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ORIGINAL PAPER

Specific Enzyme Immobilization Approaches and Their Application with Nanomaterials

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Abstract Nanotechnology-inspired biocatalysts have aroused much interest recently because nanomaterials can provide the upper limits on enzyme-efficiency-determining factors. Non-specific immobilization approaches often face problems of enzyme leaching, 3D structure change and mass transfer resistance. Here, we focus on the review of specific enzyme immobilization approaches and have separated them into non-covalent and covalent categories.

Keywords Specific enzyme immobilization · Nanomaterials · Affinity tag · Click chemistry · Polyhistidine · Biotin · Non-covalent

1 Introduction

With the development of nanotechnology in the past few decades, nanotechnology-inspired biocatalysts have aroused much interest because the supporting nanomaterials can provide high surface area/volume ratio, low mass-transfer limitation, and excellent particle mobility during reaction [1]. In general, enzyme immobilization with nanostructures can be divided into two categories: non-specific and specific [2]. Non-specific immobilization methods generally include adsorption [3], non-specific covalent binding [4, 5], entrapment [6], and encapsulation [7]. In spite of their wide application in bioconversions, non-specific immobilization approaches often encounter problems such as enzyme leaching [8], enzyme 3D structure loss [9], and strong diffusion resistance [10].

Here in this review, we focus on specific enzyme immobilization strategies with nanostructures as the supporting materials. We have divided these approaches into two categories: (i) the non-covalent immobilization methods that take advantage of the specific interaction between avidin/streptavidin and biotin or between polyhistidine and metal ions Co^{2+}/Ni^{2+}; (ii) the covalent ligation approaches—immobilization through Cys residue, "click" reaction, Staudinger ligation, and enzyme-catalyzed ligation.

2 Non-covalent Immobilization

Non-covalent specific enzyme immobilization approaches usually exploits the specific interaction between biotin and avidine/streptavidin [11] or between polyhistidine and biovalent metal ions Co^{2+}/Ni^{2+} [12]. The benefits of using non-covalent immobilization methods are: (i) the interaction between enzymes and supporting materials is specific and stable [13]; (ii) the immobilization process is reversible, i.e., supporting materials can be recycled when attached enzymes lose activity. Meanwhile, the disadvantage is that the non-covalently bounded enzymes could be easily dissociated under complex catalytic conditions.

2.1 Avidin/Streptavidin–Biotin

Biotin is a water-soluble B-complex vitamin, which is vital for cell growth [14], fatty acid synthesis [15], and amino acid catabolism [16]. Biotin has a fused ring structure containing an ureido (tetrahydroimidizalone) ring with a tetrahydrothiophene ring attached by valeric acid. Avidin, a

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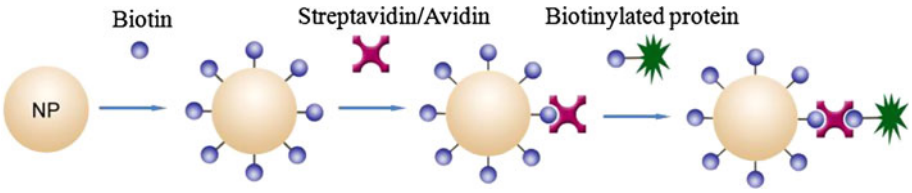

Fig. 1 Protein immobilization to nanoparticles (NP) via the interaction between biotin and streptavidin/avidin

homotetramer protein with M_W of 69 kDa, is produced from the white of bird's egg [17]. One avidin subunit can bind to one biotin molecule with a tight and specific non-covalent bond (dissociation constant $K_D \sim 10^{-15}$ M) (Fig. 1) [18], which is one of the strongest known protein–ligand interactions, approaching the covalent bond in strength [19]. In addition to their exceptional ligand-binding properties, they also have good stability against heat, extreme pHs, denaturants, and proteolysis. In general, there are two ways to prepare proteins for immobilization: (i) the biotinylation of target protein; (ii) the fusion of target protein with an affinity tag that binds to avidin or streptavidin.

Either enzyme-catalyzed or chemical biotinylation has been reported before. *E. coli* biotin ligase (BirA) can transfer endogenous biotin to the specific Lys side chain of a fifteen-residue acceptor peptide in an ATP-dependent manner, which has been developed for biological sensing or cellular labeling [20–22]. Another biotinylation enzyme is phosphopantetheinyl transferase (PPTase), which transfers phosphopantetheinyl of biotin-CoA to peptidyl or acyl carrier proteins (PCP, ACP) [23]. Yin et al. [24] immobilized PCP-tagged protein microarrays onto an avidin-coated glass slide through this method. This enzyme-catalyzed biotinylation has yet been applied to nanomaterials for biocatalysis purposes.

Other groups have used chemical biotinylation methods. Chirra et al. [25] made catalase biotinylation via succinimide chemistry. Au nanoparticles were first coated with biotin, and streptavidin was added as mediator for the conjugation of biotinylated catalase and biotin-coated Au nanoparticles, which can be used as delivery systems for therapeutic purpose. The same method was also employed for the immobilization of glucose oxidase (GOx) on apoferritin nanoparticles [26]. The activity of the immobilized GOx was well preserved while its stability was greatly improved compared to the free biotinylated GOx. About eight biotinylated GOx molecules were found attached to one apoferritin nanoparticle, and the immobilized enzyme could keep $\sim$100 % activity of free GOx. The K_M value for the immobilized GOx and free biotinylated GOx was found similar to each other, which suggested little mass transfer resistance. As for stability, the immobilized GOx demonstrated $\sim$50 % residual activity after incubation at 50 °C for 3 h, while that of free GOx was only $\sim$20 % under the same conditions. Garcia and colleagues [27] also prepared a biotinylated horseradish peroxidase (HRP-biotin), an avidin-linked HRP (HRP-Av) and a biotinylated GOx (GOx-biotin) to immobilize a multilayer of HRP-Av—(HRP-biotin–HRP-Av)$_{m-}$ GOx-biotin to magnetic nanoparticles. The immobilized enzymes showed excellent stability—the immobilized enzymes could be reused for ten cycles and stored for 14 weeks at 4 °C without apparent reduction of catalytic activity.

Strep-tag [28] and AviD-tag (Avidin-Di-tag) [29] have also been developed to bind to streptavidin and avidin/NeutrAvidin, respectively. Gaj et al. [29] have used the AviD-tag to immobilize green fluorescent protein (GFP) to NeutrAvidin resins.

The avidine/streptavidin–biotin mediated enzyme immobilization endows tighter binding of target enzyme to nanomaterials than other non-covalent immobilization methods. Moreover, a wide range of supports are commercially available for this method. The disadvantages of applying this method could be: (i) the immobilization process is relatively complicated as compared to other non-covalent immobilization methods; (ii) avidine (69 kDa) and streptavidin (60 kDa) are large proteins (tetramers) that may have detrimental effect to target enzyme [30].

