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Secrets of a covalent interaction for biomaterials and biotechnology: SpyTag and SpyCatcher

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SpyTag is a short peptide that forms an isopeptide bond upon encountering its protein partner SpyCatcher. This covalent peptide interaction is a simple and powerful tool for bioconjugation and extending what protein architectures are accessible. Here we review the origin and mechanism of SpyTag/SpyCatcher, focusing on recent innovative applications. Ligation of targeting-antibody with antigen provided a simple route to vaccine generation. SpyRings, from head-to-tail cyclisation, gave major enhancements in enzyme resilience. Linking multiple SpyCatchers gave dendrimers for T-cell activation or Spy networks forming hydrogels for stem cell culture. Synthetic biology applications include integrating amyloid biomaterials with living bacteria, for irreversible derivatisation of biofilms with enzymes or nanoparticles. We also discuss further opportunities to apply and enhance SpyTag/SpyCatcher technology.

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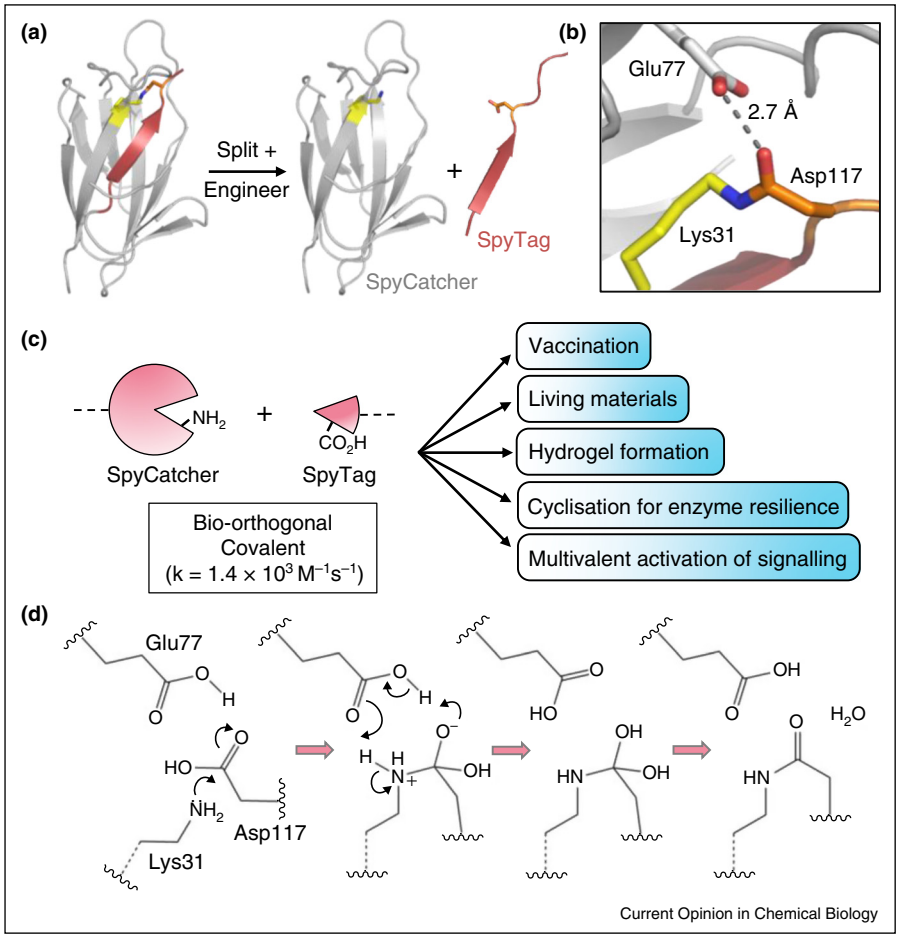
Introduction

