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Diels–Alder Cycloaddition in Protein Chemistry

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The Diels–Alder reaction is probably, together with aldol condensation, the most commonly used reaction in chemistry. The Diels–Alder approach, which involves a diene and a dienophile not present in any biomolecule, allows a chemoselective reaction without the need of protecting groups. Moreover, water has an extraordinary rate-accelerating effect on the reaction process. Thus, this chemical approach has been recognized as a promising procedure for protein bioconjugation. In this review some of the most recent advances in the application of the Diels–Alder reaction in selective surface modification, immobilization and biocatalysis will be discussed.

1. Introduction

The selective modification of proteins has been a long-standing challenge of modern chemical biology, with many applications, ranging from the study of natural post-translational modifications[1] to the development of protein-based materials. Site-selective modifications have been used in investigations of protein expression and localization, as tools in structure-function studies, pharmacokinetics of protein-based drugs as well as and to improve bioavailability, and in the development of biosensors.[2–4]

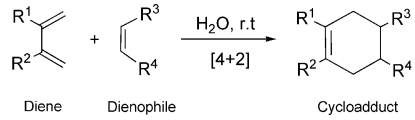
Most importantly, these reactions occur at or near physiological pH and are compatible with the complex array of functional groups commonly found in biological macromolecules including proteins, nucleotides, and carbohydrates, allowing conjugation reactions to be carried out on unprotected substrates.

Nowadays, a number of techniques for protein derivatization based on synthetic organic chemistry have been shown to have surprising utility in the context of biological molecules in aqueous media.[5]

In particular, direct modification of lysine and cysteine with an excess of label containing *N*-hydroxysuccinimide,[6] maleimide cysteine conjugation,[7] aldehyde assisted ligations,[8] aromatic amino acid side chains of tryptophan[9] and tyrosine,[10] native chemical ligation,[11] expressed enzymatic ligation,[12] expressed protein ligation,[13] Staudinger ligation[14] or 1,3-dipolar cycloaddition reaction[15] have been developed as powerful new methods makes significant advances in this field.

However, due to the multifunctionality of biomacromolecules in general and proteins in particular and the manifold applications of such techniques there is a major and continuing demand for the development of new technology providing alternatives to the methods mentioned above. The required chemistry must be compatible with the functional groups found in proteins and proceed chemoselectively under mild conditions and in aqueous solution, preferably in the absence of any potentially denaturating co-solvent.

The Diels–Alder reaction is a [4π+2π] cycloaddition between an electron-rich 1,3-diene and an electron-deficient dienophile leading to the formation of a six-membered carbocycle (Scheme 1).



Scheme 1. General Diels–Alder reaction.

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Therefore, Diels–Alder reaction is a highly selective transformation and in water turned out to be accelerated by a factor up to 10^4 when compared to that in organic solvents.[16]

Its compatibility with biological macromolecules has been explored elegantly in the conjugation of peptides to surfaces for peptide arrays,[17] small molecules[18–19] or peptides[20] to DNA, and carbohydrates to proteins.[21–22]

In this Microreview, the most recent examples of the potential application of this cycloaddition on bioconjugation and immobilization of proteins have been reviewed.

2. Site-Directed Chemical Modification of Proteins by Diels–Alder

The site-selective modification of proteins with a functional group is an important biochemical technique, but covalent attachment of a desired group to a chosen site is complicated by the reactivity of other amino acid side chains, often resulting in undesired side reactions. The Diels–Alder reaction has been presented as an elegant chemoselective methodology to introduce fluorescent labels on proteins,[23–31] to prepare neo-glycoproteins,[32] or to chemically activated proteins.[33–35]

Waldmann et al. have applied the Diels–Alder cycloaddition as a new way to label proteins with fluorophores in vitro.[23]

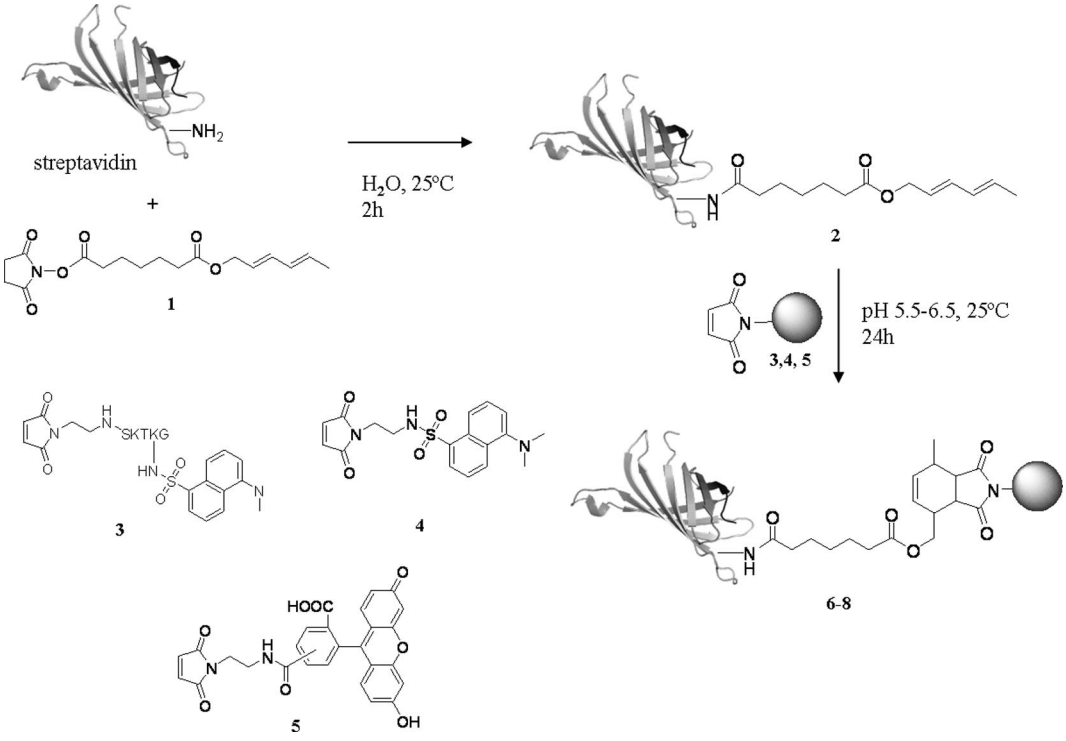
Streptavidin – composed of four biotin-binding subunits – was acylated with a hexadienyl bifunctional linker **1** on a lysine side chain. The diene-modified protein **2** was subjected to Diels–Alder ligation with three different fluorescent labeled maleimide compounds **3**, **4** and **5**, in water and room temperature respectively. The reaction was complete chemoselective producing the different cycloadducts **6**–**8** in high yield at pH around 6. Higher pH values decreased the effectiveness of the methodology (Scheme 2).[23]

The stability of the diene unit incorporated into proteins in aqueous solutions and its compatibility with the functional groups found in the proteins opens up an opportunity to combine the Diels–Alder ligation method with other ligation techniques. In such a combined strategy, a Rab 7 protein – key regulators of vesicular transport that control budding, transport, and fusion of intracellular vesicles[24] – was expressed as a truncated thioester protein[25] (**10**) and then ligated with peptide hexadienyl ester **9** under reducing conditions[26] to yield Rab hexadienyl ester **11** (Scheme 3).

