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REVIEW PAPER OPEN ACCESS

Site-specific modification of proteins by chemical/enzymatic strategies

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Abstract

Functionalization of proteins is a particular domain for the generation of modified proteins constructs which exhibit stimulating and diverse biological properties. The area has enormous potential for chemists and biologists to tune the proteins functions. The site-specific modification of proteins by installing new moieties or subunits provides an excellent opportunity to expand proteins functional abilities. In this review, we highlighted the most significant studies in protein functionalization which include chemical and enzymatic strategies. Exploration of novel methodologies is still on-going and deserves significant attention for future research in protein functionalization.

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Introduction

Chemical biology lies in the strategic position in the intercept of life and physical sciences, and its emerging trends are of significant interest to the scientific community. Cutting edge research has been carried out in the fields of genome sequencing, protein modifications, and artificial enzymes(Jeremy M Berg *et al.*, 2002). However, the site-specific modification/functionalization of a protein is also an important domain to produce new constructs of biological interests. Proteins are multitasking masters of the cells which are involved in (a) catalytic reactions, storage, and transportation (b) provide mechanical and structural support to cells, and (c) control cell growth and differentiation(Chalker *et al.*, 2011). Proteins have versatility in their functions due to presence of a vast structural diversity of amino acids and their functional groups *i.e.* amino and carboxylic acids. Proteins undergo many post-translational modifications (PTMs) either in permanent or temporary way(Wu *et al.*, 2008). Natural PTMs are undertaken by enzymes which use the target protein both as a substrate and as a template.

Apart from these challenges in adapting Nature's cue to craft modified proteins *via* site-specific functionalization of proteins was critical for many different applications which include both *in vitroand in vivo* researches. Primarily, proteins are often used as probes or as tools for understanding biological systems at the molecular level, for instance, as biosensors(Veronese & Pasut, 2005). However, proteins can also be endowed with tailor-made properties so as to act as fluorophores or spin labels during spectroscopic study of protein-protein interactions. Unnatural amino acids can also be incorporated to investigate some protein residues at sub-atomic resolution. Attachment of hydrophilic polymers *via* covalent linkage such as PEGylation can also be used to enhance biocompatibility, furtivity and stability(Veronese & Pasut, 2005). In recent time, the manufacture of medicines involves the use of monoclonal antibodies due to site specific abilities of mAbs in curing certain diseases. The receptors are effectively blocked by the 'naked' monoclonal antibodies. The next generation of biological medicines after the monoclonal antibodies was the antibody drug conjugates (ADC) that help in limiting the off target effects(Boutureira & Bernardes, 2015a).

Site-specific modifications of proteins can also offer the selective mode of treatment against deadly diseases *i.e.* cancer. This review focuses the various methods of site-specific proteins modifications which include both chemical and enzymatic protocols. The review has been divided into three major parts namely: (a) chemical/biochemical modification of proteins, (b) enzymatic modifications, and (c) their representative therapeutic applications.

The first part describes the different strategies involved in site-specific chemical protein modifications. The attachment of synthetic groups to protein templates involved the use of various traditional and advanced methods, *i.e.* acylation, methylation, reductive amination, conjugate addition *etc.*, which has been summarized indetails by Boutureira and Bernardes(Bertozzi, 2001). The different chemical functionalities such as boronic acids, azides, alkynes or aryl iodides play a pivotal role in chemical modification of proteins (Fig.1). Moreover, by using the standard organometallic catalysts, proteins can be modified in a diverse and selective way *via* various cross-coupling reactions.

In the bioorthogonal ligation approach, biomolecules are site-specifically assembled in live cells or whole organism. The term "bioorthogonal" was first introduced by Bertozzi and Kiessling(Mühlberg *et al.*, 2009), which refers to the generation of covalent bonds in high yield under physiological conditions without interfering with the universe and network of cellular molecules.The major benefit of bioorthogonal ligation reaction was the use of mild conditions to couple functional groups to form a stable covalent linkage in a biological medium. The most commonly used reactions of this type are azide-alkyne "Huisgen cycloaddition" using Cu(I) catalyst, oxime ligation, Diels-Alder cycloaddition, hydrazone formation,

Staudinger ligation (Entry 30, Table-1, Fig. 5) and native chemical ligation (NCL)(Wang *et al*., 2003).Huisgen azide-alkyne (Entry 31,32,33, Table-1) coupling was the most versatile and commonly employed *via* bioorthogonal strategy to conjugate fragments in proteins both *in vitro* and *in vivo* and presents the advantage to generate a unique product in high yields. The use of bioorthogonal conjugation, such as Cu-catalyzed azide-alkyne cycloaddition approach by Sharpless (Fig. 6, 7)(Eeftens *et al*., 2015) has obtained significant advantages in protein modification. However, the Cu (I) toxicity limits this method and requires alternative approaches such as the development of copper-free methods(Wang & Schultz, 2004).

Chemoselective conjugation methods enable the addition of probes, drugs or hydrophilic polymers to proteins. In protein functionalization, the conjugation reactions of natural amino acids involve the nucleophilic attacks of heteroatoms (N, O, S) to carboxylic acid electrophilic center to link counter parts in a peptide bond (Fig.1). Among them, the most common nucleophile found in natural protein is cysteine thiol group which usually exist in a dissulfide bridge version. The second main target for protein conjugation isL-lysineresidues that can couple with carboxylic acid moieties. The site specificity of protein was somehow difficult because of their low natural occurrence (6%) of lysine in mammalian proteins(Lang & Chin, 2014a). A variety of uniquely reactive non-natural amino acids has been incorporated into proteins with the help of particular engineered bacterial strains resulting in bioorthogonal conjugation with proteins in bioconjugate chemistry(Lang & Chin, 2014b).

The modification by enzymesis based on the chemical reactivity of naturally occurring reactive amino residuessuch as*N*-terminal modification, *C*-terminalmodification and incorporation of unnatural amino acids. Once the eukaryotic nascent polypeptide comes out from ribosomes and a template copy of RNA, a portfolio of enzymes is available for their post-synthesis modifications in the C- and *N*-terminal. The cellular protein repertoire was increased by the modification taking place in the *N*-terminal residue(Lodish, 2008). One or more processing enzymes are needed to modify a majority of eukaryotic proteins such as the peptidases, transferases and ligases acting on the first amino acid of a polypeptide. In past, the common methods used for *N*-terminal modification was targeting the side chains of native amino acid residues (cysteine and lysine). The development of new chemical and enzymatic methods enables to tune proteins properties or functions. For example, the protein labelling by the attachment of new ligands opens new opportunities in biological or therapeutic ways. The concept of photoaffinity labelling of proteins was reported early 1960s by Frank Westheimer(De Graaf *et al*., 2009). Upon irradiation, a ligand covalently modified with a photoreactive group (PG) undergoes into reactive species leading to the attachment with the targeted macromolecules (photoaffinity labelling process). The identification of unknown enzymes/ receptors, the activity of an enzyme inhibitor and interaction of ligand-receptor was investigated *via* this modification strategy(Dubinsky *et al*., 2012).The commonly used PGs were diazirines, arylazides and benzophenones *etc*.

Altogether, this review focus on some recent chemical modifications, detailed site-specific enzymatic modifications which include sialyltransferase, *O*-GlcNAc transferase, formylglycine generating enzymes, sortase, farnesyl transferase, transglutaminase, biotin ligase, *N*-myristoyl transferase,lipoic acid ligase, and therapeutic application of new protein constructs(Rashidian *et al*., 2013).

Site-specific modification of proteins

Strategies for chemical and biochemical modification of proteins

The functionalization of proteins has been carried out by different methods. Common methods involve the reaction of lysine or cysteine residues with the protein fragment in the presence of excessamount of *N*-hydroxysuccinimidyl esters (amine reactive reagent)

or maleimides (thiol reactive reagent) to afford the desired hybrid macromolecule. The strategy successfully exploits the nucleophilicity of naturally occurring amino acids such as lysine, histidine and cysteine. Cysteine, for example, can react specifically with maleimides (even in the presence of other nucleophiles) to form thioethers or with other thiols to form disulphide bonds and was often used for site-specific functionalization of proteins (Fig. 2). Induction of cysteine residues at key positions of a protein can also be achieved by site-directed mutagenesis and regioselective functionalization. This strategy was successfully used for *in vitro* and *in vivo* imaging.(Brunner *et al*., 1989).

The *ω*-amino group of lysine residues can also be targeted for protein modification with activated esters, most commonly *N*-hydroxy-succinimide esters, and aldehydes *via* amide and imine formation, respectively. Diverse types of chemical modifications of protein are provided in the table-1 (modifications *of**N*-terminus and*C*-terminus)(Boutureira & Bernardes, 2015a).Some recent strategies involve the use of transition metals for protein modification.

Table 1. Different strategies for protein modifications.

Entry	Amino acid	Protein Modification	Linkage	Ref.
1	Cysteine	Disulfide bond formation *via* exchange reaction		Hemantha (2014) (Hemantha *et al*., 2014)
2	Cysteine	Thioether formation *via* alkylation		Hemantha (2014)(Hemantha *et al*., 2014)
3	Cysteine	Thioether formation *via* conjugate addition to maleimide Michael acceptors		Massa (2014) (Massa *et al*., 2014)
4	Cysteine	Selenide (Se)-Sulfide(s) linkage formation *via* Exchange reaction		Boutureria (2012)(Boutureira *et al*., 2012)
5	Cysteine	Thiazolidine formation via *N*-terminus		Casi(2012) (Casi *et al*., 2012; Yuan & Liang, 2014)
6	Cysteine	Alkynones formation *via* addition terminal carbon of triple bond		Shiu (2009)(Shiu *et al*., 2009)
7	Cysteine	3-Arylpropionitirle formation *via* addition to nitrile group bearing triple bond		Konia (2014)(Koniev *et al*., 2014)
8	Cysteine	Oxadiazole formation by reacting with Julia-Kocienski like reagents		Toda (2013)(Toda *et al*., 2013)
9	Cysteine	Vinyl-sulphide conjugate formation *via* addition to allenamide		Abbas (2014)(Abbas *et al*., 2014)
10	Cysteine	Thioether formation *via* rhodium carbenoid		Kundu (2013)(Kundu & Ball, 2013)

11	Cysteine	Ethenethiol linakge formation *via* allene-thiol coupling	Chan (2014) (Chan *et al*., 2013)
12	Lysine	Amide formation *via* succinimide intermediate	Bailey (2014) (Bailey & Bundle, 2014; Boutureira & Bernardes, 2015b)
13	Lysine	Thiourea/Urea formation *via* Isothiscyanate/isocyanate mooiety	Bailey (2014) (Bailey & Bundle, 2014; Boutureira & Bernardes, 2015b)
14	Lysine	Amine formation *via* reductive amination process	Bailey (2014) (Bailey & Bundle, 2014; Boutureira & Bernardes, 2015b)
15	Lysine	Pyridine ring formation *via* 6π-Aza-electrocyclization reaction	Tanaka (2011) (Tanaka *et al*., 2011)
16	Lysine	Amidine linkage formation by using 2-imino-2-methoxyethyl reagents	Robinson (2004)(Robinson *et al*., 2004)
17	Lysine	Synthesis of stable iminoborates in aqueous media	Cal (2012)(Cal *et al*., 2012)
18	Lysine	Formation of triazole *via* diazonium salts	Dietheln (2014)(Diethelm *et al*., 2014)
19	Lysine	Amide formation by using ketenes	Chan (2012)(Chan *et al*., 2012)
20	Tyrosine	Diazene formation *via* diazonium salts	Gaurilyrk (2012) (Gavrilyuk *et al*., 2012)
21	Tyrosine	Amine formation *via* three component Mannich-type reaction	Lorenzi (2011) (Lorenzi *et al*., 2011)
22	Tyrosine	Protein modification *via* reaction with imines	Guo (2009) (Guo *et al*., 2009)

