Opinion

CellPress

Superglue from bacteria: unbreakable bridges for protein nanotechnology

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Biotechnology is often limited by weak interactions. We suggest that an ideal interaction between proteins would be covalent, specific, require addition of only a peptide tag to the protein of interest, and form under a wide range of conditions. Here we summarize peptide tags that are able to form spontaneous amide bonds, based on harnessing reactions of adhesion proteins from the bacterium *Streptococcus pyogenes*. These include the irreversible peptide–protein interaction of SpyTag with SpyCatcher, as well as irreversible peptide–peptide interactions via SpyLigase. We describe existing applications, including polymerization to enhance cancer cell capture, assembly of living biomaterial, access to diverse protein shapes, and improved enzyme resilience. We also indicate future opportunities for resisting biological force and extending the scope of protein nanotechnology.

The challenge of protein–peptide interaction

For a generic route to extend the applications of proteins, peptide tags are ideal. Genetically fusing a peptide to a protein of interest can allow efficient purification, detection, immobilization, and derivatization. Peptide tags are characterized by a minimal risk of disrupting the function of the attached protein [1–3]. In this opinion we focus on genetically encoded peptide–protein interactions and their optimization through spontaneous formation of isopeptide bonds (see Glossary). Protein or peptide interactions with small molecules (e.g., Ni-NTA, HaloTag, SNAP-tag, FlAsH [1]) are beyond our scope.

Peptides are generally unstructured before binding their protein partner and have a limited surface area for contacts, which presents a quandary in trying to obtain stable peptide–protein interactions [4]. The most widely used peptide–protein pairs are epitope tags (e.g., HA-, myc-, and FLAG-tags) recognized by antibodies, but more recently peptide binding to streptavidin or PDZ clamps has been developed [5–7]. The limited stability and strength of peptide–protein interactions seriously hamper the use of peptide tags, especially where the peptide–protein linkage must last for a long time, link to a nanoparticle, survive high temperature, or withstand forces [5,8–10]. Therefore, our opinion is that peptide–protein pairs forming unbreakable interactions will enable diverse new approaches for basic research and biotechnology.

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Keywords: protein engineering; nanobiotechnology; mechanobiology; synthetic biology; supramolecular assembly; biomimetic.

0167-7799/

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Existing strong protein–peptide interactions

Although advances in genetic engineering depended on a diverse array of enzymes able to ligate, edit, or recombine DNA fragments, fewer such options exist for directing covalent interactions between peptides and proteins. Native chemical ligation (NCL) and expressed protein ligation (EPL) enable peptide fragments to be joined together, but this must be at specific termini and is generally restricted to *in vitro* systems [11]. Split inteins can be fused to target proteins, reconstitute in solution, and splice themselves out to covalently attach the two polypeptides, although various side reactions may occur [12,13]. Dock-and-lock, in which proteins crosslink via cysteines on terminally attached peptide-recognition domains, also enables coupling but is not applicable in reducing environments [14].

Covalent protein coupling can also be catalyzed enzymatically. In Sortagging, an LPXTG-containing protein is covalently linked to an N-terminal pentaglycine probe via sortase catalysis [15,16]. Transglutaminases can mediate protein crosslinking between lysine and glutamine side chains, although these enzymes are frequently promiscuous [2].

Glossary

Atomic force microscopy (AFM): a high-resolution probe scanning-microscopy technique able to determine the resilience to stretching forces of biomolecules.
HaloTag: a protein tag engineered from haloalkane dehalogenase able to react covalently to form an ester with alkyl halide ligands.
Intein: a protein domain that performs protein splicing. An intein excises itself to join the remaining protein's portions.
Isopeptag: a peptide tag (TDKDMTITFTNKKDFE) derived from the C domain of the major pilin Spy0128 able to form an isopeptide bond to pilin-C.
Isopeptide bond: an amide bond connecting a side chain to a side chain or a side chain to the protein's main chain.
KTag: a peptide tag (ATHIKFSKRD) derived from the CnaB2 domain able to form an isopeptide bond to SpyTag in the presence of SpyLigase.
Pilin-C: a protein derived from the major pilin Spy0128 able to form an isopeptide bond to isopeptag.
SNAP-tag: a protein tag from engineering O^6-alkylguanine-DNA alkyltransferase that forms a covalent bond with O^6-benzylguanine derivatives.
Sortase: a class of enzyme from Gram-positive bacteria that catalyzes intermolecular amide bond formation.
SpyCatcher: a protein-binding partner derived from the CnaB2 domain able to form an isopeptide bond to SpyTag.
SpyLigase: a protein derived from the CnaB2 domain able to direct peptide–peptide ligation between SpyTag and KTag.
SpyTag: a peptide tag (AHIVMVDAYKPTK) derived from the CnaB2 domain able to form an isopeptide bond to SpyCatcher.