2.2 Polyhistidine-Co^{2+}/Ni^{2+}

The chelation between polyhistidine-tag (6 × His-tag) and bivalent metal (Co^{2+}, Ni^{2+}, Cu^{2+}, Fe^{2+} or Zn^{2+}) is another popular specific non-covalent method employed in enzyme immobilization. The imidazole ring of the histidine residue is able to coordinate with the metal ion to form stable conjugate [31, 32]. Due to its high specificity and affinity, this interaction has been widely used in protein purification, known as the immobilized metal affinity chromatography. In order to immobilize successfully, the recombinant enzyme must have a 6 × His-tag at either N- or C-terminus, which mainly depends on the location of the enzyme active site. To capture the bivalent metal ions, supporting materials must be activated first by either nitrilotriacetic acid (NTA) or iminodiacetic acid.

This specific non-covalent interaction has been mostly used in preparing biosensors by immobilizing GFP or its derivatives on functionalized nanomaterials (Table 1). It was reported that NTA-Ni^{2+} modified Fe/Pt and Co/Fe_2O_3

Table 1 Protein immobilization examples via His-tag

Protein	Supporting material	Ligand	References
Horseradish peroxidase	Au nanoparticle	Nitrilotriacetic-Co (II)	[12]
Green fluorescence protein	Au/FePt/Fe_2O_3 nanoparticle	Nitrilotriacetic-Ni (II)	[89]
Green fluorescence protein	$SmCo_{5.2}/Fe_2O_3$ nanoparticle	Nitrilotriacetic-Ni (II)	[33]
D-amino acid oxidase	Magnetic bead	Nitrilotriacetic-Ni (II)	[90]
Photsynthetic reaction center	SWCNTs	Nitrilotriacetic-Ni (II)	[13]
MS2 coat protein	SiO_2/Fe_3O_4 core/shell nanoparticle	Nitrilotriacetic-Co (II)	[91]
NADH oxidase	Nanospheres	Nitrilotriacetic-Co (II)	[43]
NADH oxidase	MWCNTs	Nitrilotriacetic-Co (II)	[44]
Glycerol dehydrogenase	SWCNTs	Nitrilotriacetic-Co (II)	[42]
Phosphopeptides	Fe_3O_4 nanoparticle	Nitrilotriacetic-Ni (II)	[35]
Green fluorescence protein	PPL-g-PEG	Nitrilotriacetic-Ni (II)	[92]
Green fluorescence protein	Gold nanoparticle	Nitrilotriacetic-Ni (II)	[93]
Peptide (AB-G5HG2)	CdSe/ZnS core/shell quantum dot	Nitrilotriacetic-Ni (II)	[94]
T4 DNA ligase	γ-Fe_2O_3 nanoparticle	Iminodiacetic-Cu (II)	[95]
Maltose binding protein	Multivalent chelator head	Tri-Nitrilotriacetic-Ni (II)	[96]
Green fluorescence protein	Agarose bead	Nitrilotriacetic-Ni (II)	[31]
Human estrogen receptor alpha	SiO_2-TMR-NTA nanoparticle	Nitrilotriacetic-Ni (II)	[36]

core/shell magnetic nanoparticles had high specificity with His-tagged GFP and the conjugates exhibited outstanding fluorescence [33, 34]. In addition to fluorescent proteins, NTA-modified nanoparticles can also be used as probes to directly detect certain proteins. For instance, super paramagnetic Fe_3O_4 nanoparticles were synthesized and coated with NTA to detect streptopain [35]. The NTA-Ni^{2+} modified silica nanoparticles demonstrated high specific binding with estrogen receptor α ligand [36].

Only a handful of enzymes have been immobilized via His-tag for biocatalysis investigation. Horseradish peroxidase (HRP) was immobilized on NTA-Co^{2+}-functionalized Au nanoparticle with favorable orientation through 6 × His-tag [12]. The immobilized HRP exhibited the same activity compared to free HRP when using 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) as substrate. Other oxidoreductases, such as NADH oxidase (NOX) and glycerol dehydrogenase [37–41], were also attached to NTA-Co^{2+} modified single-walled carbon nanotubes (SWCNTs) (Fig. 2), multi-walled carbon nanotubes (MWCNTs), nanospheres, and showed more than 80 % activity retention and notable stability improvement ([42–45]). In these cases, nanostructures were first treated with acid mixture (HNO_3/H_2SO_4: 1:3) to have –COOH groups on their surface, and added to NHS/ECS to form nanomaterial-NHS ester complex. This complex was then mixed with ANTA-Co^{2+} to produce nanomaterial-ANTA-Co^{2+} complex, which was used for enzyme immobilization. The NOX loading capacity on the modified SWCNTs (0.47 mg enzyme/mg SWCNTs) was found much higher than that of using commercially available Sepharose beads (0.01 mg enzyme/mg Sepharose beads). When the immobilized NOX and glycerol dehydrogenase were both employed in a cofactor regeneration system to produce 4-hydroxy-2-butanone (4H2B), the 4H2B yield increased to 37 % as compared 17 % of free enzyme system, which was mainly due to the enhanced enzyme stability at the reaction temperature [42].

There are several advantages of using this specific non-covalent method to immobilize enzymes on nanostructures: (i) it is not necessary to purify target enzyme before immobilization, i.e., the crude cell lysate containing His-tagged protein can be used directly for immobilization; (ii) the specific interaction between His-tag and the metal ions is strong enough to prevent protein leakage; (iii) enzymes can often preserve their native 3D structures after immobilization and hence have high activity retention; (iv) since the immobilized enzymes can be eluted off easily with imidazole, the modified nanostructures can be recycled (Table 2).

2.3 Other Affinity Tags

In addition to biotin-tag and His-tag, other affinity tags, such as gold binding peptide, iron oxide-affinity peptide, and silica-affinity peptide, have also been studied for enzyme immobilization with nanomaterials. However, these affinity tags have yet been used for biocatalysis purpose, which may be attributed to the low affinity between the affinity tags and nanomaterials [46]. However, this can be overcome by using multiple repeat tags [46] or surface-coated polymers [47].

