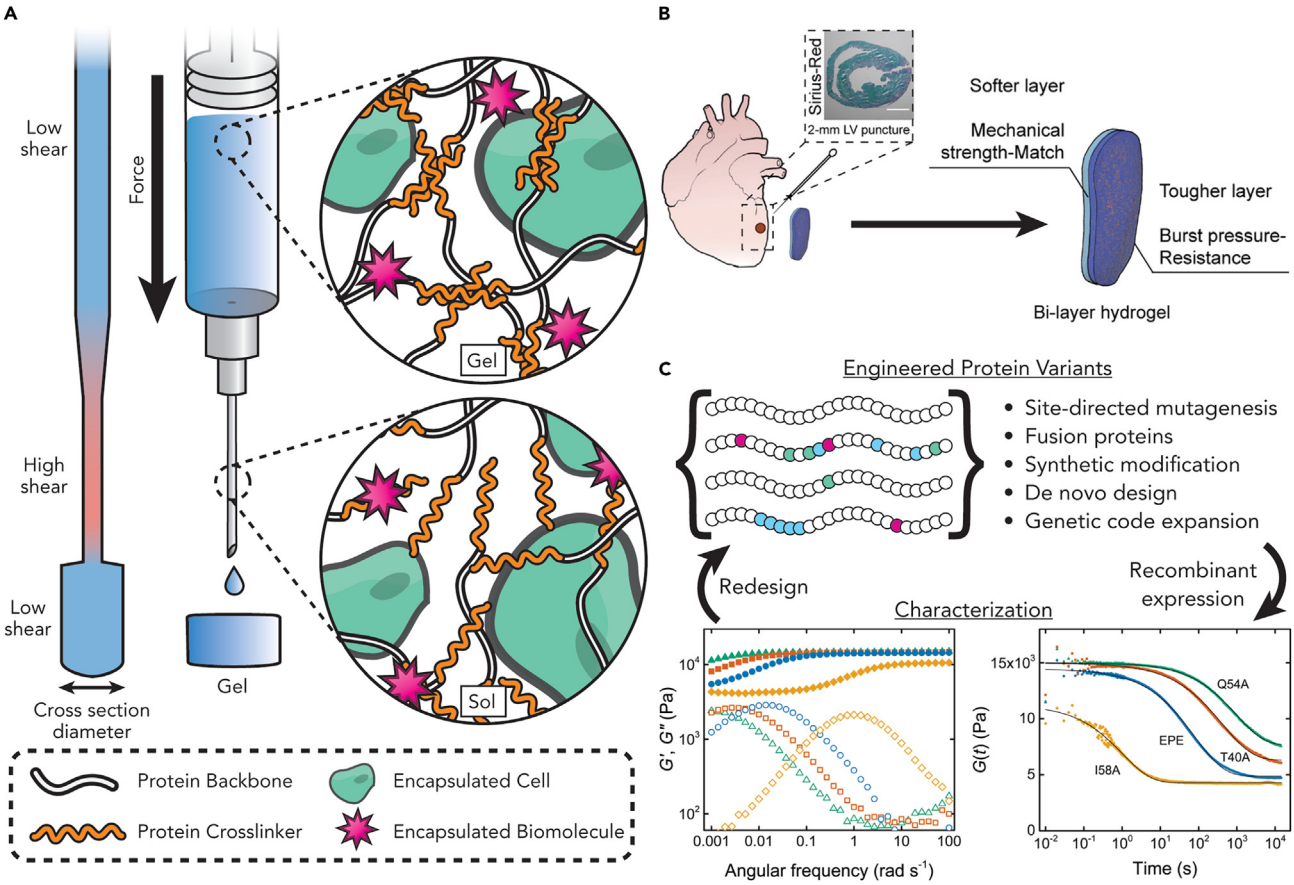



**Figure 5. Injectable and printable recombinant protein hydrogels**

(A) Shear-thinning behavior of the physical network and superior biocompatibility of many recombinant protein-based hydrogels make them attractive targets for injectable therapies and extrusion-based bioprinting applications. As these materials are pushed through narrow passages, increased shear stress causes reversible liquefication, carrying along any cellular or biochemical cargo.

(B) A burst-resistant bilayer patch uses two engineered variants of a shear-thinning leucine zipper-based hydrogel. The inner layer imitates the mechanical properties of softer native heart tissue, while the outer layer provides structural stability. Burst resistance was modulated by recombinant introduction of mussel foot protein domains Mefp3 and Mefp5 into the leucine zipper crosslinker, generating a chimeric set with an array of mechanical properties. Adapted with permission from Jiang et al.[75] Copyright 2022 Wiley-VCH.

(C) Recombinant protein hydrogels uniquely allow iterative and high-throughput screening of physicochemical and biological properties through classic and next-generation protein engineering techniques. Plots adapted with permission from Dooling and Tirrell.[76] Copyright 2016 The American Chemical Society.

sequences recognize and assemble into a thermodynamically favorable structure that yields a physically stabilized network (Figure 5A).

Seminal work by the Tirrell and Kopeček groups established the coiled-coil motif as a widely used biorecognition module and crosslink of protein hydrogels[77] and hybrid synthetic-protein networks.[78] As their name implies, coiled coils are constituted of two or more α helix motifs that recognize and assemble into a supercoil maintained by hydrophobic interactions at the interface.[79] Coiled-coil-based networks constitute most protein-enabled physical assembly chemistries, with coil domains from distinct protein origins deployed for the design of a variety of networks. This prompted a suite of investigations into the molecular engineering principles underpinning coiled-coil folding to elucidate how systematic primary sequence variation scales and translates into macroscopic changes at the network scale.[76,80] Emblematic of the strategy, a recent study by the Zhong group makes use of a recombinantly produced

coiled-coil crosslinked hydrogel to engineer bilayer cardiac patches capable of supporting cardiomyocyte proliferation, fibrosis reduction, and increased blood pumping capacity in two separate murine disease models (Figure 5B).[75]

Beyond coiled-coil biorecognition, the Heilshorn group introduced the mixing-induced, two-component hydrogel (MITCH) system for the creation of mechanically soft and fully recombinant scaffolds, originally to encapsulate, maintain, and differentiate neural stem cells.[81] MITCH systems are physically assembled from a seven-repeat WW domain from C43 (C7) and a cognate nine-repeat proline-rich sequence (P9), with heterologously expressed C7 and P9 reacting with 1:1 stoichiometry from liquid precursors to form a stable gel. In contrast to other protein-based biorecognition chemistries that either may not structurally hold under native physiological conditions or may cross-react with endogenous epitopes present on cell surfaces, the MITCH system deploys two sequences that are normally absent from the ECM. In spite of their simplicity—containing both a tryptophan-rich WW sequence and a cognate proline peptide sequence—the two components assemble with good selectivity to enable gelation in the presence of living cells. Further molecular- and sequence-level characterization and understanding of this system[82] led to expanded uses in the stem cell culture niche, including delivery of adipocyte-derived stem cells[83] and as a gel-phase ink for bioprinting.[84]

The Burdick lab pioneered a hybrid synthetic-protein-based "dock-and-lock" (DnL) hydrogel consisting of two parts: (1) the RIIa subunit of cAMP-dependent kinase A, engineered heterologously as a telechelic protein (recombinant docking and dimerization domain, or rDDD), and (2) the anchoring domain of A-kinase anchoring protein (AD), achieved via solid-phase peptide synthesis and end-functionalized on a star-PEG macromer.[85] Upon mixing, the multivalent AD domains lock into the protein dimerization dock. The resultant physical crosslinks that form enable the construction of a self-healing, shear-thinning, and ultimately injectable construct. In a subsequent report, this system was used successfully as an injectable drug delivery vehicle for interleukin-10 to treat obstructive neuropathy in a mouse model.[86]

The Kiick group harnessed the heparin-binding capability of vascular endothelial growth factor (VEGF) proteins to non-covalently synthesize hydrogels.[87] Specifically, multi-arm PEG macromers end-functionalized with heparin motifs formed hydrogels when mixed with VEGF proteins. These networks only eroded when the VEGF protein was pulled down by a presented VEGF receptor. While there have not been many examples following up on this strategy, it does highlight the potential promise of protein-polysaccharide interactions (or other forms of molecular biorecognition) in the design of therapeutically relevant hydrogels, particularly in microenvironments with a rich set of different signaling factors present.

Physical networks stabilized through protein-protein interactions hold benefit for the encapsulation and maintenance of cells given their biocompatibility. Their mechanics also result in desirable properties such as self-healing and injectability. An often-underappreciated aspect, however, is their direct amenability to systematic interrogation and evolution through sequence optimization (Figure 5C). While previous efforts have mainly focused on site-directed mutagenesis, which has admittedly resulted in powerful gains in performance, we expect the field to be energized by the emergence and widespread adoption of *de novo* protein design[59] and genetic code expansion.[88] The former approach can generate large numbers of possible sequences to be tested for expression yields and bioactivity, and the latter can move outside of a purely biochemical space to access added degrees of

functionality with a pristine regioselectivity beyond the capacity of stochastic modification. As these tools are codified, we predict many advances in biomaterial design and synthesis will become possible.

*DNA-based.* DNA biopolymers are being increasingly employed to enable new frontiers in nanotechnology and materials science.[89] Beyond its role as a genetic blueprint, DNA sequence encodes for rich structural and functional information driven through well-understood hydrogen-bond-driven base pairing that is readily co-opted for exquisitely user-definable and controllable material development. The effects of sequence variation often scale up and result in macroscopic changes, allowing tunable mechanics and stimulus responsiveness to be engineered through standard molecular biology methods. Moreover, the material stability of DNA in many biological contexts renders it highly useful as a crosslink in applications requiring long-term construct integrity. As a result, DNA-crosslinked gels have matured within less than two decades from an academic curiosity into an established area of biomaterial development.[90]

Early landmark examples have successfully exploited the governing dynamics of DNA-DNA interaction to create materials. In an early report, the Liu group synthesized networks from tri-functional Y DNA starting reactants via formation of intermolecular i-motifs,[91] bypassing the need for an enzymatic trigger for the process but leading to networks that were not stable at physiological conditions. Follow-up work established DNA hybridization of compatible "sticky" ends as a viable and potentially highly versatile approach for network synthesis.[92] By mixing tri-functionalized Y DNA precursors and di-functionalized DNA linkers, the group engineered physical networks upon mixing endowed with enhanced stimulus responsiveness and mechanical stability. The Willner group also successfully harnessed DNA self-assembly for the creation of pH-responsive DNA-based networks endowed with robust shape memory by supplementing the i-motifs with DNA duplex units generated through the usage of adenosine-rich sequences. Their networks underwent preprogrammed and reversible sol-gel transitions, whereby gelation would occur at a pH level of 5 and network dissociation at a level of 8.[93]

## Triggered hydrogel formation

As described earlier, the biomaterials field is benefitting from an expanding armamentarium of spontaneous assembly schemes for network creation. While important in a host of application spaces, these platforms often become inadequate when more nuanced control over hydrogel formation is necessary. For instance, hydrogel implants stand to benefit from spatiotemporal control over network formation. Building triggerability into gelation allows for these problems to be addressed and stands to buttress the immediate bench-to-bedside translatability of hydrogel biomaterials.[94]

Broadly, creating triggerable systems can proceed by two different methods: through the engineering of gating mechanisms (e.g., molecular "cages") into reactions that otherwise would proceed spontaneously, or alternatively deploying a reaction scheme that *a priori* requires an exogenously delivered catalyst. These stratagems will be expounded upon in detail and applications where they have proved a strong fit highlighted.

### *Triggered gel formation via covalent reaction*

*Synthetic organic-based.* Engineering gating mechanisms is of broad interest to chemists, biologists, and material scientists given the potentially afforded spatial

and/or temporal control over reaction initiation and extent.[95] While click chemistries have proved essential in the engineering of new networks, reactions typically proceed rapidly upon mixing, hampering more nuanced use cases where added control over the spatiotemporal aspects of gelation is essential. As such, efforts have been poured into taking otherwise-spontaneous step-growth chemistries and engineering gating mechanisms to control their initiation and/or progression through different triggers. Potential engineered stimuli are manifold and ultimately depend on the context of the application.

A common trigger is redox-based initiation, whereupon introduced oxidants or reductants can actuate hydrogel formation from redox-sensitive macromers.[96] Thermally triggered gelation is also a viable strategy[97]; while non-ideal for a number of biological applications involving temperature-intolerant mammalian cells, thermal actuation of gelation is useful for drug delivery platform creation. Lastly, pH can serve as a hydrogelation stimulus but is again typically precluded from applications involving live cell encapsulation.[98] Although these stimuli can afford temporal control over network formation, as well as a route to uniformly modulate hydrogel properties post synthesis, their ability to be controlled in space is limited.[99] Moreover, their applicability is hampered when minimal cellular interference is required.

Photoreactions, which can spatiotemporally dictate gelation based on when/where light is shone onto a sample, offer exciting advances in targeted hydrogel formation. This partially explains the earlier widespread adoption of photoradical-mediated chain-growth systems (e.g., acrylates, methacrylates) (Figure 2A).