Spontaneous isopeptide bond formation for protein coupling

We consider that a different type of protein chemistry – spontaneous isopeptide bond formation – might be able to overcome the above challenges. An isopeptide bond is an amide bond in a protein connecting a side chain to a side chain or a side chain to the protein's main chain (Figure 1A). Important natural examples of isopeptide bonds include ATP-dependent conjugation of ubiquitin to proteins [17] and transglutaminase-catalyzed isopeptides imparting strength to hair, skin, and blood vessels [18]. However, isopeptides bonds can also form autocatalytically, as first discovered in HK97, a bacteriophage discovered in Hong Kong from pig dung [19]. HK97's capsid is composed of 420 protein subunits that assemble noncovalently into an icosahedral particle comprising five- and six-membered rings looping through each other. Spontaneous intermolecular isopeptide bond formation between adjacent

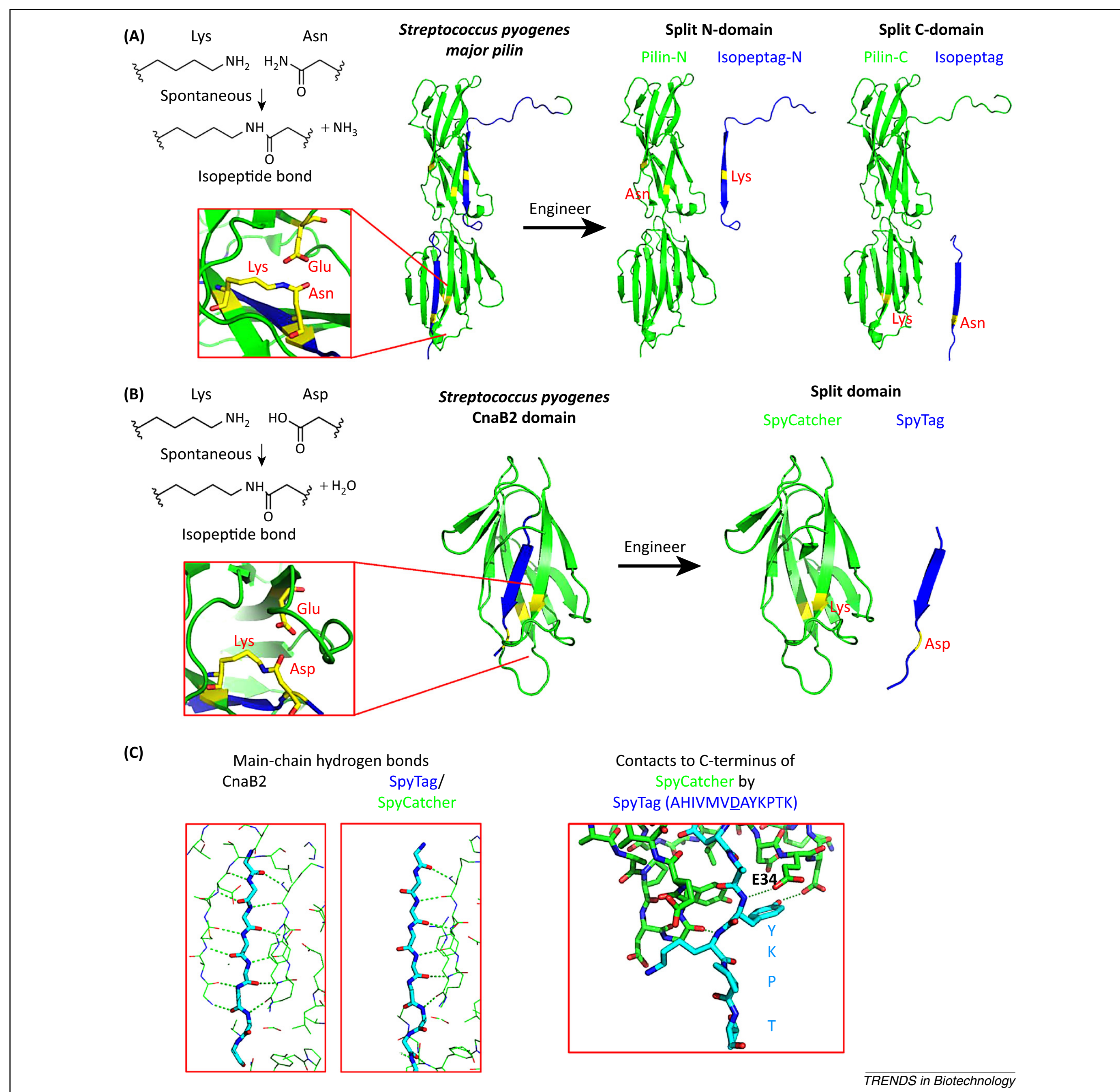

Figure 1. The generation of isopeptide bond-forming peptides. **(A)** Splitting the major pilin. Reaction between lysine and asparagine in the pilin, promoted by an apposed glutamic acid. Inset shows reactive triad in the C domain (PDB 3B2M). A cartoon shows splitting of the pilin into two separate protein–peptide pairs: pilin-N reacts with isopeptag-N, whereas pilin-C reacts with isopeptag. In blue are the peptide tags or the residues from which the tags were derived. Key residues for reaction are in yellow. **(B)** Splitting CnaB2. Reaction between lysine and aspartic acid in CnaB2, promoted by an apposed glutamic acid (PDB 2X5P). A cartoon shows splitting of CnaB2 into the protein SpyCatcher, which reacts with the SpyTag peptide. Blue marks the peptide tag and the residues from which the tag was derived. Key residues for reaction are in yellow. **(C)** Insights from the crystal structure of the SpyTag/SpyCatcher complex. Main-chain hydrogen bonds (dotted lines) form to both sides of the final β strand in CnaB2 (left panel, PDB 2X5P) but to only one side of SpyTag (center panel, PDB 4MLI). Right panel: polar contacts made by SpyCatcher (including its E34 mutation) with the C-terminal tail of SpyTag (PDB 4MLS).

subunits then locks the rings together, forming 'protein chainmail' [20].

Intramolecular spontaneous isopeptide bonds were first found in 2007 by Kang *et al.* in Spy0128, the major pilin subunit from the human pathogen *S. pyogenes* (Figure 1A) [21]. Subsequently, spontaneous intramolecular isopeptide bonds were discovered in a range of surface proteins from Gram-positive bacteria. A unique biological role was shown: intramolecular isopeptides in these domains confer proteolytic, thermal, and pH stability [21,22]. Why would disulfide bonds not suffice for high stability? Many bacteria, including important human pathogens such as staphylococci and enterococci, lack the cellular machinery for catalyzing disulfide bond formation and often inhabit hypoxic environments [23]. Another advantage of isopeptides is that disulfides are easily cleaved by reducing agents or enzymes with reactive cysteines, whereas little is known about how lysine–asparagine or lysine–aspartic acid isopeptides are cleaved. There is some precedent for reversal of lysine–glutamine linkages, such as transglutaminases acting in reverse [24] or destabilase from leeches hydrolyzing blood clots [25].