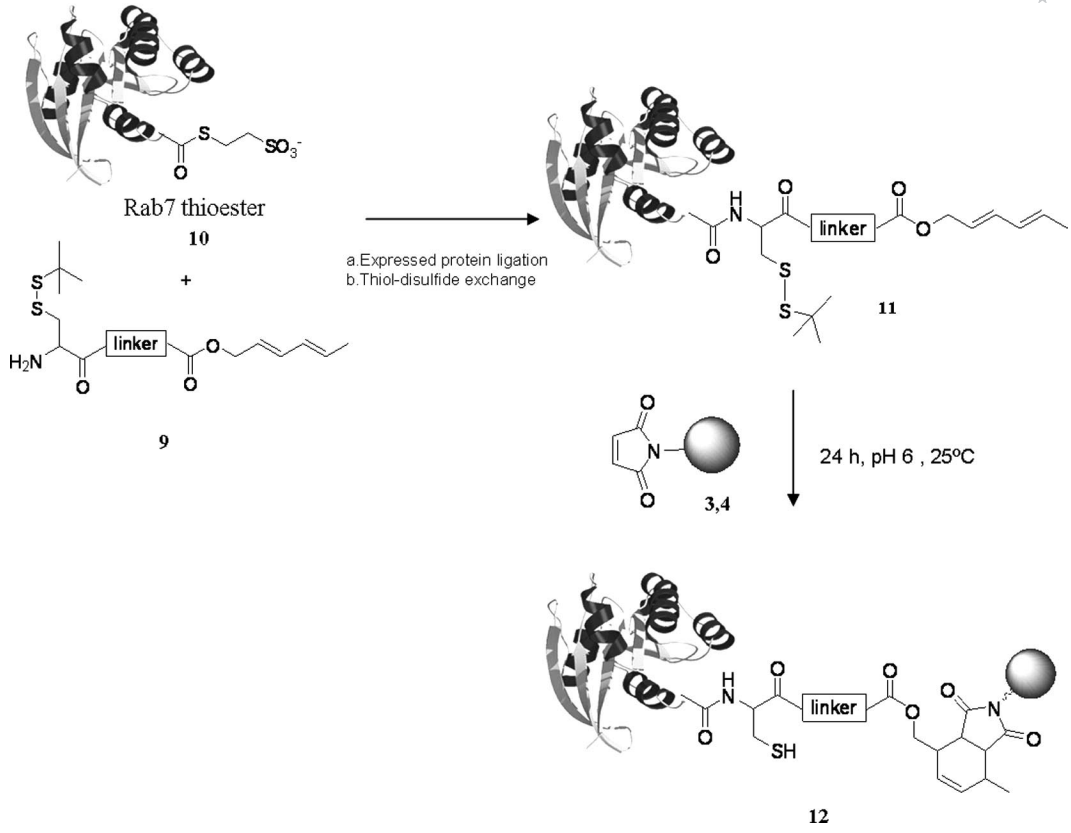
Rab hexadineyl ester was subjected to a Diels–Alder reaction with peptide-derived dienophile **3** or dansyl derivative **4** for 24 h. In the presence of a 100-fold excess of dienophile the hexadienyl protein was converted into the expected fluorescent conjugates **12** with high efficiency (approximately 90%, see Scheme 3).

More interesting was the application of this methodology to create a fluorescent-labeled new semisynthetic Rab protein **14** by the reaction of **11** with a BODIPY-labeled lipidated peptide **13**[27] (Scheme 4).

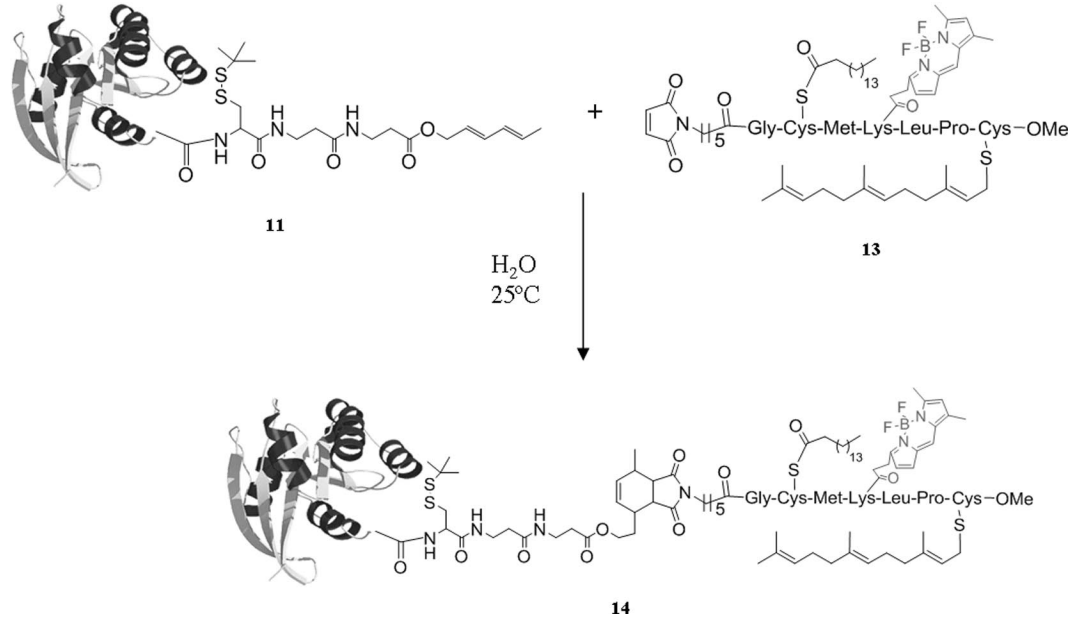
Therefore, this cycloaddition is valid even to the synthesis of sensitive protein conjugates such acid- and base-sensitive lipoproteins.[28]



Scheme 2.