While now less employed for cell encapsulation stemming from concerns over formation of cytotoxic free radicals[100] and heterogeneous network structures,[101] their reagent availability, ease of preparation and use, along with the control they afford in both triggering gelation and modifying formed networks makes photopolymerization a suitable candidate for the creation of permissive 3D scaffolds. Specifically, the combination of this chain-growth platform with a gelatin methacryloyl (GelMA) starting material was highly synergistic, as it led to the creation of networks that were amenable to cellular encapsulation and maintenance[102] as well as relatively easy photo-enabled post-synthetic modification. This galvanized reports generating hybrid-gelatin networks from methacrylated starting macromers, such gelatin-gellan gum, -PEG, -HA, and -silk fibroin.

Another popular photochemistry is the radical-mediated thiol-norbornene platform, initially developed by the Anseth group[103] for the step-growth polymerization of PEG-based hydrogels. The reaction takes place between norbornene end-functionalized macromers and thiol-terminated crosslinkers. Requiring a lower initiator and active radical concentration than typical vinyl-based chain-growth chemistries, the reaction minimizes radical-induced damage to encapsulated cells or tethered biological moieties such as proteins. Additionally, it can proceed orders of magnitude faster than a chain-growth-driven reaction and is not susceptible to oxygen inhibition. The platform was co-opted by multiple groups who applied it successfully to different base materials such as HA and gelatin.[104]

In looking to move photocrosslinking beyond radical-mediated chemistries, many labs have identified innovative ways to take step-growth chemistries previously considered uncontrollable and engineer photo-gating mechanisms into them, side-stepping radical-related cytotoxicity concerns. Toward this end, SPAAC was successfully rendered two- and three-photon activatable by the Popik and Bjerknes

groups through inactivation of its strained-alkyne group with a photocaging cyclopropenone group,[105] paving the way for SPAAC hydrogel formation and biomolecule derivatization in deep tissue. Upon photo-uncaging with near-infrared light, the alkyne group is liberated from its cyclopropenone cage to enable SPAAC reactivity. Oxime ligation was also successfully gated through photochemistry: our lab previously caged the alkoxyamine with a cytocompatible UV-light-labile 2-(2-nitrophenyl)propyloxycarbonyl (NPPOC) group, such that the photo-liberated alkoxyamine could react with a cognate aldehyde and enable photocrosslinking for hydrogel formation and patterned biomolecule immobilization.[106] The Barner-Kowollik group also recently red-shifted photocaged oxime ligation ($\lambda$ = 625 nm) through generation of an aldehyde from a furan precursor molecule in the presence of a photosensitizer, enabling transdermal initiation of photocrosslinking through a 0.5-cm-thick phantom mimic.[107]

Lastly, harnessing the [4 + 4]-photodimerization of anthracene has been shown by the Forsythe group to be a viable method to trigger network formation in the presence of cells.[108] Eliminating the need for two distinct reagents typical to click-based platforms, the group end-functionalized PEG macromers with anthracene moieties and induced network gelation via cytocompatible visible-light ($\lambda$ = 400–500 nm) illumination. While earlier reports had demonstrated network formation using anthracene dimerization, requisite cytotoxic UV light was not suitable for cell encapsulation. To overcome this limitation, the group installed electron-rich substituents (e.g., triazole, benzyltriazole) to red shift anthracene absorbance, resulting in a synthetically tractable (one form of macromer required rather than two) and cytocompatible network synthesis scheme.

Owing to the powerful synergies proffered by incorporating photoresponsiveness into bioorthogonal crosslinking chemistries, there has been a remarkable recent uptick in novel platforms that are phototriggerable.[109,110] While not all of these have yet been deployed in the context of hydrogel assembly, they are uniquely suited for many applications.

*Protein-based.* Beyond recognition and assembly of cognate pairs, protein-based platforms can act as enzymatic catalysts for the crosslinking of network precursors bearing the appropriate peptide substrate sequences. In fact, while thermal, redox, or light initiation triggers are well suited for certain applications, their usage can be sub-optimal when full bioorthogonality is desired. Given the prevalence of enzymes in all living organisms, their use as a highly bioorthogonal crosslinking reagent is potentially a powerful strategy (Table 2).

The Griffith lab first pioneered the use of transglutaminase to crosslink synthetic networks with macromers bearing appropriate recognition sequences.[111] Transglutaminase, a $Ca^{2+}$-dependent enzyme, catalyzes a transamidation reaction between the carboxamide side chain of glutamine and the amine group found on lysines, releasing ammonia as a byproduct. This early work demonstrated network formation within a couple of hours from PEG macromers functionalized with either a glutamine residue or a lysine-phenylalanine dipeptide sequence. While highly promising, the relatively slow gelation placed limitations on encapsulation efficiency and uniformity. This strategy, however, laid the groundwork for a slew of follow-up research broadening the enzymatic toolset and optimizing reaction parameters (e.g., substrate recognition, crosslinking kinetics). The Messersmith group, for instance, rationally designed substrates with high transglutaminase specificity to enable faster gel assembly kinetics.[112] Subsequent work from different groups expanded the palette

of available crosslinking enzymes with substrate recognition sequences of their own: these include horseradish peroxidase (HRP), phosphopantetheinyltransferase (PPTase), tyrosinase, lysyl oxidase, and sortase A.[113] Highlighting the promise of bioorthogonal enzymatic crosslinking, the Hwang group recently employed a tyrosinase-mediated reaction to form protective nanofilm hydrogels from monophenol-modified glycol chitosan and HA in the presence of pancreatic β cells to regulate blood glucose in a type 1 diabetes mouse model.[114]

Enzymatic crosslinking has also proved to be particularly well suited in the design and engineering of DNA-based hydrogel materials. In fact, routine enzymes in molecular biology applications have been co-opted to create hydrogels and other material structures from branched-chain DNA. This was first exemplified by the Luo group, who harnessed T4 ligase to covalently assemble branched DNA structures (X, Y, and T motifs) into hydrogels.[115] Combining enzymatic crosslinking with DNA as starting material has made possible the design of wholly novel topologies and network structures. An interesting example is the generation of hydrogels solely from pristine plasmid DNA rather than more complicated branched DNA structures. After digestion by appropriate restriction enzymes and generation of sticky ends, crosslinking through the action of a ligase leads to gelation, and in so doing solves many issues associated with material costs, synthetic intractability, and severe stoichiometric dependence.[116] Building on enzymatic synthesis even further, the Walther group[117] deployed a strategy whereby two antagonistic enzymes (i.e., urease, esterase) act in an autonomous feedback loop to regenerate a DNA hydrogel from sol to gel continuously, driven by ambient pH levels. This enabled biomaterial creation with a distinct lag-time and lifetime under closed system conditions.

*DNA-based.* While the molecular engineering of DNA has enabled spontaneous gelation from nucleotide-modified polymeric macromers, relatively simple modification to the involved reaction motifs can turn DNA-enabled platforms into chemically triggerable systems (Figure 6A). Emblematic of this approach is the use of aptamers, which are short nucleic acid sequences that are designed and engineered to bind to a particular target or family of targets. They can best be conceptualized as the nucleic acid-counterpart to protein-based antibodies, and their deployment as a material chemistry can confer several benefits. For instance, in the case of DNA aptamers, constructs can be readily synthesized through routine and economical solid-phase synthesis. Moreover, aptamers are often endowed with robust stability under different solution conditions, rendering them particularly useful as base material for material crosslinks and tethers for different cargos. Lastly, it is theoretically both straightforward and rapid to generate aptamers for a wide variety of targets spanning different biochemical classes through a now-optimized combinatorial method known as systematic evolution of ligands by exponential enrichment (SELEX).[118] A foundational example of harnessing aptamers as a material chemistry for hydrogels was demonstrated by the Tan group, where starting polyacrylamide-based macromers were end-modified with linear strands of nucleic acids and yielded network formation upon introduction of an exogenous DNA molecule, termed LinkerAdap.[119] While this was engineered to hybridize both strands of the starting macromers, it also includes an aptamer sequence for adenosine. Subsequently, introducing LinkerAdap into solution kickstarts a sol-gel transition, whereas adding adenosine will destabilize and degrade the network. This foundational example led to a flurry of downstream work that sought to apply DNA crosslink engineering for highly sensitive therapeutic delivery and biosensing applications.[120] For instance, the Wang group exploited the straightforward engineering of an anti-platelet-derived growth factor (PDGF) and incorporated it into routinely synthesized PEG-diacrylate

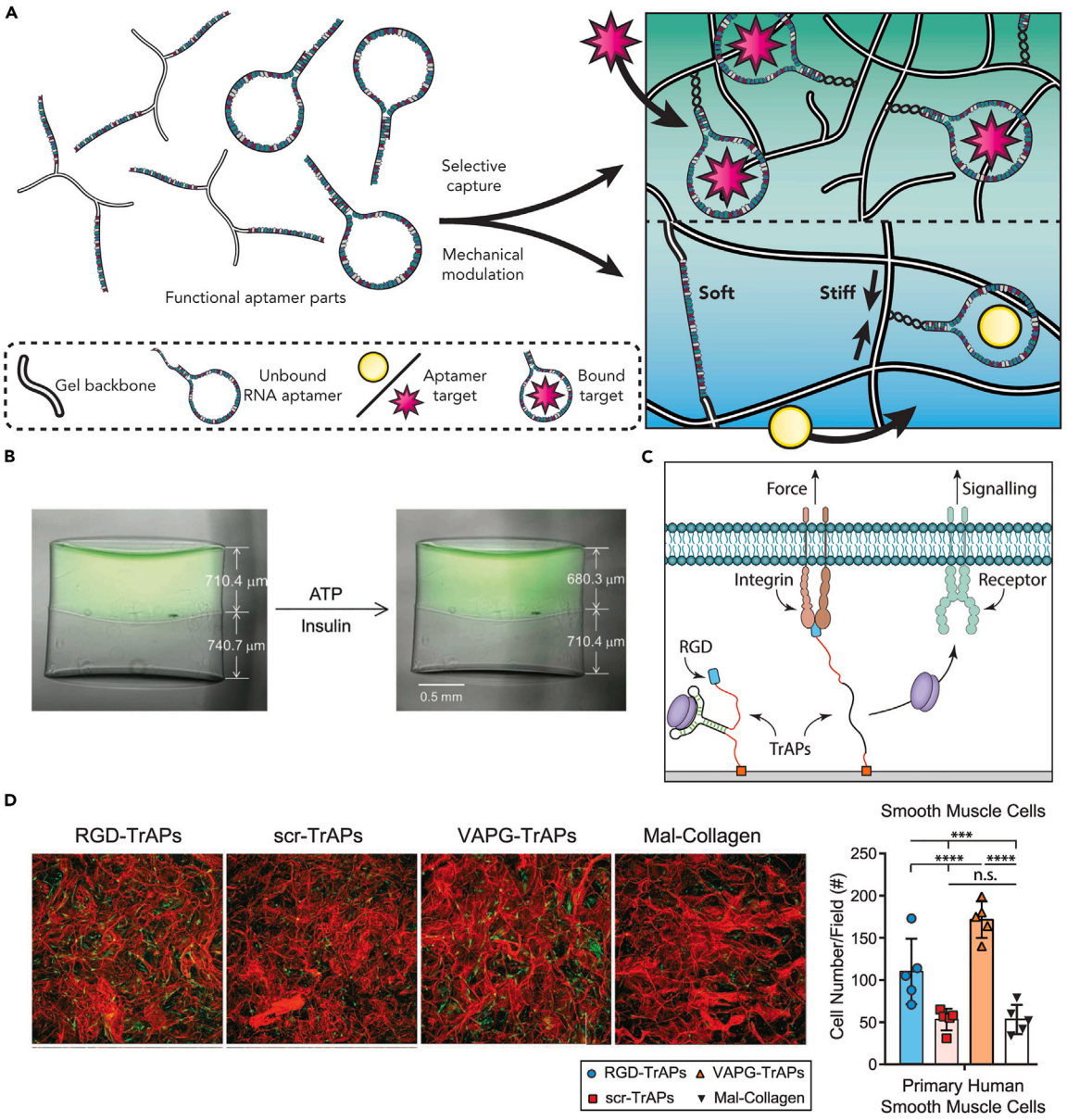



**Figure 6. Engineering dynamic biomaterials through aptamer biology**

(A) When incorporated into a hydrogel backbone, aptamers in an extended initial state can lead to macroscopic-level changes in network mechanical properties through basic biorecognition and cognate target capture.

(B) Layered hydrogels are synthesized such that the top hydrogel (fluorescent green) is crosslinked through ATP-binding extended-state aptamers and the bottom hydrogel is crosslinked through insulin-binding extended-state aptamers. When exposed to the appropriate cognate molecule (ATP in the top and insulin in the bottom network), conformational changes in the aptamer crosslink lead to significant network volume decrease. Image reproduced with permission from Bae et al.[122] Copyright 2018, Wiley-CVH.

(C) Aptamers can be engineered as force-mediated release systems. Traction-force-activated payloads (TrAPs) are designed such that an aptamer bound to a target molecule is also linked to an RGD motif that recognizes force-responsive integrin motifs. Upon local mechanosensing or application of a force stimulus, unfolding of the aptamer leads to target molecule release.