Modifying and assembling proteins is a key challenge for our ability to explore and harness living systems. Bioconjugation allows us to study and manipulate the properties of proteins, for example by allowing tracking [1], interaction sensing [2] or conferring improved therapeutic properties [3]. Additionally, bioconjugation gives the ability to create novel devices and biomaterials for anything from energy harvesting [4] and medical diagnostics [5] to cancer cell capture [6] or drug delivery [7]. Protein bioconjugation initially exploited the chemistry of natural amino acids, such as amino groups (Lys, N-terminus), carboxyl groups (Asp, Glu, C-terminus) and sulfhydryl groups (Cys). However, there is often poor control over the site and number of modifications and incompatibility with complex mixtures or living systems. For many devices or therapeutics, molecularly-defined conjugates are highly desirable [8]. Therefore a range of more selective non-covalent or bio-orthogonal covalent targeting approaches have been developed [9,10]. Non-natural amino acids (nAAs) can introduce minimally obtrusive, orthogonally reactive groups [1,11–13]. Rates vary depending on the nAA (e.g. azido-containing, alkyne-containing, norbornene-containing) from $10^{-4}\,M^{-1}\,s^{-1}$ to an impressive $10^{4}\,M^{-1}\,s^{-1}$ for inverse-electron-demand Diels–Alder cycloaddition [9,14–16]. A disadvantage is the requirement for additional cellular machinery for nAA incorporation (an engineered aminoacyl-tRNA-synthetase/tRNA pair). Peptide tags (e.g. myc and FLAG tags recognised by specific antibodies) are an alternative strategy. However, the small interface between a peptide and its cognate protein typically results in high off-rate and minimal mechanical resilience. Therefore, protein engineers have adapted natural proteins that form covalent bonds to create 'unbreakable' peptide tags, including split inteins [17], sortase [18], transglutaminase [19] and the subject of this review, SpyTag/SpyCatcher.

Generating SpyTag/SpyCatcher

Pilins and adhesins of Gram-positive bacteria sometimes contain spontaneous isopeptide bonds [20,21], often within immunoglobulin-like domains CnaB1 or CnaB2 [22,23]. We generated SpyTag and SpyCatcher by splitting the CnaB2 domain from the fibronectin-binding protein FbaB from *Streptococcus pyogenes* (*Spy*) [24,25]. CnaB2 was split into a 13 residue peptide (SpyTag) and the 116 residue complementary domain (SpyCatcher) (Figure 1a). These two parts spontaneously reconstitute to form an isopeptide bond (Figure 1b) under a range of temperatures (at least 4–37 °C), pH values (5–8), buffers (no specific anion or cation required) and even with non-ionic detergents [25]. Neither SpyTag nor SpyCatcher contains cysteine residues. SpyTag and SpyCatcher function well when fused at either the N-terminus or C-terminus; there is not enough data yet to establish the rules for their fusion within tight loops of folded domains. SpyTag/SpyCatcher at 10 μM react to high yield with a half-time of just over 1 minute (rate constant = $1.4 \times 10^{3}\,M^{-1}\,s^{-1}$). Analogously, we were able to split the major pilin Spy0128, yielding alternative reactive pairs [26]. SpyTag/SpyCatcher has now been harnessed by a range of laboratories for bioconjugation [27•,28,29] and this review will provide an update of novel directions, as summarised in Figure 1c.

Figure 1



Generation, reaction and uses of SpyTag/SpyCatcher. **(a)** Cartoon of splitting CnaB2 into SpyCatcher (grey) and SpyTag (red). Residues forming the isopeptide are shown as sticks (based on PDB 2X5P and 4MLI) [30]. **(b)** Environment of the isopeptide bond between Asp117 (carbons orange) and Lys31 (carbons yellow), facilitated by Glu77 (carbons grey). **(c)** Discussed applications of SpyTag/SpyCatcher. **(d)** Reaction mechanism. Lys31 nucleophilically attacks Asp117, followed by proton transfers involving Glu77, leading to a neutral tetrahedral intermediate and then release of water and formation of the amide bond.

