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Review

Conjugated Protein Domains as Engineered Scaffold Proteins

30th Anniversary Review

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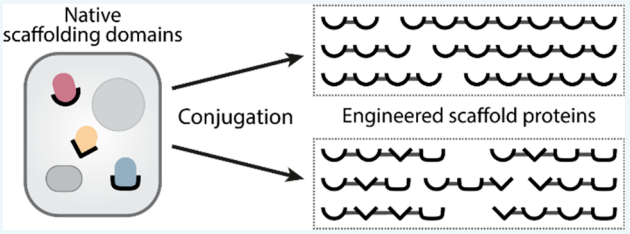
ABSTRACT: Assembly of proteins into higher-order complexes generates specificity and selectivity in cellular signaling. Signaling complex formation is facilitated by scaffold proteins that use modular scaffolding domains, which recruit specific pathway enzymes. Multimerization and recombination of these conjugated native domains allows the generation of libraries of engineered multidomain scaffold proteins. Analysis of these engineered proteins has provided molecular insight into the regulatory mechanism of the native scaffold proteins and the applicability of these synthetic variants. This topical review highlights the use of engineered, conjugated multidomain scaffold proteins on different length scales in the context of synthetic signaling pathways, metabolic engineering, liquid−liquid phase separation, and hydrogel formation.



INTRODUCTION

Cell signaling is controlled by assembly of signaling proteins into higher-order complexes, which facilitates the coexistence of multiple signaling cascades.[1] Through organization of signaling enzymes by scaffold proteins, spatiotemporal control over specific pathways is achieved.[2,3] Scaffold proteins are defined as organizing platforms that link together at least two protein partners.[4] Although these platforms typically do not possess any enzymatic activity, their specific recruitment of signaling proteins provides a tightly controlled and dynamic regulation mechanism for cellular signaling.[5,6] New cellular regulatory circuits and behaviors have been proposed to arise from recombination of the highly modular scaffold domains rather than the generation of new protein functions.[7,8] By rewiring these domains in different combinations, the finite set of native scaffold domains allows for the generation of a huge variety of signaling behavior.[9]

Multidomain scaffold proteins (MDSPs) can be divided into two classes: self-assembling or covalent scaffolds. Self-assembling scaffolds are generated through covalent coupling of scaffold domains to self-assembling units, such as peptide tags[10]—including leucine zippers,[11] or self-assembling proteins.[12] Covalent scaffolds are engineered through genetic conjugation of scaffold domains via linkers. Here, we highlight this second class of conjugated multidomain scaffold proteins. Libraries consisting of well-defined synthetic modules have been generated by conjugation, either through multimerization or recombination of native scaffold domains (Figure 1A). These precisely designed synthetic platforms aid elucidation of various molecular mechanisms, such as plasticity of pathways,[13,14] evolutionary recombination,[15,16] nonlinearity in

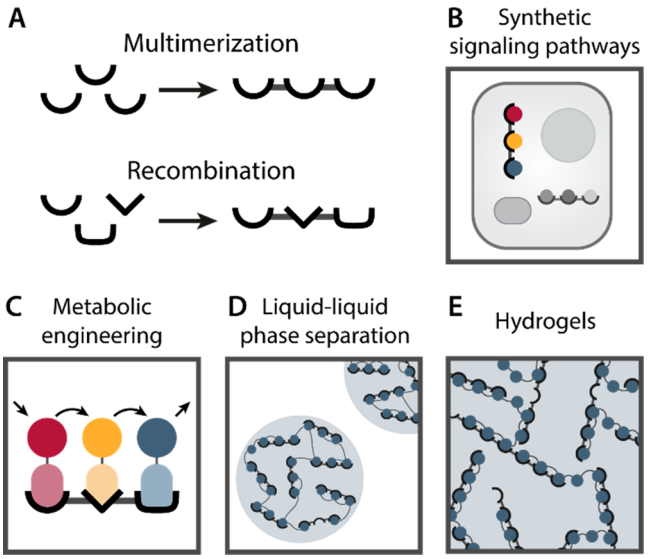


Figure 1. (A) Engineered scaffold proteins by modular domain conjugation via multimerization and recombination have applicability on different length scales in various fields such as (B) synthetic signaling pathways, (C) synthetic metabolons in metabolic engineering, (D) liquid−liquid phase separated systems, and (E) hydrogels.