(D) TrAPs enable selective activation of growth factors in 3D collagen scaffolds by primary human smooth muscle cells. Extent of release and variation between different cell types is due to the relative expression of different adhesion receptors. Images for (C) and (D) reproduced with permission from Stejskalova et al.[123] Copyright 2019, Wiley-CVH.

hydrogel matrices.[121] When gelation occurs in the presence of encapsulated PDGF, this platform can serve as a robust and synthetically tractable drug release strategy that uses affinity interactions rather than bulk material degradation to deliver its payload. Promisingly, the kinetics of the release can be dictated by controlling the degree of aptamer incorporation, wherein higher-affinity networks containing more anti-PDGF aptamers led to a slower release, while lower aptamer content led to faster release.

Aptamers can be deployed for applications that go beyond triggered payload release. One recent example of this was in fact demonstrated by the Murphy group[122]; cDNA-bound (thus initially extended) aptamers were incorporated as network crosslinks in a synthetic gel backbone, and the target motif binding capacity of these was co-opted to effect substantial volume decreases in the hydrogel (Figure 6B). They were able to show the applicability of this system both with ATP- and insulin-binding aptamers: up to a 40% volume decrease could be obtained in the case of the former and 15% in the case of the latter. This strategy is exciting as it can theoretically go much farther beyond these proofs of concept, limited only by the development of appropriate aptamer motifs that recognize relevant targets. Another powerful example by the Almquist lab showcased the deployment of aptamers as a force-responsive motif for the release of target payloads, rather than the usual scheme of "bind to release."[123] Specifically, the group developed traction-force-activated payloads (TrAPs) such that an aptamer motif was linked to both a cargo of interest (e.g., different growth factors) and an arginine-glycine-aspartic acid (RGD) cell adhesion motif that binds to cell membrane integrins. Deployment of an integrin-binding motif imparts force responsiveness to the construct, such that local applied forces lead to payload release (Figures 6C and 6D). This biomimetic approach was inspired by the large latent complex that natively controls the release of the transforming growth factor family. Excitingly, it bypasses the need for any exogenous trigger and relies on the nuances of cellular communication within the matrix to guide growth factor delivery.

*Triggered gel formation via non-covalent reaction*

*Synthetic organic-based.* Early examples of triggerable gelation of physical networks have relied on small-molecule introduction. For example, alginate polymers have historically lent themselves well to physical network assembly,[124] as the introduction of divalent cation—most typically $Ca^{2+}$—leads to the formation of ionic bridges between different chains and subsequent gelation. However, given the crucial role calcium plays in biological signaling and cell-cell communication, this method falls short of creating truly bioorthogonal systems.

Beyond the now-classic calcium-alginate gels, recent reports outlining triggered assembly of physically crosslinked networks have been relatively few, albeit powerful given the dynamism that they can encode for and the relative ease with which desired downstream properties can be achieved. For example, the Rowan group demonstrated that the thermal gelation of polyisocyanopeptide chains can yield non-covalent network formation.[125] Polyisocyanopeptides rely on a non-traditional β-helical architecture stabilized by a supportive hydrogen-bonded network. Upon heating to 37°C, the polymer fibers bundle into a stiff hydrogel, which can be further stress-stiffened—a highly desirable property that is almost always beyond the capacity of synthetic networks—in the presence of living cells in order to closely mimic cytoskeletal viscoelasticity over time. Moreover, the synthetic nature of these chains allows for the routine introduction of different epitopes for biochemical signal presentation, or alternatively modification of starting materials to enable pristine user

control over downstream properties such as ultimate stiffness and stress relaxation. A more recent example by the Zhu and He groups outlines the use of the Hofmeister effect in order to trigger PVA network formation and modulate its properties.[126] By varying ion type and concentration (thereby dictating whether salting in or salting out is occurring), the team induced hydrogelation through systematic control of PVA aggregation state, as well as encoding for the hydrogel's starting mechanics. Moreover, the ions only trigger gelation and do not play a role in maintaining network integrity, which means they can be dialyzed out post assembly and do not stand to hamper applications that require subsequent interfacing with living cells.

*Protein-based.* Most triggerable protein-enabled stratagems result in the formation of covalent networks, as seen when applying traditional enzyme-assembly methodologies or photocrosslinking of constituent residues. There are, however, some interesting examples of protein-based physical network assembly. An enzyme otherwise routinely used in molecular biology—phi29 polymerase—has been successfully applied for the physical assembly of DNA-based networks in a process termed rolling circle amplification (RCA).[127] Best conceptualized as an isothermal alternative to polymerase chain reactions (PCRs), RCA avoids the damage caused to biomolecules induced by cyclical heating and cooling, which are otherwise necessary in the case of routine PCRs. It does so by starting with a small (25–35 nucleotides) circular strand of DNA as starting material for subsequent amplification. Moreover, given that RCAs can occur at physiological temperatures, they can be deployed in the presence of living systems. This makes them ideal for the non-covalent assembly of a variety of networks through DNA chain entanglement or sequence hybridizations.[128,129]

*DNA-based.* While most DNA-crosslinked hydrogels have been limited to synthetic schemes that proceed spontaneously in a relatively uncontrollable manner, recent efforts have sought to gate or guide DNA self-assembly into macroscopic networks. Early attempts to trigger gelation were primarily focused on the introduction of $Ag^+$ ions to form duplex bridges between DNA chains.[130] However, other have sought to bypass the use of cytotoxic $Ag^+$ triggers and engineer more sophisticated spatiotemporal control into the process. Toward that end, the Willner and Fan groups reported a DNA hydrogel synthesis that is triggered by the introduction of a DNA initiator in a method they termed a "clamped" hybridization chain reaction.[131] As its name indicates, the stratagem relies on introducing a clamp into a DNA hybridization chain reaction, a process through which stable DNA fragments assemble only upon exposure to an initiator molecule. This kickstarts a series of amplification events, with ultimate amplicon size varying inversely with the size of the initiator used, in an isothermal and enzyme-free fashion.[132] Classical hybridization chain reactions occur with two starting hairpin structures and an initiator molecule to kickstart successive assembly and amplification events. In a powerful example showcasing the promise of DNA molecular engineering, simple modification of one of the starting hairpins to incorporate a repeat palindromic sequence leads to a cyclical self-assembly scheme with intermediate three- and four-arm junctions to ultimately form a DNA hydrogel, with the small initiator linker operating as stimulus for the ensuing sol-gel transition. A subsequent report by the Li and Zuo labs harnessed this system for the capture of circulating tumor cells (Figure 7).[133] Keeping the main setup of the system relatively unchanged, the groups engineered a biblock initiator-aptamer molecule that recognizes specific receptors on tumor cell surfaces. Upon aptamer-receptor binding, the initiator strand is revealed, leading to hydrogelation in the presence of the circulating hairpin structures. This "cloaks"

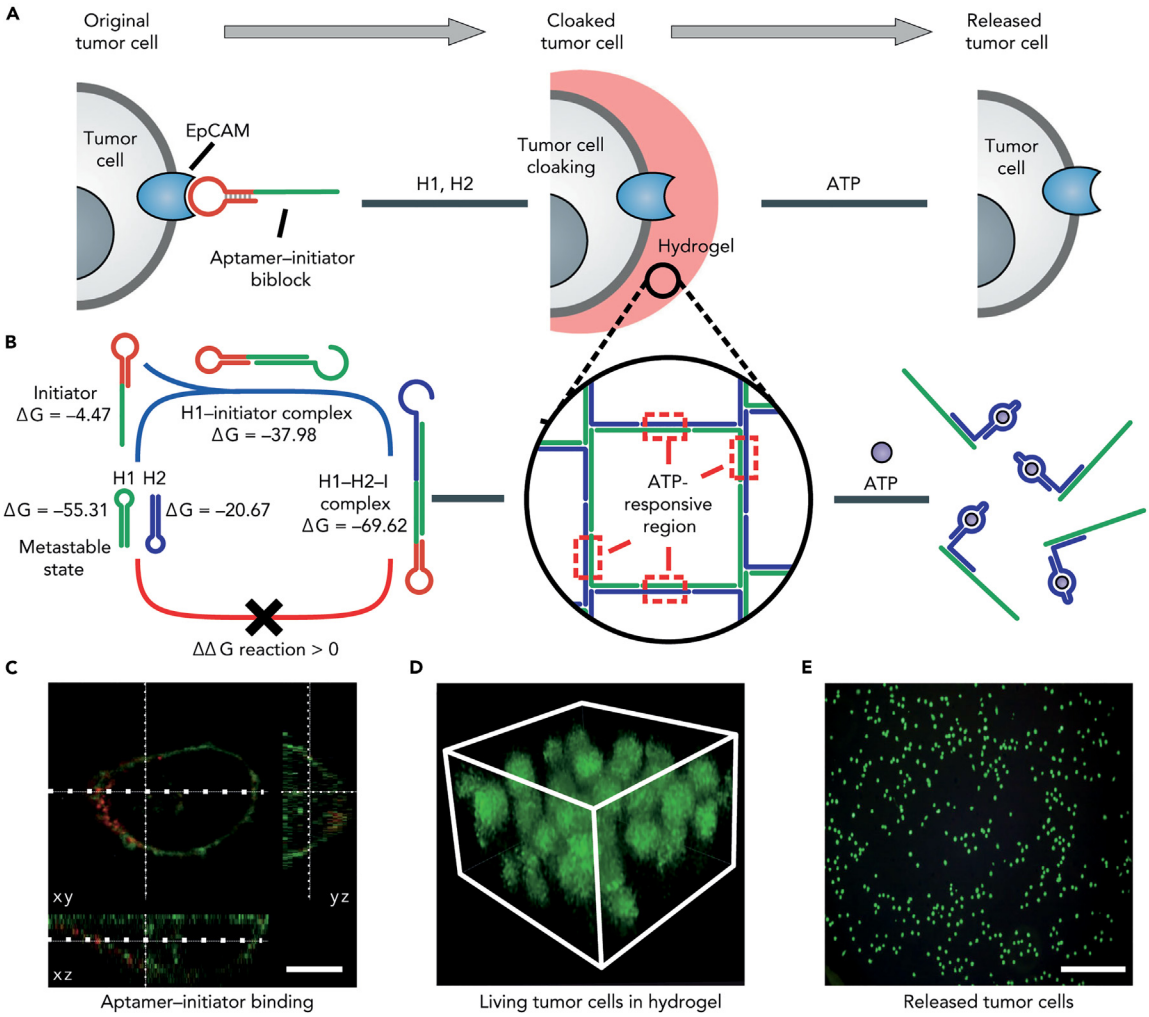



**Figure 7. Aptamer-enabled targeting and capture of circulating tumor cells**

(A) Aptamer-initiator biblock constructs are designed to specifically bind to epithelial cell adhesion molecules (epCAMs), which are highly expressed on the membranes of tumor cells. Following binding, biblocks containing an initiator trigger the formation of an encapsulating DNA hydrogel. Post encapsulation, ATP can be used to effect a conformational change within the ATP-responsive aptamer to destroy the gel, leading to tumor cell release.

(B) H1 and H2, which are step-look structured, are in a metastable state because of the protective effects of long stems in their secondary structures. In the presence of the initiator, the hybridization reaction is triggered leading to hydrogel assembly.

(C) Confocal microscopic imaging showing aptamer-initiator biblock binding to the cell surface membrane. Scale bar, 10 μm.

(D) Multi-layered cells can be found encapsulated within the DNA hydrogel when stained with FDA dyes. Stack height, 40 μm.

(E) Cells disperse in solution upon ATP-triggered release. Scale bar, 100 μm. Image reproduced with permission from Ye et al.[133] Copyright 2020, Springer Nature.

tumor cells with minimal damage, allowing for subsequent quantification and potential single-cell analysis.

## POST-SYNTHETIC MODIFICATION OF HYDROGEL BIOMATERIALS

As highlighted above, a growing toolbox of externally triggered and spontaneously proceeding reactions has birthed a wide method collection to fabricate hydrogel biomaterials. An equally if not more important thrust focuses on developing chemical methodologies that post-synthetically modulate hydrogel properties. This would enable the field to go beyond encoding an initial set of biochemical and biomechanical parameters for a hydrogel matrix and to create constructs that can

be customized on demand, potentially even in 4D. Engineering controllable dynamism into such systems would make possible longitudinal interrogation and/or control of biology, ideally in ways that capture all possible timescales of interest throughout space. In the ensuing section, we will survey different modalities that have been used to post-synthetically modify networks, with an eye toward rendering these chemistries more user controllable.

## Spontaneous hydrogel modification

Key to engineering evolvable networks is the identification and deployment of methodologies that modify hydrogels post assembly.