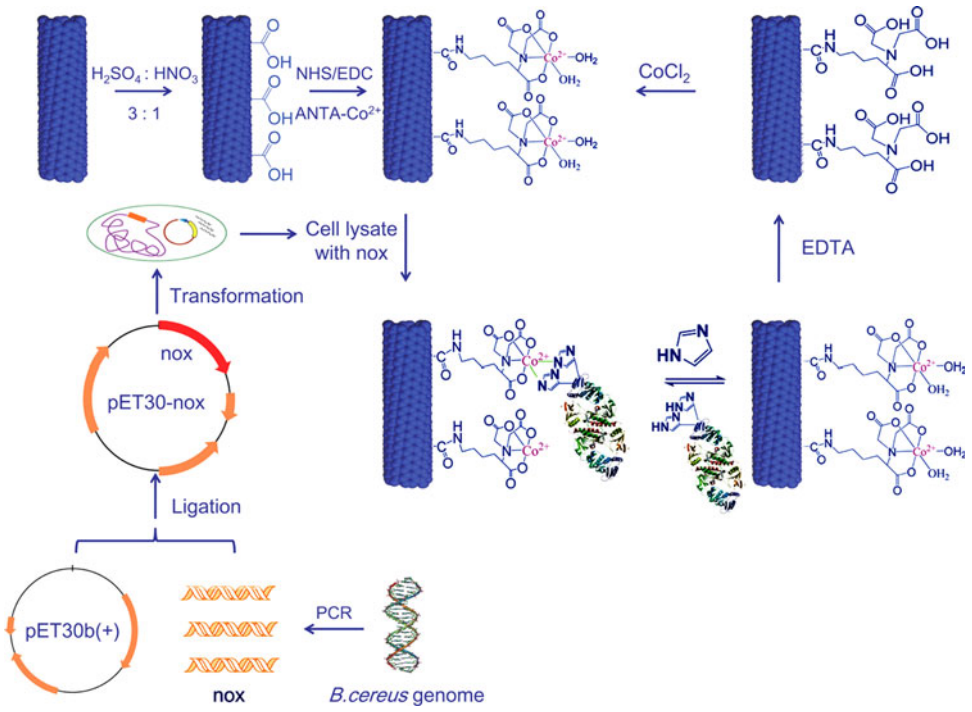

Fig. 2 Scheme of the reversible immobilization of His-tagged NADH oxidase on functionalized SWCNTs. Figure adapted with permission from Ref. [44]. Copyright 2010 Elsevier

Gold binding peptide (GBP), obtained from a cell surface display library [48], was one of the first examples of engineered inorganic-binding peptides [46]. It was found that three repeats of GBP were required for high-affinity binding [46]. Kacar et al. [49] fused multiple tandem repeats of GBP tags to alkaline phosphatase for directed enzyme self-immobilization on the gold surface. Staphylococcal protein A [50] and organophosphorus hydrolase [51] were also immobilized to Au nanoparticles for biosensing applications.

Iron oxide-affinity peptide (FeAP) and silica-affinity peptide (SiAP) were also used for the immobilization of proteins to magnetic nanoparticles. Haloalkane dehalogenase was fused with one FeAP or two repeats of SiAP for the immobilization to iron oxide surface and silica magnetic nanoparticles. The loose attachment of peptides to these nanomaterials was enhanced by surface-coated hydrophilic polyethylene glycol or 3-glycidoxypropyltrimethoxysilane molecules [47].

3 Covalent Ligation

3.1 Immobilization via Cys

Although most of the covalent immobilization methods are non-specific, some new covalent strategies, appending covalent adducts to enzymes, have overcome this problem by forming covalent bonds between the enzymes and the supporting materials specifically. One way is to use specific site-directed protein mutants—a unique amino acid residue with a special side-chain functional group could be introduced to the target enzyme via site-directed mutagenesis. The mutated enzymes can attach to the supporting materials through the side-chain functional group of this amino acid mutation, which actually forms the covalent bond with controlled enzyme orientation [52]. Among all twenty common amino acids, Cys has been widely chosen as the substitution amino acid owing to its thiol group that can be easily covalently coupled.

Cys can be introduced into any non-Cys positions via site-directed mutagenesis method for selective immobilization. For example, in order to immobilize subtilisin, a Ser residue far from the active centre was mutated to Cys, whose –SH group was attached to the membranes activated with aldehyde via cross linkers such as 4-(4-*N*-maleimidomethyl) cyclohexane 1-carboxyl hydrazide and 3-(2-pyridyldithio) propionyl hydrazide. When immobilized to PVC (polyvinyl chloride)-silica membranes, the recovered enzyme activity increased to 83 % as compared to 48 % of using random immobilization method via Schiff-base chemistry [53]. However, there are several factors that may limit the application of this method: (i) the 3D structure of target protein may be altered during mutagenesis; (ii) the immobilization cannot be specific if the enzyme has multiple Cys residues at different locations. Under certain circumstance, the immobilized enzyme could be totally inactive if the Cys is at the active site of the proteins, such as cytochrome P450 and NADH oxidase [39–41, 54]. In

Table 2 Advantages and disadvantages of various specific immobilization methods

Specific immobilization methods	Examples	Advantage	Disadvantage
Non-covalent			
Polyhistidine-Co^{2+}/Ni^{2+}	See Table 1	High activity retention (>80 %); no need to purify enzyme before immobilization; specific interaction to prevent enzyme leakage; reversible immobilization process	Non-covalent bond may dissociate under complex conditions; polyhistidine may affect certain enzyme activity
Avidine/streptavidin–Biotin	Catalase-Au nanoparticles [25]; glucose oxidase-apoferritin nanoparticles [26]; Horseradish peroxidise-magnetic nanoparticles [27]	Strong protein–ligand interaction (dissociate constant $K_D \sim 10^{-15}$ M); good stability (T, pH, denaturants, proteolysis); good activity retention	Complicated preparation process; large avidine/streptavidin tetramers (69/60 kDa) may be detrimental to certain enzymes
Covalent			
via Cys	Subtilisin-PVC-silica membranes [53]	Cys thiol group can be easily covalently coupled	Alteration of enzyme 3D structure during mutagenesis; multiple Cys residues may cause non-specific immobilization; Cys at active site may lead to enzyme inactivation
Native chemical ligation via Cys	Guanosine triphosphate hydrolyse-Au nanoparticles [57]; eGFP or CMP-sialic acid synthetase-magnetic nanoparticles [60]	Ligation under neutral aqueous conditions; no protection of amino acid residues is required	Complicated preparation process (protein must have Cys at its terminal and nanomaterials must be coated with thioester groups, vice versa)
"Click" reaction	Luciferase-Au nanoparticles [69]; lipase-Au nanoparticles [64]; trypsin-Au nanorod/mesoporous silica [66, 68]	Mild reaction conditions; irreversible reaction with high chemoselectivity; stable linkage	Instability and toxicity of copper catalyst OR reduced chemoselectivity without copper catalyst; extra reaction step to introduce azido or alkyne groups
Staudinger ligation	RGD peptide-polyamidoamine-DNA nanoparticles [76]	Mild reaction conditions; stable linkage; no toxic additives	Slower than "click" reaction; incomplete reaction; extra step to introduce azido groups
Enzyme-catalyzed conjugation	Arylmalonate decarboxylase/glutamate racemase-polystyrene nanoparticles [82]	Specific catalysis; mild reaction conditions; no need for extra reaction step to introduce ligands; high activity retention in general	Requires pure enzyme; no full conversion and relatively low selectivity for certain enzymes; low activity retention for certain enzymes

order to overcome these limitations, researchers manage to use native chemical ligation (NCL) strategy instead.