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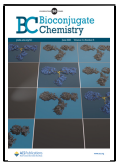

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signaling output,[17,18] phase transition,[19] and the effect of multivalency.[20,21] Fundamental insight into these mechanisms reveals how conjugated protein domains, forming engineered scaffold proteins, can be used as regulators of in- and output of signaling pathways[22,23] and generates understanding of their higher-order assembly into networks.[24]

Engineered MDSPs have been applied across multiple length scales in various fields of interest for different purposes. In synthetic signaling pathways, *in vivo* introduction of single synthetic modules allows for analysis of plasticity of native signaling pathways and the introduction of new functionalities (Figure 1B). Synthetic multienzyme assemblies are applied to optimize enzyme reaction rates and reaction efficiency (Figure 1C). Fundamental understanding of the composition and formation of liquid−liquid phase separation (LLPS) of signaling molecules into microscale biomolecular condensates can be achieved by analysis of synthetic LLPS systems (Figure 1D). Predictable tuning of hydrogel systems is achieved by detailed analysis of higher-order network formation of well-defined multidomain components (Figure 1E). As application of MDSPs within these different fields results in structures ranging from the nano- to the macroscale, the synthetic scaffold proteins at hand entail deviating requirements. This has led to complementary molecular insights into the function of native scaffold proteins, the role of recombination in evolutionary innovation, and general applicability, also outside the context of cellular signaling.

SYNTHETIC SIGNALING PATHWAYS

The role of scaffold proteins in controlling information flow within signaling pathways, whether they simply tether components or play a more active role, has been investigated extensively using synthetic variants of native scaffolds. This strategy has been widely applied to the well-characterized scaffold proteins of various mitogen-activated protein kinase (MAPK) pathways. Park et al. generated synthetic Ste5 and Pbs2 scaffolds and tested whether non-native protein−protein interactions could be used to mediate proper mating pathway function.[13] Via known mutations in Ste5, recruitment of interaction partners could be selectively abolished, which resulted in a nonfunctional mating pathway. The re-recruitment of the specific interaction partner via artificial interactions with heterologous conjugated domains resulted in restoration of the mating response. Furthermore, a diverter scaffold was generated by head-to-tail fusion of two MAPK pathway scaffolds, which was able to link the input of one pathway (α-factor) to the output of the other pathway (osmo response) (Figure 2A).

Plasticity of the MAPK pathway was further investigated by Peisajovich et al. by recombination of the various scaffold domains belonging to the different MAPK pathways. A library of chimeric scaffold proteins was generated from the domains of 11 MAPK scaffold proteins.[15] Of the 66 recombined scaffolds, 10 variants showed dynamic behaviors different from wild-type and their corresponding noncovalent coexpressed domain pairs. Of these 10 scaffolds, 7 created novel links between different signaling complexes. This high frequency of novel signaling behaviors, arising from a limited library, suggests high importance of domain recombination in the evolution of cellular signaling networks. In a later study, a library of 3375 chimeric MAPK scaffold variants—all possible conjugations of 15 domains and 3 positions—was investigated.[16] Interestingly, of the 4 recombined synthetic scaffolds

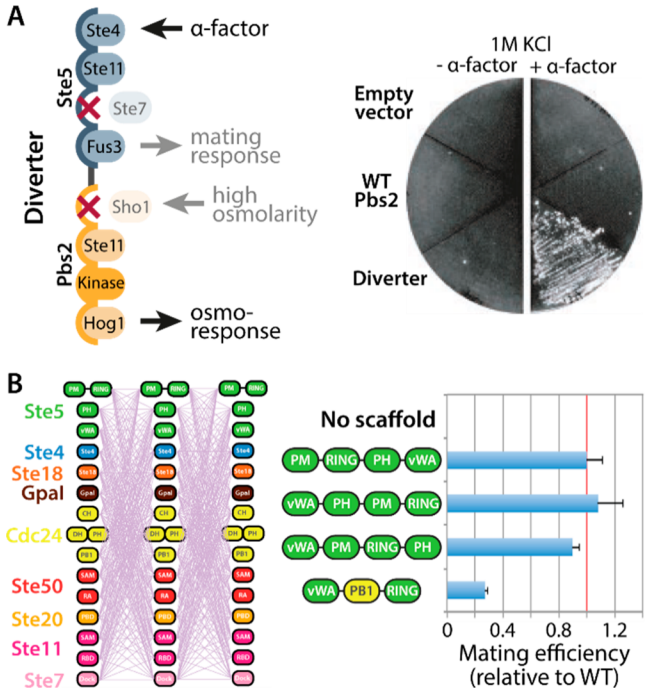

Figure 2. Plasticity in the MAPK pathway by modular recombination of MAPK scaffold domains. (A) Design of a synthetic diverter scaffold upon head-to-tail conjugation of the Ste5 (blue) and Pbs2 (yellow) scaffold and mutational disruption of the Ste7 and Sho1 binding sites. Ste11 participates in both pathways, facilitating the diversion of α-factor input to an osmo-response output. In the presence of α-factor, only strains expressing the diverter scaffold survive high-osmolarity medium. Reprinted with permission from ref 13. Copyright 2003, AAAS. (B) 3375 synthetic scaffolds created from the recombination of 15 MAPK scaffold domains. Quantitative mating efficiency for synthetic scaffolds capable of mediating a pheromone-dependent response show that the mating response can also be mediated by modular domain recombination. From ref 16. Copyright 2015, American Chemical Society.

capable of mediating a pheromone-induced response, 3 scaffolds contain the Ste5 domains (Figure 2B). These results indicate that all scaffold domains are required for a proper physiological response, but modular recombination of their order is allowed.