The mechanism of spontaneous isopeptide bond formation has been analyzed through crystallography [21], NMR [26] and quantum mechanics/molecular mechanics (QM/MM) [26,27]. Lysine forms an isopeptide bond by nucleophilic attack on asparagine with loss of NH_3 (Figure 1A) or, less commonly, by attack on aspartic acid with loss of H_2O (Figure 1B). A catalytic glutamic acid or aspartic acid is always positioned opposite the residue being attacked (Figure 1A,B), stabilizing the transition states via hydrogen bonding and donating or receiving a proton to facilitate reaction. The triad of residues directly involved in the reaction is located in the hydrophobic protein interior, which modulates the pK_a, favoring the uncharged and more reactive forms of the residues. Interestingly, synthetic organic chemists independently developed a similar design of catalyst for the environmentally friendly synthesis of amide bonds directly from amines and carboxylic acids, a reaction of great importance in the pharmaceutical and agrochemical industries [28].

Peptide tags forming spontaneous covalent bonds to proteins

A domain is not necessarily the minimal unit of a protein that is most useful for biotechnology. Splitting domains is a powerful way to control protein function. A polypeptide sequence can be strategically split into two fragments and, when expressed individually, the two fragments may recognize each other and reconstitute to form a functional protein. Split-protein reconstitution has been achieved with various scaffolds, including luciferase and fluorescent proteins [29]. Using such a strategy, peptide–protein pairs were developed that could spontaneously reconstitute and form an isopeptide bond (Figure 1A) [30]. Splitting the N domain of the major pilin generated the peptide fragment isopeptag-N, containing the reactive lysine, and the protein fragment pilin-N, containing the reactive asparagine (Figure 1A). When mixed in solution, isopeptag-N and pilin-N were able to reconstitute covalently [30]. Similarly, the C domain of the major pilin was split, with the reactive asparagine on the peptide, and further engineered to develop the more efficient isopeptag (16 residues) and pilin-C (32 kDa) partners (Figure 1A). With isopeptag in twofold excess, 98% of pilin-C reacted in 24 h [30]. The isopeptag/pilin-C reaction was robust, proceeding across a range of temperatures (4–37 °C), pH values (5–8), buffers, and detergents. Because the isopeptag/pilin-C reaction was specific and the partners were genetically encodable, they served as a facile platform for covalent labeling of a cell-surface protein for live-cell fluorescent microscopy [30].