Native chemical ligation NCL [55, 56] involves a peptide with an N-terminal Cys reacting with the C-terminal thioester of another peptide to form an amide bond. This ligation can be performed under neutral aqueous conditions, and does not require protection of any other amino acid residues including the Cys residues. For immobilization by NCL, the target protein is added with Cys residues at its N-terminal [57] or introduced with thioester groups at its C-terminal [58, 59], while the nanomaterials are coated with either thioester or Cys groups accordingly. Guanosine triphosphate hydrolase (small GTPase) [57] with the N-terminal Cys residue was immobilized onto Au-nanoparticles via a chemical linker containing a thioester group at one end and a thiol group at another. Some other simple methods have also been reported before. For instance, enhanced green fluorescent protein (eGFP) or CMP-sialic acid synthetase (CSS) [60] were fused to inteins, which could excise themselves from the fusion proteins. In the presence of mercaptoethanesulfonic acid (MESNA), a thioester could be formed at the C-terminal of eGFP or CSS, which could then be immobilized to the Cys modified magnetic nanoparticles (MNP) (Fig. 3). The loading capacity of the non-purified enzyme and the purified enzyme was found to be 94.3 and 67.2 μg/mg MNP-CSS complex, respectively, as compared to 61.9 μg by random cross-linking immobilization approach. The site-specifically immobilized CSS could keep 76.8 % of the free CSS activity, whereas that of the randomly immobilized enzyme was only 33.2 %. MNP-CSS displayed no activity drop even after repeated use of ten cycles and it could also preserve ~100 % activity even after 7 month storage at 4 °C. The shortcoming of this method is the high metabolic burden during target protein expression and folding since certain inteins could be very large in size (up to ~200 kDa, "Inbase"). Furthermore, the cleavage of inteins would be significantly affected by

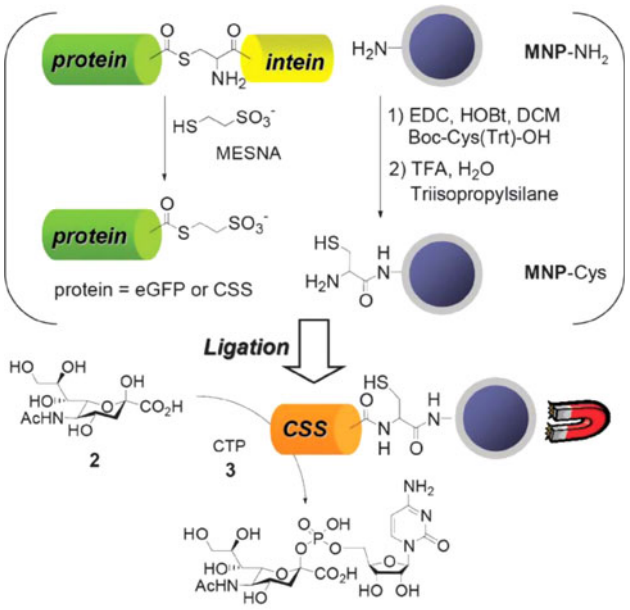

Fig. 3 Combination of the intein expression system and native chemical ligation for site-specific and covalent immobilization of target proteins. eGFP: enhanced *green fluorescent protein*; CSS: CMP-sialic acid synthetase; MNP: magnetic nanoparticle. Figure adapted with permission from Ref. [60]. Copyright 2008 Royal Society of Chemistry

target proteins. In some cases, the cleavage could hardly be observed [61].

3.2 "Click" Chemistry

The Cu(I)-catalyzed Huisgen 1,3-dipolar cycloaddition of azides with an alkyne, a well-known as the flagship reaction of the "click" chemistry family [62], has been used extensively in modern chemistry from drug discovery to materials science[63], especially for immobilizing azido- or alkyne- containing proteins onto alkyne- or azido-coated surfaces, respectively[64–69]. Azido and alkyne ligands are first attached to the target enzyme and the supporting materials, respectively, and then the reactions are carried out between azido and alkyne to form the covalent bond. This reaction has several advantages during immobilization: (i) it can be performed under mild conditions such as in water, buffered media or mixtures of aqueous/organic solvents, at room temperature; (ii) the reaction is irreversible with high chemoselectivity and the resulting linkage is stable. Despite the positive press about this reaction, it also has some shortcomings: (i) the instability and toxicity of the copper catalyst. Although new strategies have been developed to overcome this drawback, such as the "copper-free click chemistry" method reported by the Bertozzi and co-workers [70], new constraints like reduced chemoselectivity start to appear consequently; (ii) since the azido and alkyne functional groups are not present in natural proteins, it is indispensable to add an extra step to introduce the azido or alkyne groups to the target protein before immobilization. Various attempts have been carried out to achieve this goal and are listed as follows.

a) Kim et al. [71] introduced the azido group by engineering methionyl-tRNA synthetases. Other sites of the protein can also be introduced with the azido groups as long as the protein contains more than one Met residue, which makes this method non-site-specific.
b) Protein farnesyltransferase (PFTase) can transfer the farnesyl moiety of farnesyl pyrophosphate containing the azido or alkyne group to the Cys residue of *CaaX* peptides (*a* is any aliphatic residues, and *X* can be Ser, Met, Glu, Ala, or Thr). For instance, GFP, eGFP and glutathione S-transferase (GST) could be modified with an azido or alkyne group by a C-terminal-fused *CaaX* motif and then immobilized to glass slides or agarose beads [72, 73]. The immobilized eGFP showed almost the same fluorescence level as the free eGFP. The flexibility of *CaaX* peptide can minimize harmful effects on target enzyme, but it may also result in non-specific immobilization of other proteins that also have *CaaX* sequence. As such, a purification step of target enzymes may be needed before immobilization [30].
c) Lin et al. [65] fused an intein at the C-terminal of maltose binding protein (MBP) and eGFP, and an azido group was introduced into MBP and eGFP by Cys alkyne. Similar method was also used to introduce alkyne groups into eGFP. The labeled proteins were then immobilized on an alkynated or azidated glass slide. The same strategy was also used for immobilization of the *Renilla reniformis* luciferase mutant to the surface of Au nanoparticles as bioluminescent nanosensors [69].
d) Brennan et al. [64] modified the surface Lys residue of the mutated lipase (*Thermomyces lanuginosus)* by standard carbodiimide coupling chemistry to yield acetylene-functionalized lipase, which was immobilized to Au nanoparticles via "click" chemistry. It was reported that approximately seven fully active lipase molecules were attached to each nanoparticle. Similar method was employed for the immobilization of trypsin to Au nanorods and mesoporous silica [66, 68]. The amount and biocatalytic activity of trypsin attached to Au nanorods were found to be much higher than using non-selective immobilization approaches. Around 11,000 ± 1,900 trypsin molecules/nanorod was achieved via this selective technique, whereas only 6,500 ± 400 or 7,100 ± 700 trypsin molecules/

nanorod was obtained in case of electrostatic adsorption or covalent binding catalyzed by 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride. The activity retention of the immobilized trypsin via these three means was 57, 13, and 19 %, respectively. The disadvantages of this method are: (i) the modification of Lys residue may affect target enzyme; (ii) target enzyme must be purified before immobilization because other proteins may also have Lys residues. Moreover, enzymes with multiple Lys residues on their surface could cause non-site-specific immobilization.