To elucidate the prerequisites for the scaffold protein to mediate the mating response, whether sole recruitment of pathway enzymes is sufficient, Ryu and Park generated a synthetic protein scaffold.[14] The synthetic scaffold consisted of an MTD (membrane-targeting domain) conjugated to PDZ (PSD95, Dlg, and ZO-1) interaction domains, thereby generating a synthetic platform for recruitment of PDZ target peptides fused to the pathway enzymes, Ste11, Ste2, and Fus3. Only the entire synthetic scaffolds consisting of both the MTD and the PDZ domains were able to mediate a galactose-induced mating response. Additionally, a PDZ valency-dependent induction increase was observed, with a minimal requirement of 2 domains (Figure 3A). As the pathway consists of 3 enzymes, this indicates the possibility of enzyme switching or cross-activation via other scaffolds clustered at the plasma membrane.

The ability of the Ste5 scaffold protein to serve as a platform to systematically reshape output of the mating pathway was shown by Bashor et al.[17] The output of the pathway was linked to the expression of pathway modulators. Recruitment of these

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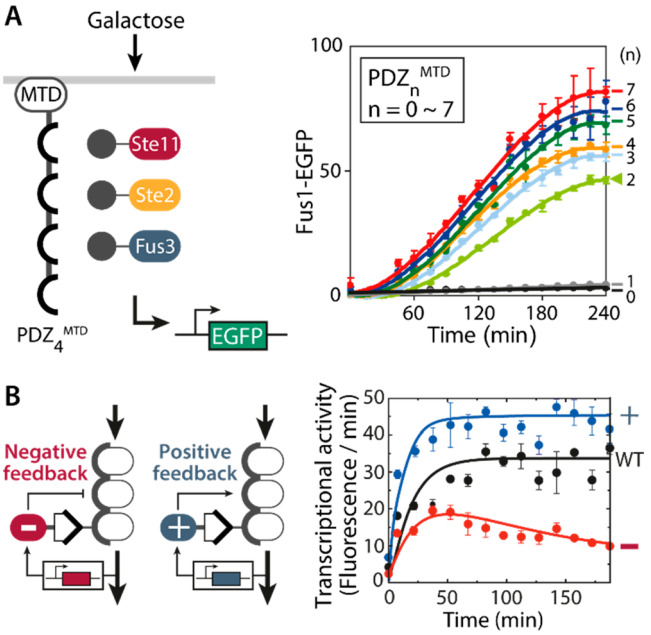

Figure 3. Synthetic scaffolds can mediate and alter pathway responses. (A) Synthetic scaffolds consisting of a membrane-targeting domain (MTD) and *n* PDZ scaffold domains (n = 0−7) were used to recruit Ste11, Ste7, and Fus3 conjugated to PDZ ligands. Time-resolved measurements of Fus1-EGFP induction upon activation by galactose for the various PDZ_n^{MTD} synthetic scaffolds show a valency-dependent response. Reprinted with permission from ref 14. Copyright 2015, AAAS. (B) Negative and positive feedback loop design. Modulators are expressed from a mating-responsive promoter which are then recruited to the Ste5-complex via an artificial recruitment domain and modulate pathway flux. The negative feedback circuit (red) shows an initial increase in transcriptional activity, followed by a decrease. The positive feedback circuit (blue) shows higher transcriptional activity compared to wild-type (WT, black) activity. Reprinted with permission from ref 17. Copyright 2008, AAAS.