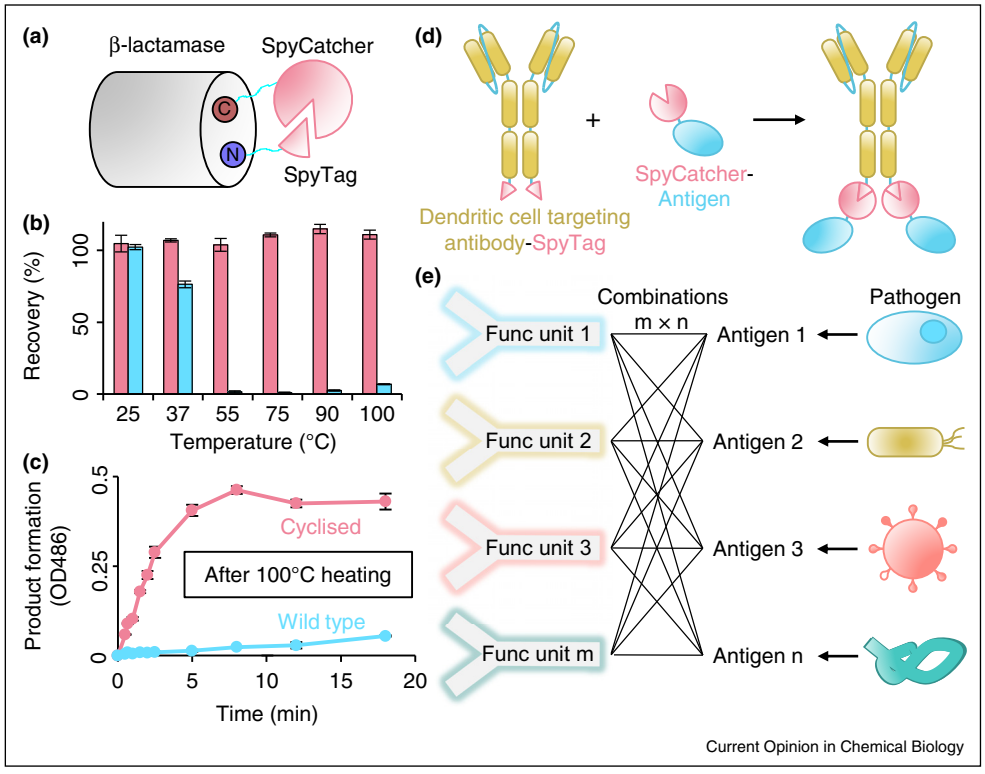
SpyTag/SpyCatcher reaction mechanism

The reaction mechanism of the intact CnaB2 domain (and presumably SpyTag/SpyCatcher) has been investigated by crystallography, NMR and quantum mechanics/molecular mechanics (QM/MM) [31,32]. The protein environment is vital for bringing the residues into optimal proximity and orientation. Also the hydrophobic surroundings alter the pK_a of the side-chains, favouring the more reactive neutral protonation states. Prior to reaction, the carboxyl groups of Glu77 and Asp117 are thought to form a double hydrogen-bond, facilitating reaction of Asp117 (Figure 1d). N_ε of Lys31 nucleophilically attacks the Cγ of Asp117, forming a zwitterionic intermediate (Figure 1d). There are then two concerted proton transfers, with the proximal Glu77 acting as a proton shuttle. A neutral tetrahedral intermediate forms, which then collapses with release of a water molecule (Figure 1d). H_2O release and formation of the amide bond is rate-limiting, with an energy barrier of 102 kJ/mol for the intact CnaB2 [31].

New uses of SpyTag/SpyCatcher for biotechnology

One area of promise is stabilising enzymes. Fusing SpyTag and SpyCatcher to the termini of a protein of interest leads to spontaneous cyclisation (Figure 2a). Our group employed this approach to generate cyclised β-lactamase and dihydrofolate reductase (DHFR) [33•]. SpyRing β-lactamase was resilient to aggregation up to 100 °C (Figure 2b) whereas the wild-type enzyme irreversibly aggregated over 37 °C. Following heating to 100 °C and re-testing at room temperature, the SpyRing enzyme retained most of its initial activity (Figure 2c), a greater impact on enzyme resilience than other cyclisation chemistries [33•].