### *Spontaneous gel modification via covalent reaction*

*Synthetic organic-based.* Synthetic schemes offer different avenues for spontaneous modification post assembly. A first category to that effect adopts off-stoichiometric ratios during the initial network formation step; by maintaining an excess of a particular reactant, biological epitopes functionalized with the appropriate handle could later be immobilized with temporal (and sometimes spatial) control. This presents a very straightforward approach that minimizes biomaterial design complexity; most often, a "mixed-mode" approach can be adopted whereby one chemistry is used for network formation and another for post-synthetic modification. As one illustrative use case, networks formed by a tetrazine-norbornene platform, for instance, can routinely be assembled such that excess norbornene groups are presented throughout the hydrogel. Biological epitopes harboring reactive thiols can then be introduced and photo-clicked in through an orthogonal thiol-ene chemistry.[25] Another example along the same line of thought was presented by the Chen group; in this report, the base platform used for network assembly was DA chemistry, and excess maleimide was left unreacted for post-synthetic patterning.[134] It is important to note that such mixed-mode methods do not necessarily always hinge on stoichiometric manipulation of starting reactants, elegant as this approach may be. Orthogonal chemical handles can be introduced into the starting macromers such that they do not influence initial network synthesis but instead present avenues for later biochemical modification. An example of this method was presented by DeForest and Anseth[135]; in that report, SPAAC was used to assemble PEG hydrogels with chemically orthogonal vinyl moieties incorporated into the starting materials and subsequently presented throughout the network. These reactive ene groups served as pendants for the later introduction and patterning of different classes of biological epitopes including small molecules and bioactive peptides through thiol-ene photochemistry. As can be seen from the examples above, owing to the diversity of chemical approaches developed for hydrogel synthesis and modification, there exist no shortage of platforms that can prove amenable to mixed approaches, where one chemistry is geared toward assembly and another tailored toward biochemical or biomechanical modification.

Another exciting development in evolvable hydrogel networks is the deployment of covalent-adaptable networks, which combine the mechanical integrity and stability of chemically crosslinked systems with the stress relaxation and enhanced viscoelasticity of physical assemblies.[136–138] By occupying this intermediate niche between the two modes of crosslinking, biomaterial scientists have been able to tap into synergies unobtainable from either strategy alone and turn the reversibility of some of these base reactions (e.g., the previously touched upon DA and oxime ligation schemes)—typically thought to be a potential weakness—into an exploitable property. Nevertheless, early iterations of these reconfigurable networks were not suited to the needs of the biomaterials science and engineering community; in fact, the

landmark report outlining the use of DA chemistry (with furan and maleimide as starting macromers) for the generation of a "re-mendable" material only did so at a transition temperature of 120°C,[139] which was the only transition point at which the adaptability of the chemistry could be accessed. A similar early landmark paper deploying hydrazone chemistry for the generation of adaptable polyethylene oxide (PEO) matrices only showed network reconfiguration within reasonable timescales at a transition pH level of 4, well below the useful range for cell studies; the linkages would break at apparent pH levels less than 4 and would reform when above that cutoff.[140] While powerful proofs of concept, rational changes had to be incorporated into the base chemistries used if this approach was to prove useful for pursuits in tissue engineering.[141] Toward that end, the first report detailing the use of a covalent-adaptable network for the encapsulation and maintenance of a cell population was presented by the Anseth group, who did so through the deployment of a hydrazone transimination platform.[142] A rapid screen of reactivities of two different aldehydes—one aliphatic and the other an arylaldehyde—with a methylhydrazine partner showed that both approaches could be viable candidates. In fact, both reaction pairs led to gelation and response profiles within reasonable timescales, and both approaches enabled the encapsulation of C2C12 myoblast cells. Since then, multiple dynamic covalent methodologies have proved useful in addition to the traditional DA[143,144] and oxime ligation[145] workhorses; among these chemistries are hydrazone,[146] thioester exchange,[147] hindered urea bond,[148] boronate transesterification,[149] among others still. Interestingly, dynamic covalent chemistries can also be used in mixed-mode approaches provided their reaction profiles and necessary solution conditions are compatible and amenable to multiplexability. One method to effect this was presented by the Anderson group, whose report used diol exchange of two kinetically distinct phenylboronic acid derivatives, 4-carboxyphenylboronic acid and *o*-aminomethylphenylboronic acid. In so doing, the authors were able to access highly nuanced mechanical and viscoelastic property ranges by mere tuning of the starting concentrations of either acid, and achieved response profiles beyond the reach of either acid alone.[150] Another method is to deploy two dynamic platforms that are fully orthogonal; a recent example of this is provided by Fustin group, who in an interpenetrating network setting were able to combine Schiff base chemistry in one hydrogel and metal-ligand coordination in another.[151]

*Protein-based.* The advent of orthogonal protein pairs has been highly enabling for the covalent post-synthetic modification of hydrogels consisting of different base materials. The fragment reconstitution-based toolkits mentioned previously have been deployed to great effect for network modulation across many synthetic platforms. To implement this strategy, the Li group photochemically crosslinked SpyCatcher-containing motifs into an underlying tandem elastomeric protein network. With these moieties in place, different SpyTagged fusions were swollen in to covalently decorate the gel with a host of proteins (i.e., fluorescent proteins, as well as cell-adhesive TNfn3 domain derived from type III fibronectin).[152] The Sun group also harnessed this approach successfully to decorate mussel foot protein-3 (Mfp-3) hydrogels displaying SpyCatcher motifs, enabling post-gelation incorporation of SpyTagged protein.[153] The West group took this blueprint further: by photochemically crosslinking different amounts of SpyTag within PEG-diacrylate hydrogels, they could specify the concentration of tethered SpyCatcher fusion proteins.[154]

Beyond fragment reconstitution, enzymes can also be co-opted for the post-synthetic modification of networks, particularly in cases where bioorthogonality and

maintenance of native bioactivity levels are essential. The Griffith lab pioneered the use of sortase-mediated transpeptidation to post-synthetically decorate hydrogel matrices. Specifically, a PEG hydrogel assembled through Michael-type chemistry was decorated with epidermal growth factor (EGF) via sortase (Figure 8A).[155] Beyond biochemical immobilization, their group also demonstrated sortase-enabled degradation of PEG networks, demonstrating gains in biocompatibility of the enzyme to the encapsulated cells when compared to more standard proteolytic gel degradation methods.[156] In the same vein, the Lin group also employed sortase to control network crosslinking density.[157] In order to do so, they exploited the inherent reversibility of sortase reactions to impart cyclical stiffening or unidirectional softening, based on the design of the constituent crosslinkers. Moreover, taking advantage of recently engineered sortases that were evolved to recognize orthogonal peptide substrates, our lab recently demonstrated the ability to spatially control degradation and cell recovery from multi-material biomaterials[158] (Figures 8B and 8C). Applications such as these highlight the extent to which genetic encodability can result in highly nuanced material responsiveness.

In a non-classical example showcasing protein-enabled network modification, the Collins group have engineered hydrogel networks with integrated nucleic acid crosslinks that cleave in response to the action of Cas12a nuclease proteins (Figure 9).[159,160] Based on the material design adopted, the group successfully demonstrated release of cargo tethered to the network through pendant DNA, bulk degradation of the network when crosslinked through appropriate DNA substrates, actuation of an electronic fuse, as well as co-opting the material for paper diagnostics endowed with remote signaling capacities. We expect recent advances in triggering CRISPR activity to further next-generation responsive materials development.

*Spontaneous gel modification via non-covalent reaction*

*Synthetic organic-based.* The earliest and arguably most robust example of a non-covalent method to confer modifiability into hydrogels is heparin, either chemically tethered via synthetic coupling, encapsulated, or included as a network crosslink.[161] Heparin is a linear polysaccharide group that was initially deployed to reduce biomaterial-induced thrombogenesis following blood contact given its anticoagulant activity. However, it was soon discovered to preferentially bind to a wide host of growth factors,[162] rendering it particularly useful for controlled the release of protein therapeutics and regenerative medicine applications.[163,164] However, in spite of its relative simplicity, routine usage of heparin has been hampered by its broad binding capabilities; its biorecognition is not limited to a singular cognate partner. Instead, it recognizes a wide family of growth factors that harbor a heparin-binding motif, which would likely complicate its use *in vivo*.

Moving beyond the broad recognition capacity of chemically immobilized/encapsulated heparin would require more judicious design or identification of target-specific docking sites that can enable the stimulus-responsive and precise release of encapsulated drugs upon analyte sensing (i.e., a more precise form of molecular biorecognition). An early example of this is the coumermycin-inducible release of drugs in loaded polyacrylamide hydrogels presented by the Weber group.[165] In fact, the group chemically conjugated a bacterially produced gyrase B protein subunit—which harbors a very strong affinity to coumermycin—to nitrolotriacetic acid-modified polyacrylamide via $Ni^{2+}$ chelation. Introducing the coumarin-based antibiotic coumermycin leads to gyrase B homodimerization, polymer crosslinking, and subsequent network assembly. Subsequent addition of the competitive antibiotic novobiocin, however, leads to dimer dissociation and resultant network degradation. This

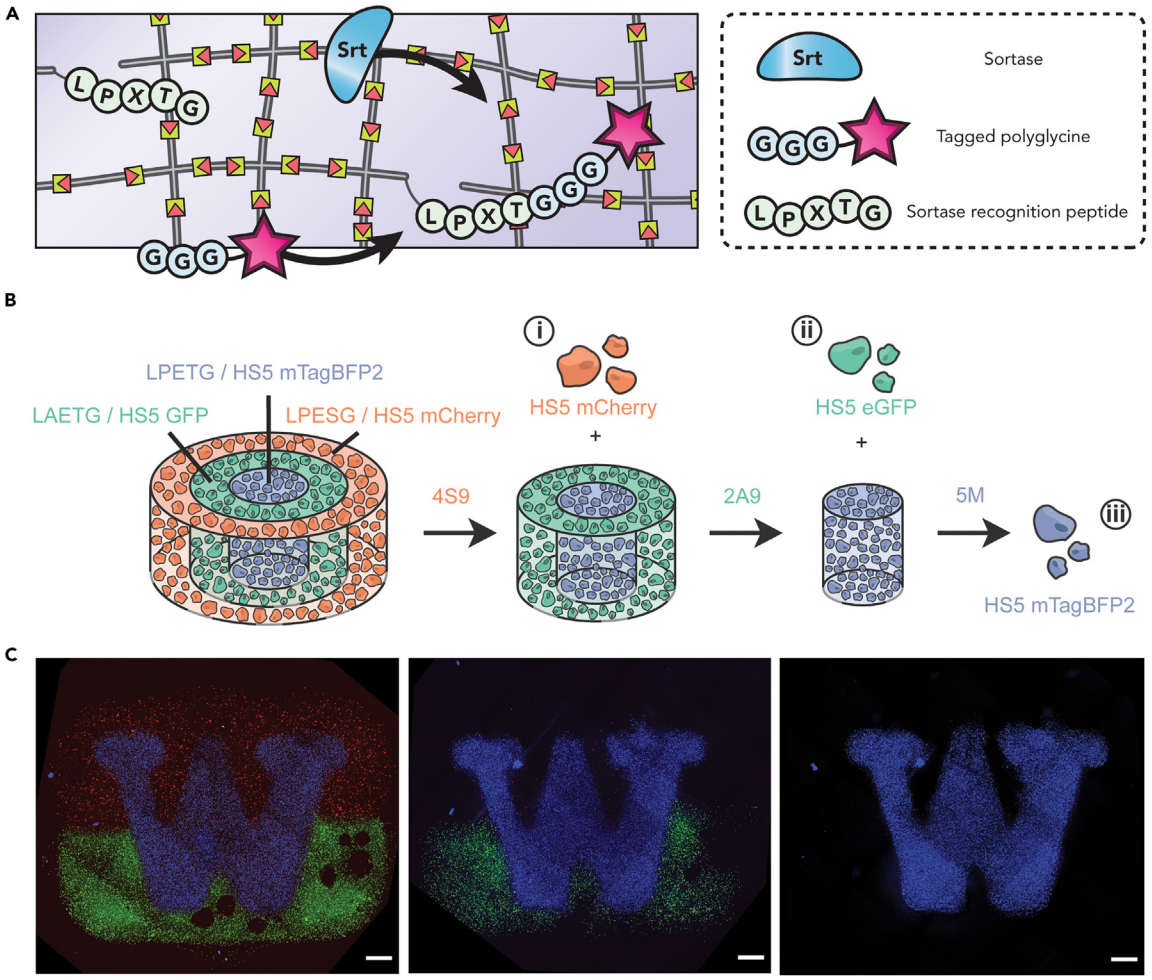



**Figure 8. Sortase-mediated gel functionalization and multi-material degradation**

(A) Sortase selectively ligates polyglycine-tagged cargo onto LPXTG-containing peptide sequences covalently bound to the polymer network.

(B) Harnessing evolved sortases' ability to recognize orthogonal peptide motifs found within crosslinkers comprising different gel regions, staged material degradation and accompanying cellular release can be achieved; for example, with eSrtA(4S9), then eSrtA(2A9), then eSrtA-5M. Adapted with permission from Bretherton et al.[158] Copyright 2023, Wiley-VCH.

