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MINI-REVIEW

Well-defined protein–polymer conjugates—synthesis and potential applications

Pall Thordarson · Benjamin Le Droumaguet · Kelly Velonia

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Abstract During the last decades, numerous studies have focused on combining the unique catalytic/functional properties and structural characteristics of proteins and enzymes with those of synthetic molecules and macromolecules. The aim of such multidisciplinary studies is to improve the properties of the natural component, combine them with those of the synthetic, and create novel biomaterials in the nanometer scale. The specific coupling of polymers onto the protein structures has proved to be one of the most straightforward and applicable approaches in that sense. In this article, we focus on the synthetic pathways that have or can be utilized to specifically couple proteins to polymers. The different categories of well-defined protein–polymer conjugates and the effect of the polymer on the protein function are discussed. Studies have shown that the specific conjugation of a synthetic polymer to a protein conveys its physico-chemical properties and, therefore, modifies the biodistribution and solubility of the protein, making it in certain cases soluble and active in organic solvents. An overview of the applications derived from such bioconjugates in the pharmaceutical industry, biocatalysis, and supramolecular nanobiotechnology is presented at the final part of the article.

Keywords Biohybrids · Protein–polymer conjugates · Nanotechnology

P. Thordarson
School of Chemistry, The University of Sydney,
Sydney, NSW 2006, Australia

B. Le Droumaguet · K. Velonia (✉)
Department of Organic Chemistry,
University of Geneva, Sciences II,
30, Quai Ernest Ansermet,
CH-1211 Geneva 4, Switzerland
e-mail: Kelly.Velonia@chiorg.unige.ch

Introduction

Among the most stimulating challenges for the scientists of our times is the understanding and exploitation of Nature's vast machinery to create novel, efficient biotechnological devices. Along these lines, the covalent attachment of single or multiple polymeric chains onto biomolecules has been an area of intense research during the last decades (Veronese 2001; Pennadam et al. 2004; Veronese and Pasut 2005; Vriezema et al. 2005).

The creation of such chimeric systems offers the exciting possibility of combining the properties of both the biological and the synthetic component in a new class of biomaterials designed to perform otherwise impossible functions/actions. Theoretically, the properties of the protein–polymer hybrids should simply be a synergistic combinatorial function of those of each individual component and should ideally lead to overcoming some of their intrinsic limitations. This assumption has been especially pursued in the area of pharmaceuticals where one of the holy grails is still the stabilization and enhancement of the action of therapeutics (proteins and drugs) without comprising their stability (Ulbrich et al. 1996; Omelyanenko et al. 1996; Maeda 2001a,b; Youngster et al. 2002; Caliceti and Veronese 2003). Furthermore, assemblies incorporating biological components are particularly attractive as supramolecular biomaterials, lab-on-a-chip components and as building blocks in microfluidics and bioprocessing applications (van Hest and Tirrell 2001; Niemeyer 2001; Shimoboji et al. 2002; Pennadam et al. 2004; Klok 2005).

The protein–polymer conjugates that are highlighted in this review are well-defined and differ from other bioadducts in the sense that the protein-to-polymer ratio is predefined and the position of the conjugation site is precisely known. From the chemical point of view, these bioconjugates are in

fact *diblock* copolymers, which, by design, have significantly higher molecular weights and volumes than their synthetic counterparts. Furthermore, as Nature synthesizes its biopolymers with high efficiency, these synthetic bioblocks have an intrinsic structural advantage over the synthetic block copolymers by possessing a monodisperse block (the protein). The incorporation of the biological component in the copolymer structure therefore offers new perspectives for creating biomacromolecules that comprise the biological properties of the protein component with the chemical and assembling properties of the polymer.