Figure 2



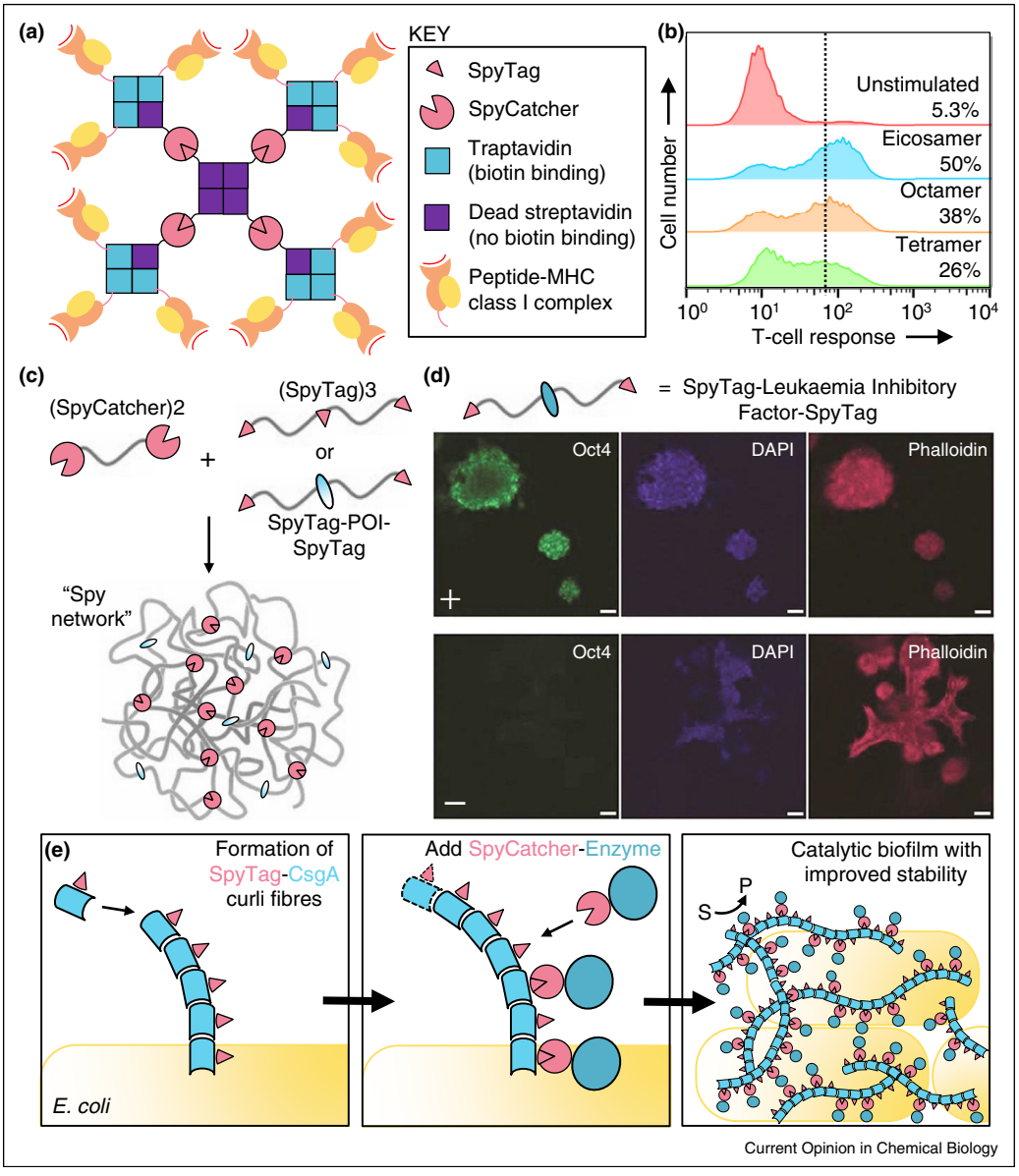
Applications of one-to-one SpyTag/SpyCatcher locking. **(a)** SpyRing generation: the protein of interest is genetically fused with an N-terminal SpyTag and C-terminal SpyCatcher (red), which spontaneously lock together. **(b)** SpyRing resilience to aggregation. Cyclised (red) or wild type (blue) β-lactamase were heated to the indicated temperature, centrifuged and soluble protein recovered (mean ± 1 s.d., $n = 3$). **(c)** Preservation of enzyme activity of cyclised (red) compared to wild type β-lactamase (blue) at room temperature, following heating to 100 °C for 10 minutes (mean ± 1 s.d., $n = 3$). **(d)** Modular vaccine production. A suitable immunoregulatory molecule, scFv-Fc (yellow) is fused with SpyTag and covalently reacts with SpyCatcher-antigen. **(e)** Production of synthetic vaccines by modular combination of function (Func) units with antigens. Classic genetic fusion requires cloning and expressing m × n possibilities; SpyTag/SpyCatcher conjugation only requires m + n expressions.

