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Nanoteamwork: covalent protein assembly beyond duets towards protein ensembles and orchestras

Anusuya Banerjee and Mark Howarth

Biological processes often depend on the harmonious interplay of multiple macromolecules. Biotechnology has had great success in applying and modifying individual components, but the building of multi-component teams is at an early stage. Cells are intelligent in sensing their environment, so manipulating just one signal can limit potency and promote side-effects for therapeutics. Here we critically assess the latest advances in irreversibly connecting individual protein units, through different spontaneous or catalysed reactions. Then we outline efforts to go beyond bipartite assembly, towards multimeric or sequence-programmed architectures. These early steps will be put in context of the enormous opportunities for synthetic protein nanomachines, focusing on catalysis and the control of cell signalling for cancer and the immune system.

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Introduction

It was once said of a Hollywood star that her acting ran the gamut of emotions from A to B. A to B is the linkage that most protein engineers have focused upon, connecting one protein unit to a second protein unit. In this review we will explore linkage of A to B to C to D, among others, linking multiple protein units to permit new opportunities for control of living systems (Figure 1). We will describe the state-of-the-art based on advances in the last 2 years and provide a critical perspective. Bipartite assembly has enabled great successes [1]. However, more complex multi-protein assemblies, approaching the sophistication of cellular machines, would bring many further benefits (Figure 1).

Why synthesise protein teams?

It is straightforward to link multiple DNA elements into a single open reading frame. However, ribosomal synthesis of polypeptide units > 100 kDa is frequently defeated by translation errors and misfolding. Modular coupling permits each unit to be independently expressed (including in different hosts), validated (for folding, size and function) and chemically modified. In addition, modular assembly allows diverse coupling orientations and temporal control of coupling. We will focus on covalent assembly of protein units, because non-covalent interactions limit robustness. Here we will not cover nucleic acid nanotechnology [2]. Natural protein 'orchestras' provide extraordinary examples of catalytic speed and fidelity (in cellular DNA replication) or sensitive integration of multiple positive and negative inputs (at Natural Killer cell immune synapses) (Figure 1). The lack of comparable *synthetic* protein orchestras emphasises the motivation to enhance protein-directed assembly.

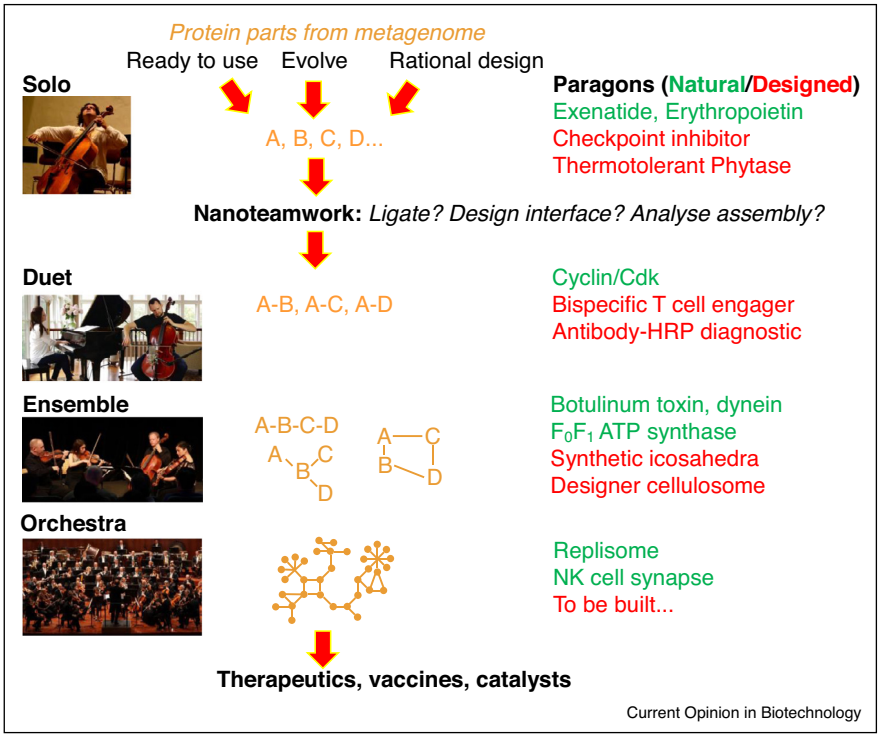
Tools for protein ligation for multi-protein assembly

We will introduce methods to join one protein covalently to another protein, focusing on those applied for multi-protein assembly (Figure 2). We emphasise coupling of genetically-encoded building blocks, operative under mild conditions (4–37 °C, pH 6–8, <5% organic solvent), consistent with the majority of proteins retaining their fold and activity and applicable for living cells. Other reviews nicely describe protein reaction with non-protein ligands [3] or chemical synthesis of proteins [4].