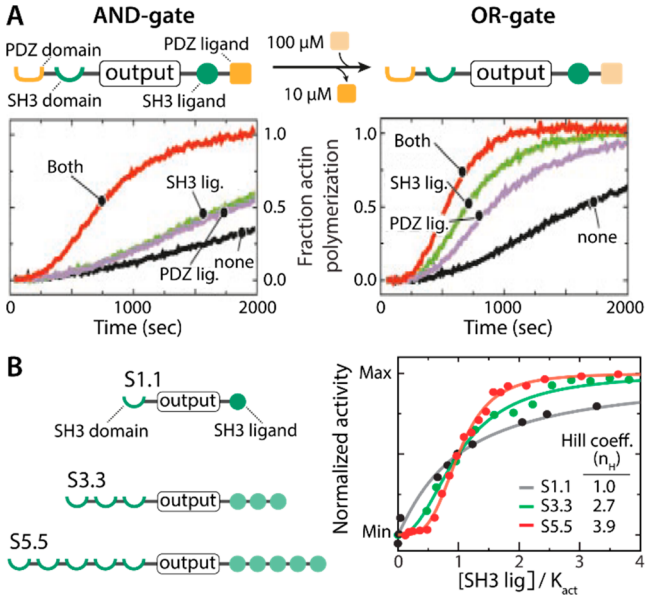

Figure 4. Synthetic modules altering output response. (A) The PDZ/SH3 switch resembles an AND-gate; strong activation is observed only upon addition of both SH3 and PDZ ligands. By interchanging the intramolecular PDZ ligand (10 μM affinity) with a weaker binding PDZ ligand (100 μM affinity), the switch resembles an OR gate (right), in which the individual ligands already yield relatively strong activation. Reprinted with permission from ref 26. Copyright 2003, AAAS. (B) Ultrasensitive switch designs. Comparisons of input/output functions for switches S1.1, S3.3, and S5.5; each switch's relative activity is plotted as a function of the concentration of input ligand normalized by K_{act}. Observed ultrasensitivity scales with the number of autoinhibitory interactions. Reprinted by permission from ref 18. Copyright 2007, Springer Nature.

modulators to an artificial binding site on Ste5 resulted in synthetic positive- and negative-feedback loops, as the transcriptional activity was either increased or decreased with respect to wild-type activity (Figure 3B). By further expansion of this modulator recruitment toolkit, diverse response behaviors such as acceleration, delay, pulse generation, and ultrasensitivity could be engineered. Using similar synthetic modulator recruitment scaffolds, Wei et al. studied the effect of recruitment of bacterial virulence to the Ste5 and Pbs2 scaffolds.[25] These pathogen effector proteins induced alterations in the pathway time-dependent dynamics, making them valuable synthetic biology tools.

Collectively, the application of conjugated multidomain scaffolds to generate synthetic MAPK signaling pathways has shown the higher-order role of scaffold proteins as signal-processing hubs. Serving as the target of feedback loops, scaffold proteins alter signaling amplitude and timing. Furthermore, recombination of the scaffold domains has shown that scaffolds are modular and flexible organizing centers of which the response can be modulated by simple alterations or rearrangements of the recruitment domains.

Besides synthetic variants of MAPK scaffolds, various other synthetic modules have also been engineered to study signaling circuits. Synthetic equivalents of complex allosteric gating signaling switches—such as the actin regulatory switch N-WASP (neuronal Wiskott-Aldrich syndrome protein)—were generated by Dueber et al.[26] These allosteric switches consist of an output domain conjugated to a PDZ domain with a SH3 (SRC Homology 3) domain conjugated to their respective ligands (Figure 4A). Intramolecular recognition induces a conformational change of the output domain, thereby switching the module to the OFF-state. Addition of high-affinity ligands results in dissociation of the intramolecular ligands, switching the module to the ON-state. The resulting switches are functionally modular; simple substitution of the high-affinity intramolecular PDZ ligand (10 μM) for a lower-affinity PDZ ligand (100 μM) showed transition in behavior from an AND-gate to an OR-gate (Figure 4A). A range of different gating behaviors was obtained by altering parameters such as linker length, output domain, and intramolecular ligand affinities. This strategy was further expanded toward ultrasensitive input/output control upon introduction of tandem SH3 domains and intramolecular ligands, in which the switches showed a valency-dependent increase in sensitivity (Figure 4B).[18] Intramolecular scaffold−ligand interactions can also be exploited for the generation of a synthetic autoinhibited scaffold. Aper et al. used the native interaction between the natural bivalent scaffold 14-3-3[27] and one of its ligands, ExoS, to generate covalently conjugated, autoinhibited scaffolds.[28] By incorporation of protease recognition motifs in the linker between the domains, protease-activatable scaffold proteins were created. Versatility of these scaffolds was shown in context of synthetic signaling networks[29] and self-activation. Collectively, these synthetic scaffold switches provide insight

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into functionality and modularity and how complex natural switches facilitate cellular gating behaviors.