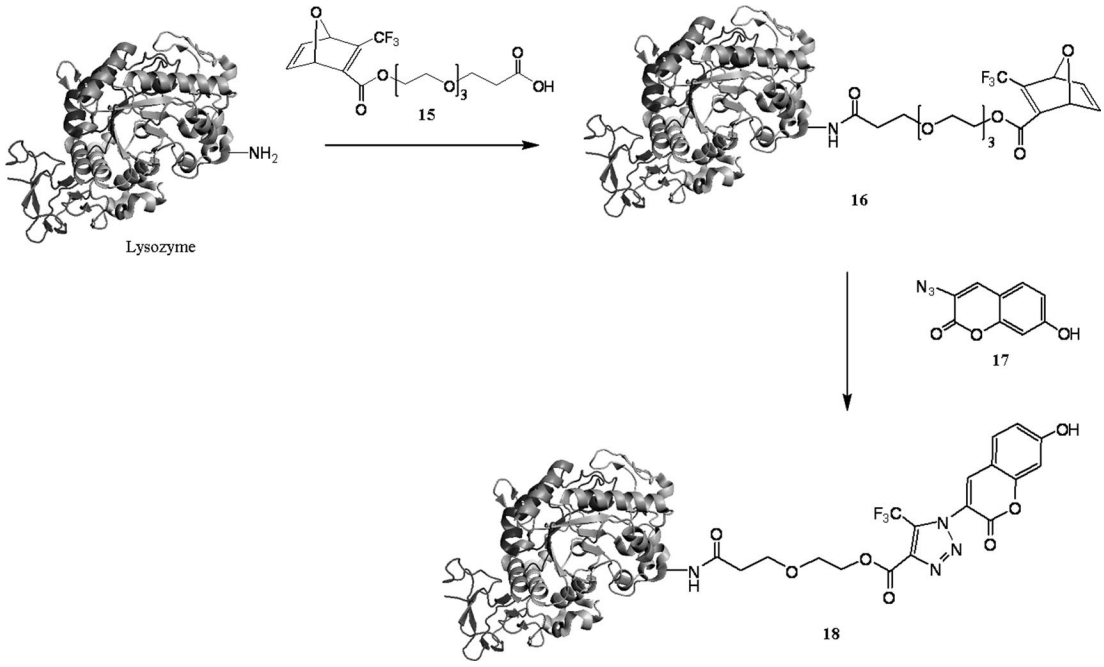
Scheme 3.



Scheme 4.

A tandem [3+2] cycloaddition/retro-Diels–Alder (tandem crDA) reaction has been recently described as a potentially useful metal-free bioconjugation for the incorporation of a fluorescent label molecule to a protein (Scheme 5).[29]

The applicability of the methodology was demonstrated using hen egg white lysozyme as model protein. It was functionalized with an oxanorbornadiene moiety by coupling derivative **15** to its lysine residues. Then this functionalized



Scheme 5.

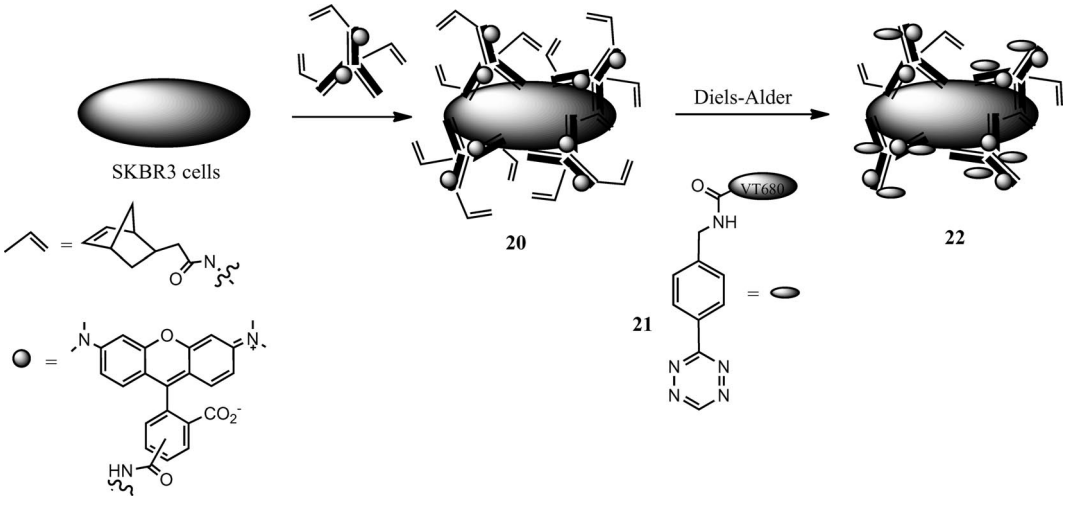
lysozyme **16** was mixed with 3-azido-7-hydroxycoumarin (**17**) for 36 h at 25 °C producing the desired adduct **18**. The formation of this adduct was evaluated by SDS-PAGE with coomasie staining and UV detection at 366 nm.

The modification of antibodies to live cell labeling is another interesting application of this approach.[30–31]

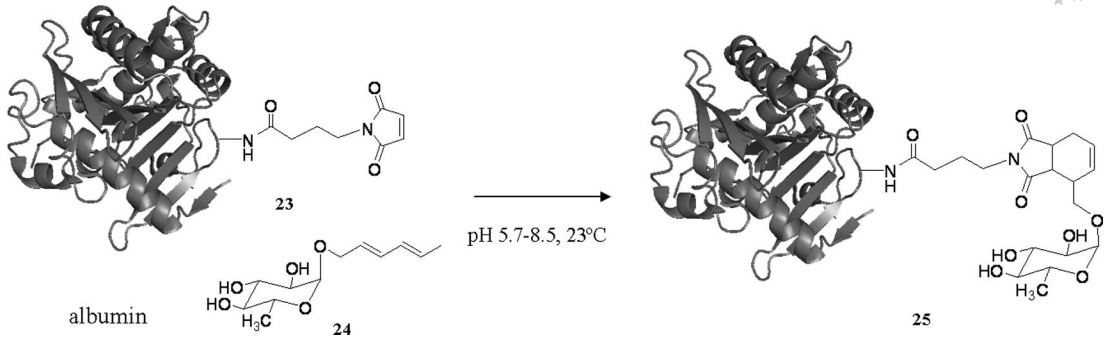
Her2/neu receptors on live human breast cancer cells were targeted with a monoclonal antibody modified with the norbornene **19**. Tetrazines conjugated to a near-infrared fluorochrome (**21**) selectively and rapidly label the pretargeted antibody **20** in the presence of serum forming dihydropyrazine products **22** by a Diels–Alder reaction in high yield (Scheme 6).[30] These findings indicate that this chemistry is suitable for in vitro labeling experiments, and suggests that it may prove to be a useful strategy for in vivo pretargeted imaging under numerous modalities.