Split inteins interact spontaneously and through a series of reactions splice themselves out, to achieve traceless peptide bond linkage between two protein units (Figure 2a). Some split inteins depend on Cys reactivity, which complicates their use on disulfide-containing proteins. Also, there are multiple steps of reaction so side-products are often seen. Split inteins show good specificity in the cellular environment; after consensus design and mutagenesis, the latest generation react fast and have improved temperature-tolerance [5]. Simultaneous use of two orthogonal split intein pairs allowed multi-part assembly, although limitations were identified in solubility for SspDnaB reactive precursors [6].

SpyTag is a 13 amino acid peptide that reacts spontaneously to form an isopeptide bond with the protein module SpyCatcher (Figure 2b) [7]. This pair was engineered from a *Streptococcus pyogenes* adhesin domain. SpyTag and

Figure 1



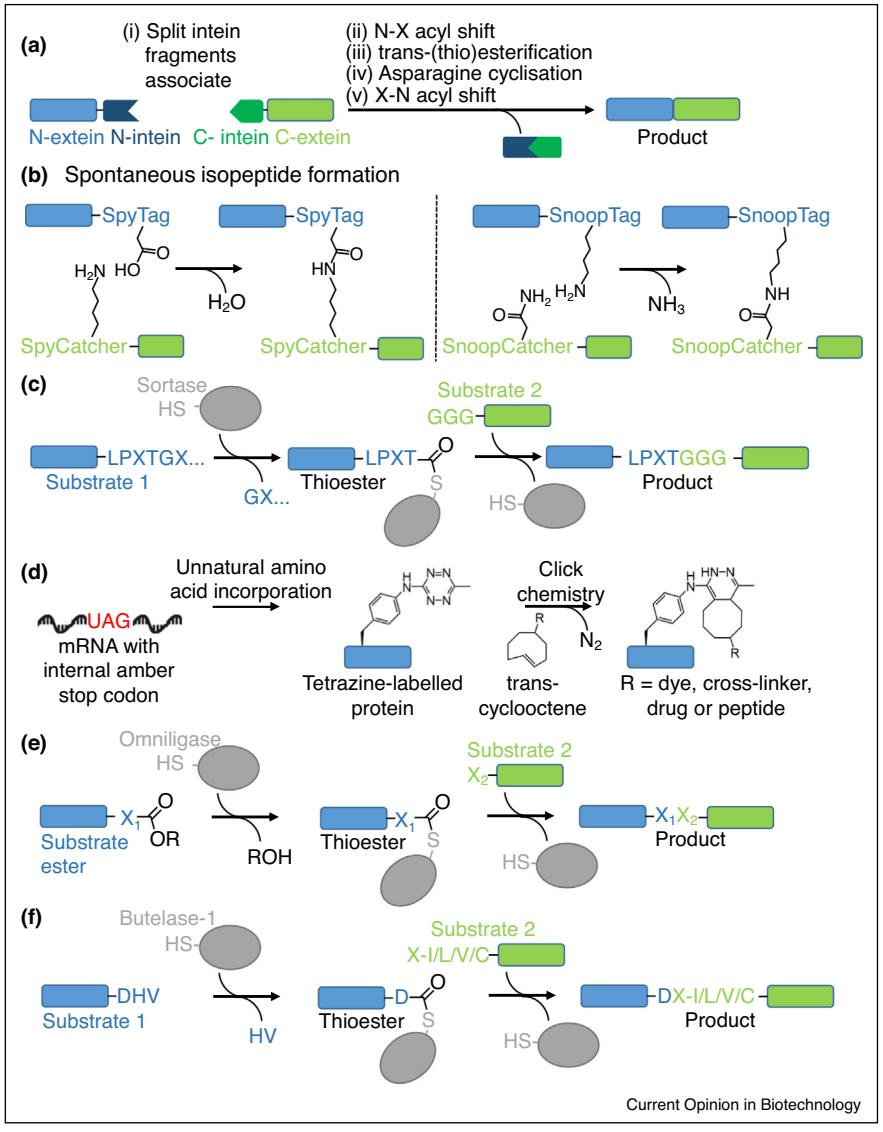
Concept of nanoteamwork in synthetic protein assembly. Outstanding protein units have been harnessed from natural diversity. These units sometimes work well in isolation (solo). However, by improving our ability to combine protein units (protein duet, ensemble, orchestra), new important properties may be achieved.