3.3 Staudinger Ligation

Staudinger ligation yields an amide bond between an arylphosphine moiety and an azide group, which was first reported in 2000 [74]. Although it has been used widely for different bio-conjunction applications including immobilization [75], there are very few reports related with nanomaterials. Only RGD peptides with azido groups were immobilized to polyamidoamine-DNA nanoparticles for improving their cellular uptake [76]. Compared with "click" chemistry, staudinger ligation requires no toxic additives such as copper catalyst, but its drawbacks are as follows: (i) the reaction is slower an "click" chemistry and often does not run to full conversion; (ii) requires an extra step to introduce azido groups to target protein before immobilization [77].

3.4 Enzyme-Catalyzed Conjugation

Compared with the aforementioned covalent immobilization reactions catalyzed by chemical catalysts, enzyme-catalyzed covalent conjugation is more specific and milder. On top of that, no extra step is required to add ligands to target proteins. Enzymes such as sortase A [78, 79], transglutaminase [80, 81], and PPTase [82, 83] have been investigated but only a few immobilization applications with nanomaterials have been reported so far.

Sortase A from *Staphylococcus aureus* is a transpeptidase that attaches surface protein to cell walls: it cleaves between the Gly and Thr within a conserved LPXTG motif near the C-terminus and catalyzes the formation of an amide bond between the carboxyl-group of Thr and the amino-group of polyglycine as well as aliphatic amines. The fibronectin-binding protein and glycosyltransferase with LPXTG tag have been immobilized on biosensor chips and Sepharose beads previously (Fig. 4) [78, 79]. The immobilized glycosyltransferases retained 90 % activity as compared to its soluble form. It was reported before that the sortase-catalyzed reaction was reversible and might result in low ligation efficiency. This could be overcome by addition of β-hairpin structures around the LPXTG tag [84], but the β-hairpin structures influence on enzyme activity was not mentioned in the report.

Transglutaminase catalyzes the formation of a covalent bond between a free amine group and the γ-carboxamide group of protein- or peptide-bound glutamine. Bonds formed by transglutaminase exhibit high resistance to proteolytic degradation. This enzyme can recognize specific amino acid sequences such as LLQG and MLAQGS. Many proteins have been immobilized via transglutaminase to different supporting materials (Fig. 5). For example, Sugimura and colleagues [80] immobilized GST and single-chain fragment antibody to chemically amine-terminated gels catalyzed by transglutaminase. Moriyama et al. [81] also have reported immobilizing bacterial alkaline phosphatase to magnetic particles via transglutaminase.

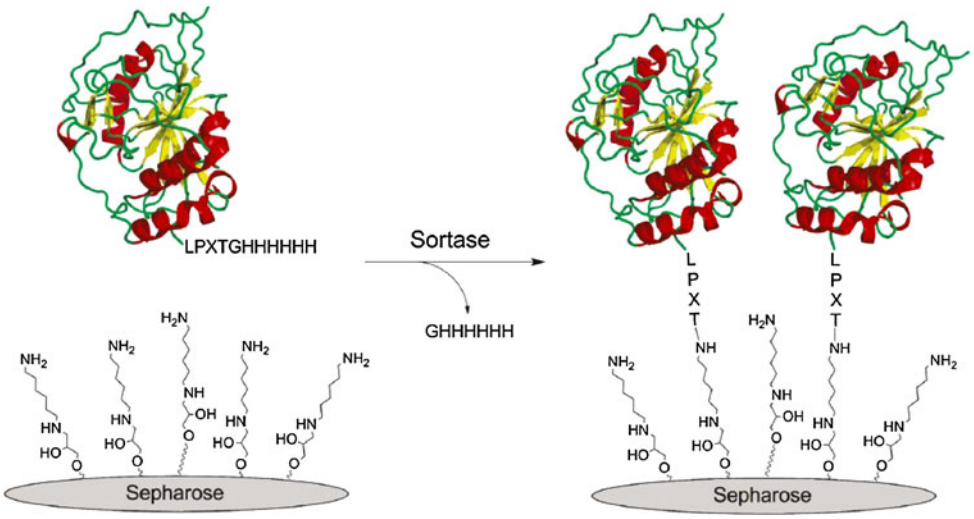

Fig. 4 Sortase A-based site-specific immobilization of glycosyltransferase. Figure adapted with permission from Ref. [79]. Copyright 2010 American Chemical Society

Nevertheless, since transglutaminase has broad substrate specificity, it may catalyze the aggregation of target proteins or lead to immobilization at more than one location.

Sfp is a relatively promiscuous PPTase from *Bacillus circulans*. The Sfp-catalyzed immobilization attaches target protein through a Ser residue of the fused ybbR-tag to phosphopantetheine moiety of CoA attached to supporting materials. The very first example was the immobilization of luciferase and glutathione-S-transferase on resin and the activity retention of luciferase was found to be around 90 % [2]. Wong et al. [82] immobilized the ybbR-tagged arylmalonate decarboxylase (AMDase) and a glutamate racemase (GluR) to polystyrene nanoparticles catalyzed by Sfp (Fig. 6). The k_{cat} of the immobilized AMDase and GluR was 1.70 and 0.01 s^{-1} respectively, as compared to 358 and 44.51 s^{-1} for free ybbR-tagged enzymes, implying both enzymes lost most of their activities after immobilization, which probably was due to, as stated by the authors, "the electrostatic repulsion of the negatively charged substrate attempting to approach the nanoparticles that were also negatively charged due to the unreacted surface carboxylates". It was suggested that the surface group of supporting materials was important to Sfp-catalyzed immobilization. The shortcomings of this method could be: (i) the immobilization of CoA to supports is unstable; (ii) When target enzyme is directly harvested from cell lysate, endogenous CoA may interfere with immobilization [30].