Furthermore, a Diels–Alder reaction has been employed on the preparation of neo-glycoprotein.[32] A saccharide-linked conjugated diene **24** were selectively coupled to dienophile activate albumin **23** (Scheme 7) at room temperature in pure water with a reaction half-life of approximately 2 h. Therefore, this could represent an interesting extensive methodology to other proteins.

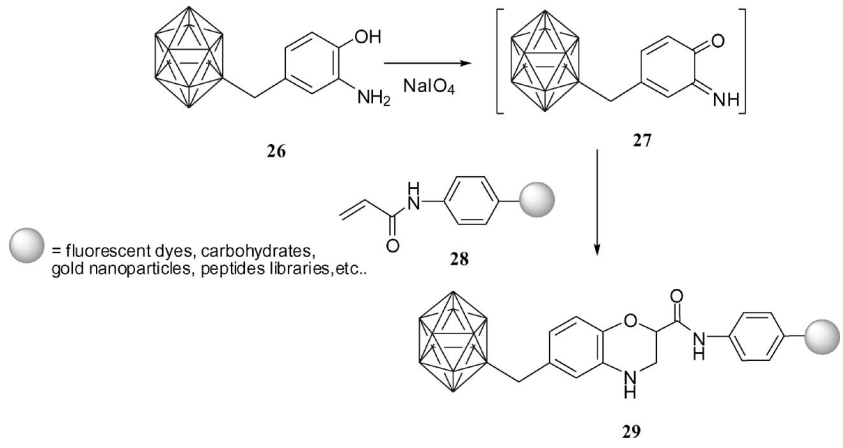
Francis et al. have described an efficient strategy based on a hetero Diels–Alder reaction for the attachment of olefin substrates to modified tyrosine residues on bacteriophage MS2.[33] A three-step synthetic sequence was used to convert tyrosine residues on the phage particle firstly to *o*-



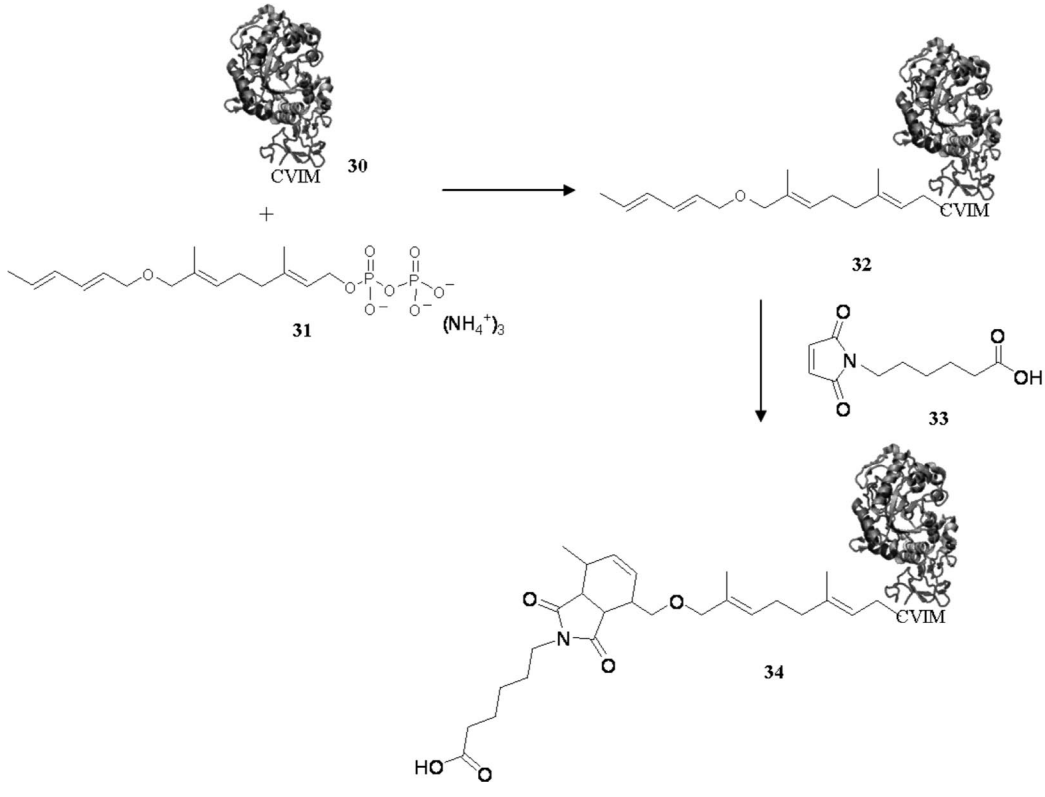
Scheme 6.



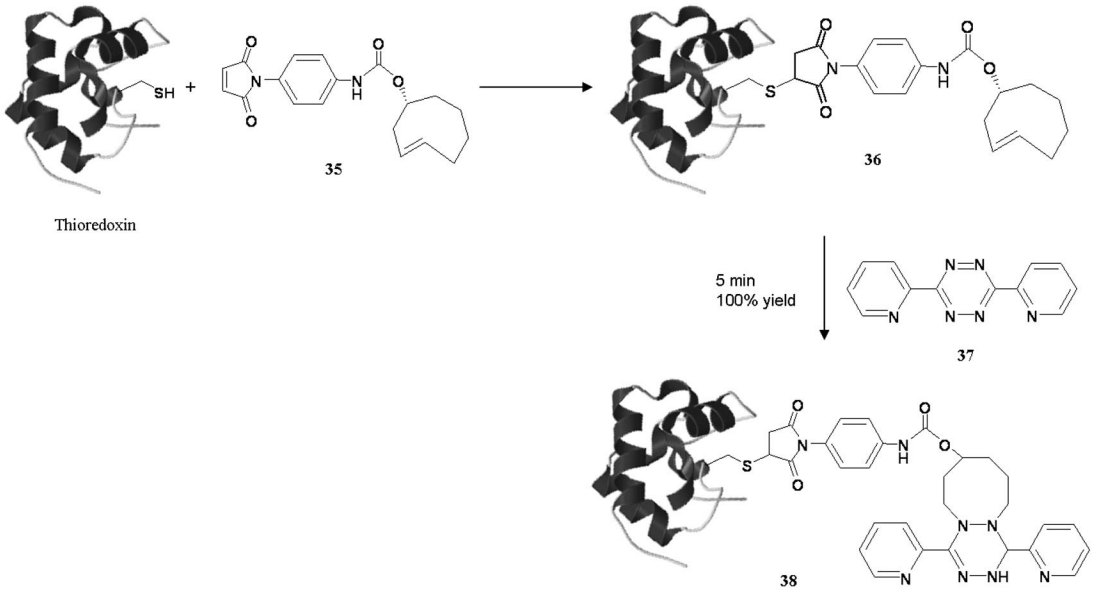
Scheme 7.