SpyCatcher have now found diverse applications [8]. Key features are efficient reaction at low micromolar concentration of each partner and robust reaction with SpyTag or SpyCatcher at any protein location, as long as the units are sterically accessible [7]. The simple reaction mechanism allows high coupling yield [9•]. Various other Tag/Catcher modules have been published (e.g. isopeptag, SdyTag) [10–13], with SnoopTag/SnoopCatcher showing complete orthogonality to SpyTag/SpyCatcher and high yield (Figure 2b) [9•]. A notable advance in bipartite assembly was in-field manufacture of protein pairs. Adding water led to *in vitro* transcription and translation, so that combinations of recognition-modules (DARPin or nanobody) covalently joined with the output module (fluorophore, enzyme, toxin), via spontaneous SpyTag/SpyCatcher reaction [14••].

Sortase has become a popular tool for protein ligation, principally *Staphylococcus aureus* sortase A, which links C-terminal LPXTG to N-terminal oligoglycine (Figure 2c). An orthogonal sortase has been applied from *S. pyogenes* [15]. A challenge for sortase applications is the high micromolar K_m of natural and engineered sortases, which is problematic for many protein substrates. Furthermore, competing thioester hydrolysis or back-reaction may limit product yield. To ligate substrates at low concentration, Ni^{2+}-chelating lipids helped bring together His-tagged sortase and His-tagged target proteins at the surface of liposomes [16]. Alternatively, the recognition site — LPXTG was linked to SpyTag, while sortase was linked to SpyCatcher; SpyTag/SpyCatcher is then cleaved off as the final product is generated [17•].

Unnatural amino acid (UAA) incorporation is an elegant strategy for protein modification. Through codon reassignment, one amino acid in a protein can be replaced with an amino acid with bio-orthogonal reactivity. Improvements in click chemistry have enabled incorporation of fast reacting functional groups, that is, conformationally-strained trans-cyclooctene reacts with a tetrazine at $>10^6\ M^{-1}\ s^{-1}$ (Figure 2d) [18]. Complication of the suppressor codon being read as a stop codon has been reduced in *Escherichia coli* by removing 314 amber stop codons from the genome [19], or in mammalian cells by engineering eukaryotic Release Factor 1 [20]. The focus of UAA-incorporation has been linking proteins to small molecules (for imaging or cross-linking), more than linking to other proteins. Incorporating multiple distinct UAAs was advanced by *in vitro* translation with purified components and pre-charged tRNAs recognising three redundant codons [21••].

Figure 2



Tools for covalently linking one protein to another protein. **(a)** Split intein ligation, **(b)** spontaneous isopeptide bond formation by SpyTag and SnoopTag, **(c)** sortase, **(d)** unnatural amino acid incorporation, **(e)** omniligase, and **(f)** butelase.

Proteases can be prompted to work in reverse, such as subtiligase and more recently trypsiligase, peptiligase and omniligase (Figure 2e). Proteolysis is usually thermodynamically favourable, so ligation is driven with ester substrates [22••]. Generating protein–ester substrates raises its own challenges, but the small recognition motifs present great opportunities for linking proteins to modified peptides.