The CnaB2 domain of the fibronectin-binding protein FbaB from *S. pyogenes* provided the building blocks for a smaller, faster-acting pair of partners [26,31–33]. CnaB2 was initially split into peptide and protein partners, surface-exposed hydrophobic residues were removed, and interactions at the binding interface were enhanced. This generated the optimized 13-residue SpyTag and 116-residue SpyCatcher binding partners (Figure 1B) [34]. Reconstitution between SpyTag and SpyCatcher was rapid, achieving an approximately 50% yield in 1 min and complete reaction of SpyCatcher with longer incubation times. SpyTag/SpyCatcher still reacted together efficiently across various pH values (5–8), buffers, temperatures, and detergents [33]. Reconstitution was specific in *Escherichia coli* and mammalian cells, allowing genetically encodable protein coupling intracellularly and extracellularly [33]. Crystallography of the SpyTag/SpyCatcher complex revealed that the reconstituted protein had extra regions of flexibility compared with the parental domain, as well as hydrogen-bonding patterns that may explain the rapid binding of SpyTag and the role of the I34E mutation of SpyCatcher in facilitating docking (Figure 1C) [34]. This study also revealed that SpyCatcher could be truncated at each terminus, to form an 84-residue partner, without significantly reducing reaction efficiency [34].

Peptide–peptide ligation by SpyLigase

Preferably, specific connections between two proteins would be achieved simply by modifying each protein with a peptide tag. Adding SpyTag is a small modification, but adding an 84-residue SpyCatcher tag is not ideal. To circumvent this problem, CnaB2 was split into three parts to generate a covalent peptide–peptide ligation system. SpyLigase was engineered by excising the β strand from SpyCatcher that contains the reactive lysine, resulting in a new 10-residue peptide tag (KTag) (Figure 2) [35]. SpyTag was unmodified, whereas the remaining CnaB2 scaffold, containing the catalytic glutamic acid, was circularly permuted to yield the 107-residue SpyLigase (Figure 2). SpyLigase was able to dock with both SpyTag and KTag, reconstituting the catalytic triad and directing isopeptide formation between the two peptide tags [35].

However, this technology is not yet optimal. SpyLigase ligation is slower and lower yielding than SpyTag/SpyCatcher, requires a specific buffer, and has not yet been demonstrated in cells [35]. Nevertheless, the unique properties for peptide–peptide linkage make this system a promising starting point for linking protein building blocks with minimal modifications.

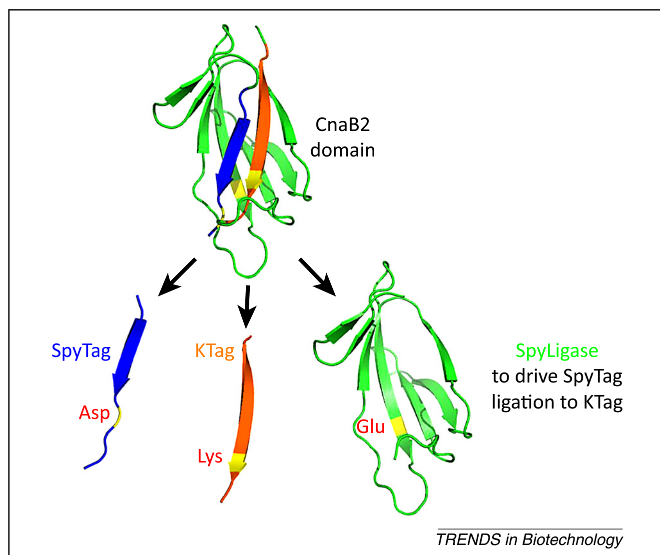

Figure 2. Peptide–peptide ligation by splitting CnaB2. Cartoon of the splitting strategy: β strands were excised to obtain two peptide tags – SpyTag (blue) with the reactive aspartic acid and KTag (orange) with the reactive lysine. The remaining protein domain (SpyLigase, green) could direct isopeptide bond formation between SpyTag and KTag. Residues involved in reaction are colored yellow.