Scheme 8.



Scheme 9.



Scheme 10.

amino tyrosine **26** and then, to *o*-imino-quinone **27**, which served as the diene platform for the subsequent Diels–Alder reaction with dienophiles **28** (Scheme 8).

These modified viral capsids present potential applications as chemical reagents for the formation of nanometer-scale circuity upon the appropriate immobilization of metal particles,[36] the ability to encapsulate drugs or inorganic species in viral cages,[37] the possibility of creating isolated catalytic chambers inside viral capsids, and the use of virions as scaffolds for the polyvalent display of bioactive ligands.[38]

Proteins containing the CAAX motif can be chemoselectivily modified by a Diels–Alder reaction, by a previous prenylation with isoprenoid analogues.

A diene-functionalized phosphoisoprenoid **31** was bound to Ypt7 protein (**30**), a small GTPase, specifically by a farnesyltransferase (FTase) via to a cysteine unit in the context of a short C-terminal sequence that can be easily grafted onto recombinant proteins. In this way the prenylated protein **32** could be derivatized with 6-maleimidohexanoic acid **33** (Scheme 9). With 40 equiv. of reagent and a reaction time of 15 h the reaction yield was approximately 30% of **34**.

A new method for bioconjugation based on inverseelectron demand Diels–Alder chemistry has been described as another interesting example.[35] Thioredoxin, an 11.7 kDa protein, was functionalized with *trans*-cyclooctene derivative **35** by cysteine conjugation at pH 6.

The derivative **36** was combined with tetrazine **37** and a very fast retro-[4+2] cycloaddition was performed on aqueous solution, with a 100% of adduct **38** formed in only 5 min (Scheme 10). Also this reaction tolerates a broad range of biological functionality.

Thus, all these examples show that the Diels–Alder cycloaddition represents a promising methodology to create new artificial enzymes.

3. Site-Directed Immobilization of Proteins by Diels–Alder

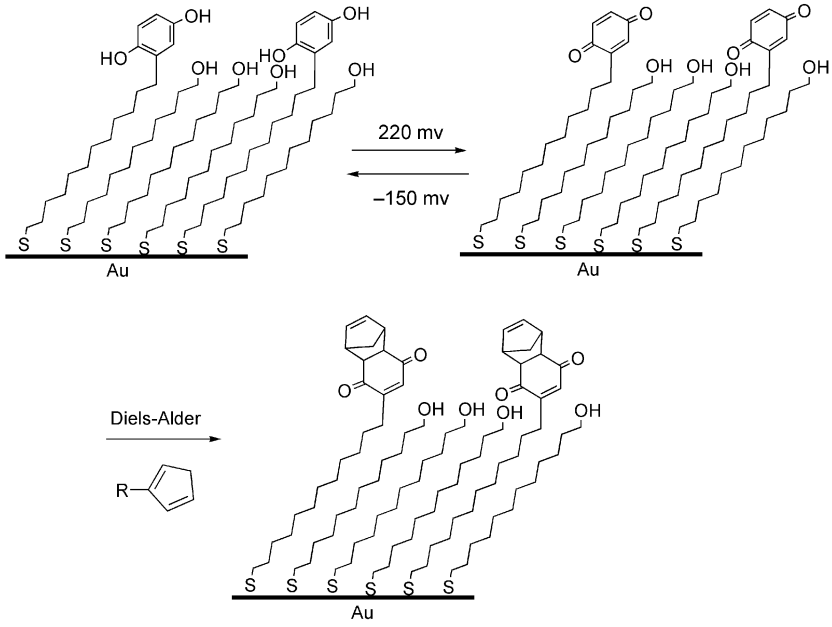
Chemical selective immobilization of biomolecules onto solid surface has been a target of numerous synthetic endeavors as this process facilitates many potential applications of biomolecules. For example, microarrays, microbeads, nanoparticles, biosensor chips, and surface functionalization of medical devices play increasingly important roles in basic biological research and biomedical applications. A series of techniques for biomolecule immobilization have been established.[39–42] However, conventional methods for surface bioconjugation are limited by low efficiency, selectivity, and harsh reaction conditions.

Therefore, it remains a demand for the development of alternative approaches, in which the chemistry is compatible with the functional groups found in biomolecules and proceeds chemoselectively under mild conditions and in aqueous solution, preferably in the absence of any potentially denaturating co-solvents and catalysts.

Diels–Alder cycloaddition has been recognized as a promising procedure for surface immobilization of biomolecules.

Mrksich et al. described a first immobilization of biomolecules in glass slide by Diels–Alder condensation.[43] This strategy was applied to prepare peptides and protein microarray by a maleimide functionalized surface. However, the methodology of the process was optimized for the preparation of a peptide chip with the kinase substrate AcIYGEFKKKC-NH_2 immobilized onto a SAM on gold to be used for the characterization of enzymatic activity (Scheme 11).[44]

The first version of microarray proteins by a Diels–Alder reaction in aqueous media was presented by Waldmann et al.[45] where a simple and selective method for immobilization of proteins and enzymes was described.



Scheme 11.

Avidin or strepavidin previously activated by the incorporation of a cyclopentadiene were chemoselectively immobilized on different maleimide-activated glass slides (Scheme 12). The immobilization of the protein was determined by the incorporation of Cy5-biotin.