The interplay of scaffold proteins and kinases was investigated by Hobert and Schepartz, who reported a miniature-protein-based scaffold to template phosphorylation of a latent substrate, hDM2, by the Hck kinase.[30] Similarly, Taz phosphorylation was directed by Whitaker et al. upon conjugation of the kinase to tandem SH3 scaffold domains.[31] A bridging module consisting of a SH3 ligand and a leucine zipper was used to recruit complementary leucine zippers conjugated to kinase substrates. Via systematic alterations of the scaffold and bridging module, autoinhibition and combinatorial inhibition were shown. By applying the classic principles of proximity-induced reactions, via the introduction of orthogonal interaction domains, the dynamics of kinase activity could be altered. These results indicate that such passive protein scaffolds can play an active role by directing enzyme activity. Additionally, certain scaffold proteins, such as 14-3-3, bind phosphorylated ligands, which can be exerted to engineer synthetic modules. Kinase activity sensors were generated by Xu et al. comprising 14-3-3 conjugated to small NanoBiT.[32] Phosphorylation of a bivalent kinase recognition motif conjugated to large NanoBiT resulted in binding to the 14-3-3 scaffold, thereby complementing the full luciferase.

METABOLIC ENGINEERING

Synthetic scaffolds can also be exploited for the generation of artificial metabolic pathways. Within synthetic enzyme complexes, enzymes exerting different activity levels can lead to suboptimal pathway flux through the accumulation of intermediates. Optimization of enzyme stoichiometry can be used to overcome this flux imbalance. To achieve such regulatory control over stoichiometry, Dueber et al. introduced a synthetic scaffold consisting of three orthogonal scaffold domains—GBD (GTPase binding domain), SH3, and PDZ.[33] By conjugation of their respective ligands to the three enzymes of the mevalonate pathway, selective recruitment of those enzymes to the synthetic scaffolds was achieved. The resulting complex ($GBD_1SH3_1PDZ_1$) showed a 1.4-fold increase in mevalonate production over the unscaffolded pathway. By varying the stoichiometry of the different scaffold domains, the production levels could be increased, with an optimum (77-fold) for the $GBD_1PDZ_2SH3_2$ scaffold (Figure 5A). Using similar synthetic multidomain scaffolds, the biosyntheses of, e.g., glucaric acid,[34] resveratrol,[35] butyrate,[36] (deoxy)-violacein,[37] and L-serine,[38] have been optimized, showing the modularity of this scaffold.

Of special interest within metabolic engineering are pathways inspired by native cellulosomes. The central feature within these metabolons is the cohesin−dockerin pair, which is a high-affinity protein complex that allows for position-specific incorporation of enzymes (Figure 5B).[39] Although the function of native cellulosomes is to degrade cellulose, the various cohesin scaffolds and dockerin ligands allow for modular assembly of synthetic cellulosomes and new metabolic pathways. The potential utilization of cellulosome hybrids and recombination of its domains for various applications was first acknowledged by Bayer et al.,[40] who also generated the first *in vitro* synthetic cellulosome.[41] Innovations within this field have led to the construction of higher-order cellulosomes,[42] the introduction of noncellulosomal enzymes,[43] and their introduction within organisms, such as bacteria[44−46] and *S. cerevisae*.[47,48] The cohesin−dockerin systems have also been