Protein assemblies through spontaneous isopeptide bond formation

Despite the immense sophistication of multicomponent machines in nature (e.g., the ribosome, replisome, and spliceosome), synthetic biologists remain at an early stage in assembling artificial protein structures with desired modularity and stability [10,14]. To generate diverse nonlinear protein architectures, Zhang *et al.* elegantly harnessed different arrangements of SpyTag and SpyCatcher linked to elastin-like polypeptides (ELPs) (Figure 3A). Cyclized, tadpole-shaped, star-shaped, and H-shaped structures were produced in good yield simply on mixing [36].

Linear polymers thinner than natural protein fibrils were generated by Matsunaga *et al.* using isopeptag/pilin-C [37]. Pilin-C's isopeptag-binding pocket was engineered so that a disulfide bond inhibited binding of another isopeptag, thus generating a redox-sensitive 'cap' (Figure 3B). A reducing agent uncaged the isopeptag-binding pocket and enabled self-polymerization, ingeniously forming pilus-like 'hyperthin nanochains' in an inducible manner (Figure 3B). C- and S-shaped polymers of >200 nm were observed by atomic force microscopy (AFM) [37]. Isopeptag/pilin-C were also exploited for irreversible assembly of protein conjugates with spatial control by Abe *et al.*, enabling Förster resonance energy transfer (FRET) applications between fluorescent proteins (Figure 3C) [38]. To link proteins into chains in which the protein is modified only with peptide tags, affibodies (non-immunoglobulin-binding proteins) or antibodies were genetically fused to SpyTag and KTag. SpyLigase could then link these components into 'protein tentacles' over 20 units long (Figure 3D) [35]. The small size of SpyTag and its ability to react even when placed internally in proteins opens new routes for the controlled and stable assembly of proteins, reaching structural complexities infeasible with standard chemical conjugation strategies. Future applications of these assemblies include force-resistant biomaterials for tissue engineering, capsules to regulate drug delivery, and functionalizable hydrogels to control cell adhesion, growth, and differentiation [36–38].

Current applications for SpyTag-based technology

Force has widespread effects on biological processes such as regulation of cell division, blood clotting, and metastatic spread of cancer cells [39–41]. Single-molecule measurement of force usually employs optical tweezers or AFM. It is challenging to form specific force-resistant linkages to probe how biomolecules respond under force. Often-undefined hydrophobic adhesion or disulfide bonds are used as such linkages. Therefore, there is a great need for force-resistant, specific, and genetically encodable linkages. Spontaneous isopeptide formation may have evolved to resist force [22]. AFM revealed the inextensible nature of the major pilin of *S. pyogenes*; the presence of isopeptides in both N- and C-terminal domains enabled the pilin to withstand forces of up to 800 pN without unfolding [42]. By contrast, a myosin power stroke in muscle contraction generates approximately 2 pN [43]. Similarly, the interaction between SpyTag and SpyCatcher has been analyzed at the single-molecule level (Figure 4A) and was stable under forces of up to 1900 pN [33], making it the strongest measured protein interaction. This mechanostability is approximately 20 times higher than streptavidin–biotin, one of the strongest noncovalent interactions in nature [9,44]. Therefore, SpyTag peptide fusion may be a good potential tool for interrogating the mechanical behavior of proteins. This interaction may be superior to interactions that drive existing approaches, which use less-specific disulfide bonds or the larger protein fusion HaloTag or SNAP-Tag [45,46]. SpyTag/SpyCatcher not only allows precise immobilization onto AFM tips, but may also provide the opportunity to study the effect of greater forces on specific sites at the cell surface. In addition, amide bonds survive greater forces than disulfides [47]. SpyTag could also be a valuable route for the formation of protein tandem repeats for AFM, which would accelerate data acquisition [48].

Another application of SpyTag in synthetic biology has been to create functionalizable amyloid fibrils. CsgA is secreted from bacteria and spontaneously assembles into amyloid fibrils, facilitating bacterial surface adherence and biofilm formation. Coculturing bacteria expressing CsgA and CsgA-SpyTag led to growth of mixed fibrils bearing surface-exposed SpyTags [49]. The fibrils could then be decorated with SpyCatcher-linked quantum dots and imaged by fluorescence microscopy (Figure 4B) [49].