23	Tyrosine	Protein modification by reacting with azomaleimides		Ban (2010)(Ban *et al.*, 2010)
24	Tyrosine	Protein modification *via* metal (Pd) catalysed allylic *O*-alkylation of amino acid (tyrosine)		Tilley(2006)(Tilley & Francis, 2006)
25	Tryptophan	Protein modification via Rhodium carbenoids		Antos (2014) (Antos & Francis, 2004)
26	Unnatural Amino acid			
27		Aldehyde and Ketones	Protein modification *via* oxime formation	Lundblad (2014) (Boutureira & Bernardes, 2015b)
28		Aldehyde and Ketones	Protein modification *via* hydrazone formation	Lundblad (2014) (Boutureira & Bernardes, 2015b)
29		Aldehyde and Ketones	Protein modification *via* Wittig reaction	Agarwal (2013) (Agarwal *et al.*, 2013)
30		Azide	Protein modification *via* Staudinger reaction	Lundblad (2014) (Boutureira & Bernardes, 2015b)
31		Azide	Protein modification *via* metal free click reaction	Debetes (2011) (Debets *et al.*, 2011)
32		Azide	Protein modification *via* Cu(I) mediated click reaction	Rostovtsev (2002) (Rostovtsev *et al.*, 2002)
33		Azide	Protein modification *via* Ru(II) mediated click reaction	Tam (2007) (Tam *et al.*, 2007)
34		Isonitrile	Formation of isonitriles *via* tetrazine intermediates	Stöckmann (2011) (Stöckmann *et al.*, 2011)

The role of Palladium in the chemical modification of protein has been summarized in details by Brik *et al.*(Brik & Jbara, 2017) A ruthenium-based organometallic compound has been employed in sub stoichiometric amount for protein medications *via* alkyne hydrosilylation. The coupling reaction has been optimized under physiological conditions which were comparable with well-known bioorthogonal reactions (Fig. 2).

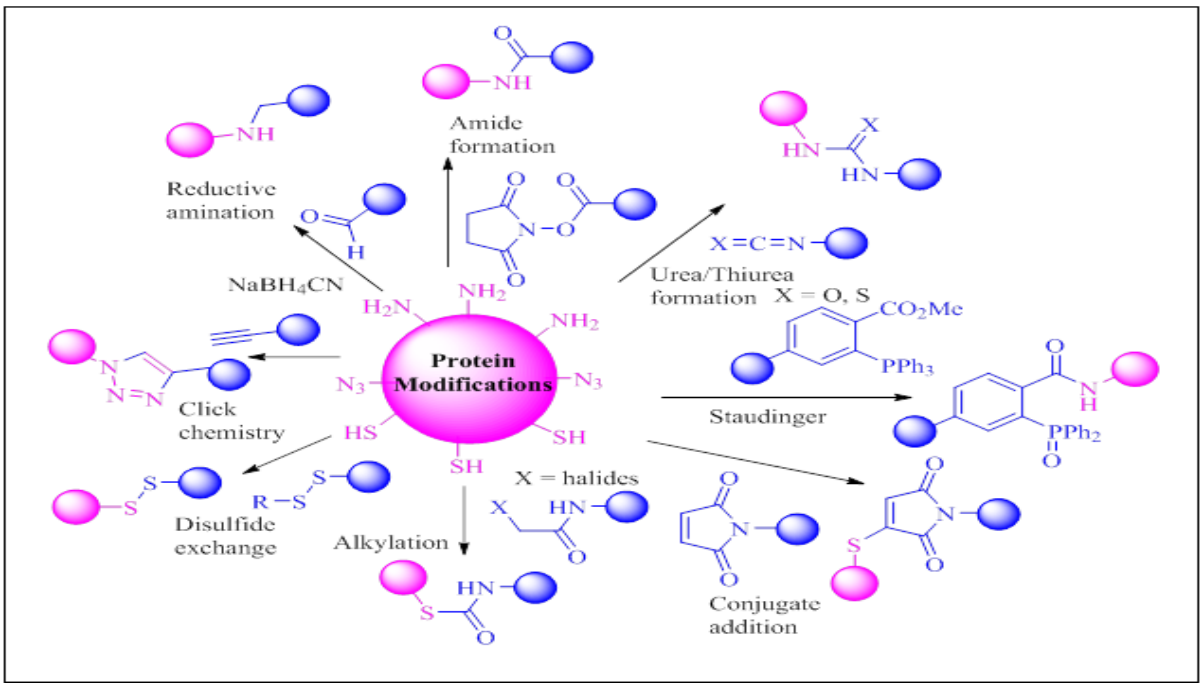

Fig. 1. Scope and potential of chemical site-selective protein-modification methods(Boutureira & Bernardes, 2015a).

Chemical modifications *viaN*- or *C*-terminus are the key points of protein functionalization. *C*-terminal modification of proteins usually occurs *via* two different types such as prenylation and GPI anchors.

Prenylation**:** It is an irreversible covalent PTM which is composed of farnesylation and geranylgeranylation that are found in all eukaryotic cells. The first protein prenylation was discovered in fungi in 1978. The first prenylated protein, farnesylated lamin B was found in 1988. This modification has gained importance due to its impact on the cellular activity of numerous proteins.

Three prenyltransferase enzymes, namely, farnesyltransferase (FTase), geranylgeranyltransferase type 1 (GGTase-1), and geranylgeranyl (20 carbons) isoprenoid group. Some of the known prenylated proteins are the subunits of trimeric G proteins, protein kinases, Ras and Ras related GTP-binding protein, fungal mating factors, nuclear lamins and one viral proteins(Zhang & Casey, 1996).

Mechanism of Prenylation: Protein prenylation involved: i) farnesylation, ii) proteolytic cleavage of aaX residues, and iii) carboxymethylation. The peptide motif at the *C*-terminal of the protein known as the CaaX-box motif was identified by FTase and GGTase-1(Lane & Beese, 2006). The *C*-terminal aaX tripeptide was cut from the newly prenylated protein by an endoprotease, Ras-converting enzyme 1(Rce1) or Ste24p. The prenylcysteine residue at the new *C*-terminus was methylated by the isoprenylcysteine carboxylmethyltransferase. The above three steps increased the protein hydrophobicity resulting in the association of the plasma membrane. The prenylated proteins were more stably localized by the presence of a polybasic domain upstream of the CaaX box or

palmitoylation at one or two cysteine residues (Palsuledesai & Distefano, 2015). The formation of site-specific modification of proteins such as PEGylated proteins, protein-DNA conjugates and dual labeled proteins are one of the important applications of the prenylation-based labelling strategy.

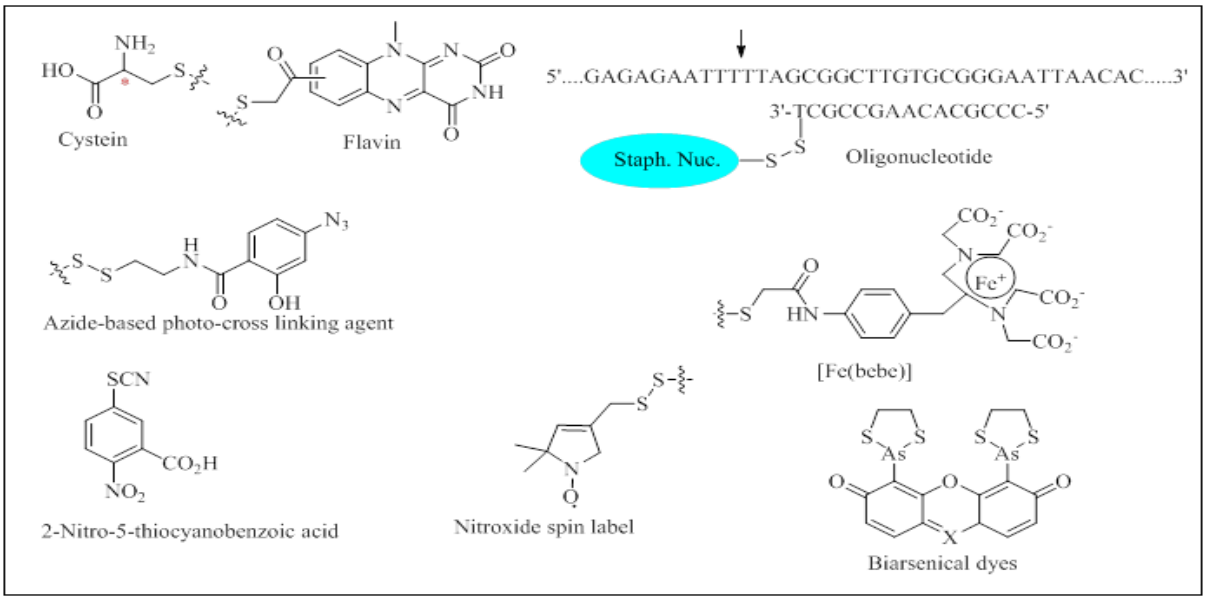

Fig. 2. Cysteine residues modified for the introduction of a wide range of nonpeptidic groups.

GPI anchors: The surface proteins and glycoproteins anchored to the cell membranes by a class of complex glycolipids termed as glycosylphophatidylinositols (GPIs).

The *C*-terminal GPI anchors about 10-20% of eukaryotic membrane proteins in the outer lipid leaflet of the plasma membrane(Orlean & Menon, 2007). The polypeptide *C*-terminus of all GPI anchored proteins and glycoproteins are attached to the phosphoethanolamine [(P)-EtNH$_2$] moiety at the mannose-III (Man-III) 6-*O*-position of the GPI anchors structure(Ferguson, 1999).

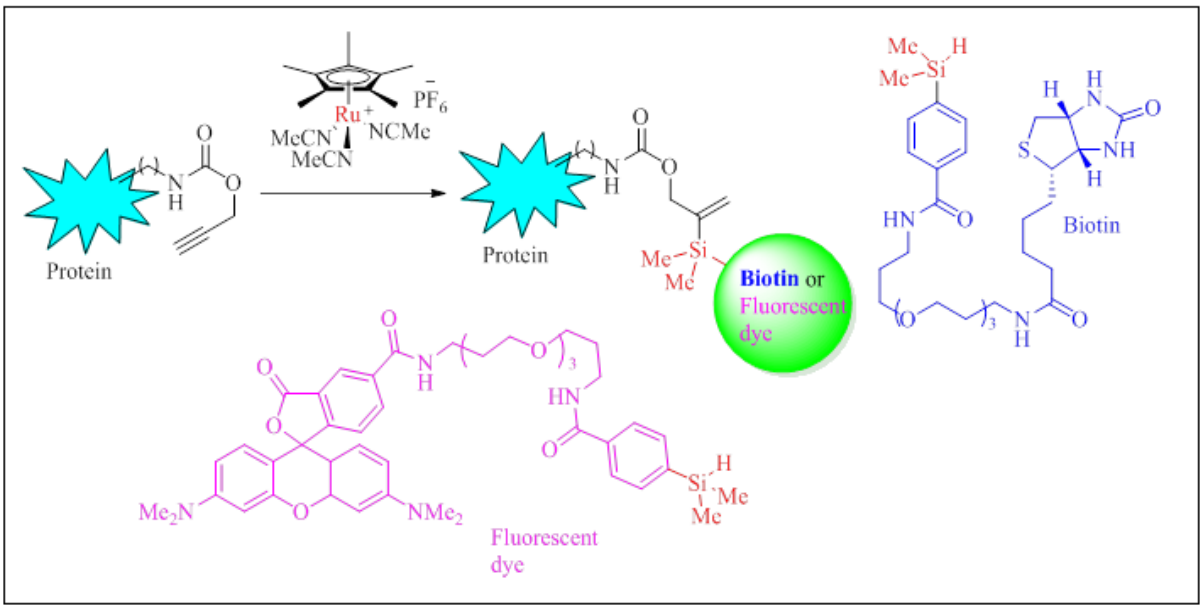

Fig. 3. Example of functionalization of proteins*via* hydrosilylation.