SpyTag/SpyCatcher was recently established for vaccine optimisation [34••]. An antigen is covalently linked to an immunoregulatory 'function' unit, such as an antibody targeting to the optimal location (e.g. dendritic cells, the most efficient antigen-presenting cell-type). SpyTag was fused to a single-chain Fv-Fc specific for the dendritic cell-surface marker DEC205 (Figure 2d). SpyCatcher was fused to the model ovalbumin antigen or a tick-borne encephalitis virus domain and then conjugated to anti-DEC205-SpyTag to create a full vaccine. The modular vaccine generated efficient cytotoxic T-cell and antibody responses [34••] and may overcome the frequent time-consuming challenges in finding expression hosts and folding conditions compatible with both the targeting unit and the antigen. The modular strategy also vastly reduces the number of constructs that need to be cloned and purified (Figure 2e).

Creation of new protein architectures and biomaterials using SpyTag/SpyCatcher

One current limit in the ability to engineer proteins is the construction of large, multi-component architectures in a defined manner. The specific and covalent SpyTag/SpyCatcher interaction provides a powerful way to build and link proteins into such assemblies [28]. One simple solution for higher-order assembly is to bring together SpyTag/SpyCatcher with one of the most widely used non-covalent assembly tools: streptavidin/biotin. We combined traptavidin (a streptavidin mutant with 10-fold decreased off-rate for biotin) with SpyTag/SpyCatcher, creating SpyAvidin nanohubs (Figure 3a) [35•]. Here, dead streptavidin subunits that are incapable of binding biotin are fused to SpyTag or SpyCatcher. Denatured subunits were mixed with traptavidin subunits; refolding and ion-exchange chromatography generated defined hetero-tetramers containing 1 to 4 SpyTags or SpyCatchers. When combined, tetramers spontaneously reacted to form specific assemblies with different biotin-binding capacities. Tetramers with 0–3 biotin-binding sites, octamers with up to 6 biotin-binding sites and even eicosamers with 12 biotin-binding sites were created (Figure 3a). The importance of these defined assemblies was shown by attaching biotinylated peptide-MHC class I complexes, an activator of T-cell signalling. The response

Figure 3



Creation of new protein architectures and protein-based materials using SpyTag/SpyCatcher. **(a)** SpyAvidin hubs for multimerisation, by integrating streptavidin/biotin with SpyTag/SpyCatcher. The eicosamer (20 streptavidin-based subunits) binds 12 biotin-containing ligands. **(b)** Stimulation of T-cell line using SpyAvidins with different numbers of peptide-MHC class I; Eicosamer = 12; Octamer = 6; Tetramer = 4 (proportion of cells above threshold marked). **(c)** Hydrogels for cell growth. ELPs fused with multiple SpyCatchers and SpyTags spontaneously form Spy networks. **(d)** Maintenance of pluripotency in a Spy network. Embryonic stem cells in a Spy network with (top row) or without (bottom row) SpyTag-leukaemia inhibitory factor-SpyTag were stained in green for the pluripotency marker Oct4. DAPI marks nuclei in blue and phalloidin marks filamentous actin in red. Reproduced with permission [37••]. **(e)** Catalytic biofilms. *E. coli* secreting SpyTag-fused CsgA make curli, decorated with SpyCatcher fused to the enzyme of interest and conferring increased stability to the enzymes.

of T-cells increased with increasingly multivalent stimulation (Figure 3b) [35•]. Given the huge range of biotinylated ligands commercially available, SpyAvidins should be a plug-and-play route to a range of stable defined assemblies.