The observed equilibrium constant for noncovalent binding in antibody–antigen or streptavidin–biotin interactions can be shifted by orders of magnitude toward dissociation when one of the partners is attached to a quantum dot or other nanoparticle [8]. To address this challenge, biomolecules may be linked to nanoparticles covalently [50,51]. SpyTag has been used for irreversible protein linkage to magnetic particles (Figure 4C). Magnetic microparticles or nanoparticles are the most common way to isolate rare cells. Magnetic capture of circulating

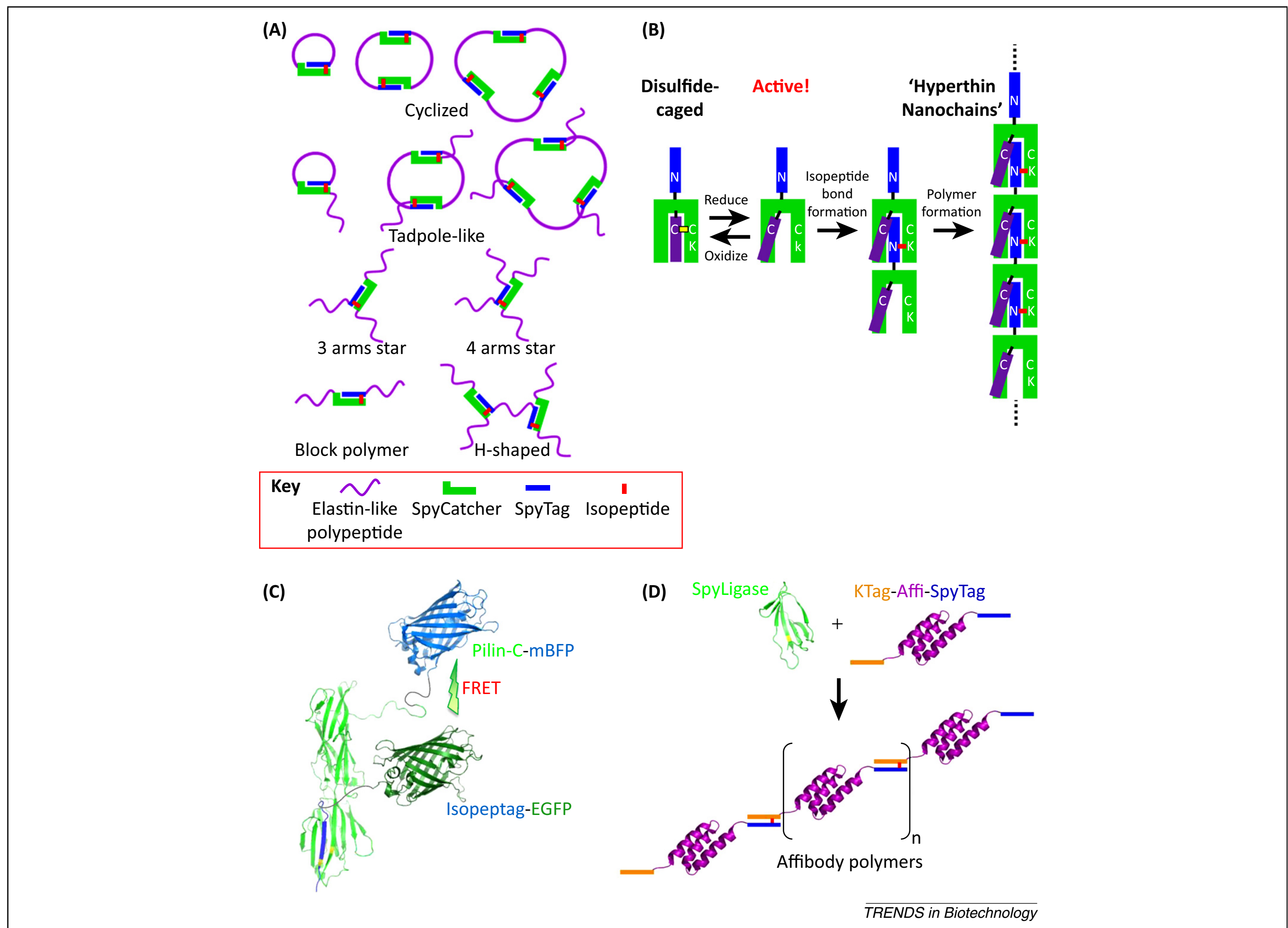

Figure 3. Artificial assemblies created via spontaneous isopeptide bonds. **(A)** Diverse protein architectures from positioning SpyTag/SpyCatcher in elastin-like polypeptides. **(B)** Hyperthin nanochains via switchable covalent self-polymerization. Isopeptag (blue) was added to pilin-C to enable polymerization, but pilin-C reactivity was masked by a strand (purple) forming a disulfide bond (yellow). On reduction, the isopeptag of one subunit could fit in the groove of another subunit, leading to formation of an isopeptide bond (red) and chain extension. Chains were much thinner than amyloid or collagen fibrils. Adapted from [37]. **(C)** Cartoon of the conjugation between pilin-C and isopeptag, each linked to different fluorescent proteins [monomeric blue fluorescent protein (mBFP) or enhanced GFP (EGFP)], enabling Förster resonance energy transfer (FRET). **(D)** Protein tentacles: cartoon of SpyLigase locking together affibodies (purple), which were tailored with SpyTag (blue) and KTag (orange), into multivalent chains.