The requirement for efficient GPI anchoring was that the hydrophobicity of natural GPI signals was around the range of ΔG_{app} = 0 kcal.mol^{-1}(Galian *et al.*, 2012). The first total synthesis of an intact GPI anchor was reported by Ogawa and co-workers in 1991.Using native chemical ligation, short peptides were coupled with simple GPI analogues to obtain the first GPI-peptide conjugates (Tanaka *et al.*, 2003).

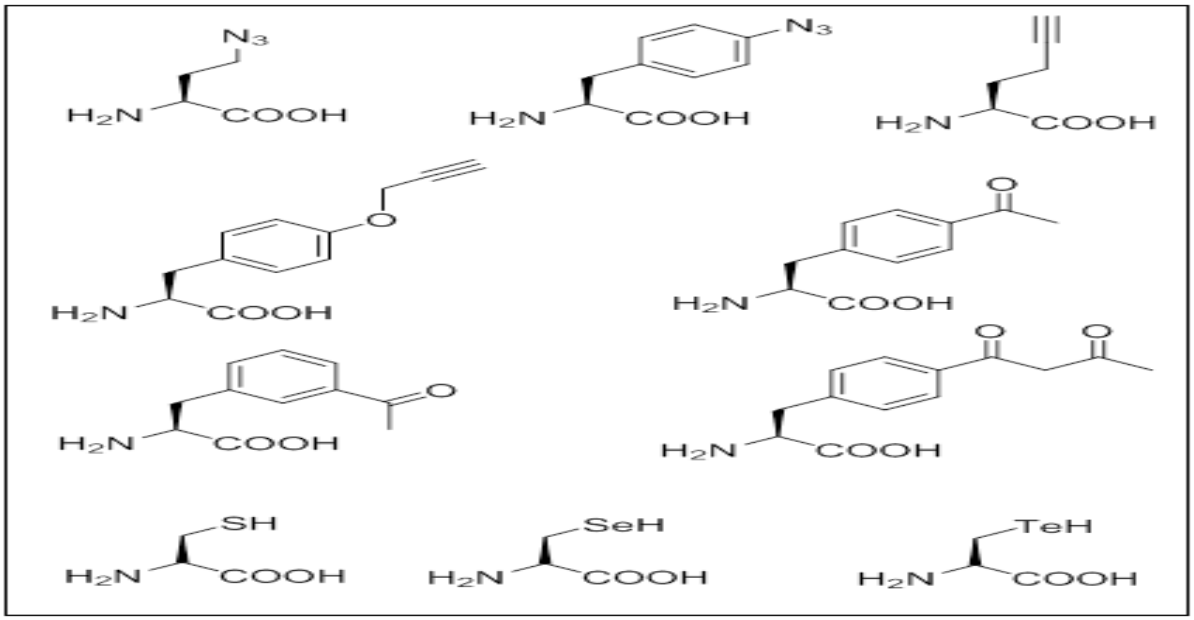

Fig. 4. Most commonly employed unnatural amino acids in specific-protein functionalization.

The first GPI-anchored protein with prion protein linked to a GPI anchor was prepared by Seeberger and co-workers (Becker *et al.*, 2008). The regiospecific coupling of extensively protected peptides/glycopeptides carrying free *C*-termini with GPIs carrying a free (P)-EtNH$_2$ moiety, followed by global deprotection was an alternative method for the preparation of GPI-linked peptides and glycopeptides(Xue *et al.*, 2003).

GPI chemical synthesis: The isolation of natural GPIs was difficult due to the presence of carbohydrates, lipids and other moieties in heterogeneous forms. Therefore, to get the homogenous and structurally well characterized GPI for certain bioactivity purpose, chemical synthesis modes were utilized to prepare the desired GPI. The GPI synthesis takes place by two different methods. The first method involves the construction of GPI skeleton and then *via* manipulation of protecting groups to install regioselectively the phosphate and lipid groups. The strategy suffers with the late installation of phosphate groups. While in the other strategy, trimannose, pseudodisaccharide and phosphorylating reagent are used for the assembly of the target GPI.

This was called convergent strategy and has been successfully applied in the synthesis of many GPIs and their derivatives(Swarts & Guo, 2010).For additional structural diversity, branched GPI anchors have also been synthesized using orthogonal protecting groups(Yu *et al.*, 2013). Among the orthogonal approaches, native chemical ligation (NCL) has been widely employed where a *C*-terminal thioester reacts with an *N*-terminal cysteine(Sun *et al.*, 2013).

The thiol of the cysteine first undergoes a transesterification, leading to a new thioester. Subsequently, an S→N acyl shift leads to the formation of a new peptide bond(Chin *et al.*, 2003).This technology expands the semi-synthesis of proteins to approximately 100 to 200 amino acids. However, the major limitation of this approachis the need ofa specific enzyme-substrate complex and methods of purification (Zhang *et al.*, 2003).

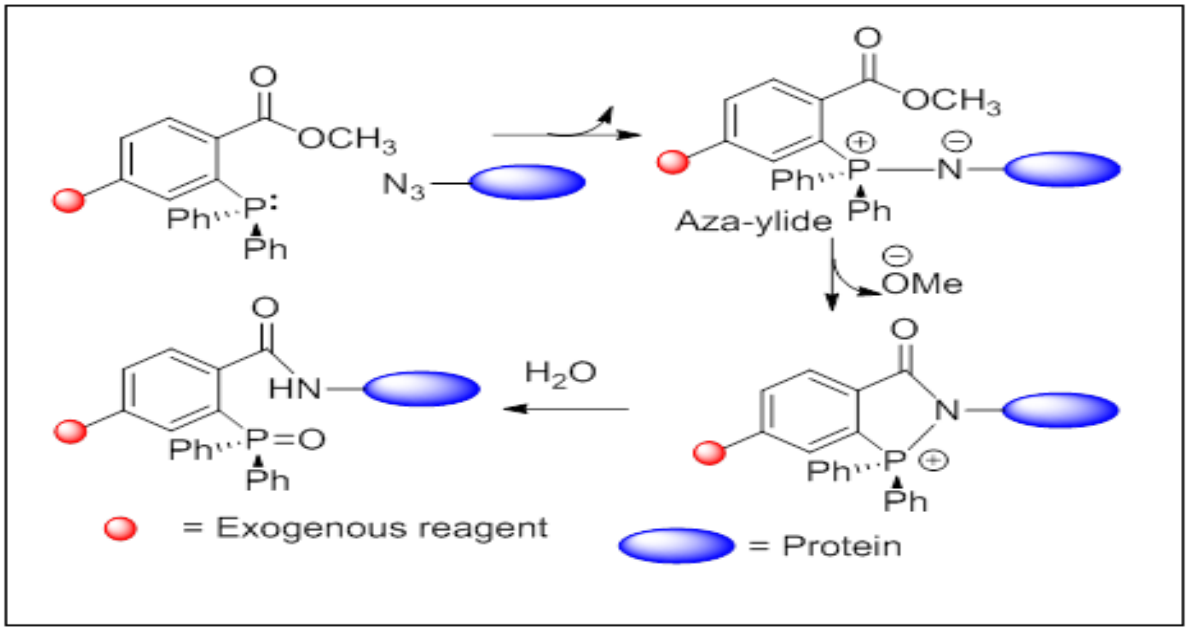

Fig. 5. Staudinger ligation between a protein bearing azide moiety and phosphine reagent.

Incorporation of unnatural amino acids (uaas) by protein engineering

The Schultz group has developed a powerful methodology for the incorporation of unnatural amino acids during ribosomal protein biosynthesis. Using an auxotrophic strain of a particular amino acid has been considered as a classical method employed for the incorporation of unnatural amino acids (UAAs) (Fig. 4)(De Graaf *et al.*, 2009).

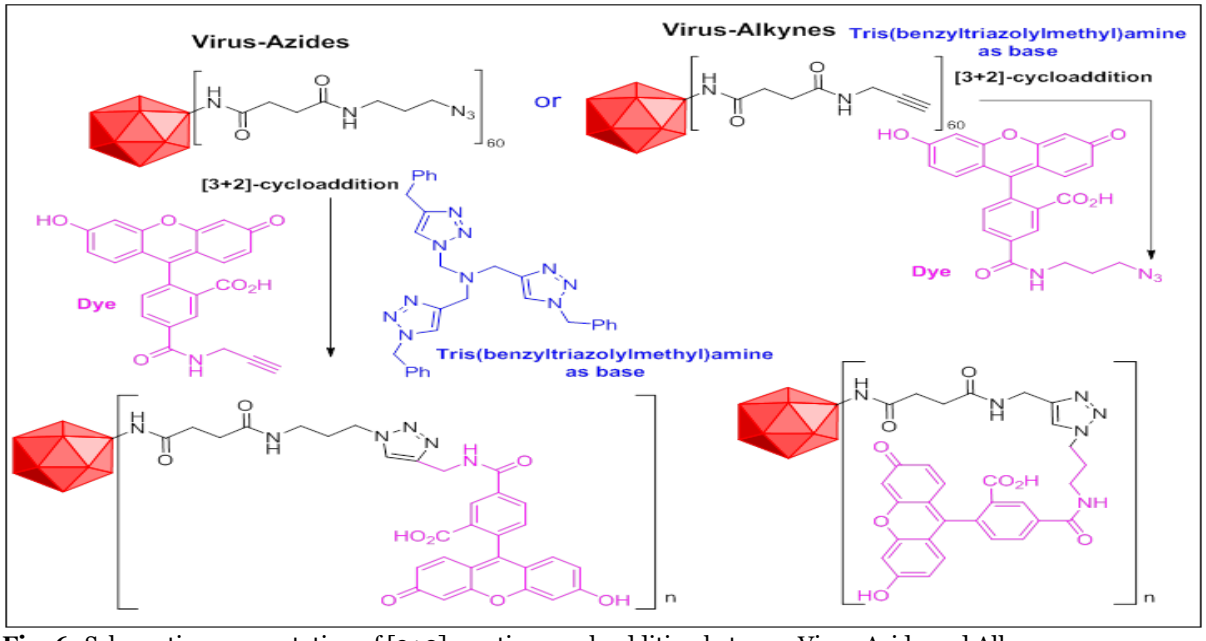

Fig. 6. Schematic representation of [3+2] reactions cycloaddition between Virus-Azide and Alkyne.

This method has opened up new avenues for the development of recombinant proteins where a single unnatural amino acid can be incorporated into the protein at a specific position. Mutated tRNA in which anticodon loop with capability of identifying a stop or a 4 bp frame shift codon has also been developed. After connecting the unnatural amino acid to the mutated tRNA *via* chemical or enzymatic reaction, site-specific incorporation was performed leading to the corresponding recombinant protein. Amino acids

bearing different bioorthogonal groups, such as alkyne, alkene, azido, selenol, and tellurol were successfully used. The limit of this method lies in the bulkiness of the side residues limiting their acceptance by the ribosome. Nevertheless, the development of recombinant ribosome with different sizes of gatekeeper has been a promising way for selective incorporation of unnatural amino acids.