(C) Sequential sortase treatment enables user-defined control over cell-laden multi-material degradation. Maximum intensity projections of the University of Washington logo, comprising cells constitutively expressing one of three fluorescent proteins, are shown prior to degradation (left), following treatment with eSrtA(4S9) treatment (center), and following eSrtA(2A9) treatment (right). Scale bars, 1 mm. Reproduced with permission from Bretherton et al.[158] Copyright 2023, Wiley-VCH.

was used successfully for the encapsulation of a VEGF protein during encapsulation and controlled release, which could be tuned by varying the relative concentrations of the different parameters at play. Given the simplicity of the platform as well as its reliance on commonly used and US Food and Drug Administration (FDA)-approved antibiotics (rendering potential downstream translation all the more promising), the group built upon their strategy further by porting it into a more bioinert PEG-based network crosslinked through Michael-type chemistry,[166] applying it for the release of a hepatitis B vaccine,[167] and exploiting its amenability for pharmacological regulation to build a cell-growth-supporting matrix.[168,169]

*Protein-based.* Analogously to covalent schemes for the post-synthetic modification of networks via proteins, non-covalent chemistries also hold significant promise for the reversible modulation of network properties, be they mechanical or

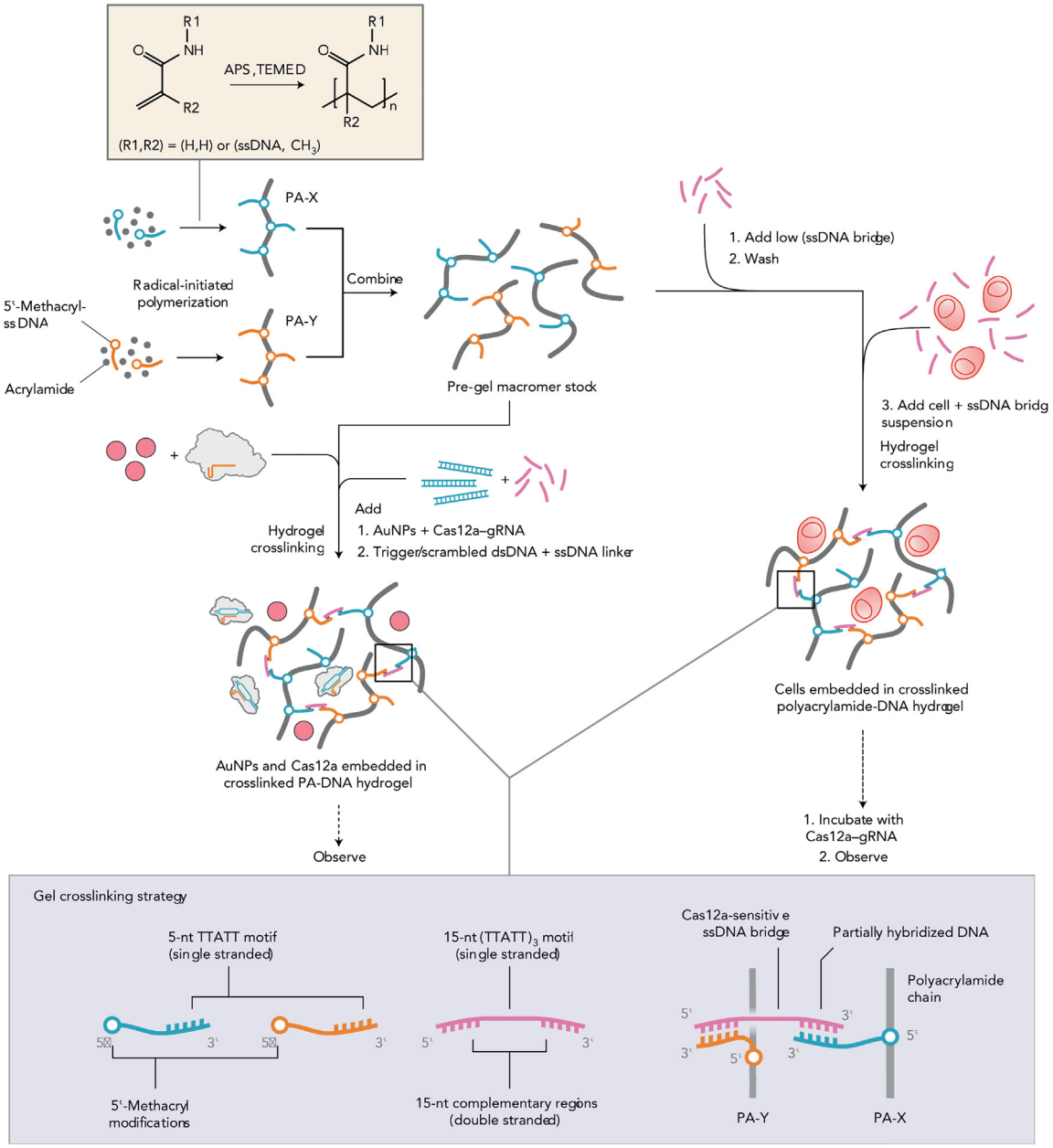



**Figure 9. Design and synthesis of Cas-responsive hydrogel networks**

Methacryl-functionalized DNA is incorporated into polyacrylamide chains (PA-X, PA-Y) during starting macromer polymerization. This enables different routes to gel actuation and response modes. In one example, shown on the left, the Cas12a-gRNA is added to the gel precursor with the nanoparticle cargo, before the addition of double-stranded DNA (dsDNA) cues and single-stranded DNA (ssDNA) crosslinker. In another example, shown on the right, cell encapsulation is triggered through the addition of a small amount of ssDNA bridge crosslinker to the macromers mixed in solution. This thickens the pre-gel solution and minimizes losses incurred during the washing step. More ssDNA linker is then added at the same time as the cells to fully crosslink the hydrogels. Finally, the experiment is initiated by exposing the gels to gRNA-complexed Cas12a and dsDNA. Additional details of the crosslinking strategy (bottom of the panel): the two ends of the DNA bridge hybridize with distinct ssDNA anchors incorporated into polyacrylamide macromers, while the central AT-rich portion remains single stranded and sensitive to Cas12a collateral activity. Reproduced with permission from Gayet et al.[160] Copyright 2020, Springer Nature.

biochemical in nature. Early examples were provided by the Li group, who incorporated what they termed a mutually exclusive protein (MEP) to act as a redox-controlled crosslink that can switch between folding and unfolding.[170] After initial

photochemical crosslinking, the MEP—a GL5CC-I27 domain—can exhibit an unfolded state when exposed to a reductant (i.e., DTT) and refolded when re-oxidized (with $H_2O_2$). This translates to direct changes in resultant stress-strain curves, whereby different patterns can be cycled through reversibly based on environmental redox activity. Through these, the group also showcased the granular-level changes in properties such as the network Young's modulus, resilience, and swelling ratio. While modulating redox activity may not be a promising bio-orthogonal avenue, it does lend itself well to insightful investigations of gelation and hydrogel architecture; promisingly, the deployment of this stimulus is not limited to the modulation of properties at the macro scale but can also at the nano scale. Specifically, the Dougan group co-opted the aforementioned strategy and applied it to photochemically crosslinked bovine serum albumin (BSA) networks, which are tied together further by disulfide-bonded "nanostaples." The existence of this secondary crosslink enabled the group to harness a diverse array of protein characterization techniques—most notably circular dichroism (CD) and small-angle scattering (SAS)—to identify the formation of self-similar (i.e., fractal) nanoclusters upon local unfolding, leading to force-labile crosslinks.[171] Going beyond modulating redox activity to control downstream network properties, another robust strategy to cycle through different network profiles consists of reversibly folding-unfolding protein crosslinks through denaturing agents. In a representative example, the Li group showcased a hydrogel consisting of the highly folded globular GB1 or a *de novo*-designed FL domain.[172] Harnessing the reversible folding-unfolding of folded domains upon exposure to a denaturant (guanidine hydrochloride in this report), the group demonstrated solid shape memory and, impressively, were able to construct different multi-material geometries.

Physically dynamic systems can also be codified into fragment reconstitution-type chemical platforms. Although covalent schemes such as isopeptide bond formation between SpyTag and SpyCatcher are irreversible, those based on non-covalent reconstitution such as the GL5CC region of GB1 are dynamic by nature.[173] Splitting the aforementioned GL5CC domain leads to two fragments, termed GN and GC, that predictably assemble non-covalently in solution.[174] Owing to the redox dependency of such a linkage material dynamism can again be imparted by cycling through different redox states, with oxidative environments and low temperatures (approximately 4°C) leading to disulfide bond formation and reduction (coupled with temperatures above 37°C) yielding a more dynamic physical network amenable to subsequent re-patterning. This remains the only example of a dual-component non-covalent protein-based modification that is controlled via a redox mechanism, and it was applied with success for the repeatable tethering and release of an enhanced cyan fluorescent protein (ECFP) and the Tnf3 protein from the tenascin domain. We envision that the identification of similar motifs—either inspired by nature or developed *de novo*—stands to energize work in this space by providing more robust tether-and-release profiles as well as removing the necessity for rather stark temperature changes.

*DNA-based.* Owing to well-established paradigms governing DNA interactions, DNA crosslinks are well suited for modification through different modalities (e.g., heat, pH, enzyme) without extensive redesign of starting macromers. Beyond unidirectional modification, shape memory is a feature implicit to many DNA-based networks, allowing reversible state switching based on underlying gel conditions.

In addition, aptamer technologies have proved uniquely enabling for creating dynamic physically assembled networks that recognize highly specific molecules

(e.g., signaling factors, metabolites, toxins). Harnessing aptamers as DNA antibodies, various groups have successfully built hydrogel vehicles for either therapeutic delivery or alternatively biosafety and bio-detection units. Unlike conventional antibodies, however, aptamers hold the added benefit of construct stability across harsh physicochemical conditions. Beyond aptamer-based technologies, there exist examples of groups who have harnessed the complementarity of designed nucleic acids to impart modifiability into networks. A powerful example of this is provided by the Mooney lab, who exploited nucleic acid complementarity to create a refillable drug depot for extended therapeutic release *in vivo*.[175] As DNA synthesis continues to gain in speed and efficiency, high-throughput screening of aptamer libraries will enable the identification of newer aptamer-target pairs.

## Triggered hydrogel modification

Moving beyond spontaneous reaction schemes for hydrogel modification, triggerable platforms enable temporally and/or spatially controllable avenues for network modulation. Most often, these advances are made possible through photochemical methods, which enable dose-dependent responses and reaction specification in both space and time.

### *Triggered gel modification via covalent reaction*

*Synthetic organic-based.* User-triggered modification of network properties post assembly is a necessary precondition for gaining 4D control. To achieve this capability, stimulus responsiveness is engineered into the hydrogel during assembly in the form of stimuli-responsive crosslinks or, alternatively, stimuli-responsive excess moieties for post-synthetic epitope patterning and biochemical/biomechanical modulation.

Representative of the first strategy, our group engineered a PEG-based hydrogel that could flexibly exercise Boolean logical response,[176] whereby preprogrammed combinations of environmental cues would yield specific downstream outputs (e.g., gel dissolution, therapeutic delivery) (Figure 10A). Showcasing all possible YES/OR/AND logical outputs for a three-input system (i.e., enzyme, light, reductant), 17 distinct stimuli-responsive materials were synthesized, wherein differences in linker structure translated into different material outputs. This logic-predicated response strategy was then extended by our group for release of living cells,[176] proteins,[177] and small-molecule payloads.[178]

For the triggerable introduction of biological signaling factors, our group also harnessed a photocaged oxime ligation strategy to post-synthetically modify PEG-,[180] collagen-, and fibrin-based networks.[181] Post network formation, user-directed light exposure liberates NPPOC-caged alkoxyamines, enabling the tethering of aldehyde-functionalized proteins and anisotropic biological signaling to take place within the network. This strategy is encouraging given the tunability it has afforded photopatterning of natural protein-based gels, typically thought to be more limited in their configurability than their purely synthetic counterparts (Figures 10B and 10C).

Photocontrolled reaction schemes are also a viable strategy for the modulation of networks, enabling either stiffening or softening based on the underlying design (Figure 10D). For softening matrices, an early example was presented by Kloxin and Anseth, who designed photocleavable crosslinks and network tethers based on an *ortho*-nitrobenzyl group moiety, enabling post-gelation modification of the hydrogel in the form of either softening or biomolecule release.[182] Our group also coupled this photochemistry with multiphoton lithography, enabling the creation