tumor cells (CTCs) from blood is having a major impact on our understanding of human cancer biology and is starting to be used for cancer prognosis and personalized cancer therapy [52]. Any weakness in the link between magnetic microparticles and their biomolecule partners can reduce the sensitivity of cell capture; secondary antibodies and even streptavidin–biotin interactions can be limiting [9,53]. Covalently linking SpyCatcher bearing an N-terminal cysteine (Cys-SpyCatcher) to magnetic beads enabled SpyTag-linked affibody polymers to be attached entirely through covalent bonds (Figure 4C). Affibodies ligated into covalent chains using SpyLigase enabled capture of cells bearing low levels of tumor antigen without increasing nonspecific capture [35]. These polyaffibodies or polyantibodies may find future applications in increasing the speed and sensitivity of capture of other rare eukaryotic cell types (e.g., stem cells, T cell populations, *Plasmodium*) as well as viral or bacterial pathogens, using both magnetic and microfluidic capture.

Enhancing the resilience of enzymes can also be achieved using SpyTag/SpyCatcher technology. Instability of enzyme activity often limits diagnostic devices, biofuel production, or biotransformations. Circularization of enzymes using early approaches often had only a modest effect on thermal tolerance [54]. By fusing their termini with SpyTag and SpyCatcher, β-lactamase and dihydrofolate reductase were cyclized; the enzymes could be heated to 100 °C and on cooling retained high solubility and catalytic activity (Figure 4D) [55]. Differential scanning calorimetry indicated that this cyclization did not change the temperature for enzyme unfolding but increased refolding after thermal stress [55].

Regarding limitations, SpyTag is a genetically encoded tag and therefore must be cloned onto the protein of interest case by case. Our experience is that SpyTag and SpyCatcher are well tolerated at the N- or C-termini of a wide range of proteins, ideally linked through a glycine–serine spacer [33,35,49,55], but there can be instances in which fusion interferes with folding or protein interactions. Moreover, although the reaction of SpyTag with SpyCatcher can occur in minutes with high yield using micromolar protein concentrations, the