All of the above mentioned covalent immobilization methods share one advantage in common–the covalent attachment of proteins to nanomaterials can prevent enzyme leakage, which is crucial for the application under interfering conditions. However, some covalently immobilized enzymes may experience significant activity drop during covalent bonding. It's difficult to find one immobilization method to fit all applications, hence different strategies should be selected based on different applications.

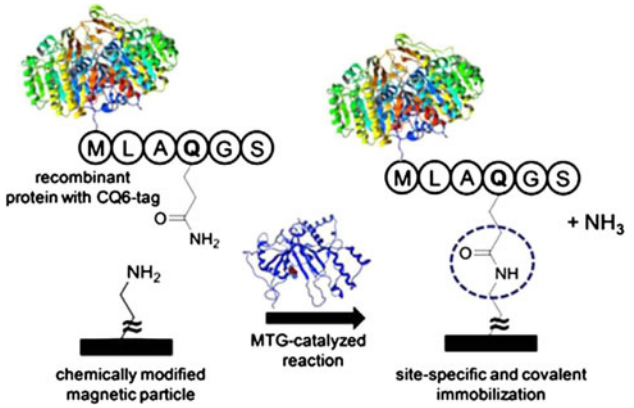

Fig. 5 Schematic illustration of site-specific and covalent immobilization of a recombinant protein tagged with a Gln-containing substrate peptide on chemically modified magnetic particles. Figure adapted with permission from Ref. [81]. Copyright 2011 Elsevier

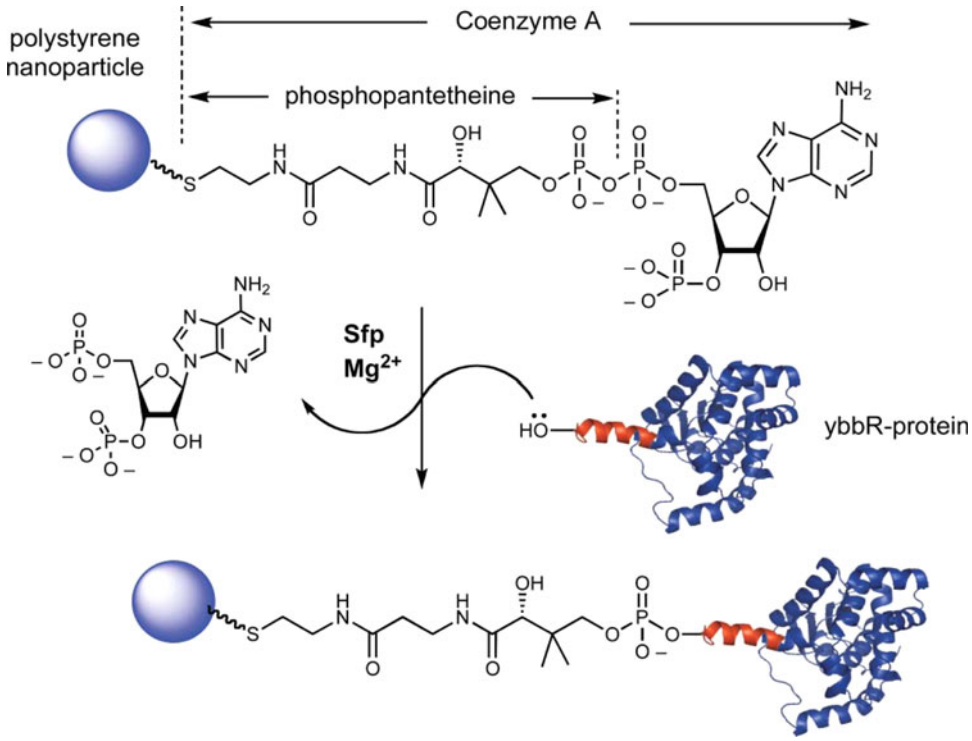

Fig. 6 Strategy for the immobilization of ybbR-protein fusion on to CoA-functionalised polystyrene nanoparticles mediated by phosphopantetheinyl transferase (Sfp). Figure adapted with permission from Ref. [82]. Copyright 2010 Royal Society of Chemistry

4 Other Specific Immobilization Methods

There are other specific immobilization approaches that do not fall into the two categories we have listed here. For example, Chu et al. [85] fused eGFP with the N-terminal segment of DnaE intein, and associated the C-terminal segment of DnaE intein, to the liposomes or lipid-coated silica nanoparticles. DnaE intein, is a special intein—the C- and N-intein fragments of DnaE intein, are able to self-assemble spontaneously [86] (Fig. 7). Excision of DnaE intein, resulted in the immobilization of eGFP to the liposomes or lipid-coated nanoparticles. Additionally, substrate analogues have also been explored for enzyme immobilization purpose. Phosphonate is the substrate analogue of esterase cutinase, which can be attacked by the catalytic serine residue, resulting in the formation of a stable covalent adduct resistant to hydrolysis. Hodneland et al. [87] constructed a cutinase-calmodulin fusion protein, which was immobilized to self-assembled monolayers (SAM) on the gold surface via the reaction of cutinase and SAM-attached phosphonate. GST can bind to Au nanoparticles coated with a mixed surface of thiol-terminated tri(ethylene glycol) and glutathione in a similar way with high specificity [88].

5 Conclusions

Enzymes are often evaluated for their activity retention and stability enhancement after immobilization. Compared with free enzymes, immobilized enzymes usually have improved stability during operation and can be easily recovered, which applies to not only nanoscale but also macroscale supporting materials. If enzymes are immobilized via affinity tags such as His-tag, even supporting materials can be recycled. As for activity retention, enzymes immobilized via specific covalent binding (Cys mutagenesis and "click" chemistry) are likely to preserve much better activity than using non-selective covalent approaches. If enzyme immobilization is based on specific non-covalent interactions, the enzyme-nanomaterial conjugate can achieve even higher activity retention ($\geq$80 %). Enzyme loadings with nanomaterials were also found higher than that of macro-scale supporting materials, probably owing to their high surface area. Some specific immobilization methods mentioned here have never been applied to nanomaterials for biocatalysis purposes, such as enzyme-catalyzed biotinylation and Staudinger ligation, which leaves space for further explorations in this area.