Enzymes from cyclotide-producing plants also allow efficient protein–protein ligation. Butelase-1 ligates proteins with a C-terminal D/N-H-V motif to those with an N-terminal X-I/L/V/C motif (Figure 2f). Butelase-1 has been limited by the need to purify enzyme from butterfly peas and the moderate reaction-yield, because of reversal by the cleaved HV dipeptide [23]. OaAEP1 is a ligase with the asparaginyl endopeptidase fold, reacting through a thioester intermediate, which can be expressed successfully in *E. coli*. OaAEP1 showed good activity in ligating Ubiquitin with C-terminal –N-G-L to targets with G-L- at their N-terminus [24].

Distinct ligation technologies may be combined: HaloTag, SpyTag and streptavidin-binding peptide permitted simultaneous organisation of different enzymes on bacterial outer membranes [25].

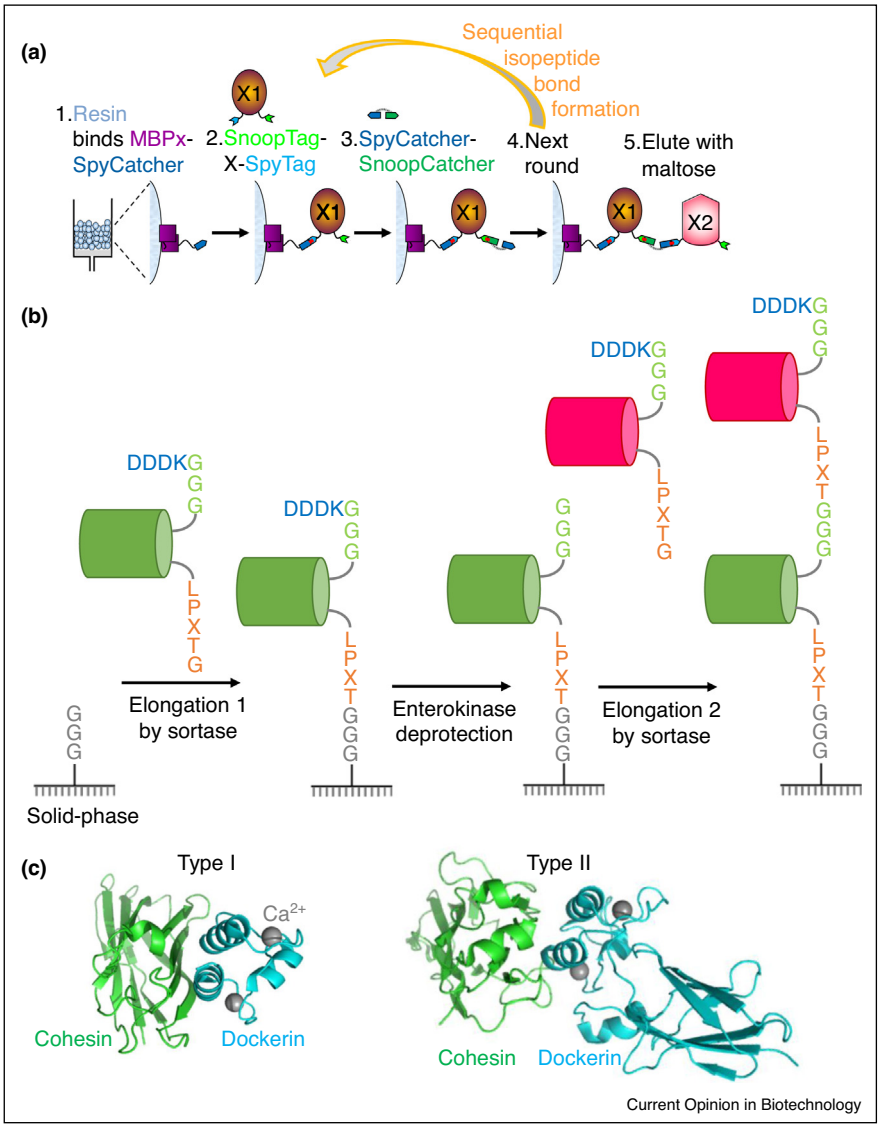
Multi-protein assembly

There are a few different strategies for covalent multi-protein assembly. In the ideal situation, one would have simultaneous coupling: a one-pot reaction. Since reactions never go to 100% conversion and there are limited different coupling reactivities, sequential reaction is more common for assembly of >3 components. Sequential reaction is facilitated by solid-phase attachment, avoiding purification at each stage, while excess reagent drives reaction towards completion. However, solid-phase attachment makes it harder to scale up and effort is needed to produce the excess reactant.