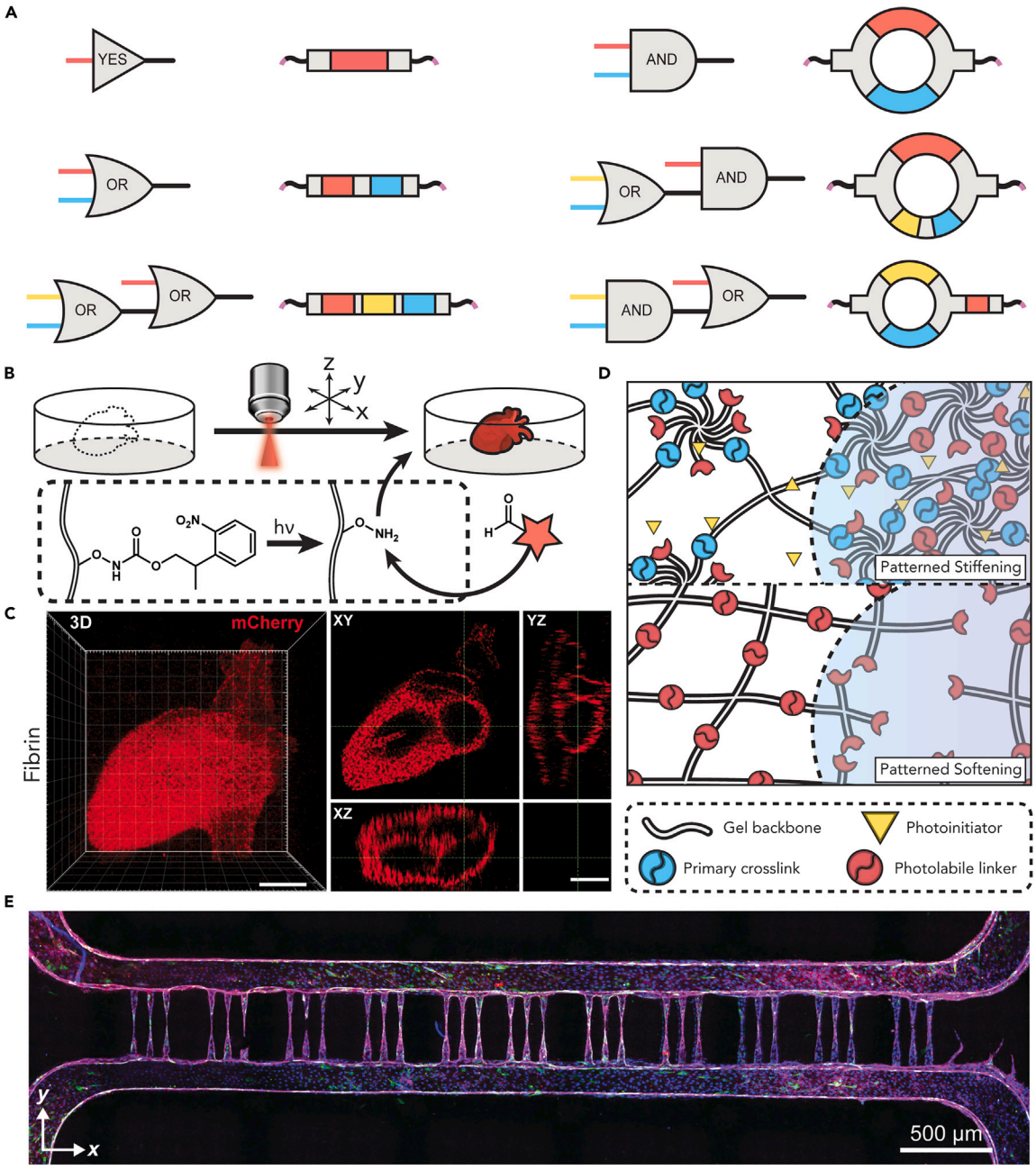



**Figure 10. User-engineered and directed biomaterial responsiveness**

(A) Material inputs such as light, enzyme, and reductant responsiveness can be codified as Boolean logic crosslinkers. Adapted with permission from Badeau et al.[134] Copyright 2018, the authors.

(B) Biological epitopes can be photo-patterned with pristine spatiotemporal control in order to recapitulate native physiological structures *ex vivo* (reaction platform shown here is a photomediated oxime ligation).

(C) Three-dimensional patterning of an anatomical heart is achieved in a fibrin-based hydrogel network through photomediated oxime ligation, showcased with 3D and cross-sectional cut views (mCherry-CHO is shown in red). (Scale bar, 50 µm).

(D) Hydrogel networks can be engineered to reversibly photostiffen/photosoften through judicious engineering of a secondary photolabile linker.

(E) Photodegradation of hydrogel networks can yield shapes and geometries with pristinely conserved features such as endothelialized 3D vascular networks. Adapted with permission from Arakawa et al.[179] Copyright 2020, the authors.

of pristine microvoid geometries at the capillary scale and beyond (Figure 10E).[179,183] Given the broad interest that photomodulation chemistries have garnered, and their limitations with regard to tissue penetration, recent efforts have poured into the development of more red-shifted and more photolabile groups. An example of this was recently demonstrated by our group, wherein, through the design of novel ruthenium-based crosslinks and tethers, hydrogel modulation was successfully effected up to several centimeters deep in tissue.[184]

For stiffening networks, a recent study by the Anseth group details the use of an SPAAC-based network for the study of injury-mediated stiffening environments.[185] Strained alkynes can undergo secondary photocrosslinking in the presence of a suitable photoinitiator, enabling matrix stiffening on demand from a commonly employed reaction scheme. Another example of this strategy is the reversible [2 + 2] photocycloaddition reaction of pyrenes.[186] First harnessed by the Forsythe group to engineer PEG networks using styrylpyrene groups, the light-triggerable nature of the reaction permitted a bidirectional modulation of resultant mechanical properties by controlling the number of formed crosslinks.[187] Additionally, its reversible nature allowed for a potential network disassembly upon exposure to 340-nm light. Subsequent work substituted the styrylpyrene groups for acrylamidylpyrenes,[188] a substitution that enabled the decoupling of light wavelength needed for network formation and network stiffening. The Anseth group also built upon anthracene-enabled [4 + 4]-photodimerization—previously demonstrated as a viable network synthesis strategy[108]—to construct a longitudinally modifiable hydrogel well suited for the interrogation of cellular behavior in stiffening environments.[189] Illumination with cytocompatible 365-nm light induces gelation, and further exposure to the same wavelength leads to progressive network stiffening, resulting in a compositionally simple single-starting reagent tool to study mechanobiology in 4D.

A step beyond one-shot network functionalization would enable repeatable biomolecule patterning in order to fully recapture ECM dynamism and biochemical heterogeneity. An example of this, previously demonstrated by DeForest and Anseth, employs orthogonal photochemistries, with one—the thiol-ene reaction—deployed for biomolecule tethering and another—the photocleavage of an incorporated *ortho*-nitrobenzyl group—toward subsequent release.[190] While robust, such approaches are ultimately limited by cycle repeatability. Toward that end, the Anseth group developed an allyl sulfide-based platform for the theoretically repeatable introduction, exchange, and removal of different biochemical epitopes,[191,192] all of which can occur while retaining the synthetic tether functionality. Taking inspiration from reversible addition-fragmentation chain transfer (RAFT)-based polymerizations,[193] during which the main transfer agent can be natively regenerated, allyl sulfide functional moieties can enable the reversible addition and removal of virtually any reactive thiol-containing compound. This platform has also made possible the amplified photodegradation of hydrogels at light intensities much more attenuated than those used for typical degradations.[194] However, free radicals are prone to degrade proximal proteins over time,[195] and concerns remain over their potentially cytotoxic effects on encapsulated cells, which may limit more widespread use of this platform in spite of its promise. In addition, theoretical cyclability in the aforementioned system is harshly limited because of ligand exchange with the underlying network anchor motif, leading to the degradation and release of the reactive allyl sulfide moieties over time.

*Protein-based.* While spontaneous protein-based chemistries have proved useful in generating networks decorated with different polypeptide moieties, biological

interrogation at multiple timescales can be greatly enhanced with triggerable platforms. Protein-enabled chemistries, particularly "smart" constructs with well-established input-output responses, are suited to that goal.

In the same vein, enzymatic modification workflows can also be engineered such that they present avenues for user-directed photocontrol. A prominent early example of this was demonstrated by the Lutolf group. Building on their prior work showing uniform gel protein patterning with transglutaminase,[196] the group then installed an Nvoc lysine photocage on its cognate peptide substrate such that the ensuing enzymatic ligation reaction is fully abrogated until cage photorelease.[197] This enabled the group to successfully guide protein patterning to user-defined sub-volumes of the network, showcasing arguably the first controllable and fully bioorthogonal hydrogel functionalization strategy targeting full-length proteins such as VEGF and a fibronectin domain. Inspired by this strategy, our group has also looked to control protein presentation in hydrogels through methodologies that preserve native-level bioactivity.[198] Prior to protein immobilization onto gels, sortase-enhanced protein ligation (STEPL) was employed to install a variety of synthetic tethers onto a protein library. These synthetic tethers can be engineered to enable clicking onto virtually any network chemistry. A caged alkoxyamine, for instance, can be used to guide protein patterning to user-definable matrix sub-volumes via a photomediated oxime ligation. Moreover, judicious installation of stimuli-responsive chemical groups, such as photoscissile moieties, can enable spatiotemporally guided protein release within the network.

Beyond enzymes, tools derived from non-opsin optogenetics have proved to be well suited for photoresponsive material development. A prominent example of this is the photocleavable protein (PhoCl), which is the first fully genetically encoded polypeptide with a photoconversion mechanism that leads to backbone scission.[199,200] Derived from the photoconvertible protein mMaple,[201] PhoCl harbors a Kaede green-to-red chromophore within its backbone that undergoes beta-elimination upon illumination with 400-nm light. Rather than photoconvert, as is the case with its predecessor protein, PhoCl was evolved to cleave instead, leaving behind an empty barrel that is no longer fluorescent and a small peptide scar. Moreover, its main value proposition lies in its amenability to expression as a chimera with a wide host of C-terminal fusion partners, which partially explains its widespread adoption both within the optogenetics community and beyond. By recombinantly expressing PhoCl with a suite of C-terminal proteins and N-terminally tagging PhoCl with an azide handle, our group generated SPAAC networks uniformly decorated with photoreleasable proteins.[202] The West lab also harnessed the photoresponsiveness of PhoCl to extend their prior work tethering SpyCatcher fusion proteins into PEG-diacrylate networks, enabling post-synthetic tethering of biological moieties and their subsequent photorelease.[203] Given the versatility of PhoCl as a fusion partner, it has found a prominent application space within hydrogel biomaterials, where it has also been co-opted for the design of networks with tunable mechanical properties[204] and the creation of a solid-phase protein display system.[205]

Beyond the release of bioactive molecules, recapturing the native cellular niche at different scales of spatiotemporal resolution would stand to benefit from full user control over protein activation. Toward that end, our group recently established a versatile method to photocontrol protein ligation via a light-activated SpyLigation (LASL).[206] This method relies on the use of amber codon suppression to introduce an *ortho*-nitrobenzyl photocaged lysine at the critical residue within the SpyCatcher protein, preventing ligation until photo-uncaging. When used to

photoassemble otherwise inactive split protein fragment pairs, this method can reconfer bioactivity onto the stably reassembled protein product. While a promising avenue for future work in protein-protein complementation, especially when considered in tandem with elegant computational approaches for the generation of split proteins from full-length precursors,[207,208] this approach is limited by the following constraints: (1) the parent protein should be amenable to splitting at a site such that both fragments are separately inactive; (2) its split fragments should not spontaneously reassemble; (3) each split fragment should be amenable to expression as either a SpyTag or a SpyCatcher fusion, and lastly (4) it may still prove refractory to systems that collapse into an unfavorable orientation upon complementation, preventing the recapture of native-level bioactivity. Ongoing efforts in our lab seek to sidestep these existing limitations while permitting direct photoactivation of many diverse protein classes in solution, throughout biomaterials, and within living cells.

### *Triggered gel modification via non-covalent reaction*

*Synthetic organic-based.* The application of an exogenous stimulus to direct network modification—ideally in a fully bidirectional manner—is a highly desirable feature to engineer into next-generation hydrogels. Triggerable non-covalent reactions can potentially afford this desired reversibility in hydrogel customization.

In the context of host-guest chemistry-based gels, light responsiveness can be engineered in a straightforward way by having the guest molecule be a stimulus-responsive construct rather than a simple aliphatic chain. For instance, the Stoddart group used an azobenzene as guest within a cyclodextrin host. After spontaneous gelation, they were able to photocontrol reversible sol-gel transitions based on the wavelength of light delivered.[66] In fact, azobenzene in its *trans* conformation docks into the cyclodextrin cavitand, resulting in gelation; upon illumination with 350-nm UV light, the azobenzene adopts a *cis* conformation and results in disassociation from the cyclodextrin core, leading to a gel-sol transition. Subsequent exposure to 450-nm visible light results in a reversion to a *trans* configuration and docking, thereby recapturing the gel state and showcasing the reversibility of the procedure. Engineering photoresponsive host-guest systems has led to the conceptualization of smarter therapeutic delivery vehicles. For example, the Anseth lab has developed a PEG-based azobenzene-cyclodextrin hydrogel for the encapsulation of a model fluorophore-tagged peptide.[209] Inducing a *trans-cis* isomerization by 450-nm light exposure leads to an accelerated rate of peptide release, motivating the potential and possibility for on-site therapeutic release using relatively simple formulations.