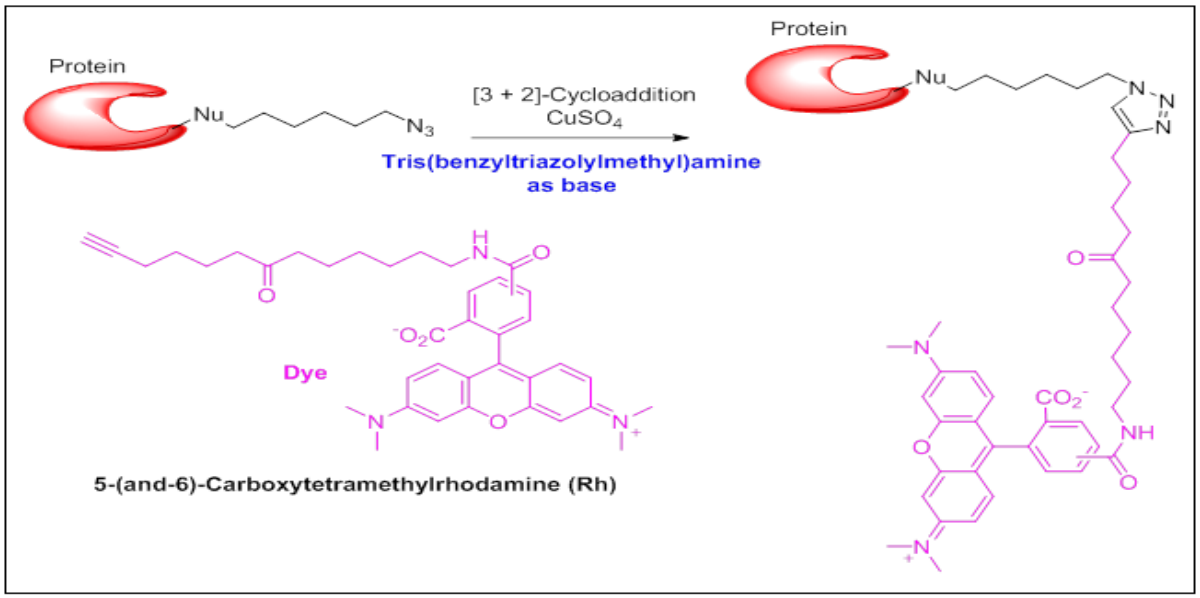

Fig. 7. Schematic representation of [3+2] reactions cycloaddition between Protein-Azide and Alkyne group.

More recently, this methodology was successfully extended to *in vivo* studies using recombinant strains of *E. coli* auxotrophic for the incorporation of unnatural amino acids to recombinant proteins(Wang & Schultz, 2005). The newly synthesized proteins from stable cell line lysates were identified to label the cell surface of *E.colivia* CuAAC techniques(Hudis, 2007). An alternative approach was proposed by Uttamapinant *et al.* where chelating azides are used for loading a reduced amount of the required metal that allowed cell-compatible labelling (Lee *et al.*, 2009). The advantage of using CuAAC for *in vitro* protein modification was its fast reaction rate and easy to use in nature. The construction of highly-valent protein nanoparticles, QuaNCAT-quantitative method for primary cell proteomics, creation of PEGylated proteins, production of dual PTM glycoprotein mimics due to its orthogonality with the presence of cysteine. Cellular proteomic analysis (BONCAT) is the most significant application of CuAAC(Spicer & Davis, 2014).

Also, unnatural azido- and alkyne-containing amino acids werealso incorporated into methionine auxotrophic *E. coli* strains by the above method (Kiick *et al.*, 2002). The disadvantage of this method includes limitation *in vivo* studies and causes of cellular toxicity at higher concentration. The tyrosine tRNA/aaRS of archaebacteria Methanococcus jannaschii and the pyrrolysine tRNA/aaRS of Methanosarcina barkeri/mazeri are the two commonly used systems for the incorporation of UAAs(Liu & Schultz, 2006). An incredibly diverse range of over 150 UAAs possessing varied structures, functionalities and reactive handles were incorporated with the help of the above two machineries. The requirement for an easy access to the public of the required plasmids for all protein expression systems are some of limitations for this method.The incorporation of unnatural 'tags' into proteins that undergoes to site-selective modifications was carried out by the suppression of an amber-stop codon and was one of the indispensable tool in the recent advances that increased the efficiency of suppression by knocking out the gene Rf1(Release factor one) in *E. coli* and suppression of amber in living animal(Johnson *et al.*, 2011).

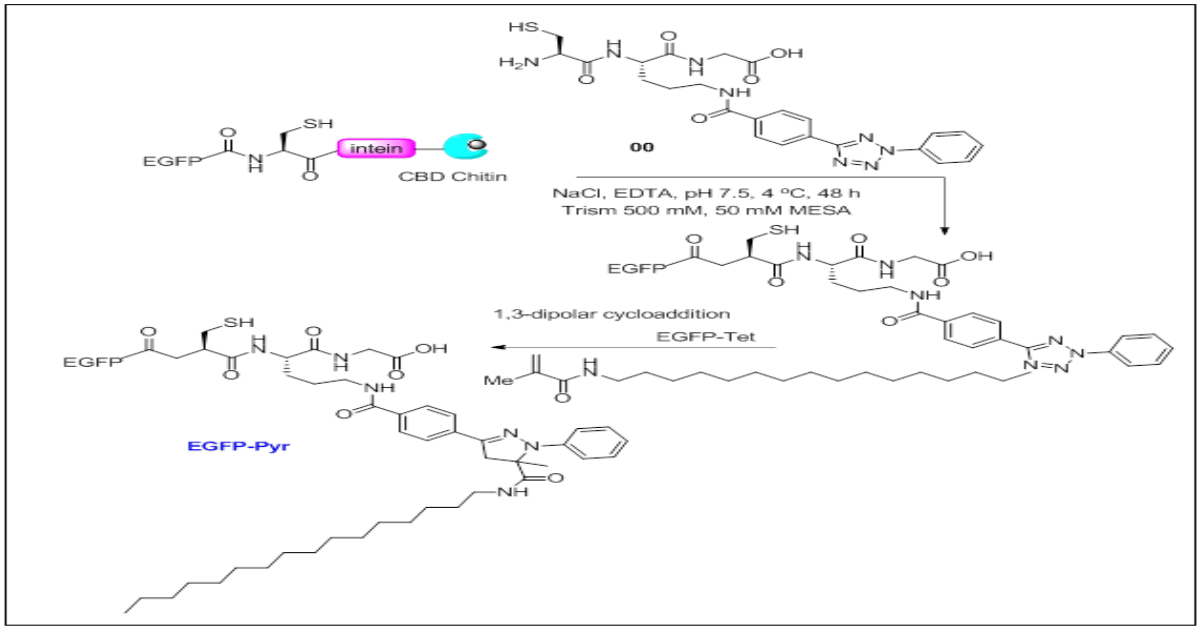

Fig. 8. Analysis of EGFP-Pyr through photoinduced 1,3-dipolar cycloaddition.

The first metabolic labelling of proteins with ^{15}N-labeled tyrosine in rat was reported by Schoenheimer(Ngo & Tirrell, 2011).

The other non-naturally occurring amino acids can be incorporated into protein fragments. Earlier, the idea of unnatural amino acid incorporation was reported by Munier, R. *et al.* in 1956 by using selenomethionine to modify bacterial proteins(Munier & Cohen, 1959).

Using this approach, the cowpea mosaic virus wascoated with azido or alkyne-containing fluorescent moieties by nonspecific lysine labelling of the caspide (Fig.6)(Wang *et al.*, 2003).

The breakdown in capside structure, ion specific labelling, and requirement for organic co-solvent, incomplete conversions and the regioselectivity for the triazole formation are some of the limitations of this method. Later, the activity-based profiling of intracellularly labelled proteins was undertaken by CuAAC for highselectivity in cellular lysatesindicating the possible tolerance for cellular components (Fig. 7)(Speers *et al.*, 2003).

Prior to Bertozzi's work in 1960s, highly strained cyclooctynes reacted with azide-'tagged' glycoproteins at room temperature without any exogenous ligands or catalysts(Kim *et al.*, 2013). The CuAAC reaction showed similarly slow kinetic compared to Staudinger ligation(Agard *et al.*, 2006).During Zebrafish embryo growth, DIFOs was used to envisage the glycans development that showed 'bio-orthogonality' at moderate rate than the Staudinger ligation(Laughlin *et al.*, 2008).

The limitation involved in this method is the difficulty in the synthesis of unstable compounds and the low reaction rate and speed (Lang *et al.*, 2012b).

When the reactive dienes *trans*-cyclooctene and norbornene reacted with suitable tetrazine dienophiles, the rate of protein labelling was found to be1000 times faster than CuAAC(Devaraj *et al.*, 2008).

The isomerisation of trans-cyclooctenes takes place in the presence of thiols, formation of reaction mixture of regioisomers that prevented the essential syntheses of functional structures and inherent instability of tetrazines are the disadvantages of this method(Lang *et al.*, 2012b). Some tetrazoles reacted as a latent source of nitrile imines that undergo [3+2]-cycloadditions with unactivated alkenes was another procedure for cycloadditions on proteins (Fig.8).

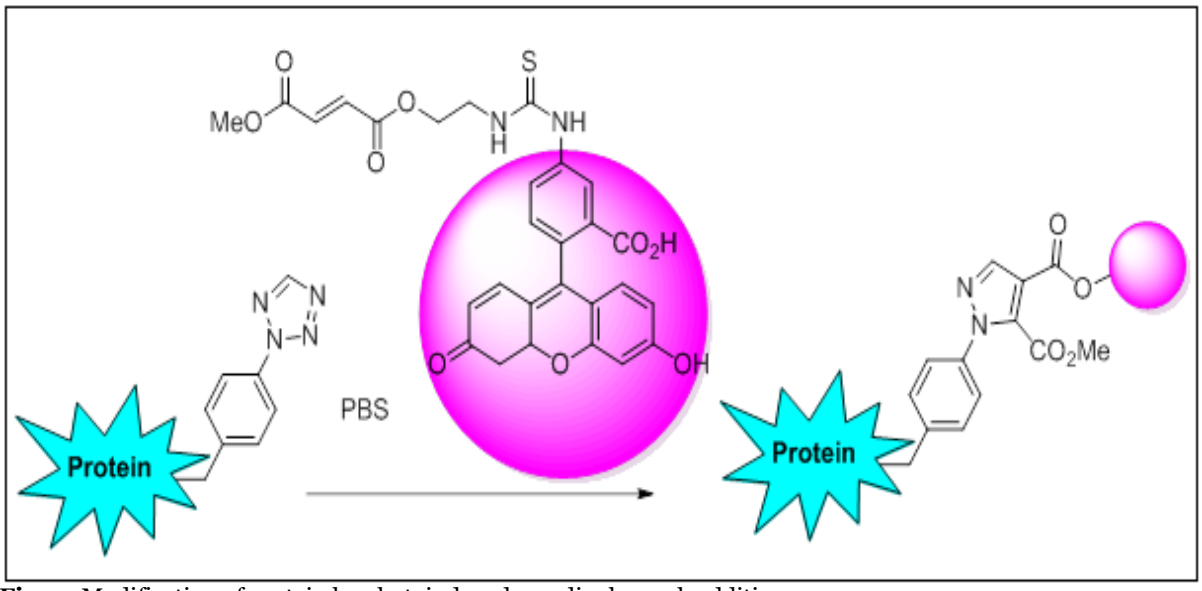

Fig. 9. Modification of protein by photoinduced 1, 3-dipolar cycloaddition.