For chain synthesis by isopeptide bond formation, extension was performed by sequential SpyTag/SpyCatcher reaction and SnoopTag/SnoopCatcher reaction (Figure 3a) [9•]. The growing 'polyproteam' chain was anchored to amylose resin by a tandemly-repeated maltose binding protein, so elution occurred under mild conditions (addition of maltose). Although the Tag/Catcher modules increased the overall size of the conjugates, up to 10-unit protein teams were assembled with good yield, validated by SDS-PAGE, mass spectrometry and size-exclusion chromatography [9•].

Step-wise sortase reaction employed a single sortase and, after coupling, unveiled N-terminal oligoglycine for the next reaction step using enterokinase cleavage (Figure 3b) [26].

Figure 3



Nanoteam assembly strategies. **(a)** Iterative isopeptide bond formation. **(b)** Iterative sortase coupling. **(c)** Cohesin/dockerin interaction for one-pot multi-module assembly. Type I complex (PDB 1OHZ) and Type II complex (PDB 5K39).
Source: Adapted from Refs. [9•,26].

Multi-protein assembly can also be achieved by adapting the modules organising enzymes in the cellulosome. Even though dockerin:cohesin interactions are non-covalent (Figure 3c), they are worth describing here because dissociation constants are often picomolar, they have strong mechanical stability, and there are numerous orthogonal pairs [27]. However, the calcium-dependence of dockerin (Figure 3c) may be an issue for intracellular application [28].

Specific applications of nanoteamwork

Enzyme organisation for catalysis

An area of enormous interest is re-engineering pathways to enhance production of fine chemicals or biofuels. In a cell, organisation of adjacent enzymes in a pathway can occur through: (i) specific enzyme:enzyme interfaces; (ii) binding to a protein scaffold or protein microcompartment; or (iii) tethering to the cytoskeleton or a membrane [29]. Enzyme organisation may facilitate flux down a particular pathway (vs a competing branch), maintain cofactor pools or redox balance, and avoid accumulation of toxic intermediates (often electrophiles, like aldehydes). Classic examples are assembly-line enzymes like fatty acid synthase or polyketide synthase, 'catalytic cathedrals'. When pathways are re-engineered, there may be mix-and-match of enzymes from different species. Therefore, natural spatial relationships between enzymes may not apply and should be engineered. Tandem fusion of enzymes is often damaging to folding and catalysis. Note that the simple act of immobilising enzymes can sometimes change kinetic parameters and enhance stability [29].

Dockerin:cohesin modules have been applied for engineering the difficult task of cellulose digestion. In *Clostridium thermocellum*, the primary scaffoldin uses the carbohydrate-binding module to anchor to cellulose fibres and then type I cohesin:dockerin interactions to anchor various enzymes. The anchoring scaffoldin links to the surface of the bacterium via an S layer homology module and type II cohesin:dockerin interactions to the primary scaffoldin [30•]. Designer cellulosomes have focused on primary scaffoldin engineering, but a scaffoldin containing >6 cohesins became difficult to express. However, making different adaptor scaffoldins has enhanced degradation by assembly with 8 enzymes (Figure 4a) [30•].

For catalytic microcompartments, phage MS2 coat could be fused with SpyTag on an internal loop and co-assembled with two enzymes from the indigo biosynthetic pathway; resultant nanoparticles were catalytically active and the enzyme retained higher activity than free enzyme one week later [31]. Enzyme positioning can also be valuable in sensing. Ultra-sensitive detectors of Prostate Specific Antigen were assembled covalently, achieving amplification via 4 functional units (nanobody, SH3 domain, SH3-binding peptide and TVMV protease) and three separate proteolytic activities [32].