*Protein-based.* Controllable protein-based modulation of hydrogel networks is rendered possible primarily by optogenetics-derived dimers that assemble and/or disassemble upon directed light exposure.[210] Given the tunability of these constructs and their amenability to rational optimization, multiple groups have recently sought to co-opt them as hydrogel crosslinks to modify network properties on demand. A report by the Weber group, for example, covalently coupled a monocysteine-containing bacterial phytochrome (Cph1)[211] to a multi-arm PEG through a routine vinyl sulfone reaction.[212,213] Illumination of the protein-PEG conjugates with 660-nm light led to Cph1 dimerization, increasing gel crosslink density and stiffening; 740-nm light shifts the protein construct back to a monomeric state, leading to softening. These changes in the underlying network mechanics—as shown by changes in hydrogel storage and loss moduli—depend on illumination wavelength, light dosage, as well as the amount of incorporated Cph1 dimers. Illustrating the power of this approach, the storage modulus of an example network with 70 mg/mL of incorporated Cph1 dimers can drop by approximately 40% (from a starting point of

approximately 2,500 Pa down to 1,500 Pa) when exposed to 740-nm light (at which most Cph1 components turn monomeric), and regains its initial value upon re-exposure to 660-nm light and subsequent network stiffening. This method is powerful owing to its reversibility—hydrogels can be cyclically stiffened and softened on demand—and the wavelength of light delivered is red shifted, obviating any cytotoxicity concerns. Moreover, it is a compositionally simple platform requiring one protein to be generated and purified. Recent work has extended the dynamic range achievable by this initial iteration—termed CyPhyGel by the group—through the introduction of a single amino acid mutation R472A identified by benchmarking against other phytochrome variants.[214] While a simple mutation, this change increased the dynamic range of achievable stiffness states—measured through the network storage modulus—by approximately 12%. While powerful, both reports fell short of achieving reversible sol-gel transitions, a staple of non-covalent synthetic- and DNA-based crosslinking approaches and often beyond the reach of protein-enabled platforms. Most recently, the group successfully showcased reversible sol-gel transitions by introducing judicious changes to their starting macromers.[215] Eight-armed PEG macromers were deployed (instead of four-armed, as in the earlier reports), and the system was predicated on the heterodimerization of the red/far-red light photoreceptor phytochrome B (PhyB) and phytochrome interacting factor 6 (PIF6). The starting reactants do not gel upon mixing; rather, it is illumination with 660-nm light that leads to heterodimerization and subsequent gelation. Exposure to 740-nm light then reverts the system back to a sol state, with a storage modulus dynamic range of 0–800 Pa.

Another example of reversible modulation of network mechanical properties was showcased by our group. By incorporating either a photoresponsive LOV2-Jα[216] or calcium-responsive calmodulin-M13[217] fusion motif as a material crosslink, we were able to effect cyclic stiffening/softening of PEG-protein gels, respectively blue light or calcium ion exposure.[218] Other strategies looking to achieve similar reversible modulation outcomes have used other optogenetic constructs such as Dronpa145N[219] and UVR8.[220] Many of these non-opsin optogenetic pairs can be scanned for performance and appearance in prior reports in a regularly updated community database named OptoBase (https://www.optobase.org/).[221] While evolved both in nature and at the lab bench with drastically different use cases envisioned, these opto-proteins hold great promise as material-forming chemistries and will likely continue to serve as inspiration for biomaterial scientists looking to create more user-controllable cellular niches.

*DNA-based.* While a relatively new frontier, triggered modification schemes for DNA-based networks are gaining traction in the field. The governing dynamics of DNA stimulus responsiveness are getting increasingly elucidated and codified. Applying these rules to material design may result in constructs with switchable properties such as dynamic ligand presentation and cyclical stiffness states. In arguably the most powerful example showcasing the potential tunability of DNA-crosslinked networks, the Willner group introduced a multi-triggered supramolecular DNA/bipyridinium dithienylethene (DTE) that can be modulated reversibly over time through light, redox switching, or introduction of a crown-ether molecule.[222] This network can be in one of two states depending on the photoisomerization of the DTE group: a closed state in which it acts as an electron acceptor and an open state in which it loses its electron-acceptor properties. Practically, this entails that control of DTE isomerization dictates the downstream triggerability and modifiability of the network through photochemistry, redox changes, or introduction of a crown-ether molecule for electron transfer. Given the ease of cycling through

different DTE states through illumination with UV-visible light, the system can enable near-effortless cycling through different stiffness states while remaining endowed with robust shape memory. Another report by the group showcased photochemical or small-molecule control over a polyacrylamide-based network cooperatively bridged by stimuli-responsive DNA chains.[223] While technically consisting of boronate ester-crosslinked polyacrylamide, the initial hydrogel matrix is cooperatively bridged by nucleic acid sequences that serve as the launchpad for further stimulus responsiveness. In one design, an azobenzene motif is incorporated into the intercalator unit. As discussed previously, azobenzene exhibits robust optical switching properties, basically operating as a robust optical gear to switch the system from higher (60 Pa in storage modulus at wavelengths beyond 420 nm) to lower (20 Pa at a wavelength of 365 nm) stiffness states. In another design, G-quadruplex units stabilized by K+ ions serve as further cooperative crosslinkers instead of the azobenzene intercalator. This allows the group to then cycle through the same high- and low-stiffness states by repeated introduction or removal of a crown-ether small molecule. Another recent example by the Di Michele group deploys G-quadruplex units to physically crosslink hydrogel networks—which the group terms quad-stars—in a one-pot, isothermal, and enzyme-free manner.[224] The G-quadruplex units can then serve as cation-responsive (e.g., K+) crosslinks enabling the cyclical assembly or disassembly of the hydrogel. Alternatively, UV-light exposure in the presence of a porphyrin photosensitizer can also lead to matrix photocleavage.

## TRANSLATIONAL CONSIDERATIONS IN BIOMATERIAL DEVELOPMENT

We hope our above discussion has successfully highlighted the diverse array of chemical and biological platforms that can be deployed to assemble and subsequently modify hydrogel biomaterials. However, we also want to switch our lens and critically assess the translational promise and actual market traction that these bioengineering advances have successfully driven. While all nascent technological (and specifically biotechnological) platforms should be given time to develop at pilot scales before getting stress tested on the market, the time is ripe for hydrogel biomaterials to mature and transition from bench to bedside. Hydrogels first came to the fore as a market reality more than six decades ago in the form of contact lenses,[225] and, promisingly, have since witnessed consecutive foundational landmarks in their study that have promised to expand their use cases far beyond their beachhead application. Among these is the initial Langer and Folkman proof of concept for the deployment of amphipathic and/or hydrophilic polymer matrices for the release of macromolecular drugs (arguably the origin of controlled release as a field proper)[226] and the critical extension and formalization of the space as well as supplementation with predictive mathematical modeling[227,228] put forth by Peppas. In addition, the foundational theoretical-experimental work developed by Bissell in the early 1980s highlighted the importance of the extracellular niche as a critical regulator of gene expression[229] and cellular differentiation and tissue development[230]; in so doing, her work catalyzed the framing and deployment of hydrogel biomaterials as powerful scaffolds for the recreation of the native cellular niche, what we now broadly refer to as tissue engineering.[231,232]

The advances we have surveyed throughout the core of this review expand upon how rational deployment of novel chemical and biological crosslinking platforms stand to sharpen and optimize the use of hydrogel matrices as controlled release depots, implants, and tissue engineering scaffolds, among other adjacent applications.

However, despite a burgeoning palette of seemingly promising crosslinking platforms, comparatively very few hydrogel-based therapies have made it to the clinic; tellingly, in searching for clinical trials (clinicaltrials.gov) that mention "hydrogel," approximately 50% of search results point to contact lenses or variations on ocular implants. Understandably, this leaky translational pipeline has led to much debate and discussion, with an increasing number of manuscripts now dedicated to discussing platforms with concrete translational promise[233] or charting the development of hydrogels in the clinic.[4]

In an effort to fix this leaky pipeline, biomaterial scientists and engineers need to first understand the regulatory hurdles that they will face in looking to transport their technologies to the clinic. Following this exposé, we hope to highlight some market applications and promising technologies for next-generation hydrogel biomaterial deployment.

## Regulatory approval constraints and practical hurdles

A challenging regulatory landscape is the predominant reason for a leaky translational pipeline. Hydrogels are considered a medical device as per section 201(g) of the Federal Food, Drug, and Cosmetic Act, with ultimate product class (i.e., class I, II, or III) dictated by the number and type of payload and additives within the formulation. When a hydrogel biomaterial is designed to be delivered as a standalone construct, as is usually the case for wound healing or anti-inflammation therapies, product champions contend with a development timeline on the order of 1–5 years, as the product is categorized as a less risky class I or II device. The existence of safety predicates owing to older hydrogel products on the market accelerate this regulatory navigation substantially as a result. However, newer-generation hydrogels are typically co-opted for the delivery of therapeutic cargos (e.g., nucleic acids, proteins, living cells, or combinations thereof). For these indications, cases for safety predicates become considerably weaker; as a result, these biomaterials are marketed as class III devices and considered "combination products" in FDA parlance. Practically, this engenders the need for a 510(k) Pre-Market Notification Submission for legal marketing rights within the US, entails a development timeline on the order of 7–10 years, and results in drug development costs that range from $50 million up to $800–900 million, often on the upper end. For perspective, Medtronic's INFUSE—typically considered the landmark hydrogel biomaterial in the clinic—peaked at $750 million in sales in 2011, with revenue levels varying substantially year on year since. Such high development costs severely compromise the translational potential of otherwise promising technologies that may have a pressing clinical need but no substantial market to allow them to recoup initial development costs, as is often the case with many orphan indications.[234]

## Translational promise beyond injectable therapies

While earlier discussion centered on injectable therapies is likely the most broadly relevant, the translational promise of hydrogels should not remain entirely predicated on controlled release and directly adjacent application spaces. Many of the chemistries touched upon throughout this review stand to impart higher-order stimulus responsiveness than that required for smart drug delivery systems, which may render newer constructs over-engineered for controlled release but otherwise uniquely suited for the recreation of pristine ECM niches *ex vivo* for avenues such as disease modeling, value-added chemical bioproduction, and bioprinting. For instance, one of the most pressing hurdles to clear in modern biomedicine is the path toward regulatory approval, which currently stands at a 2023 approval rate of 7.9%.[235] Even when successful, navigating through all requisite phases through to

FDA clearance is a decade-long procedure that routinely incurs multiple hundred millions of dollars in development costs. Clearly, this is a large pain point in the engineering of new therapies, and one to clear rapidly if healthcare innovation is to keep its requisite momentum. A powerful solution to this quandary lies in the development and engineering of organoid models—ideally in a massively parallel manner—that would sidestep the need for lengthy clinical trials by providing an avenue to collecting the same (if not higher) quality clinical data from reproducible tissue biology models.[236,237] A key advantage to well-engineered organoid models is the generation of patient-specific clinical insights based on the recapitulation of near-native tissue signature, which promises to catapult personalized medicine from an academic dream to a market reality.[238] Galvanized by a foundational manuscript showcasing the engineering of a lung "organ on a chip" that successfully mimicked key physiological markers of native lung tissue,[239] multiple startups have emerged and ventured into organ-on-a-chip and organoid model engineering, including Emulate Bio, Herophilus, Chinook Therapeutics (now acquired by Novartis), Organoid Therapeutics, among many others still, each with a slightly different base platform chemistry and/or organoid portfolio in development. Large-cap pharmaceutical companies have also looked to infiltrate and grow organically within this niche; for example, the Institute for Human Biology (IHB) was started at Roche to engineer reproducible disease models at scale for faster drug lead identification. The number of players competing within this space highlights its market promise. Importantly for our discussion in this review, enabling technologies such as controllable bioorthogonal chemistries coupled with pristine biochemical and biomechanical patterning will prove crucial to the development of these technologies, where precise user 4D control and reproducible findings emblematic of native tissue biology are of the highest order.[240,241] Beyond disease model engineering, hydrogels have also proved to be powerful scaffolds for the encapsulation of a wide range of microorganisms, rendering them uniquely suited for biomanufacturing pursuits. Common use cases involve the freeze-drying of hydrogel constructs that harbor a target expression strain, abrogating the need for stringent cold chains in their transport, followed by rehydration and reconstitution at the site of interest. This then enables biomanufacturing at target locations of a variety of value-added chemicals (predominantly hard-to-transport pharmaceuticals) all while bypassing otherwise limiting supply chain constraints.[242,243] Advances in the sourcing of gel materials and the impact of different crosslinking chemistries on encapsulated culture viability and long-term maintenance can then directly translate into more powerful hydrogels-as-living materials for bioproduction.[244]

## Hydrogel biomaterials in the market

Previous reviews have presented significantly valuable contributions in surveying biomaterials that have progressed to the clinic and/or market. Prominent examples of these include a broad landscape of injectable therapies[4,245] as well as relatively recent deep dives into hydrogel biomaterials geared toward musculoskeletal therapy,[246] orthopedic implants,[247] skin-tissue wound healing,[248] and cardiac tissue engineering.[249] Herein, we provide a focused survey of some of the most prominent hydrogel biomaterials that have been marketed across as wide a range of indications and mechanisms of action. We also highlight some promising recent technologies in various stages of development. Beyond listing the platform name and developer, we look to provide some clarity with regard to material composition and crosslinking chemistry wherever possible. Prominent injectable therapies that have been broadly marketed are surveyed in Table 3, and we call out the nuances of the delivery method or underlying mechanism where appropriate.