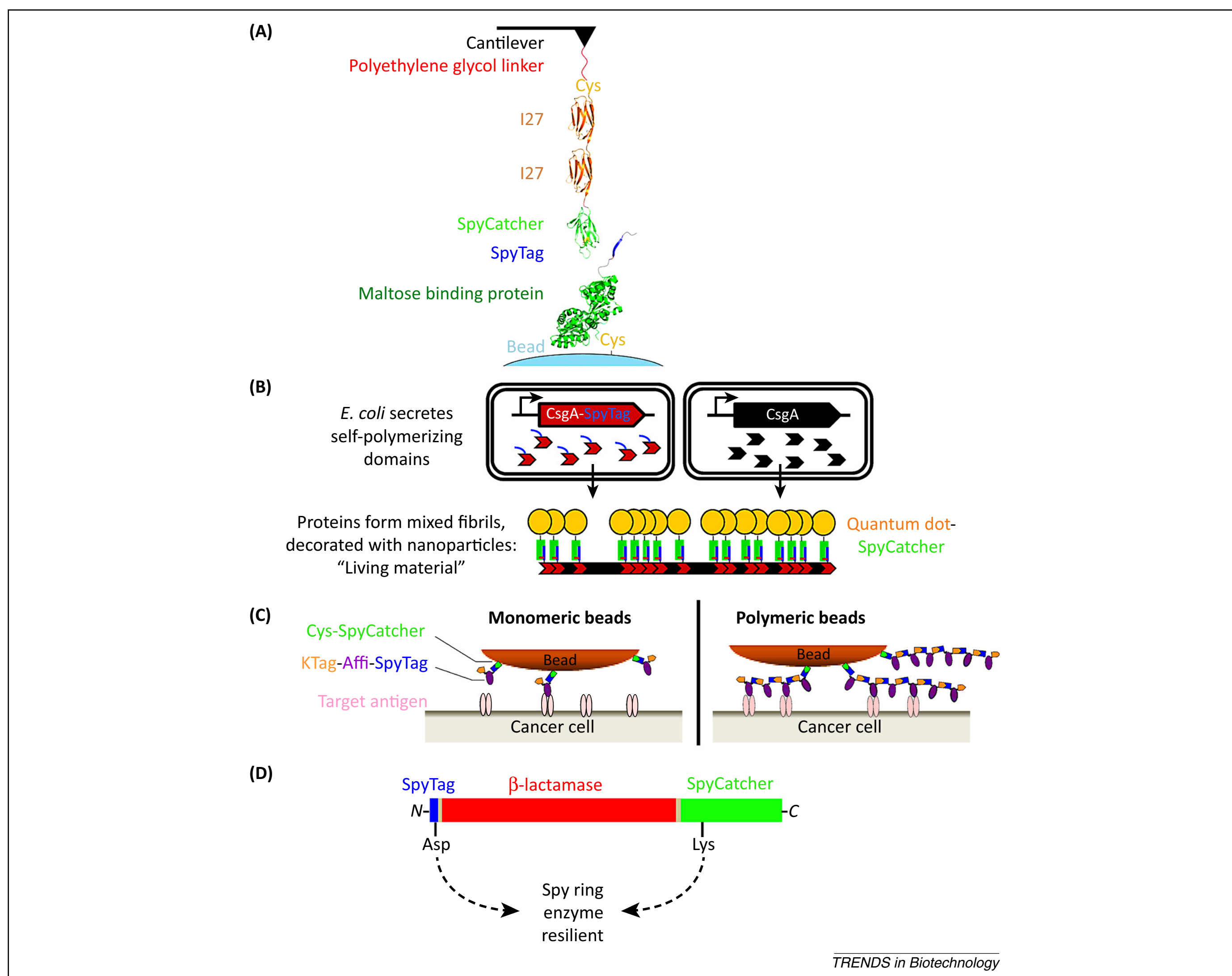

Figure 4. Application of isopeptide-assembled chains. **(A)** Cartoon of the construct used to study SpyTag/SpyCatcher mechanical stability by atomic force microscopy (AFM). Adapted from [33]. **(B)** Mixed fibrils generated from *Escherichia coli* CsgA (major curli subunit) and linked to nanoparticles via SpyTag/SpyCatcher. **(C)** SpyLigase-mediated polymerization of affibodies for enhanced cancer cell capture. Cartoon comparing the interface between a cancer cell and a magnetic bead coated with affibody monomers or SpyLigase-assembled affibody polymers. **(D)** Cartoon of cyclization of β-lactamase to enhance resilience to thermal inactivation.

reaction rate of 10^3 M^{-1} s^{-1} for SpyTag/SpyCatcher is orders of magnitude below the diffusion limit [33]. Therefore, systems such as biotin–streptavidin, with on rates close to the diffusion limit [56], remain superior for capturing a tagged species at nanomolar concentrations or below.

Concluding remarks and future perspectives

Here we have described the emerging role of spontaneous isopeptide bonds in forming ultrastable peptide–protein interactions. Three different isopeptide bond-forming systems have been engineered, but the size and rapid reaction of SpyTag with SpyCatcher makes this the preferred system. SpyTag/SpyCatcher's resilient interaction, which forms under a wide range of conditions and is able to react at any suitably exposed site, is already enabling exciting applications. SpyTag/SpyCatcher has allowed facile assembly of diverse nonlinear protein architectures, conferred resilience to boiling on enzymes, and enhanced magnetic cell capture. SpyTag/SpyCatcher also showed its mettle when expressed in cells for fluorescent microscopy and labeling biofilms. Future directions include resisting forces from molecular motors inside cells and providing unbreakable anchors between specific cellular components and surfaces or cantilevers. The modularity of SpyTag/SpyCatcher may allow proteins to be assembled irreversibly like building blocks within living organisms, creating new opportunities for constructing evolvable and robust nanomachines.

Acknowledgments

G.V. was funded by the Medical Research Council and Merton College Oxford, B.Z. by the Clarendon Fund, Oxford University Department of Biochemistry, and St. Peter's College, Oxford, and M.H. by the European Research Council (ERC-2013-CoG 615945-PeptidePadlock). The authors thank Dr Jacob Orry Fierer and Mr Christopher Schoene for their stimulating comments.

Disclaimer statement

B.Z. and M.H. are inventors on a patent application regarding isopeptide bond-forming peptides (UK Patent Application No. 1002362.0).

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