Signalling teams

Signal transduction is sometimes by simple receptor dimerisation (e.g. Epidermal Growth Factor Receptor), but sometimes by interplay of multiple inputs with defined spatial relationships, (e.g. T cell activation at immune synapses). Biotechnology is adept at mimicking the first type of signalling but amateur at the second. Isopeptide-linked polyproteam assembly allowed combinatorial clustering of different ligands to modulate apoptosis signalling (Figure 4b). Death Receptor signalling is sensitised on cancer cells, but no therapeutic has had the combination of potency and specificity to gain clinical approval. Linking a nanobody to Death Receptor 5 and affibodies to different growth factor receptors identified a combination with unexpected potency in cell death induction (Figure 4b) [9•].

Modular vaccine assembly

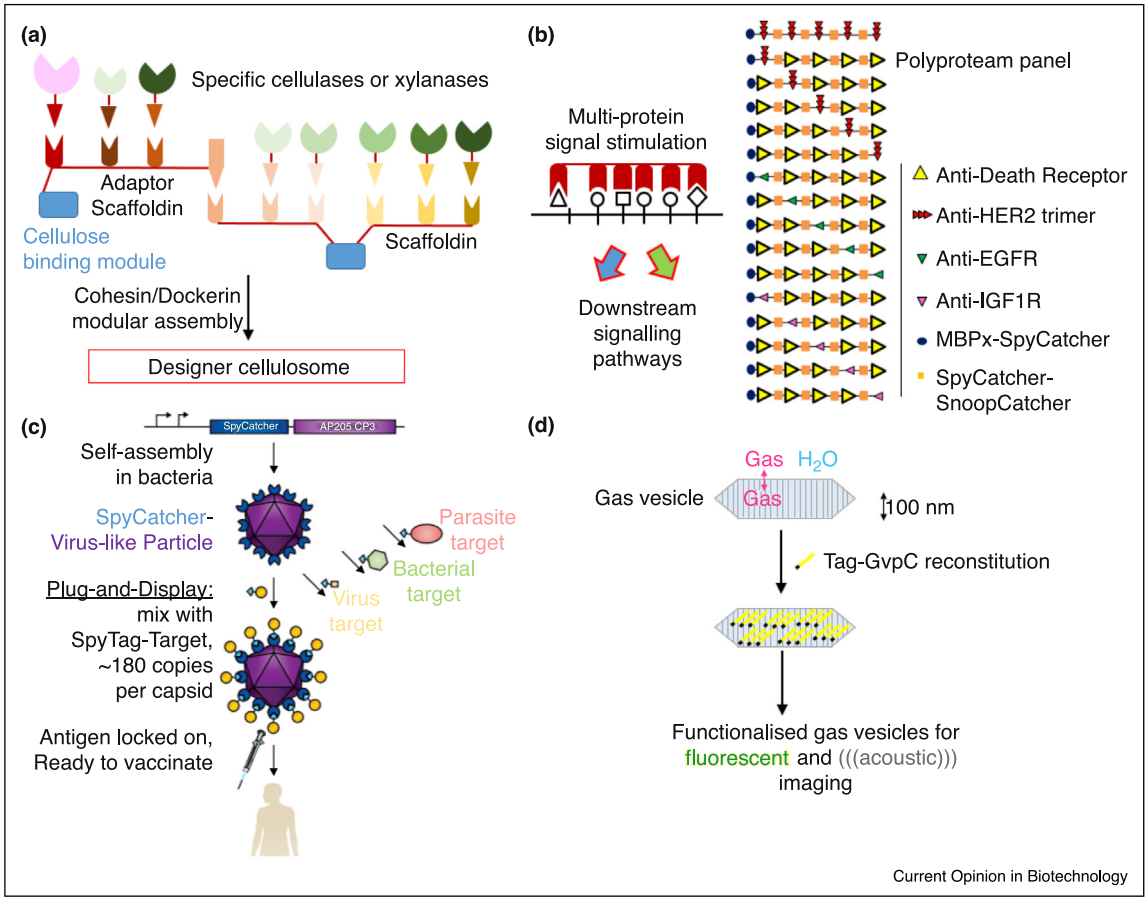
An important aspect of signalling is manipulating the immune system, notably for vaccines or the revolution in cancer treatment via immunotherapy [33]. Ligand clustering is a major stimulant to the immune response, resembling viral or bacterial surfaces. Ligation methods enable monomeric proteins from pathogens to be assembled on multimeric scaffolds, so that they resemble viruses and stimulate strong antibody responses against that antigen. SpyCatcher linked to the coat protein of the phage AP205 can couple with a range of SpyTag-fused disease-associated proteins (malaria, tuberculosis, cancer, allergy) (Figure 4c) and induced potent antibody responses [34,35]. The precision of isopeptide-mediated coupling versus chemical coupling (from Lys to Cys) enhanced potency for blocking transmission of malaria to mosquitoes [36]. Modular VLP coupling was also achieved with sortase on phage P22 particles [37], Cowpea Chlorotic Mottle Virus [38], or split Hepatitis B Virus core [39].