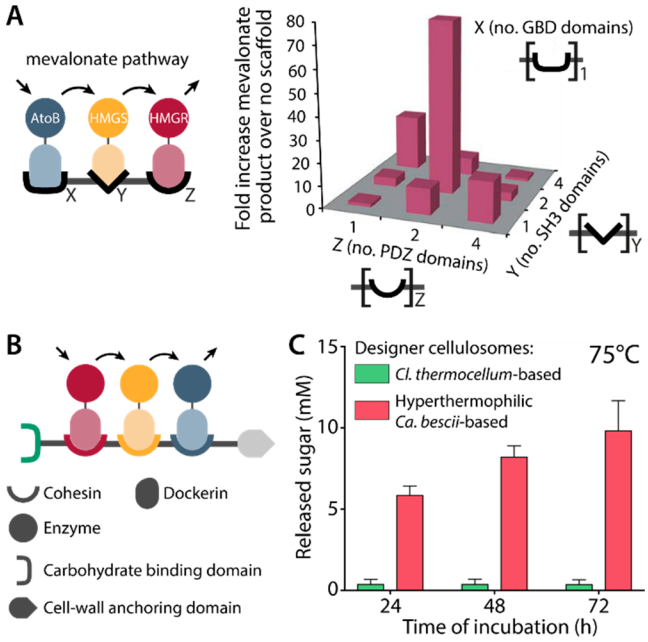

Figure 5. Synthetic scaffolds provide modular control over metabolic pathways. (A) Synthetic scaffolds to control the mevalonate pathway constructed by conjugation of three interaction domains (GBD, SH3, and PDZ), where *x*, *y*, and *z* represent the number of repeats, respectively. Optimizing the number of recruitment domains ($GBD_1SH3_2PDZ_2$) for maximum pathway flux resulted in a 77-fold increase in product titer compared to the non-scaffolded pathway. Reprinted with permission from ref 33. Copyright 2009, Springer Nature. (B) Schematic overview of synthetic cellulosomes, consisting of conjugated cohesin domains, often with a carbohydrate binding domain and, if required, a cell-wall anchoring domain. Dockerin-fused enzymes can be recruited to the scaffold via interaction with the cohesin domains. (C) Comparison of the activity of *Cl. thermocellum*-based designer cellulosome (green bars) versus hyperthermophilic *Ca. bescii*-based designer cellulosome (red bars) at 75 °C, using the same enzymes. The designed hyperthermophilic cellulosome shows better thermostability, with higher concentrations of product formed at 75 °C. From ref 57.

utilized for nonmetabolic purposes, such as protein purification,[49,50] biosensors,[51] and building blocks for *in vitro* synthetic biology projects.[52] Early systems used cellulose as the substrate; later, modularity of cohesin-scaffolds and dockerin-fused enzymes was utilized in the field of sustainable biosynthesis.[53−55] Recent advances in the field of cellulose degradation have focused on transferring the cellulosomal technology to industrial settings. For example, cellulose systems are introduced into microbes which lack the required biosynthesis ability but are tolerant of low pH and ethanol.[56] Additionally, hyperthermostable cellulosome variants are generated to remain stable during exothermic processes.[57] Kahn et al. used cohesin-dockerin pairs from the thermophilic microbe *Ca. bescii*, the resulting designer cellulosome showed higher activity at 75 °C than the native *Cl. thermocellum* (Figure 5C). Since in both systems the same enzymes were used, these results highlight the importance of the scaffold stability on the whole cellulosome complex.

The field of metabolic engineering has led to insights into the generation of various synthetic metabolons relying on pathway assembly of enzymes onto conjugated multidomain scaffold proteins. Recombination of these domains allows for optimization of metabolic flux and the introduction of new

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biosynthetic pathways, thereby greatly expanding the functional application of synthetic protein complexes.[58]

LIQUID–LIQUID PHASE SEPARATION

Cellular signaling is tightly regulated in time and space through various mechanisms such as classic organelles, scaffold proteins, and membraneless organelles. These membraneless compartments, also called biomolecular condensates, function to concentrate proteins and nucleic acids. A review by Banani et al. summarizes both the cellular and biochemical assays that have provided insight into the molecular regulation of these biomolecular condensates.[24] Here, we focus on the molecular insights that have been gained, specifically via the application of conjugated multidomain scaffold proteins. Multidomain proteins allow for precise control of valency and monovalent affinity, thereby serving as ideal model systems.

Li et al. generated multidomain scaffolds by conjugation of 1 to 5 SH3 domain repeats, which interacted with the ligand composed of 1 to 5 conjugated repeats of PRM (proline-rich motif) ligand.[59] At low concentrations and low valency, these solutions were clear, while at high concentrations and higher valency they showed the presence of phase-separated droplets (Figure 6A). The phase-separation behavior of the multivalent natural nephrin–Nck–N-WASP system was analyzed both *in vitro* and *in vivo*, which showed the requirement of all three components for the formation of droplets. In an artificial system, Banjade and Rosen observed a sharp transition in clustering of Nephrin, Nck, and N-WASP upon increasing concentration.[61] This behavior is indicative of a critical concentration required for clustering, resulting in phase separation. It was found that this behavior is highly dependent on the valency of the proteins and the interaction strength between the proteins.