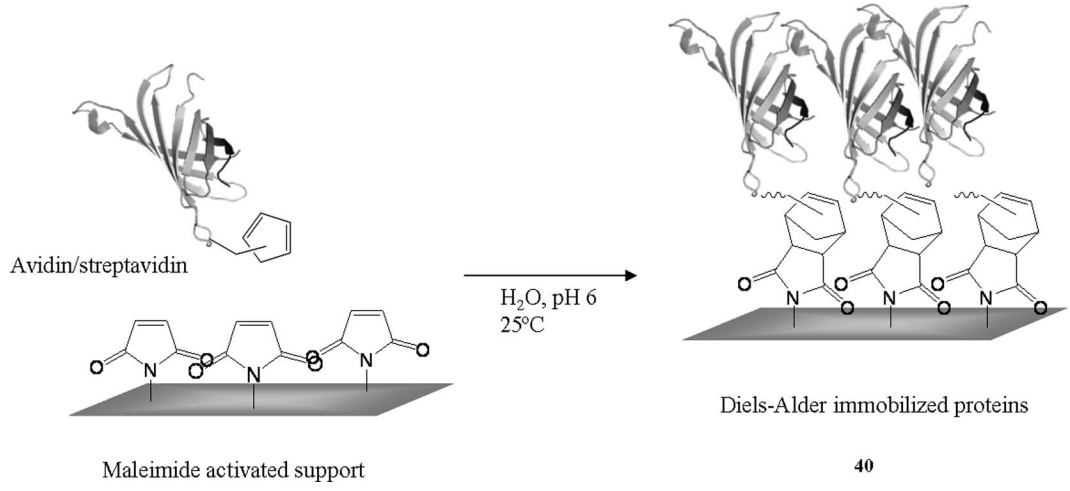
A second strategy of protein immobilization was based on a previous PEGylation of the protein.[46]

Sun et al. modified protein A by a chemical reaction between a poly(ethylene glycol) (PEG) spacer (**41**) incorporating a cyclopentadiene on the side to the amino groups of the lysines on the protein. This modified protein was selectively immobilized on a maleimide-functionalized glass slide in high yields on aqueous media at neutral pH (Scheme 13).

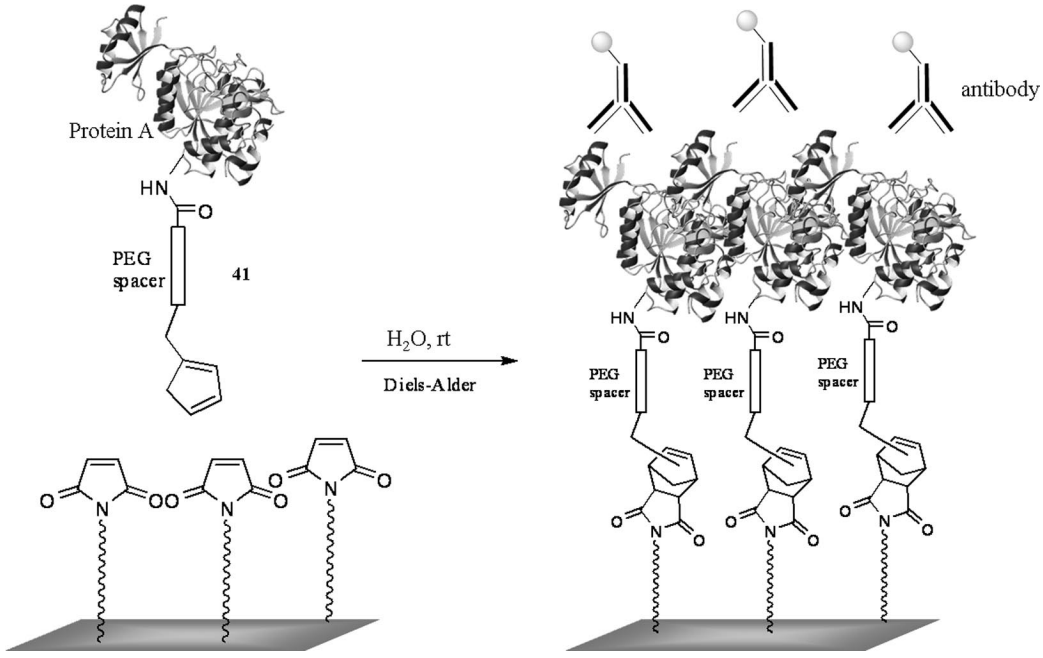
Also this methodology was successfully used to immobilize other proteins by a Diels Alder coupling of a selective modified substrate, biotin derivative **42** for streptavidin immobilization or anomeric position activated lactose **43** for lectin immobilization (Scheme 14).

The current approach will find wide application in the functionalization of solid surface such as microchip, sensor, and nanoparticle as well as diagnostic and therapeutic medical devices.

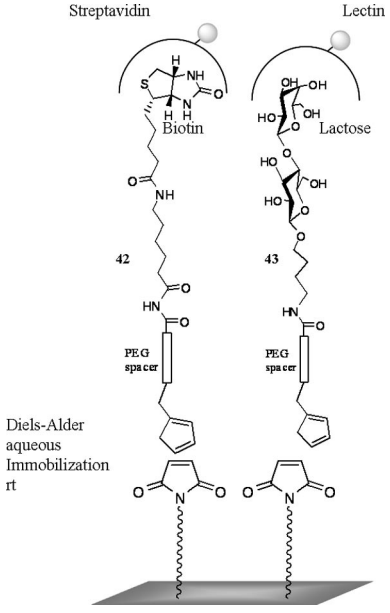
The immobilization of proteins throughout these strategies, besides these results, still present margin of improvement, specially on the application of biocatalytic methods, as immobilization of enzymes, purification strategies, etc.



Scheme 12.



Scheme 13.



Scheme 14.

Another interesting application was the preparation of immuno polymeric nanoparticles by chemoselective immobilization of antibodies.[47]

Antibodies *anti*-HER2 (a therapeutic antibody used to treat breast cancer) functionalized with maleimide (**44**) in the carbohydrate chains within the Fc region were specifically incorporated on furan-functionalized micellar nanoparticles derivatized by Diels–Alder in aqueous solution (Scheme 15). This was efficient binding with breast cancer HER2-over-expressing cell lines in vitro.

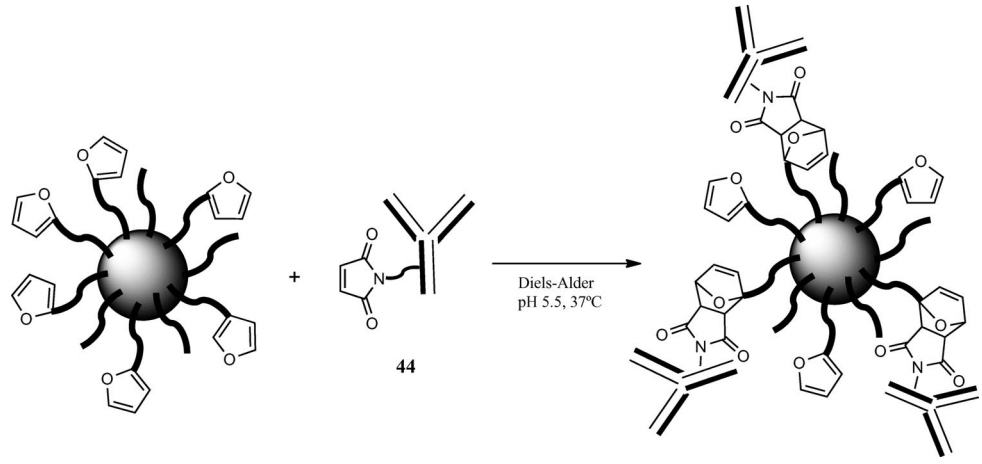
The versatility of the nanoparticle system can be extended to create complex and multiple functional delivery vehicles by taking advantage of the Diels–Alder reaction to immobilize molecules appropriate for imaging, diagnostics, or the targeted delivery of therapeutic age.