**Table 3. Survey of representative hydrogel injectable therapies**

| Platform | Company | FDA approval status/year if marketed | Approved indications | Source material | Base chemistry | Delivery method | Mechanism | Other notes |
|---|---|---|---|---|---|---|---|---|
| INFUSE | Medtronic | 2002 (for first indication in spinal fusion) | Spinal fusion, orthopedic trauma surgeries, maxillofacial correction, and currently in clinical trials for further indication | Collagen | Non-covalent interaction between base collagen and encapsulated BMP-2 | Spinal injection | Passive and diffusion-controlled delivery of BMP-2 | Commercial market leader in hydrogel drug delivery systems, achieving peak sales of $750 million |
| Vantas | Endo Pharmaceuticals | 2004 (for first indication in palliative prostate cancer treatment) | Palliative advanced prostate cancer treatment, treatment for early-onset puberty | Poly(hydroxyethyl methacrylate) and poly(2-hydroxypropyl-methacrylate) | Methacrylate chemistry for initial crosslinking with passive non-covalent encapsulation of histrelin acetate (a gonadotropin-release hormone agonist) | Subcutaneous injection (at inner aspect of upper arm) | Passive and diffusion-controlled delivery of histrelin acetate | Discontinued indefinitely as of September 2021 because of manufacturing issues |
| Bukamid | Searchlight Pharma | 2006 | Injected to aid in treating female stress urinary incontinence | Polyacrylamide | Chemically crosslinked polyacrylamide with no further additives or delivered cargo | Transurtheral injection | Acts as a bulking agent when injected. Procedure is a series of injections (three or four), no incisions are necessary, and treatment is long lasting (order of years) | One of two female stress urinary incontinence hydrogel therapies on the market, wit the other (Coaptite) based on a cellulose matrix |
| Belotero Balance | Merz Pharmaceuticals | 2011 | Injected to aid in the correction of moderate-to-severe facial wrinkles | HA | Chemically crosslinked hyaluronic acid with no further additives or delivered cargo | Dermal injection | Acts as a filler designed to integrate into facial skin tissue | First approved in the EU in 2004 making it the first marketed HA-based therapy. Other therapies marketed since have used a similar starting formula or supplemented HA with lidocaine for local anesthesia |
| TraceIT | Augmenix (now acquired by Boston Scientific) | 2013 | Installed to improve tissue alignment to streamline image-guided therapy | PEG | Chemically crosslinked PEG with no further additives or delivered cargo | Percutaneous injection | Acts as a filler; improves tissue alignment for image-guided therapy | |

(*Continued on next page*)

**Table 3. *Continued***

| Platform | Company | FDA approval status/year if marketed | Approved indications | Source material | Base chemistry | Delivery method | Mechanism | Other notes |
|---|---|---|---|---|---|---|---|---|
| SpaceOAR | Augmenix (now acquired by Boston Scientific) | 2015 | Installed as a rectal spacer designed to minimize the side effects of radiation therapy for prostate cancer | PEG | Chemically crosslinked PEG with no further additives or delivered cargo | Rectal injection | Acts as a rectal spacer; no payload to deliver | Remains at the injection site for approximately 3 months post injection |
| OTX-TKI | Ocular Therapeutix | currently in phase II | Injected to treat wet AMD | PEG | Chemically crosslinked PEG with incorporated axitinib, a small tyrosine kinase inhibitor | Intra-vitreal injection | Acts as a delivery agent for the sustained (6 months+) release of axitinib | there currently exists no sustained-release platform for the treatment of wet AMD |
| Neo-Kidney Augment (NKA) | InRegen (now acquired by ProKidney) | Currently in phase II | Injected as a type 2 diabetes treatment as well as kidney disease treatment (both indications are currently pursued) | Gelatin | Chemically crosslinked gelatin with encapsulated renal cells | Kidney injection | Acts a cell therapy scaffold for kidney tissue regeneration | InRegen has also deployed a similar cell therapy strategy for the treatment of chronic kidney disease |
| VentriGel | Ventrix | Currently preparing for phase II | Injected as a repair treatment after myocardial damage | Native myocardial ECM | Unmodified, porcine-sourced native myocardial ECM | Cardiac injection | Acts as a repair scaffold for damaged myocardium microenvironments | – |

Representative hydrogel-based injectable therapies are surveyed and platform specifics such as source material, base chemistry, and delivery method are discussed where appropriate. BMP, bone-morphogenic protein; AMD, age-related macular degeneration.

As can be seen from the highlighted injectable therapies above, a disproportionate number are simple and very often uni-compositional (e.g., hydrogels as fillers, bulking agents, or spacers). Recently, more groups have designed more complex injectables harboring protein, oligonucleotide, or cellular therapies with varying rates of success. Anecdotes of costly and high-profile failures (e.g., recently, FX-322 from Frequency Therapeutics) often deter hopeful entrants with similar material platforms, especially when the target pathophysiology is located at a challenging injection site. This harsh regulatory landscape has contributed to an unsurprising trend whereby relatively simple injectable hydrogels that have already gained FDA clearance for a specific indication get tested for as broad a palette of indications as possible. For instance, Bulkamid, initially developed as a treatment for female stress urinary incontinence, is now undergoing regulatory tests for multiple incontinence indications. In the same vein, TraceIT, first approved for bladder tumor image guiding, is now being tested for a variety of tumors such as pancreatic, rectal, and oropharyngeal.

Going beyond injectable therapies, we also provide an overview into some of the most promising organoid and organ-on-a-chip companies (Table 4), as we believe this niche to be an often-overlooked killer application when looking at hydrogel biomaterial commercial promise. As part of our survey, we highlighted therapeutic focus areas and close with thoughts on the path forward for the field.

What stands out in the case of most organoid platforms has been their historical over-reliance on off-the-shelf source material as the underlying matrix, with Matrigel being the most prominent.[250] While highly enabling because of sourcing simplicity and relative straightforwardness in work-up and preparation, such materials can be limiting across many different aspects. Most prominently, they suffer from severe batch-to-batch variation, leading to a reproducibility crisis in cell culture experiments that routinely deploy them. Moreover, they are not readily amenable to downstream modification, rendering them practically refractory to many of the chemistries and related platforms that can be engineered to create dynamic cell culture environments and, in the process, generate actionable biological insight.[251] We hope to impress the notion that the field stands to benefit greatly from a steady migration from sourced ECM alternatives to "blank-slate"-like starting materials that are amenable to downstream biochemical and biomechanical modulation. The explosion in chemistries that are uniquely suited for spatiotemporal modulation promises to energize the field by imparting both more confident and reproducible results, as well as the possibility to fully recapture the nuances of the extracellular niche *in vitro*.[252] Naturally, aspects such as synthetic tractability and ease of use will come to the forefront of discussion as these chemistries look to achieve market traction.

## CHALLENGES AND FUTURE OUTLOOK

As highlighted in this review, advances in materials chemistry have catapulted hydrogel biomaterials well beyond static frameworks for the simple encapsulation of cells and therapeutics and into the world of 4D customization. Central to this shift has been the enabling power of emerging click, bioorthogonal, and chemoenzymatic reactions that have allowed researchers a much-increased breadth of properties such as tunability of kinetics, biocompatibility, triggerability, and multiplexability. The palette of assembly and post-synthetic modification schemes available for use are manifold, and we hope that this work galvanizes the necessary form of convergent science that bridges different areas of expertise together.

**Table 4. Survey of organoid and organ-on-a-chip companies**

| Company | Year founded | Tissue model portfolio | Brief description of platform technology | Notes |
|---|---|---|---|---|
| Chinook Therapeutics | 2011 (now acquired by Novartis) | Focused on kidney organoid preclinical models for lead generation | Primary kidney cells are induced into organoids when grown in a Matrigel 3D support | Chinook's kidney models have proved immediately fruitful as they have led to six possible drug leads that have cleared phase 1 |
| Emulate Bio | 2013 | Brain-chip, colon-intestine chip, duodenum-intestine chip, kidney chip, lung chip, liver chip | Microfluidic PDMS harboring tissue-mimicking hydrogel culture. Source hydrogel material and crosslinking specifics varies chip to chip, although most likely relies on either Matrigel or a proprietary form of decellularized ECM | Chips for further tissue models are underway; first mover in disease modeling |
| Sengine Precision Medicine | 2015 | Proprietary PARIS test is theoretically applicable to all solid tumors | Primary cancer cells are grown in a Matrigel network to recapture native and patient-specific solid tumor intricacies. Cells are then assayed for particular genotypic and phenotypic markers through bioinformatics analyses | Clinical Laboratory Improvement Amendments (CLIA)-certified platform for identification of optimal chemotherapy drugs or drug combinations for therapeutic intervention |
| Organoid Therapeutics | 2019 | Focused on the engineering of: (1) glandular organoids for clinical implantation as an alternative to pharmacological therapy, and (2) organoids as clinical models | Primary cells are grown on a first-of-its-kind ECM substrate sourced from pancreatic ECM, with advantages being a more familiar and natural microenvironment for encapsulated cellular populations compared to Matrigel | Organoid Therapeutics is one of very organoid engineering las that have side-stepped the use of Matrigel as supporting scaffold |
| Herophilus (previously System1 Biosciences) | 2021 | Focused on the development of primary-cell derived brain organoids for the modeling of neurodevelopmental, neurodegenerative, and neuropsychiatric diseases | Patient-derived neuronal populations are induced into organoids when grown in a Matrigel scaffold | Most leads are still in development across different indications, with a promising candidate for Rett syndrome furthest in development |
| IHB – Roche | 2023 | NA | NA | This represents the first greenfield investment by a large pharmaceutical company looking to develop in-house tissue models |

Notable organoid and organ-on-a-chip companies are surveyed and platform nuances are highlighted where appropriate. PDMS, polydimethysiloxane; NA, not available.

However, we do want to highlight the multiple challenges standing in the way of broad commercial adoption of modern biomaterial systems. While the past decades of research have made possible dozens of novel chemical platforms encoding expanded biomaterial functionalities, the pipeline from bench to market has proved to be problematically leaky. In large part, this can be explained by first-order constraints—defined here as the near-inevitable engineering design problems and necessary nuanced tradeoffs—such as the difficulty of achieving an optimal set of mechanical properties (e.g., in the case of injectable therapies, exquisitely tuned shape memory has to be engineered *a priori* into the material) or showing good biocompatibility and avoiding unwanted immune responses (particularly important in the case of implants or drug delivery depots). Second-order constraints, however, are also highly important in the broader context of hydrogel biomaterials achieving their commercial promise; we define these as more general problems and perhaps non-obvious concerns that should be on the minds of scientists and engineers looking to push their materials into broader commercial applicability. These constraints include, but are not limited to, (1) limitations of the underlying material platform with regard to breadth of applicability, generally due to over-engineering; (2) the particularly challenging regulatory landscape that hydrogel biomaterials have to navigate en route to approval; and (3) the difficulty of achieving meaningful scale-up of materials with many chemistries in academic laboratories.

## Complexity versus simplicity in biomaterial design

The increased dynamism and stimulus responsiveness engineered into newer hydrogel networks has necessitated a steady concomitant increase in their design complexity. While highly enabling of biological interrogation, such systems have often fallen beyond the reach of labs that would make the most use of them and have inadvertently dimmed their own translational potential. As it stands, many next-generation constructs are powerful but over-engineered, necessitating a near-perfect confluence of artificial experimental conditions or parameters, beyond which they do not hold much value. Debates have ensued over whether complexity as a design principle should be embraced in biomaterial engineering[253] or whether efforts should be put into creating systems that are simpler and more tractable by comparison instead.[254] The answer most likely falls somewhere in the middle: researchers should note how enabling newer and more "complex" platforms—either operating alone or in tandem with other chemistries—have been for different use cases. However, new developments in the space should also be evaluated on their portability and ease of use across different contexts for a more holistic assessment of true translational promise.

## Regulatory landscape for hydrogel biomaterials

A key roadblock for hydrogel biomaterials is the particularly complicated regulatory landscape they are required to navigate. With frustratingly few exceptions, all hydrogel-based products are categorized as devices in the eyes of the FDA, as mentioned earlier. When used as depots for drug or cell therapies, they are designated as combination products. Practically, this implies that any potential hydrogel-encapsulated therapies have to undergo an additional 510(k) Pre-Market Notification submission and are looking at close to a decade prior to approval for their indications, severely hampering any commercial viability. The solution to this goes beyond the bench and should take the form of lobbying for faster approvals, particularly in cases where promising precedents have been established with regard to safety, efficacy, and biocompatibility.

## Achieving material scale-up

Should an enabling chemical platform for the synthesis of evolvable networks show promise beyond the lab bench, an immediate consideration should be whether

achieving meaningful material scale-up is possible in the first place. Highly costly materials will likely not find much traction in the marketplace, and many biomaterials, while elegant on paper and in particular killer applications, are severely compromised with regard to translational promise when their synthetic intractability or other adjacent caveats (e.g., shelf life, ease of use) are brought to the fore. Ready material availability and overall portability thus should be critical factors when evaluating the promise of novel chemistries used for biomaterial engineering.

### Parting thoughts

Biology is a dynamic discipline at every level, from singular signaling pathways to broader systems-level views of organismal development. Proper interrogation of such a complex and multi-layered environment necessitates a convergence of disciplines and areas of expertise to guide the design and engineering of environments that enable proper design, sensing, and interrogation of biology in 4D. We believe the selfsame multi-disciplinarity that initially conceptualized and defined the field of hydrogel biomaterials will provide it its requisite impetus to pose and answer the next generation of questions and develop newer theranostic modalities. Specifically, we envision the next frontier of biomaterials science and engineering to be enabled by disciplines previously thought to be fully orthogonal, such as optogenetics, *de novo* protein design, high-throughput materials discovery, and advanced analytics tools. While challenging to bridge together all these sets of expertise within the umbrella of a singular lab, it will become all the more necessary in the medium to long term for these synergies to be captured if the field is to converge onto solutions to its longest standing problems.

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## AUTHOR CONTRIBUTIONS

R.G. and C.A.D. discussed and wrote this review with feedback from R.M.F. Figures were prepared by R.M.F. with input from R.G. and C.A.D.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

## INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

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