A photoactivated 1,3-dipolarcycloaddition reaction between 2,5-diphenyltetrazole and methyl crotonate was reported by Huisgen and his co-worker. A rapid and highly selective modification of proteins carrying a diaryl tetrazole group in biological media was reported by using a bioorthogonal, photoinducible 1,3-dipolar cycloaddition reaction. This reaction was tolerant of some protein moieties and was extremely fast. The tetrazole group was attached to the protein by semi-synthesis of the protein and treatment with a simple set of alkenes. This reaction was useful in influencing the function of the modified protein in living systems(Song *et al*., 2008). An irradiation with ultraviolet light was required for this technique and was termed as 'photo-click'. A number of alkenyl-UAAs such as homoallylglycine and cyclopropenes were modified by this reaction (Fig. 9)(Wang *et al*., 2010). The site-selective protein modification with unnatural amino acids was improved by combining a reassignment of sense and nonsense codon (Cui *et al*., 2017).

Site-specific mutagenesis and chemical modifications

The UAA *p*-iodophenylalanine was proposed as a Palladium coupling partner by incorporating it into proteins via the suppression of an amber-stop codon(Santoro *et al*., 2002).Brustad *et al.* demonstrated that *p*-boronophenylalanine embarked Suzuki couplings. But the yield was low and high temperature was required for the reaction to cause the denaturation of the protein(Brustad *et al*., 2008a). Through the discovery of water-and-air-stable ligand, 2-amino-4,6-dihydroxypyrimidime (ADHP,L1) assuming the Suzuki-Miyaura cross-couplings at 37 ℃ in water at pH=8, the first efficient Pd-mediated reaction on a protein was demonstrated by Chalker *et al.*(Chalker *et al*., 2009b).

It was found that the coupling on the cell surface of *E.coli* was relevant to the Suzuki-Miyaura reaction(Spicer *et al*., 2012). The glycoproteins in a cellular synthetic glycocalyx are mimicked by the coupling of carbohydrate-boronic acids to cell surfaces(Spicer & Davis, 2013).A high-yielding site-specific PEGylation of protein was achieved by using the self-liganding boronic acids with $Pd(OAc)_2$(Dumas *et al*., 2013).The allyl sulfides act as a substrate for the aqueous cross-metathesis with Hoveyda-Grubbs II catalyst through a proposed sulfur-relayed mechanism(Lin & Davis, 2010). The prognostic rule for this protein reaction was self-metathesis, reagent reactivity and discovery of sensitiveness to handiness(Lin *et al*., 2010). The significant increase in the reaction rate and expanded the substrate scope are due to the tune modification of heteroatoms (S--Se) 135 and was used to a chemically controlled 'write-read-erase' histone protein alteration cycle.

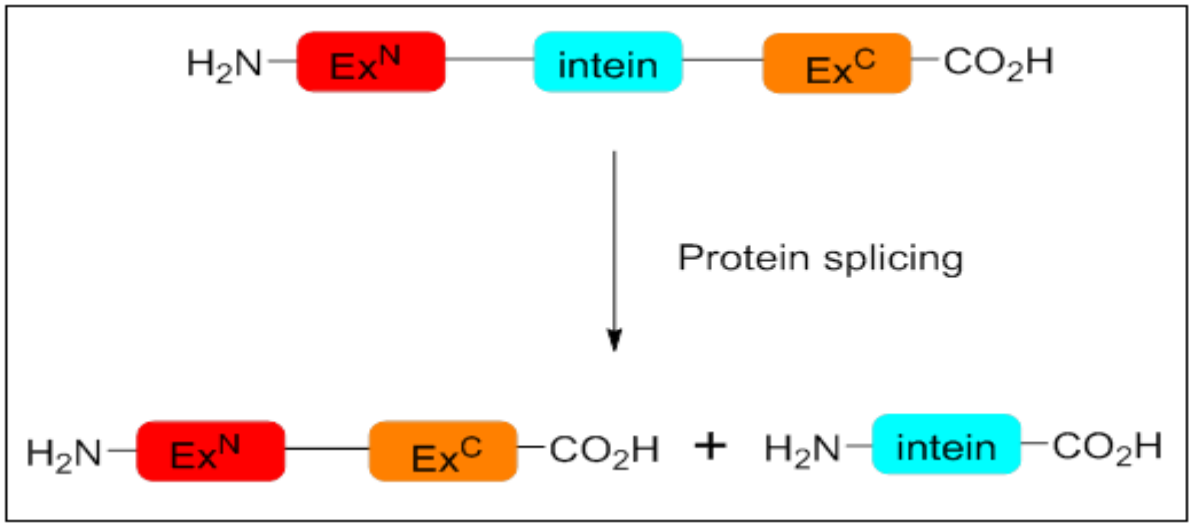

Fig. 10. Intein-mediated splicing of protein: Intein self-catalyzes its splicing. The intein-mediated splicing connects together the two flanking regions ExN and ExC.

Another representation of interesting TM catalysis was using rhodium-generated carbenoids formed from diazo reagents for modifying the tryptophan residues[44]. Using a structure-directed approach, rhodium-bound metallopeptides was used to catalyze the modification of tryptophan(Popp & Ball, 2010).The limitation of this method was that high specific interaction was required for directing this reaction.

The aldehydes and ketones were found to play an important role in the conversion of hydrazines and hydroxylamines respectively into hydrazones and oximes under acidic environment andthis reaction was accelerated by nucleophilic catalysts(Dirksen & Dawson, 2008).The site-specific protein tagging was carried out by periodate cleavage of *N*-terminal Ser/Thr residues leading to a terminal aldehyde(Geoghegan & Stroh, 2002).The selective modification of antibodies and filamentous phage are permitted by a range of amino acids supported by this reaction(Carrico *et al.*, 2012).

The ketone-containing amino acids are genetically incorporated by amber-stop codon deletion through chemical acylation of a tRNA synthetase. Without chemical acylation,ketone-containing amino acids,*p*-acetylphenylalanine and *m*-acetylphenyalanine, were successfully incorporated into proteins of E.coli and eukaryotic cells(Geoghegan & Stroh, 2002).

An improved reaction kinetics was displayed by the incorporation of aliphatic ketone-containing amino acids and an increased stability of the oxime products was reported in diketone-containing amino acids(Zeng *et al.*, 2006). A natural formylglycine-generating enzyme in both prokaryotic and eukaryotic cells was directed by a six-residue sequence tag(Cuculis *et al.*, 2016). The drawback of the reaction involving aldehydes and ketones are the existence of a range of carbonyl-containing substrates in cells but was not suitable for *in vivo* applications(Popp & Ball, 2010). The alkyl cysteine analogues was generated by the UAA dehydroalanine (Dha) that acts as a Michael acceptor to react with sulfur nucleophiles and creating an electrophilic secondary to nucleophilic reaction of cysteine(Chalker *et al.*, 2009a). The elimination of active-site serines, moderate oxidative removal of cysteine with sulfonylhydroxylamine reagents and oxidative discharge of unnatural selenocysteine amino acids are the number of routes through which Dha was accessed(Bernardes *et al.*, 2008). The number of thioether mimics of natural protein modifications such as phosphorylation, lysine methylation/acetylation, lipidation and glycosylation were set up by the addition of functionalized thiols to Dha (Wang *et al.*, 2012).

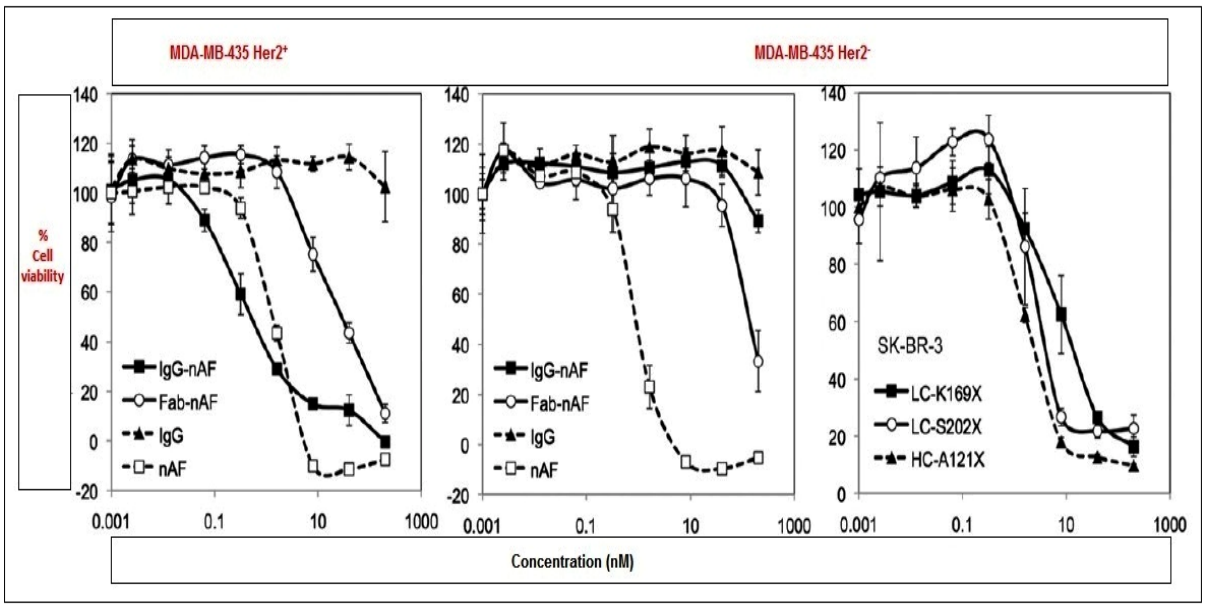

Fig. 11. Cytotoxicity *in vitro* assays. (a) Anti-Her2-IgG was more cytotoxic than unconjugated auristatin-linker and Fab on MDA-MB-435/Her2+ cells, (b) IgG was less active on MDA-MB-435/Her2- cells, (c) IgG was less active on MD-MBA-435/Her2-cells.

The first reaction of chemical mutagenesis was carried out in 1965 by Wilchek *et al*(Zioudrou *et al*., 2002) by taking advantage of the conversion of serine to cysteine residues in polypeptides. The advantages of using chemical approach for the rapid modulation of the side-chainstructure are the introduction of cysteine and unnatural analogues by tosylation of serine and SN_2 displacement. The chemical transformation of one particular amino-acid side chain into other was first reported by Koshland in 1966(Polgar & Bender, 1966). The cysteine residue was obtained by the chemical conversion of the active-site serine of subtilisin in this method. Clark and Lowe(Clark & Lowe, 1978) showed the first example of mutational divergence in the modification of different side chain of amino-acids was provided by the chemical mutation of papain. It was carried out to produceglycine and serine fromcysteine. Using phenylacyl bromide, the reactive active-site cysteine of papain was selectively alkylated and mutated. The phenacyl-inhibited protein was converted into the desired thioaldehyde product by repeated photolysis. The formylglycine was formed by the slow hydrolysis of the corresponding thioaldehyde after the release of hydrogen sulfide. A retroaldol reaction was then performed by reducing formylglycine with $NaBH_4$in the presence of serine residue at pH=9leading to the production of a glycine residue(Wright *et al*., 2016). The mutants of protein structure-function studies were generated by an easy and effective site-directed mutagenesis using two single-primer reactions(Edelheit *et al*., 2009).

The central role played by phosphocarrier protein IIIGlc, a major signal-transducing protein in E. coli was studied by site-directed mutagenesis.