4. Natural and Artificial Diels–Alderases

Regarding to the versatility of enzymatic catalysis and occurrence of enzyme-catalyzed pericyclic rearrangements, it is entirely possible that Nature could also orchestrate this transformation. Indeed some circumstantial evidence suggests the existence of enzymes that catalyze the Diels–Alder reaction. To date, only two enzymes have been purified to homogeneity and demonstrated to catalyze reactions where the substrates and products are consistent with a Diels–Alder cycloaddition: lovastatin nonaketide synthase (LovB) and macrophomate synthase.[48]

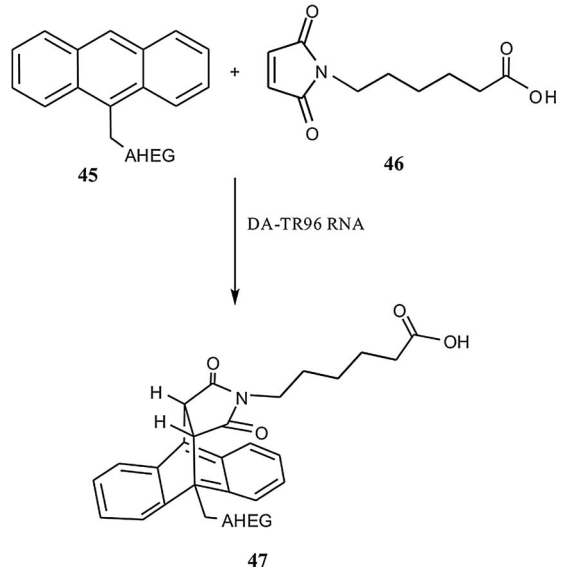
However, in Nature biocatalysts other than enzymes with Diels–Alderase activity in water have been found such as catalytic antibodies[49] or ribozymes.[50–51] Recently, new classes of RNA enzymes or ribozymes containing 5-(4-pyridylmethyl)uridinecarboxamides have been selected for its ability to promote a Diels–Alder cycloaddition reaction.[52] DA22 RNA sequence family, which was selected with pyridyl-modified nucleotides, accelerates a cycloaddition reaction between anthracene and maleimide derivatives with high turnover.[53–54]

The free unmodified RNAh 39M49 was the most active biocatalyst promoting the Diels–Alder reaction between the diene, 9-anthracenylmethyl hexaethylene glycol (AHEG) **45**



Scheme 15.

with the dienophile, 6-maleimidocaproic acid (**46**). The reaction was followed by decreasing the absorbance of the diene and formation of Diels–Alder product **47** (Scheme 16).[53a]

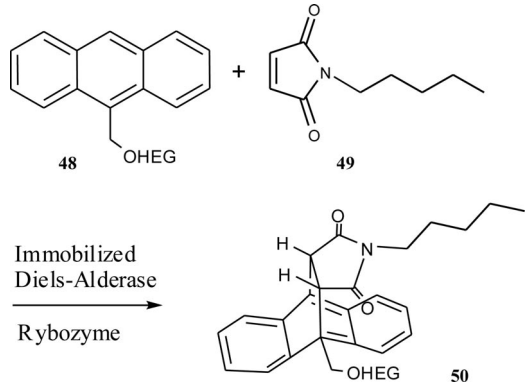


Scheme 16.

The 39M49 RNA structure, folding and substrate interactions have been well characterized by X-ray crystal structures of the RNA both, free and bound, to a Diels–Alder product, fluorescence resonance energy transfer (FRET) and photoaffinity cross-linking, amongst other techniques.[55–59]

Another interesting application was the Diels–Alder reaction catalyzed by immobilized Diels–Alderase ribozymes.[60]

49-Nucleotide RNA molecule was quantitatively immobilized on an agarose matrix by periodate oxidation of the 3′-terminal ribose and coupling to a hydrazide moiety and used as stereoselective catalysts on the Diels–Alder reactions between OHEG anthracene dienes **48** and maleimide dienophile **49** with high enantioselectivities (Scheme 17). Their catalytic activity and selectivity was maintained for many cycles of catalysis with an excellent long-term stability.



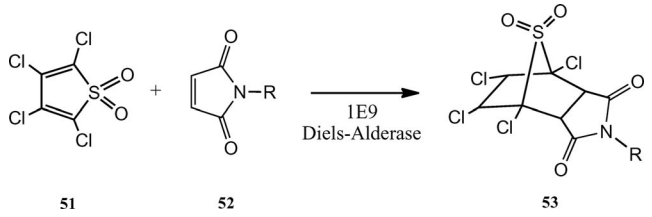
Scheme 17.

Antibodies featuring Diels–Alderase activity were obtained by challenging the immune system with transition state analogues.[61–64]

These antibody-catalyzed reactions display Michaelis–Menten kinetics featuring unexceptional KM values in the 10^{-3} M range for both the dienes and the dienophiles as expected for normal organic host–guest complexation in water.

Antibody 1E9 catalyzes the Diels–Alder reaction of thiophene dioxide **51** and maleimide **52**.[61–62] The remarkable catalytic ability of this antibody has been explained by the high degree of shape and electrostatic complementarily with the transition state evidenced by hydrogen bonds and other polar interactions and by the presence of a key asparagine residue that can form a hydrogen bond with one of the carbonyl groups of the dienophile (Scheme 18).