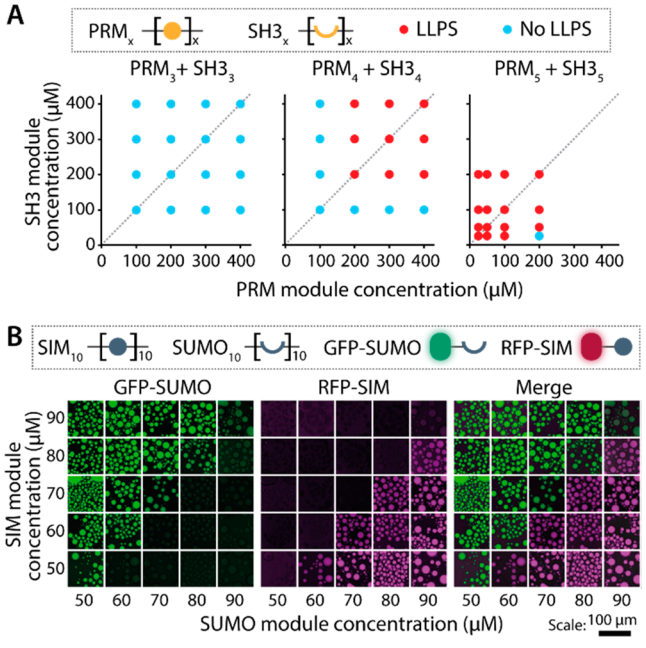

Figure 6. Synthetic multidomain scaffolds allow systematic analysis of prerequisites for liquid–liquid phase separation (LLPS). (A) Phase diagrams of multivalent PRM_{3-5} and $SH3_{3-5}$ proteins. Red circles indicate phase separation, and blue circles indicate no phase separation. Reprinted by permission from ref 59. Copyright 2012, Springer Nature. (B) Phase diagram position dictates client recruitment. Solutions of multivalent scaffolds plus the indicated clients were imaged for client fluorescence. GFP-SUMO (green) and RFP-SIM (magenta) (100 nM each) were mixed with the indicated module concentrations of polySUMO and polySIM. Reprinted with permission from ref 60. Copyright 2016, Elsevier.

The compositional regulation within these cellular bodies was investigated by Banani et al. via the introduction of low valency clients.[60] By varying the concentrations of a synthetic $SUMO_{10}$ (small ubiquitin-like modifier) scaffold and SIM_{10} (SUMO interacting motif) ligand, partitioning of monovalent GFP-SUMO and RFP-SIM was followed (Figure 6B). A sharp transition in recruitment was observed based on the relative stoichiometries of the scaffold and ligand. Additionally, droplet composition is strongly influenced by client valency, as larger magnitudes of maximum partitioning were observed for di- and trivalent clients compared to their monovalent equivalents. Recruitment of GFP-SUMO and -SIM clients into endogenous cellular bodies (promyolocytic leukemia nuclear bodies) in U2OS cells showed selective and valency-dependent partitioning analogous to the synthetic model system.

Collectively, these synthetic systems show that sharp transitions observed in liquid–liquid phase separation are driven by multivalent interactions between scaffolds and their respective ligands and provide a model for the subsequent recruitment of lower valency clients within these droplets. The partitioning of cellular signaling molecules within these phase separating systems thus provides a regulatory mechanism for generating nonlinearity in signaling pathways.

HYDROGELS

Protein-based supramolecular hydrogels allow for tuning and tailoring the viscoelastic properties by molecular-level design, as both the interaction strength between the domains and the amount of scaffold domains per chain can be varied. Various protein-based hydrogels exist;[62,63] however, here we specifically focus on the application of conjugated scaffold domains. Wong Po Foo et al. engineered protein-based hydrogels from conjugated WW scaffold domains (C), which interact with conjugated proline-rich ligand peptides (P) (Figure 7A).[64] Mixing-induced two-component hydrogels (MITCH) are formed upon mixing of the scaffold CX (where X represents the amount of conjugated domains) with the ligand PX. Microrheological measurements of various CX and PX mixtures demonstrated clear differences in viscoelastic properties. The low functionality mixtures—C3:P3, C3:P9, and C7:P3—showed liquid-like behavior, in contrast to the high functionality mixture—C7:P9—which showed hydrogel behavior (Figure 7B). This behavior is dependent on the interaction between both components, as omitting either component results in liquid-like behavior (Figure 7C).

In a follow-up study, tunability of this gel was investigated by determining the viscoelastic properties for various component densities and ratios.[19] The density of the two components greatly affects the hydrogel-forming ability, as only higher weight percentages of components—7.5 and 10% w/v—showed gel-like behavior (Figure 7D). The hydrogel-forming ability is less dependent on the C:P ratio, as hydrogel formation is observed across various C:P ratios. Interestingly, gels with a C:P ratio of 0.5—rather than 1:1 stoichiometry—exhibited the highest degree of elasticity and formed the strongest network. Isothermal titration calorimetry analysis of the C7 scaffold with either single ligand (P1) or the ligand chain (P9) revealed 7.46 or 2.32 apparent binding sites per C7