Zhang and colleagues also built unfamiliar protein architectures, using SpyTag-fused and SpyCatcher-fused elastin-like polypeptides (ELPs) [36]. A small number of components generated architectures of diverse shapes, including star and H-shaped. Sun *et al.* expanded this strategy to synthesise hydrogels [37••]. Mixing ELP fusions of two terminal SpyCatchers with terminally and internally fused SpyTags, robust protein matrices termed Spy networks form spontaneously (Figure 3c). Spy networks were customised by introducing a protein of

interest internally: a Spy network containing leukaemia inhibitory factor (LIF) encapsulated and maintained the pluripotency of embryonic stem cells (Figure 3d) [37••].

Living systems often depend upon dynamic reversible interactions, but real-world applications of synthetic biology may require integration of living systems with irreversible assemblies, hence the interest in living materials. SpyTag/SpyCatcher has been applied to construct catalytic biofilms, which hold promise for complex industrial transformations. The self-assembling fibril subunit CsgA was fused to SpyTag and secreted from *Escherichia coli* [27•,38••]. The biofilm bearing the tagged curli filaments could then be functionalised with an enzyme (or multiple enzymes in a reaction pathway) site-specifically tagged to SpyCatcher to create a catalytic biofilm (Figure 3e). Incorporation of amylase into such biofilms increased resilience to harsh pH and solvents and could improve long-term stability [38••].

Future challenges

SpyTag/SpyCatcher has shown a range of intriguing applications, but every nascent technology requires optimisation. In our hands, fusion to SpyTag rarely disturbs function or yield. However, SpyCatcher is a larger fusion, even after we reduced the size from 116 to 84 residues [30]. SpyCatcher is also a split protein (and may have dynamic tertiary structure), so even though SpyCatcher is a satisfactory fusion tag in different proteins using bacterial and mammalian expression, there can still be a reduction in expression yield or surface display efficiency. One route we explored was to split SpyTag/SpyCatcher further into three parts: two peptide tags (SpyTag and KTag) and the third part, SpyLigase, to direct linkage of the two peptides. SpyLigase generated affibody polymers to enhance the sensitivity of magnetic cancer cell capture [39•]. The reaction rate of SpyTag/SpyCatcher is usually effective for *in vitro* ligation, but cellular proteins of interest may be at subnanomolar concentrations and so the required reaction times may be slower than cellular events. Therefore, work is ongoing to advance SpyCatcher towards an ideal fusion tag and the SpyTag/SpyCatcher reaction rate towards the diffusion limit.

Conclusions

SpyTag/SpyCatcher has now established itself as a simple route to covalent protein conjugation and is quickly finding application by a range of laboratories. This review has highlighted some exciting uses of SpyTag/SpyCatcher in biotechnology and synthetic biology. Many of the examples given are proofs-of-principle, so it will be exciting to see how the techniques will mature. For example, it will be important to see how far SpyTag/SpyCatcher cyclisation may be generalised for enhancing resilience, to facilitate molecular evolution and industrial enzyme application. Also, customisable hydrogels may be extended to regenerative medicine, transplantation and tissue engineering. Although precise nanoscale assembly can now be achieved with DNA, programmed nano-assembly with proteins is still in its infancy, but such initial applications suggest that protein nano-assembly will contribute to addressing major biological challenges.

Conflict of interest

M.H. is an inventor on a patent regarding isopeptide bond-forming peptides (European patent EP2534484).

Acknowledgement

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- •• of outstanding interest

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