Furthermore, hardly anything is known about the existence of naturally occurring hetero-Diels–Alderase enzymes. Reymond et al. described the preparation of monoclonal antibodies that catalyze the hetero-Diels–Alder (HDA) reaction of a nitroso heterodienophile with 1,3-pentadiene with good control of regio- and enantio-

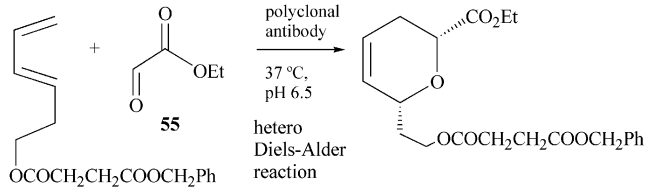


51 52 53

Scheme 18.

selectivity.[65–66]Antibody-catalyzed retro hetero-Diels–Alder reactions have also been reported releasing HNO as the heterodienophile.[66]

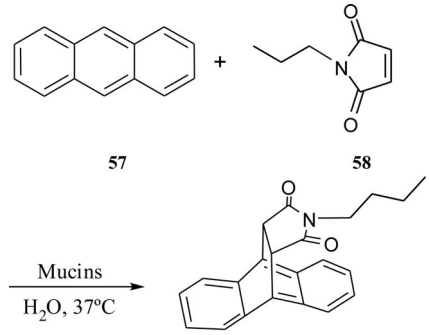
Wu et al. reported the only example of an antibody-catalyzed HDA reaction involving a carbonyl dienophile.[67] They generated a mixture of polyclonal antibodies raised against a hapten mimicking an *endo* approach of ethyl glyoxylate towards a poorly activated diene **54**. The *endo* approach as programmed in the transition state analogue (TSA), resulted in the exclusive formation of the *cis*-3,4-dihydropyran derivative **56**. Although showing moderate rate enhancement, the antibodies allowed for an excellent level of diastereocontrol in favour of the *cis* isomer (Scheme 19). The same authors also reported an example of an antibody-catalyzed aza-Diels–Alder reaction leading to the *trans* adduct as programmed by the hapten, a TSA that was mimicking in this case an *exo* approach of the imino dienophile toward the diene.[68]



54 55 56

Scheme 19.

Other proteins have shown Diels–Alderase activity.[69] Gozin et al. demonstrated for the first time that under physiological conditions, two representative mucins, bovine submaxillary mucin type I (BSM) and porcine gastric mucin type III (PGM) promoted a Diels–Alder reaction between anthracene **57** and *N*-propylmaleimide **58** (Scheme 20) with yields between 47–65% of **59**. Also, the reaction was accelerated in phosphate buffer solution 39- and 24-fold by BSM and PGM, respectively, relative to the rate of the same reaction in chloroform.



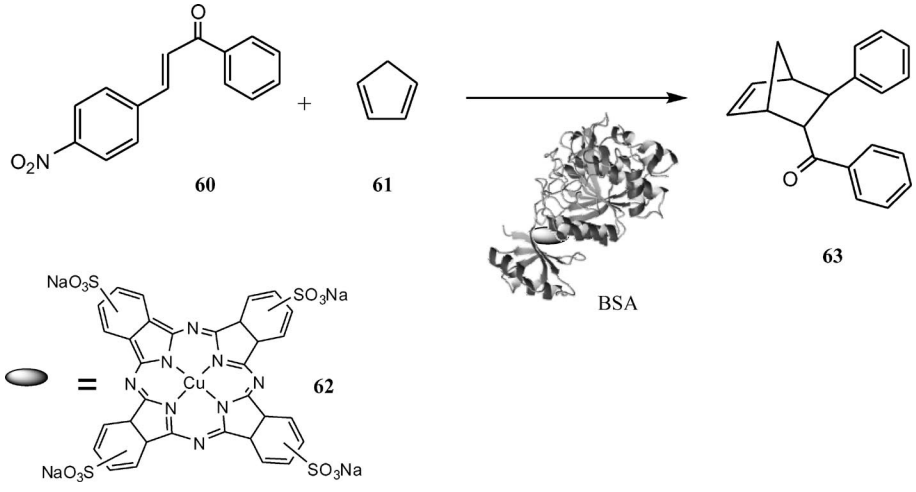
57 58 59

Scheme 20.

Mucins consist of branched oligosaccharide chains attached to a protein backbone.[70–72] This unique structure was discovered to be critically important to the rate acceleration, as various cyclic and noncyclic oligosaccharides were far less efficient in promoting the same reactions.

However, currently many research groups have been concentrated on the creation of artificial enzymes, hybrid systems which perform a chemoselective Diels–Alder to mimic a naturally occurring Diels–Alderase.[73–75]

For example, Reetz et al.[73] have described the preparation of protein conjugates by treating serum albumin BSA with the phthalocyanine–Cu^{II} complex **62** by specific noncovalent anchoring of the metal. These provide Lewis acidic catalysts for highly enantioselective Diels–Alder reactions of azachalcone **60** with cyclopentadiene (**61**) in an aqueous medium leading to products **63** with surprisingly high enantiopurity (98% *ee*) (Scheme 21).



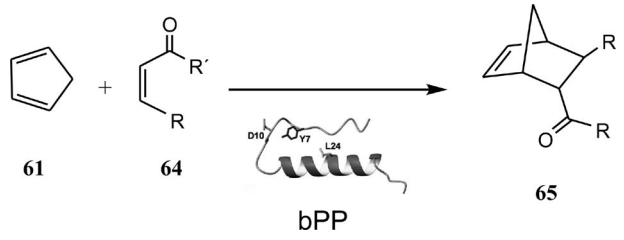
60 61 62 63

Scheme 21.

This opens the door for other types of enantioselective Lewis acid catalyzed reactions with these hybrid catalysts, but also for other Lewis acidic transition metals.

Roelfes et al. have developed a novel strategy towards the design of artificial metalloenzymes, which involves grafting of an active site onto an existing small natural protein scaffold by incorporation of a nonproteinogenic amino acid that is capable of binding a transition-metal ion.[75] Bovine pancreatic polypeptide (bPP), a member of the pancreatic polypeptide family of peptide hormones, was selected as the protein scaffold.

A key strength of the present approach is that an existing binding pocket in the protein is not required; this greatly expands the choice of protein scaffolds for artificial-metalloenzyme design. By using bPP-based catalysts, good enantioselectivities were obtained in the Cu^{2+}-catalyzed Diels–Alder reaction in water (83 to 86% *ee*) between cyclopentadiene **61** and different dienophiles **64** (Scheme 22). A particularly interesting feature of the present system is the high substrate selectivity, which is reminiscent of natural enzymes.



Scheme 22.

Conclusions

The examples present in this review describe the enormous potential of this chemical approach. Nevertheless, the development of new methodologies for incorporation of diene to the biomolecule, reaction conditions optimization or simple methods for incorporation of dienophile to the support, biomolecules, polymer, etc are still demanding. This strategy could be a key tool on the preparation of hybrid catalysts or on the protein–protein bioconjugation for tandem catalysis.

Therefore, combining this selective methodology together genetic methods, other synthetic methods and immobilization approaches could be possible to create the best catalysts demanding for the modern chemistry with applications in fine chemistry, food chemistry, nanotechnology, biomedicine, biofuels production, etc.

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