The form I of green fluorescent protein (*GFP*) was stabilized by the double mutant Thr203Val/Glu222Gln *via* site-directed mutagenesis(Wiehler *et al*., 2003).A family of 'brightening' genetically encoded calcium indicators (GECis) with voltage sensitivity than the parent probe; ArcLight was produced by three rounds of site-directed mutagenesis. The directed evolution was also used to create the first GFP-based genetically encoded voltage indicators (GEVis) showing a positive slope relationship in $\Delta F/\Delta V$ (Platisa *et al*., 2017).

The fundamental working of the T3SS protein and three types of secretion system were studied by pDM4-based site-directed mutagenesis strategy using Yersinia as model.

The point mutation and in-frame deletion mutations was identified by this method in order to understand better the molecular action between the various components of the Yersinia type III secretion system(Nilles *et al.*, 2017).A Harobin double mutant, NIIIR/R230G was constructed by site-directed mutagenesis. It was found to be expressed and active in high level during hypertension and thrombosis. As a result, it was concluded that NIIIR/R230G was an appropriate candidate for biotechnological applications (Huang *et al.*, 2017).

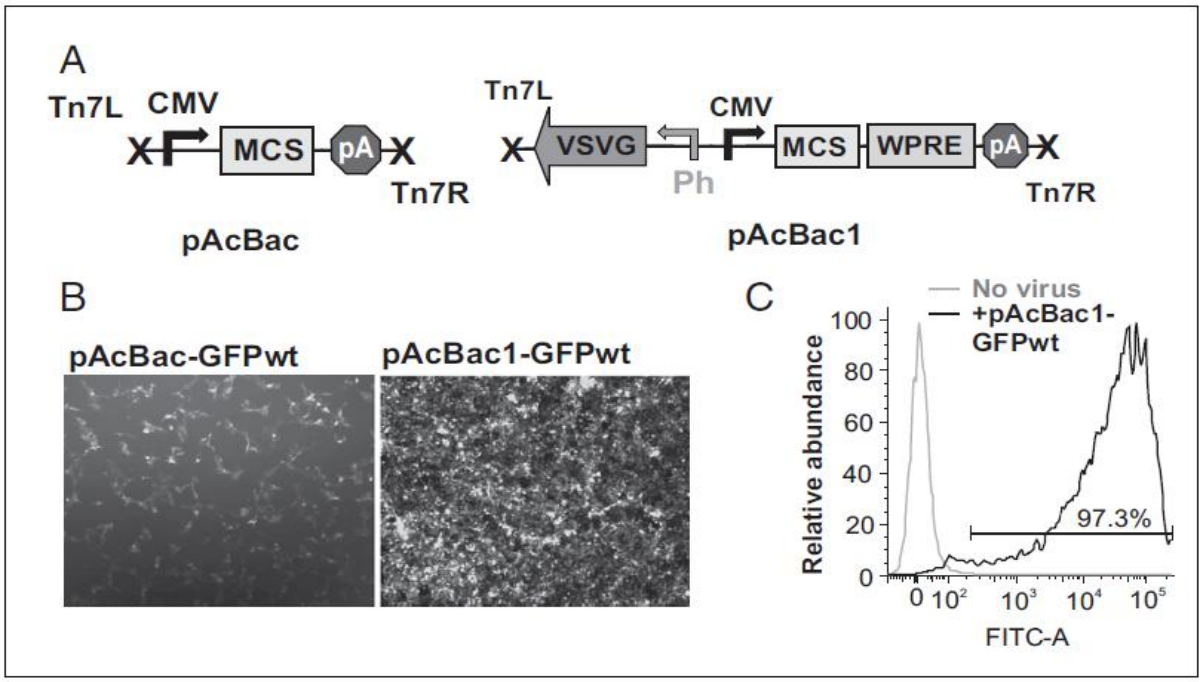

Fig. 12. Production of baculovirus vector. (A) Baculovirus plasmid with a CMV promoter (B) Using pAcBac-GFPwt and pAcBac1-GFPwt derived baculovirus, eGFP(wt) was expressed in HEK293 cells, (C) 48h post-infection of HEK293 cells with pAcBac1-GFPwt FACS analysis. (Reproduced from ref (Wang *et al.*, 2013) with permission of PNAS).

The synthesis of the antitumor agent, epothilone A was used for the synthesis by 2-deoxyribose-5-phosphate aldolase (DERA) by the condensation of two aldehydes (Pei *et al.*, 2011). A hepatocyte growth factor (HGF) was engineered by site-directed mutagenesis to study the role of cell surface heparan sulfate glycans(HS) in biological signaling and ligand-receptor interaction. It was concluded that this protein plays a central role in the HGF-driven mitogenesis, metastasis and tumor growth. The characterization of Talaromyces thermophilus F1208 xylanase was engineered by the extension of the *N*-terminal and site-directed mutagenesis in the *C*-terminal resulting in a particular hydrolytic feature (Li *et al.*, 2017). The peptide containing site specific advanced glycation endproducts (AGEs) was synthesized by site-specific mutagenesis to obtain a new conjugated glycoprotein in high yield. This modified AGEs protected the native protein from proteolytic digestion(Kaur *et al.*, 2016).

Lucia *et al.* reintroduced two missing copper ligands in the SOD-like protein (superoxide dismutase) of *Bacillus subtilis* to convert an inactive prokaryotic SD homologue into an active protein(Banci *et al.*, 2005).Another study involved a series of ubiquitin mutants created by site-directed mutagenesis by exchanging the lysine residues with asparagine residues. Later, evaluation of the mutants was carried out by aselective non-covalent adduct protein probing-mass spectrometry (SNAPP-MS) to ascertain the absolute contribution of each lysine as a binding site for 18-crown-6 (18C6)(Liu *et al.*, 2008). The role of Serine-60 (Ser-60) in a catalytic process studied

using site-directed mutagenesis. Ser-60 was considered as a conserved residue on one rim of the substrate binding cleft and found to reduce or eliminate the enzymatic activity of Drosophila protein L-isoaspartyl methyltransferases (dPIMT), site-directed mutagenesis(Bennett *et al.*, 2003).In another interaction study involving protein-carbohydrate in human lysozyme was probed using a combination of site-directed mutagenesis and affinity labelling. An intrinsic conformational rigidity was observed in the structures of disaccharides(Muraki *et al.*, 2000). Site-directed mutagenesis was used to evaluate the phospholipid transfer activity and HDL binding ability in wild-type human plasma phospholipid transfer protein (PLTP) and in 16 PLTP variants produced by replacing 12 charged amino acids. It was found that the N-terminal pockets was critical for PLTP transfer activity and the reduction of PLTP activity was carried out by amino acid substitutions by decreasing the affinity of PLTP for high-density lipoproteins (HDLs)(Ponsin *et al.*, 2003).

The easiest and most straightforward way to induce gerrmline mutations at high frequency was provided by chemical mutagenesis. The different mutagenic portfolios are diethyl sulfate (DES), diepoxybutane (DEB), acetaldehyde, nitrosoguanidine (NTG), trimethylpsoralen with ultraviolet light (UV/TMP), formaldehyde, nitroguanidine (NTG), diepoxyoctane (DEO), ethyl methanesulfonate (EMS), *N*-ethyl-*N*-nitrosourea (ENU) and a cocktail of EMS/ENU. The EMS and UV/TMP have been the most widely used mutagens in *C.elegans*(Thompson *et al.*, 2013).

Photoaffinity labelling: The commonly used photoreactive groups (PG) for photoaffinity labelling are benzophenones, arylazides and diazirines. Upon irradiation, benzophenones produced reactive triplet carbonyl states. The advantage of using benzophenones was its inertness to the solvent and long wavelength of irradiation. A long period of irradiation was required for the use of benzophenones and has been one of its drawback(Prestwich *et al.*, 1997).The next commonly used PG was arylazides are easily prepared but the short wavelength at which it was excited will damage biological macromolecules(Platz, 2002).The reactive carbenes are generated by diazirines upon irradiation leading to the cross-coupling to the neighbouring atoms(Gilchrist, 1969).The most important characteristic of carbenes was their ability to quickly form a covalent bond with the nearest target molecule by the insertion of C-C, C-H, O-H and X-H (where X = heteroatoms). These diazirines are stable at room temperature and to nucleophiles butthehigh reactivity upon irradiation and the brief lifetime of the generated carbenes upon irradiation are the advantages of using diazirine groups for photoaffinity labelling. The damage to the targeted biological system is reduced by its absorption at longer wavelength at both acidic and alkaline conditions.

Enzymatic modifications: Post-translational modifications (PTMs) occur naturally in proteins such as cysteine oxidation, phosphorylation, ubiquitination, methylation, protein splicing, proline *cis-trans* isomerization, nitrosylation, acetylation and glycosylation(Karve & Cheema, 2011).In addition, there are other straightforward classes of enzymes involved in the functionalization of proteins after translation such as ligases, transferases and transpeptidases.

The most prevalent and complex PTMsare glycosylation, in which the carbohydrate chains are covalently attached to the proteins(Walsh & Jefferis, 2006). In protein splicing, intein domain was removed by self-excision and rejoined by native peptide bond formation, resulting in the restore function of the host protein (Wu *et al.*, 2009).Protein trans-splicing was a hybrid system where two intein domains are split into two fragments IntN and IntC(Wu *et al.*, 2009).These fragments could be recombinant or synthetic. A typical example of mini-intein is Ssp DnaB.

The translation of intein-containing mRNA leads to a protein, which further leads to a shorter and intein-free protein after ripening and splicing. The intein sequence was delimited by two flanking regions

named *N*- and *C*-Exteins (ExN and ExC, respectively), which are joined together after splicing. The splicing was described by the following steps (Fig. 10):

Step 1: Nucleophilic amino acid residue at the *N*-terminal splice junction was involved in the formation of a linear ester immediately by N→O or N→S acyl rearrangement.

Step 2: Nucleophilic residue at the C-terminal splicing junction on the linear ester intermediate targeted resulting in the branched ester intermediate formation.

Step 3: Excised intein with a C-terminal amino-succinimide residue and the two exteins joined by an ester bond was formed by cyclization of the asparagine residue adjacent to the C-terminal splice junction coupled with the cleavage of the branched ester intermediate.

Step 4: the aminosuccinimide residue is hydrolyzed and the resulting ester is rearranged by linking the exteins to form the more stable amide bond. The intein catalyzes the first three steps while the last step is irreversible and spontaneous.

The effect of PTMs on the structure or activity of proteins are achieved by chemical modification of cysteine residues (Rowan *et al.*, 2014). The incorporation of photo-caged oNBTyr unnatural amino acid enjoys the benefit of NMR labelling at specific tyrosine residues and specific binding surfaces without altering the protein sequence. This study opens up new avenues for site-specific labelling and hence makes NMR studies of large proteins possible (Cellitti *et al.*, 2008).

The unnatural amino acid, *p*-boronophenylalanine BF was used for the study of the evolution of glycan binding proteins in Boro-X-E. Coli bacteria from developing phage-based system (Liu *et al.*, 2009). The incorporation of reactive functional groups maleimide onto the surface of nanoparticles formulated using polyD, L-lactide-co-glycolide PLGA copolymer in a syngeneic mouse model in vivo by Interfacial Activity Assisted Surface functionalization IAASF resulted in a biologically active cRGD peptide conjugation to the surface of nanoparticles resulting in enhanced accumulation in tumor tissues(Toti *et al.*, 2010).