Highly focused immune responses may be susceptible to immune evasion or pathogen diversity. Therefore precise coupling of >1 type of pathogenic protein per particle is valuable, as shown with two malaria antigens via Spy and Snoop reaction [40].

Synthetic biomaterials

Spider silk has long been an object of fascination, but recombinant expression of silk proteins has been challenging. However, modules from a large silk protein were expressed in *E. coli*, refolded, and connected by split inteins to improve mechanical properties [41]. Split intein-containing silk proteins were also expressed in tobacco seeds, where a 37 kDa monomer multimerised to >500 kDa and was stable for >1 year [42].

Figure 4



Functional examples of multi-protein teamwork. **(a)** Catalysis, illustrated by a designer cellulosome, **(b)** Signalling, illustrated by a polyproteam for inducing cancer cell-death. **(c)** Vaccination, with modular VLP decoration against diverse pathogens. **(d)** Biomaterials, with nanobubble decoration. *Source*: Adapted from Refs. [9•,30•,34,46].

Amyloids play a role in many diseases but also provide synthetic biology opportunities. *E. coli* can secrete CsgA, which spontaneously assembles into amyloid nanofibres. Although assembly is non-covalent, CsgA-SpyCatcher amyloid resisted washes with 8 M guanidinium hydrochloride and 5% sodium dodecyl sulfate and then could still be functionalised with SpyTagged partners, with potential for filtration matrices [43].

Synthetic hydrogels can mimic the extracellular matrix, facilitating growth of stem cells or tissue patterning. Mild chemistries which do not react with encapsulated cells are a major benefit, as is regulated assembly/disassembly. $CarH_C$ domains were expressed with SpyTag or SpyCatcher at each end. Mixing these units quickly caused linear chain formation. Adenosylcobalamin triggers $CarH_C$ to tetramerise (so the chains gel), while green or white light cleaves the C–Co bond and subunits monomerise. Therefore, entrapped human stem cells could be tunably released from the hydrogels [44•].

Decorating oil-in-water microcapsules, SpyTag-hydrophobin proteins bound to the oil/water interface, so particles remained monodisperse for weeks and could be covalently decorated [45]. Gas-containing nanobubbles can also be maintained by 2 nm-thick cyanobacterial protein shells. The shells can be stripped of native GvpC and redecorated with recombinant GvpC, bearing RGD for cell-targeting or SpyTag for simultaneous acoustic and fluorescent imaging (Figure 4d) [46].

Conclusions

We hope that this review will convey some of the exciting work in this field so far, the great potential for nanoteam engineering, and the challenges as synthetic biology improves its scope in complex assembly synthesis. Apart from advances in ligation, other research areas are important for nanoteamwork. Characterising your nanoteams is often as difficult as their synthesis. Therefore, the recent revolution in atomic-resolution cryoelectron microscopy should advance the field [47]. Solubility will change,

when units are ligated into nanoteams. Therefore, experience from building diverse nanoteams will help to generate predictive power, as well as illuminating how natures giant assemblies evolved high solubility. *De novo* design has landmark successes in designing individual protein units. It has also engineered specific interfaces for 2D (surfaces) [48] and 3D (icosahedral) assemblies [49]. As important is the design of linkers with defined spacing, curvature and rotation [50]. Beyond engineering arbitrary spatial relationships, the subsequent challenge will be nanoteam regulation, following advances on switching protein units by light or small molecules.

Conflict of interest

MH is an inventor on patents filed regarding isopeptide bond-forming peptides (European patent EP2534484 and United Kingdom Patent and Trademark Office 1509782.7, 1705750.6, and 1706430.4) and a founder of SpyBiotech.

Acknowledgements

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