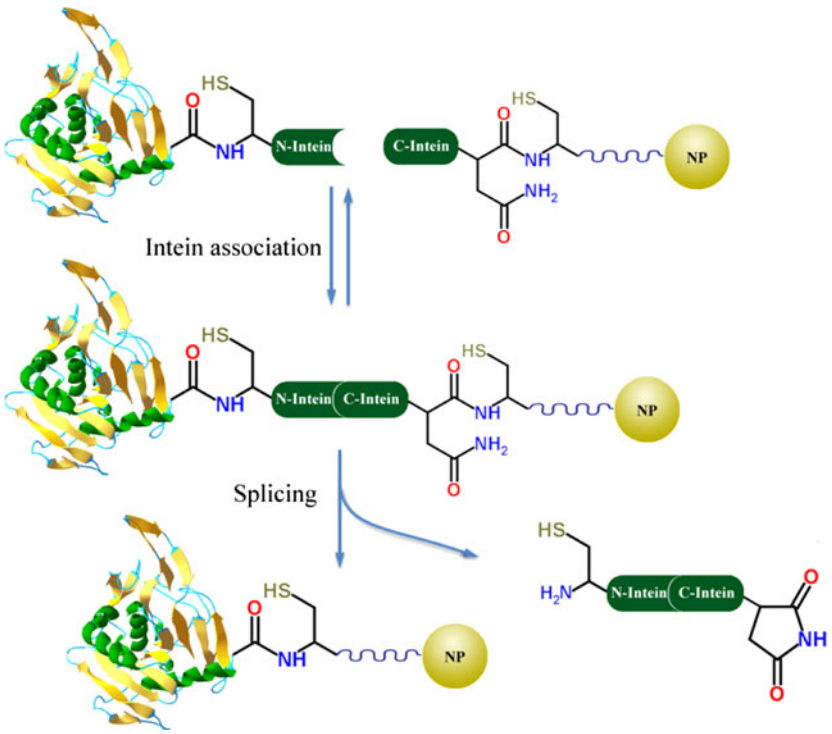

Fig. 7 Site-specific immobilization of protein onto a solid support through split-intein-mediated protein trans-splicing

References

1. Wang P (2006) Curr Opin Biotechnol 17(6):574
2. Wong LS, Thirlway J, Micklefield J (2008) J Am Chem Soc 130(37):12456
3. Besteman K, Lee JO, Wiertz FGM, Heering HA, Dekker C (2003) Nano Lett 3(6):727
4. Letant SE, Hart BR, Kane SR, Hadi MZ, Shields SJ, Reynolds JG (2004) Adv Mater 16(8):689
5. Pierre SJ, Thies JC, Dureault A, Cameron NR, van Hest JCM, Carette N, Michon T, Weberskirch R (2006) Adv Mater 18(14): 1822
6. Kim MI, Kim J, Lee J, Jia H, Bin Na H, Youn JK, Kwak JH, Dohnalkova A, Grate JW, Wang P, Hyeon T, Park HG, Chang HN (2007) Biotechnol Bioeng 96(2):210
7. Luckarift HR, Spain JC, Naik RR, Stone MO (2004) Nat Biotechnol 22(2):211
8. Manyar HG, Gianotti E, Sakamoto Y, Terasaki O, Coluccia S, Tumbiolo S (2008) J Phys Chem C 112(46):18110
9. Hong J, Gong P, Xu D, Dong L, Yao S (2007) J Biotechnol 128(3):597
10. Hudson Sarah, Cooney Jakki, Magner Edmond (2008) Angew Chem Int Ed 47(45):8582
11. Wang SH, Zhuang HS, Du LY, Lin SL, Wang CT (2007) Anal Lett 40(5):887
12. Abad JM, Mertens SFL, Pita M, Fernandez VM, Schiffrin DJ (2005) J Am Chem Soc 127(15):5689
13. Graff RA, Swanson TM, Strano MS (2008) Chem Mater 20(5): 1824
14. Messmer TO, Young DV (1977) J Cell Physiol 90(2):265
15. Graham IA, Eastmond PJ (2002) Prog Lipid Res 41(2):156
16. Gravel RA, Narang MA (2005) J Nutr Biochem 16(7):428
17. Rosano C, Arosio P, Bolognesi M (1999) Biomol Eng 16(1–4):5
18. Takeuchi T, Tham SY, Rechnitz GA (1991) Anal Chim Acta 251(1–2):291
19. Laitinen OH, Hytonen VP, Nordlund HR, Kulomaa MS (2006) Cell Mol Life Sci 63(24):2992
20. Schatz PJ (1993) Bio-Technology 11(10):1138
21. Medintz IL, Anderson GP, Lassman ME, Goldman ER, Bettencourt LA, Mauro JM (2004) Anal Chem 76(19):5620
22. Chen I, Howarth M, Lin WY, Ting AY (2005) Nat Methods 2(2):99
23. Gehring AM, Lambalot RH, Vogel KW, Drueckhammer DG, Walsh CT (1997) Chem Biol 4(1):17
24. Yin J, Liu F, Li XH, Walsh CT (2004) J Am Chem Soc 126(25):7754
25. Chirra HD, Sexton T, Biswal D, Hersh LB, Hilt JZ (2011) Acta Biomater 7(7):2865
26. Zhang Y, Tang Z, Wang J, Wu H, Lin C-T, Lin Y (2011) J Mater Chem 21(43):17468
27. Garcia J, Zhang Y, Taylor H, Cespedes O, Webb ME, Zhou DJ (2011) Nanoscale 3(9):3721
28. Schmidt TGM, Skerra A (1993) Protein Eng 6(1):109
29. Gaj T, Meyer SC, Ghosh I (2007) Protein Expr Purif 56(1):54
30. Wong LS, Khan F, Micklefield J (2009) Chem Rev 109(9):4025
31. Khan F, He MY, Taussig MJ (2006) Anal Chem 78(9):3072
32. Gupta M, Caniard A, Touceda-Varela A, Campopiano DJ, Mareque-Rivas JC (2008) Bioconjug Chem 19(10):1964
33. Xu CJ, Xu KM, Gu HW, Zheng RK, Liu H, Zhang XX, Guo ZH, Xu B (2004) J Am Chem Soc 126(32):9938
34. Xu CJ, Xu KM, Gu HW, Zhong XF, Guo ZH, Zheng RK, Zhang XX, Xu B (2004) J Am Chem Soc 126(11):3392
35. Li YC, Lin YS, Tsai PJ, Chen CT, Chen WY, Chen YC (2007) Anal Chem 79(19):7519
36. Kim SH, Jeyakumar M, Katzenellenbogen JA (2007) J Am Chem Soc 129(43):13254
37. Wang L, Chong H, Jiang R (2012) Appl Microbiol Biotechnol. doi:10.1007/s00253-012-3919-1
38. Zhang H, Lountos GT, Ching CB, Jiang R (2010) Appl Microbiol Biotechnol 88(1):117
39. Lountos GT, Jiang R, Wellborn WB, Thaler TL, Bommarius AS, Orville AM (2006) Biochemistry 45(32):9648
40. Jiang RR, Bommarius AS (2004) Tetrahedron Asymmetry 15(18):2939
41. Jiang RR, Riebel BR, Bommarius AS (2005) Adv Synth Catal 347(7–8):1139
42. Wang L, Zhang H, Ching C-B, Chen Y, Jiang RR (2011) Appl Microbiol Biotechnol. doi: 10.1007/s00253-011-3699-z
43. Wang L, Xu R, Chen Y, Jiang RR (2011) J Mol Catal B Enzym 69:120
44. Wang L, Wei L, Chen Y, Jiang R (2010) J Biotechnol 150:57
45. Wang L, Chen Y, Jiang RR (2012) J Mol Catal B-Enzym 76:9
46. Sarikaya M, Tamerler C, Jen AKY, Schulten K, Baneyx F (2003) Nat Mater 2(9):577
47. Johnson AK, Zawadzka AM, Deobald LA, Crawford RL, Paszczynski AJ (2008) J Nanopart Res 10(6):1009
48. Brown S (1997) Nat Biotechnol 15(3):269
49. Kacar T, Zin MT, So C, Wilson B, Ma H, Gul-Karaguler N, Jen AKY, Sarikaya M, Tamerler C (2009) Biotechnol Bioeng 103(4):696
50. Park H, Park TJ, Huh YS, Choi BG, Ko S, Lee SY, Hong WH (2010) J Colloid Interface Sci 350(2):453
51. Yang M, Choi BG, Park TJ, Heo NS, Hong WH, Lee SY (2011) Nanoscale 3(7):2950
52. Turkova J (1999) J Chromatogr B 722(1–2):11
53. Viswanath S, Wang J, Bachas LG, Butterfield DA, Bhattacharyya D (1998) Biotechnol Bioeng 60(5):608
54. Urlacher VB, Girhard M (2008) Trends Biotechnol 26:126
55. Dawson PE, Muir TW, Clarklewis I, Kent SBH (1994) Science 266(5186):776
56. Tam JP, Lu YA, Liu CF, Shao J (1995) Proc Natl Acad Sci USA 92(26):12485
57. Becker CFW, Marsac Y, Hazarika P, Moser J, Goody RS, Niemeyer CM (2007) ChemBioChem 8(1):32
58. Camarero JA, Kwon Y, Coleman MA (2004) J Am Chem Soc 126(45):14730
59. Helms B, van Baal I, Merkx M, Meijer EW (2007) ChemBioChem 8(15):1790
60. Yu CC, Lin PC, Lin CC (2008) Chem Commun 11:1308
61. Ghosh I, Considine N, Maunus E, Sun L, Zhang A, Buswell J, Evans TC Jr, Xu MQ (2011) Methods Mol Biol 705:87
62. Rostovtsev VV, Green LG, Fokin VV, Sharpless KB (2002) Angew Chem Int Ed 41(14):2596
63. Moses JE, Moorhouse AD (2007) Chem Soc Rev 36(8):1249
64. Brennan JL, Hatzakis NS, Tshikhudo TR, Dirvianskyte N, Razumas V, Patkar S, Vind J, Svendsen A, Nolte RJM, Rowan AE, Brust M (2006) Bioconjug Chem 17(6):1373
65. Lin PC, Ueng SH, Tseng MC, Ko JL, Huang KT, Yu SC, Adak AK, Chen YJ, Lin CC (2006) Angew Chem Int Ed 45(26):4286
66. Gole A, Murphy CJ (2008) Langmuir 24(1):266
67. Polito L, Monti D, Caneva E, Delnevo E, Russo G, Prosperi D (2008) Chem Commun 5:621
68. Schlossbauer A, Schaffert D, Kecht J, Wagner E, Bein T (2008) J Am Chem Soc 130(38):12558
69. Kim YP, Daniel WL, Xia ZY, Xie HX, Mirkin CA, Rao JH (2010) Chem Commun 46(1):76
70. Agard NJ, Prescher JA, Bertozzi CR (2005) J Am Chem Soc 127(31):11196