Without disrupting antigen binding, *p*-acetylphenylalanine pAcPhe unnatural amino acid was incorporated into an antibody antigen binding fragment Fab targeting human epidermal growth factor receptor 2 Her2 by site-specific conjugation method. The biological, physical and pharmacological properties of homogeneous antibody conjugates anti-Her2 Fab-Saporin in Her2 positive breast cancer cells generated *in vitro*a number of constructs with distinct conjugation sites were quantitatively assessed and optimized by recombinant method(Hutchins *et al.*, 2011).

The genetically encoded unnatural amino acids with an orthogonal chemical reactivity were also used to synthesize a homogenous antibody-drug conjugates ADCs with specific control of conjugation site and stoichiometry. The potent *in vitro* cytotoxic activity against Her^{2+} cancer cells, excellent pharmacokinetics and complete regression of cancer in rodent xenograft treatment models was demonstrated by the resulting conjugates (Fig.11)(Axup *et al.*, 2012).The liposomes are site-specifically modified byenhanced green fluorescent protein eGFP by Sortase A mediated transpeptidation reaction, which can increase the efficiency of delivery of liposomesusing proteins(Wu & Guo, 2012).

The unnatural amino acid UAAswere used for other PTMs and incorporated site-specifically into targeted proteins in *E. coli*using the highly efficient suppressor plasmid, pUltra single copy each of the tRNA and an aminoacyl-tRNA synthetase. This incorporation by pUltra exhibits a highactivity suppression than the others which help for the mutagenesis of single and multiple-UAA in *E. coli*(Chatterjee *et al.*, 2013a).The orthogonal *t*RNA o-*t*RNA and the non-natural amino acids nnAAs-modified protein are produced

simultaneously in 50-88% suppression efficiency by site-specific incorporation of nnAAs into proteins by cell-free protein synthesis CFPS method and it was used for screening scissile ribozymes for better catalysis,(Albayrak & Swartz, 2013)cell-based assays for – *in vitro* and *in vivo* studies,(Wang *et al.*, 2013) sun frequency generation SFG vibrational spectroscopy, Time of flight secondary ion mass spectroscopy ToF-SIMS and SFG combined with near-edge X-ray absorption fine structure NEXAFS spectroscopy, molecular dynamic MD simulations,(Weidner & Castner, 2013) and tyrosine-sulfated protein are the other technologies involved in the study of post translational modification of proteins. The proteins and peptides are modified using nanometallic reagents at their cysteine amino-acid residues(Maynard, 2015).

A semisynthetic lipase with superior enantioselectivity in the desymmetrization reaction with enantiomeric excess (*ee*) of 99% was synthesised by site-directed introduction of tailor-made peptides on the lipase lid site(Palomo, 2017). A reduced level of Ahp1 urmylation and mcm^5s^2U34 is maintained by the sulfur transfer defects linked to Tum1, Uba4 enzymes that are required for Urm1 activation by thiocarboxylation. The above feature can be found in ubiquitin-like modifier system Uba4, Urm1 but not in canonical ubiquitin family members(Judes *et al.*, 2016).The malate dehydrogenase (MDH) can be acetylated by acetyl-phosphate or acetyl-CoA and deacetylated by *E.coli* acetyltransferase YfiQ(Venkat *et al.*, 2017). The *S*-GlcNAcylation on cystein residues glycoproteins modified with *S*-(GLcNAc) (N-acetylglucosamine) is found to be involved in cell-cell adhesion and gene expression(Xiao & Wu, 2017). A decreased pancreatic cancer patient survival rate is significantly associated with the loss of phosphorylation site pfsSNV (protein function site affecting single nucleotide variations) at the position 105 in MEF2A(Pan *et al.*, 2017).

A short affinity tag is included in the fluorophore-containing peptide to obtain stoichiometrically labelled protein for purification(Sarpong & Bose, 2017).A rapid and specific protein *S*-glutationylation of a protein can be performed by glyoxalase II interactin and by using its natural substrate S-d-lactoylglutatione(Ercolani *et al.*, 2016). A differential regulation of histone acetylation is achieved by inhibiting the kinase using site-specific acetyl lysine antibodies. The antibodies can be used to study the role of the acetylation of lysine in the new signalling pathways (Chen *et al.*, 2017). The autosomal recessive keratoderma-ichthyosis-deafness syndromeis caused by the mutation in the VPS33B sequence (a Sec1/Munc18 family protein that interact with Rab11a and Rab25 protein) affecting the interaction of Rab protein and modification of collagen (Gruber *et al.*, 2017). The human dimethylarginine dimethylaminohydrolase-1 (DDAH1) is inactivated by diverse fragment-sized 4-halopyridines through covalent modification of the active-sitecysteine(Schardon *et al.*, 2017). The transpeptidase activity of carboxypeptidase Y was performed to label theC-terminalmotif of the protein with an affinity biotin tag for an easy isolation usingavidin beads. The identification by mass spectrometer by an approach termed as Profiling of Protein C-Termini by Enzymatic Labelling (ProC-TEL) was done for a full characterization. The isolation of the*C*-terminal motif of peptides from *E. coli* was successfully carried out by Proc-TEL method. The work will help in the identification of proteolytic cleavages in complex biological systems(Duan W *et al.*, 2016).

The Tiki protein cleaves a fragment of the amino terminus of Wnt proteins by acting as a membrane-tetheredmetalloproteases and Notum inhibits the modification of theconservedlipophilic motif which is essential for Wnt activities. The above activities by the two extracellular enzymatic antagonists, Tiki and Notum resulted in the modification and inactivation of Wnt proteins(Zhang & He, 2016).Astrong binding property to*β*-cyclodextrin (*β*CD) was exhibited by a proline variant of an evolved sortase A (SrtA 7M) after labelling it with lithocholic acid (LA) at the *N*-terminal position(Rosen *et al.*, 2016). The terminal deoxynucleotidyl transferase (TdT) added Z-QG-

modified deoxyuridine triphosphate (Z-QG-dUTP) and deoxynbucleoside triphosphates (dNTPs) to the 3'-end of a DNA aptamer. The resultant (Z-QG)m-(dN)1-aptamer served as stickers for microbial transglutaminase (MTG)(Takahara *et al.*, 2016).The enzymatic digestibility of carbohydrates is improved and the solubility and stability of proteins in the Brewer's spent grain is affected by the steam explosion(Kemppainen *et al.*, 2016).

Modification by metabolic labelling

In this *in vivo* study, multiple unnatural amino acids were incorporated based on frequency of genetic occurrence(Rodriguez *et al.*, 2006).Frameshift and non-sense codon suppression method, photoremovable groups, fluorescent labels and photo cross linkers are other methods adopted for the genetic incorporation ofseventy novel amino acids.The specific labelling of AGT fusion protein with chemically diverse compounds in living cells and *in vitro* was performed using specific reaction of O^6–alkylguanine-DNA alkyltransferase AGT with O^6–benzylguanine BG derivatives.

Two regions of the protein present outside of the active site were found to influence the activity of the protein towards BG derivatives (Gronemeyer *et al.*, 2006).

In neutral aqueous solution, N$\mathcal{E}$–allyoxycarbonyl-N$\mathcal{E}$–methyl-L-Lysine was genetically encoded in*E. coli*and transferred into methyl lysine with catalyst ruthenium resulting in the site-specific introduction of methyl lysine in proteins. The role of lysine methylation in signalling, epigenetic and protein structure was investigated by this method(Ai *et al.*, 2010). The site-specific conjugation of pyrroline-carboxy-lysinePcl with polyethylene glycol, oligonucleotides, fluorescence, peptides, oligosaccharides and biotin labels in *E. coli* and mammalian cells was found to enhance the pharmacology and functionality of proteins(You *et al.*, 2011).Genetic encoding of a norbornene amino acid using the pyrrolysyl tRNA synthetase/tRNA CUA pair in *E. coli* in *vitro* and mammalian cells are used for direct site-specific intracellular protein labelling by bioorthogonal reaction(Lang *et al.*, 2012a).A variety of unnatural amino acids with novel chemical and biological properties was inserted site-specifically into the proteins by inserting two poly-specific tRNA/ aminoacyl-tRNA synthetase pairs into the baculovirus. The incorporation of genetic elements from mammalian viruses significantly improves the transduction efficiency of baculovirus(Chatterjee *et al.*, 2013b).

The rapid assessment of engineered proteins was carried out by chemical customization of protein sites specifically through intein-linked yeast display(Marshall *et al.*, 2013). New mutant proteins are created with distinct spectral properties after the substitution of histidine analogues pyrrolysyl-tRNA synthetase PylRS/tRNAPyl pair with His66 in blue fluorescent protein BFP. The structural and chemical diversity of unnatural amino acids genetically encoded in prokaryotic and eukaryotic organisms was better understood in this study(Xiao *et al.*, 2014).

The biochemical and cellular studies of protein structure and function were described by biosynthetic incorporation of a low-molecular-weight fluorescent amino acid2-amino-3-5-dimethylaminonaphthalene-1-sulfonamidepropanoicacid dansyl-alanine into Saccharomyces cerevisiae proteins at a well-defined site (Summerer *et al.*, 2006).

The site-specific labelling of proteins for single-molecule fluorescence resonance energy transfer smFRET was developed for the construction of dual-labeled T4 lysozyme variants that allowed the T4 lysozyme folding study by single-molecule resolution(Brustad *et al.*, 2008b).

The concentration of Ca^{2+} present in distinct locations of living cells are measured by linking Ca^{2+}sensitive dye Indo-1 to SNAP-tag fusion protein by fluorescence spectroscopy(Bannwarth *et al.*, 2009).An environmentally sensitive fluorescent amino acid was incorporated into proteins site-specifically in retort to the amber nonsense codon

TAG which resulted in high fidelity and good yield in yeast. Conformational changes inprotein structure induced by ligand binding are also studied (Lee *et al*., 2009). The dynamics, structure and interactions of protein was studied by the combined application of ultrafast two-dimensional infrared 2D IR vibrational echo spectroscopy and site-specific incorporation of two vibrational dynamics labels VDLs (Thielges *et al*., 2011). The intramolecularly quenched probes SNAP-Tag fluorogenic probes are used for wash-free labellingof cell surface localized epidermal growth factor receptor EGFR and SNAP-tagged *β*-tubulin in cell lysates quantification(Sun *et al*., 2011).

Using a polyspecific aminoacyl-tRNA synthetase, fluoro-containing tyrosine residues were incorporated into ribonucleotide reductase generating in a good yield of fluorotyrosine-containing proteinsfor the identification and detection of the newly introduced tyrosyl radicals by EPR spectroscopy (Minnihan *et al*., 2011). Based on the specific conjugation of ligands to SNAP-tag fusion proteins, the ligand-receptors interactions were analyzed by SNAP-based TR-FRET assay, S-CROSS assay, cell imaging assay which permittedto study the strong and weak interactions(Haruki *et al*., 2012).

Using the advantages of the regioselectivecontrol of the conjugation, next generation of protein therapeutics and the optimization of the physical and biological properties of protein conjugates were identified by genetically encoded amino acids containing ketone, alkyne, azide and tetrazine side chains in reaction to the nonsense and frame shift codons(Kim *et al*., 2013).

Using sortase-mediated reactions, C-terminal loop labelling of proteins at an internal specific site was carried out and the yields were found to be high(Guimaraes *et al*., 2013).

Recombinant green fluorescent protein, GFP, glutathione *S*-transferase GST and antibody-binding protein G bearing a *C*-terminal CVIA motif and modified bioorthogonal alkyne functional group was covalently andregioselectively immobilized on dithiocarbamate DTC self-assembled monolayers SAMs by a Huisgen cycloaddition reaction with minimal non-specific binding on a gold surface(Choi *et al*., 2014).