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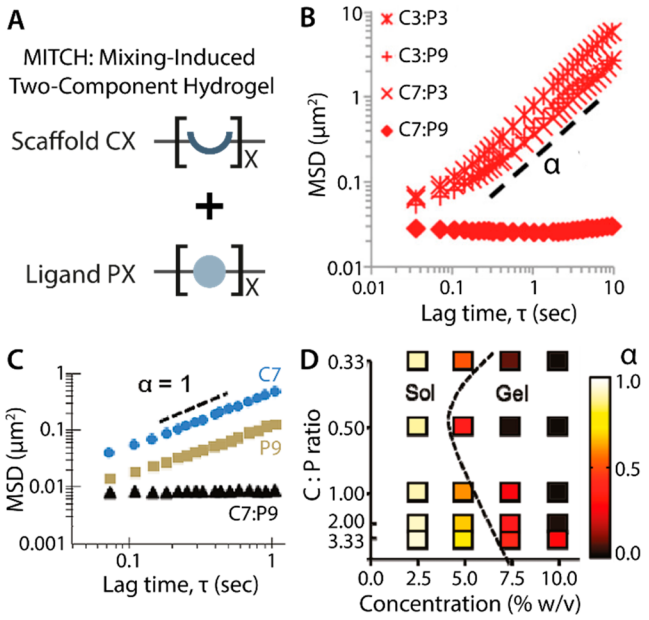

Figure 7. Tunable hydrogels via synthetic multidomain scaffold proteins. (A) MITCH consists of scaffold CX and ligand PX (X denotes the amount of repeats). (B) Microrheology of C3:P3, C7:P3, C3:P9, and C7:P9 (7.5% w/v). Reprinted with permission from ref 64. Copyright 2009, National Academy of Sciences. (C) Microrheology of C7, P9, and C7:P9 (10.0% w/v). From ref 19. Copyright 2011, American Chemical Society. (D) Phase diagram of C7:P9 mixed in different ratios and at different % w/v. The dotted line is a visual guide to separate the liquid phase ($\alpha > 0.55$) from the hydrogel phase ($\alpha < 0.55$). From ref 19. Copyright 2011, American Chemical Society.

molecule, respectively. These results indicate that conjugation of ligands affects the binding between the individual ligands and the scaffold. Therefore, the effect of linkers should be considered when designing multidomain scaffolds and ligands, as this may alter the macroscopic properties of the resulting hydrogel network.

The MITCH system has been used to control the codelivery of cells and growth factors for regenerative medicine therapies. Mulyasasmita et al. showed that the MITCH-system provided significant protection from cell damage after injection through a syringe compared to PBS.[65] Additionally, a MITCH composite hydrogel with hydroxyapatite nanoparticles was used to encapsulate and immobilize adipose-derived stem cells within a macroporous scaffold to stimulate bone regeneration.[66] In summary, protein-based scaffolds and ligands in hydrogels allow for specific tuning of the viscoelastic properties, which facilitates its translation toward clinical application.

CONCLUSION

Engineered multidomain scaffold proteins have provided substantial fundamental insight into molecular mechanisms facilitating complex signal transduction. Conjugation of modular scaffold domains via multimerization and recombination allows for rapid diversification of existing scaffold proteins. The four highlighted application fields have provided complementary molecular insight into scaffold functioning. First, *in vivo* introduction of synthetic scaffold proteins showed plasticity of native signaling pathways and the application of these scaffolds for designing new functional pathway outputs. Modular recombination and multimerization allows for optimization of pathway flux and connection of otherwise unrelated input and output responses, which has enormous potential in the field of biosynthesis and metabolic engineering. Additionally, cellular liquid−liquid phase separation is dictated by multivalent interactions between scaffold proteins and ligands, which leads to partitioning of cellular signaling molecules within a confined space. Finally, detailed analysis of the well-defined scaffold proteins within hydrogel systems allows for predictable tuning of the viscoelastic properties.

Altogether, synthetic multidomain scaffold proteins are valuable tools in synthetic biology both to gain fundamental understanding and in terms of application. MDSPs can be applied to engineer therapeutic or diagnostic functionalities in synthetic cells or to tune hydrogels for specific regenerative medicine therapies. Additionally, integration of self-assembling properties results in behavior deviant from the isolated components, similarly to that observed within the described LLPS and hydrogel systems. Therefore, we envision future application of covalent scaffolds to template self-assembly, for example, in capsid formation[67,68] or in combination with other biomolecules such as RNA to study biomolecular condensate formation.[69] Within these complex higher-order structures, modularity of these covalent scaffolds would allow for tunability of network formation.

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Notes

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ABBREVIATIONS

(E)GFP, (enhanced) green fluorescent protein; GBD, GTPase binding domain; LLPS, liquid−liquid phase separation; MAPK, mitogen-activated protein kinase; MDSP, Multidomain scaffold proteins; MITCH, mixing-induced two-component hydrogel; MTD, membrane-targeting domain; N-WASP, neuronal Wiskott-Aldrich syndrome protein; PDZ, PSD95,

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Dlg, ZO-1; PRM, proline-rich motif; SH3, SRC homology 3; SIM, SUMO interacting motif; SUMO, small ubiquitin-like modifier; WT, wild-type

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