71. Kiick KL, Saxon E, Tirrell DA, Bertozzi CR (2002) Proc Natl Acad Sci USA 99(1):19
72. Gauchet C, Labadie GR, Poulter CD (2006) J Am Chem Soc 128(29):9274
73. Duckworth BP, Xu JH, Taton TA, Guo A, Distefano MD (2006) Bioconjug Chem 17(4):967
74. Saxon E, Bertozzi CR (2000) Science 287(5460):2007
75. Kohn M, Breinbauer R (2004) Angew Chem Int Ed 43(24):3106
76. Parkhouse SM, Garnett MC, Chan WC (2008) Bioorg Med Chem 16(13):6641
77. Schilling CI, Jung N, Biskup M, Schepers U, Brase S (2011) Chem Soc Rev 40(9):4840
78. Clow F, Fraser JD, Proft T (2008) Biotechnol Lett 30(9):1603
79. Ito T, Sadamoto R, Naruchi K, Togame H, Takemoto H, Kondo H, Nishimura SI (2010) Biochemistry 49(11):2604
80. Sugimura Y, Ueda H, Maki M, Hitomi K (2007) J Biotechnol 131(2):121
81. Moriyama K, Sung K, Goto M, Kamiya N (2011) J Biosci Bioeng 111(6):650
82. Wong LS, Okrasa K, Micklefield J (2010) Org Biomol Chem 8(4):782
83. Waichman S, Bhagawati M, Podoplelova Y, Reichel A, Brunk A, Paterok D, Piehler J (2010) Anal Chem 82(4):1478
84. Yamamura Y, Hirakawa H, Yamaguchi S, Nagamune T (2011) Chem Commun 47(16):4742
85. Chu NK, Olschewski D, Seidel R, Winklhofer KF, Tatzelt J, Engelhard M, Becker CFW (2010) J Pept Sci 16(10):582
86. Kwon Y, Coleman MA, Camarero JA (2006) Angew Chem Int Ed 45(11):1726
87. Hodneland CD, Lee YS, Min DH, Mrksich M (2002) Proc Natl Acad Sci USA 99(8):5048
88. Zheng M, Huang XY (2004) J Am Chem Soc 126(38):12047
89. Gu HW, Xu KM, Xu CJ, Xu B (2006) Chem Commun 9:941
90. Kuan I, Liao RJ, Hsieh H, Chen K, Yu CY (2008) J Biosci Bioeng 105(2):110
91. Liao YQ, Cheng YJ, Li QG (2007) J Chromatogr A 1143(1–2):65
92. Zhen GL, Falconnet D, Kuennemann E, Voros J, Spencer ND, Textor M, Zurcher S (2006) Adv Funct Mater 16(2):243
93. Lee JK, Kim YG, Chi YS, Yun WS, Choi IS (2004) J Phys Chem B 108(23):7665
94. Kim J, Park HY, Kim J, Ryu J, Kwon DY, Grailhe R, Song R (2008) Chem Commun 16:1910
95. Herdt AR, Kim BS, Taton TA (2007) Bioconjug Chem 18(1):183
96. Lata S, Reichel A, Brock R, Tampe R, Piehler J (2005) J Am Chem Soc 127(29):10205