Representative applications

Therapeutic antibodies: Absorption, distribution, metabolism and excretion ADME are the factors influencing the protein therapeutics *in vivo*. In drug delivery, polyethylene glycol, PEG was considered as a gold standard resulting in hydrodynamic size increase and reduction in immunogenicity due to camouflage of proteins for the immune system (Veronese & Mero, 2008). In 1983, antibody formation against PEG conjugates was discovered (Richter & Åkerblom, 1983). The other polymers that are used for related research were biodegradable poly amino acids, non-biodegradable poly*N*-2-hydroxypropylmethacrylamide, poly-2-oxazoline,polyglycerols, polyvinylpyrrolidone and monoclonal antibodies(Knop *et al*., 2010).

The efficacy of antibody therapy was also determined by biodistribution. The renal clearance of antibodies was found to be influenced by immunogenicity, proteolysis, glycosylation and interaction between the Fc region and the FcRn receptor antibodies (Roopenian & Akilesh, 2007). Due to the difference in the Fc region (fragment crystallizable region), it was found to vary according to each immunoglobin (Ig) class. The bio distribution was found to be influenced by the interstitial hydrostatic pressure, the fluctuation in the lymphatic darning and pegylated antibodies (Pedley *et al*., 1994).

Antibody drug conjugates: The antibody drug conjugates (ADCs) were considered as the next generation of biological medicines. The benchmark of ADCs was set by Herceptin, based on monoclonal antibody-trastuzumab, which targets the over-expression of Hex2 on certain types of breast cancer(Hudis, 2007).The major drawback in using ADCs: unspecific antibody was found to be accumulated in healthy tissue, the heterogeneous

population pharmacokinetics were distorted before the cytotoxic agent reached the target cells, it was misplaced (Kim *et al.*, 2012). Tumor specific antigens and T-cells are recognized by bispecific antibodies instantly. T-cells recruited by bispecific antibodies were found to be effective for cancer therapy(Chames & Baty, 2014).Bispecific antibodies are found to be effective for the treatment for cancer *in vitro*, but *in vivo* studies need to be carried out in order to know its efficacy.The neutralization of antigens was carried out by immune complexes based on its antibody composite. The binding of the tumor–specific antigen to the antibody inducesa cascade of blockage and receptor down-regulation (Martinelli *et al.*, 2009). DNA scaffolds decorated with single-domain antibodies, polymersomes, avidin, liposomes, and gold are the best examples for a diverse set of scaffolds(Rosen *et al.*, 2016).

The advantage of using ADCs was the simplicity of the process and the non-requirement of protein engineering techniques. The first generation of ADCs was made by the two classical bioconjugation methods namely non-specific acylation of lysine residues with activated esters and alkylation of cysteine thiols with maleimides(Senter & Sievers, 2012).The product produced by conjugation to lysine was found to be heterogenous(Ponsin *et al.*, 2003),while the heterogeneity for maleimide conjugation was established to bewith a least potential in the case ofmaleimide conjugation(Sun *et al.*, 2005).

The maleimide-linked doxorubicin conjugate of BR96 was prepared by Trail *et al.* by using the interchain disulfide bonds reduction approach and conjugating it to the resulting eight cysteine residues(Agarwal & Bertozzi, 2015).

The simplified production of cysteine-alkylated ADCs was made possible by partial reoxidation or full reduction of cysteine residues with Ellman's reagent preceded by conjugation to maleimido-auristatins or by partial reduction of the residues by either dithiothreitol (DTT) or tris(carboxyethyl)phosphine (TCEP)(Sun *et al.*, 2005).

The low drug antibody ratio DARs0 was achieved by using each disulfide as a single point of attachment from conjugation at interchain disulphide (Shaunak *et al.*, 2006). The inherent instability was prevented in these conjugates due to the dual attachment sites. The clickable conjugate with a single point of attachment was obtained in high yield by treating the reduced Fab fragment with an *N*-propargyldibromomaleimide (Agarwal & Bertozzi, 2015). The fairly low accumulation of antibody at tumor sites after intravenous administration was the disadvantage of using the first generation of ADCs(Gebleux & Casi, 2016). The maytansinoids or auristatins are the two most widely used drug platform for the development of ADC. The two FDA-approved ADcs are KADCYLA™ (maytansinoid) and ADCETRIS™ (auristatin)(Krop & Winer, 2014).

The calicheamicins, camptothecins, duocarmycins, anthracyclines and pyrrolobenzodiapines represented the class of cytotoxic compounds.

The alkylation, scission or cross-linking of the corresponding nucleic acids strands were promoted by the binding of some of the above drugs to the minor groove of DNA(Gebleux & Casi, 2016). The multidrug resistance (MDR) was reckoned on the overexpression of a protein that actively participated in the efflux of drugs from intracellular space(Yu *et al.*, 2015).The antiproliferative activity in HER2-expressing/MDR-expressing human tumor cell lines at picomolar concentrations was carried out by amatoxins conjugated to Herceptin(Anderl *et al.*, 2014).

The influx of leukocytes was mediated to the location of disease by pro-inflammatory payloads such as IL-2, TNF, IL-12. The therapeutic index of immunocytokines was increased by the localization of thecytokine effects at the site of disease(Hemmerle *et al.*, 2013).The clinical trials involving the antibody-cytokine fusions directed against component of the tumor sub-endothelial extracellular matrix (ECM)

was found to have advanced into the next stage(Neri & Sondel, 2016). The interstitial fluid pressure in the tumor environment for the uptake of other therapeutic agents was lowered by the vasoactive properties of certain cytokines (IL2 and TNF) (List *et al.*, 2014).

The CEA, A33, HER2, CD20, PSMA, the alternatively-spliced A1 domain of tenascin-Cand the EDB domain of fibronectin and A was the best characterized targets for antibody based pharmacodelivery (Larson *et al.*, 2015). The ADCs target was identified as the sub-endothelial ECM that did not require internalization. The non-internalizing antibodies coupled to the photosensitizers weredegraded by non-internalizing antibodies uponnear-infrared irradiation (Palumbo *et al.*, 2011).

The accumulation in the tumor site at late stage was increased by using the traditional antibodies in the full IgG format because of its half-life long circulatory in blood and high cupidity for the target antigen(Teicher & Chari, 2011). At the expense of sub-optimal tumor: blood and tumor: organ ratios, the IGG format increased the tumor uptake (Tian *et al.*, 2014). The different pharmacokinetic properties with individual applications were featured by the recent advancement in development of engineered mAb fragments(Schmidt & Wittrup, 2009). The impact on ADC efficacy was controlled by the number of cytotoxic molecules coupled to the antibody. The promising results in preclinical models were exhibited by homogenous preparation of ADC with higher ADC values with hydrophilic linked-payload combinations (Strop *et al.*, 2015). The undisrupted interchain disulfide and drug conjugation with defined stoichiometry was possible by engineering the reactive cysteine residues at specific sites in the antibody backbone(Panowski *et al.*, 2014). The highly homogenate antibody-conjugates was generated by the intrinsic substrate specificity of bacterial transglutaminase (BTG)(Strop *et al.*, 2013).

The biodistribution and *in vivo* stability of ADC products had direct implication in the nature of the linker(Nolting, 2013).The performance of ADC was based on the hydrophobicity, drug release mechanisms, polarity and coupling site on the antibody molecule(Lyon *et al.*, 2015). The cleavable and the non-cleavable linkers were the two categories of linkers used. The first acid-labile linkers like hydrazones were used for the rapid development of ADCs(van der Velden, 2001). Upon internalization, the disulfides linkers released the payloads inside the cells and the stable nature of disulfides linkers at physiological pH was the main reason behind the use of disulfides linkers(Kovtun *et al.*, 2010). The stability of the corresponding ADC product was modulated by the coupling site on the antibody backbone (Doronina *et al.*, 2003).A better control of drug release was seen on the use of peptide linkers. A promising result of high specificity and toxicity was exhibited by optimized dipeptide-based linkers (Doronina *et al.*, 2003).

The incorporation of the ADC in the target cell accompanied by the lysosomal degeneration of the antibody to amino acid level by catabolic degradation (Erickson *et al.*, 2006).

The formation of thioethers and amide bonds was due to the use of SMCC bifunctionalcross-linker (Succinimidyl-4-[*N*-maleimidomethyl] cyclohexane-1-carboxylate), a common non-cleavable linkers in ADC(Lambert & Chari, 2014).A possible impact on drug potency and immunogenicity was established by the use of bifunctional linkers that coupled either to the antibody or to the drug after cleavage(Buskas *et al.*, 2004). No traces of the original drug coupling was witnessed by the use of “traceless” linkers(Bernardes *et al.*, 2013).

The disulfides-based ADC was released by the maytansinoid thiol intracellularly with-standing S-methylation and triggered a derivate with increased potency (Erickson *et al.*, 2010). Initially at first, the precise quantification of ADC targeting in pre-clinical methods was carried out by dual labelling approach of antibody and drug moiety (Cohen *et al.*, 2014). More than 40 ADCs was under the clinical development

recently. The high-affinity small ligands particular to tumor-associated antigens targeted tumors more swiftly and effectively(Srinivasarao *et al.*, 2015). The use of DNA-encoded chemical libraries facilitated the isolation and medicinal Chemistry improvement of ligands(Litovchick *et al.*, 2015). CD^{8+} T-cells destroyed the minimal residual disease by a process known as "immunogenic cell death"(Gerber *et al.*, 2016)The potent activity *in vivo* was exhibited by the ADC products in conjgation with immunocytokines in fully immune competent tumor-bearing mice(Casi & Neri, 2015).A non-toxic model of an ADC was found to be antibody-fluorophore conjugate (AFC). The binding of dansyl sulfonamide ethyl amine (DSEA)-linker maleimide on intrachain cysteines of trastuzuman was used a standard antibody with the basis of AFC(Wagner-Rousset *et al.*, 2014).The site-specific ADCs was engineered by the formation of spontaneous isopeptide bond between two peptide recognitions, SpyTag and KTag(Levengood *et al.*, 2017).

Conclusion

Plenty of researches works have been done for tuning the functionality of a peptide,and chemo selectivity have been deeply explored. However, site-specific modification and regio-selectivity of peptidesremain a challenging task. A lot of efforts have been previously used to circumvent these shortcomings by utilizing solvent accessible thiols, N-terminus of lysines, carboxylates, aspartate and glutamate and targeting C-terminus. Due to the presence of multiple protein functionalitieson the protein surfaces, these approaches presented massive limitations.

The development of native chemical ligation, intein and sortase methodologies have enhanced the synthesis of higher peptidesize and proteins of about 200 amino acids. In fact, the resulting product-pools contained congeners having various degrees of modifications at various sites. Different challenges in site-specific functionalization need to be overcome; in this regard, the development of site-specific functionalization utilizing enzymes or different chemical reagents that modify specificresidues without modifying the others will be a promising approach. In addition, the development of labile protecting groupscompatible with the protein stability is also needed to simplify site-specific functionalization.

The insertion of unnatural amino acids whichhave been developed in the last decade, are also reported as another promising tool for protein functionalization.

The directed evolution of proteins was also an efficient approach for altering or improving protein functionality, but apart site-directed saturation mutagenesis, most of commonly used techniques lead to random functionalization. Another perspective consists in the development of site-specific targeting using peptides, foldamers or small molecules bearing a pending catalyst or reactive moieties for the site-specific modification of proteins. Continuous major shortcomings need an expansion of the research for more effective methodologies. These modifications will help for the diversity and expand thenature-inspired ability to explore new functionalities and patterns to a wide range of applications.

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