From the biological point of view, the conjugation of a polymer can either alter or preserve many of the important protein biological functions, such as enzymatic activity or receptor recognition, depending on the overall resulting structure. In principle, such conjugation masks the protein surface and increases its molecular size in ways that reduce its renal ultrafiltration, prevent the approach of antibodies or antigen processing cells, and reduce the degradation by proteolytic enzymes. Finally, upon conjugation the synthetic polymer is expected to convey its physico-chemical properties and thus modify also the biodistribution and solubility of the protein or enzyme. This offers the possibility of creating synthetic enzymes that are soluble and active in organic solvents, of developing new techniques in biocatalysis, and most importantly, of advancing in pharmaceutical technology and supramolecular nanobiotechnology.

It is worth mentioning that the synthesis of well-defined protein–polymer biohybrids falls under the umbrella of specific protein functionalization studies. This area has been in the spotlight of academic research for several decades now and continues to flourish and serve as one of the main knowledge foundations for the understanding of biomolecules to successfully employ them. The bibliography in this area is vast (Matthews et al. 1991; Hermanson 1996; DeSantis and Jones 1999; Qi et al. 2001) but out of the scope of this review paper.

Methods for specific protein functionalization

The holy grail of bioconjugate chemistry is to develop efficient methods for the modification of proteins and other biological molecules with a high degree of specificity *in vitro* and *in vivo*. No such magic bullet universal technique exists to date, but significant advances have been made in the development of techniques that fulfill the above criteria under specific conditions. Proteins can be chemically modified using a range of methods (Hermanson 1996). In this review, we will only focus on those that have been successfully used to modify proteins with polymers in a specific, programmed manner. We can broadly divide these into three major categories: a) methods for direct specific

functionalization, b) methods for indirect specific functionalization, and c) methods for specific functionalization via cofactors/ligands (Fig. 1). It should be noted that the only difference between methods a) and b) is that in the former, the polymer is introduced directly onto the protein structure while in the latter, a heterobifunctional spacer is first linked to the protein to create a reactive biohybrid, which is subsequently further functionalized by the attachment of a polymer onto the spacer.

Choosing the appropriate method to functionalize a protein is no easy task. The most useful protein functionalities for specific chemical modification are the natural amino acids side chains of cysteine (Cys), lysine (Lys), tyrosine (Tyr), and glutamine (Gln) together with the α -N terminus (α -N) of the peptide backbone. Directed techniques for the introduction of non-natural amino acids into proteins using methods such as non-ribosomal peptide synthesis, peptide ligation (Hodgson and Sanderson 2004), and tRNA engineering methods (Hodgson and Sanderson 2004; Budisa 2004) have somewhat broadened the range of available functionalities, although their use is still quite limited. Recently, proteins have also been selectively modified via their genetically engineered His-Tags (Meredith et al. 2004).

Another consideration in this paper is the complementary reactive group(s) for these natural and non-natural amino acids (*vide supra*), which must be available on the target polymer or heterofunctional spacer. Practical considerations such as chemical compatibility or economic factors may often rule out certain bioconjugate routes. More often, it is the availability of the target functional group on the protein that determines the route taken. The target has to be reasonably exposed on the surface of the protein and not buried in the interior of the ternary protein structure. In the case that two or more amino acid side chains (e.g., Lys) are exposed, it becomes very difficult to control the bioconjugation reaction in such a way that only one specific linker between the protein and the target is created. Finally, the modification should ideally have little or no effect on the protein conformation and function, e.g., catalytic activity and specificity. It is, therefore, clear that detailed structural information is a pre-requisite for the successful selective functionalization of proteins. In practice, this usually requires information about their three-dimensional structure via single-crystal X-ray and/or solution NMR studies, although the protein sequence might be sufficient, provided that some structural information is available.

The most popular but least specific of the above-mentioned methods is coupling to the ϵ -amino group on a lysine residue (Lys) or the N terminus (α -N) of the protein (Fig. 2). The ϵ -amine group on Lys and α -N can react very efficiently with a number of functional groups, including aldehydes and activated carboxylic acids. The reaction of

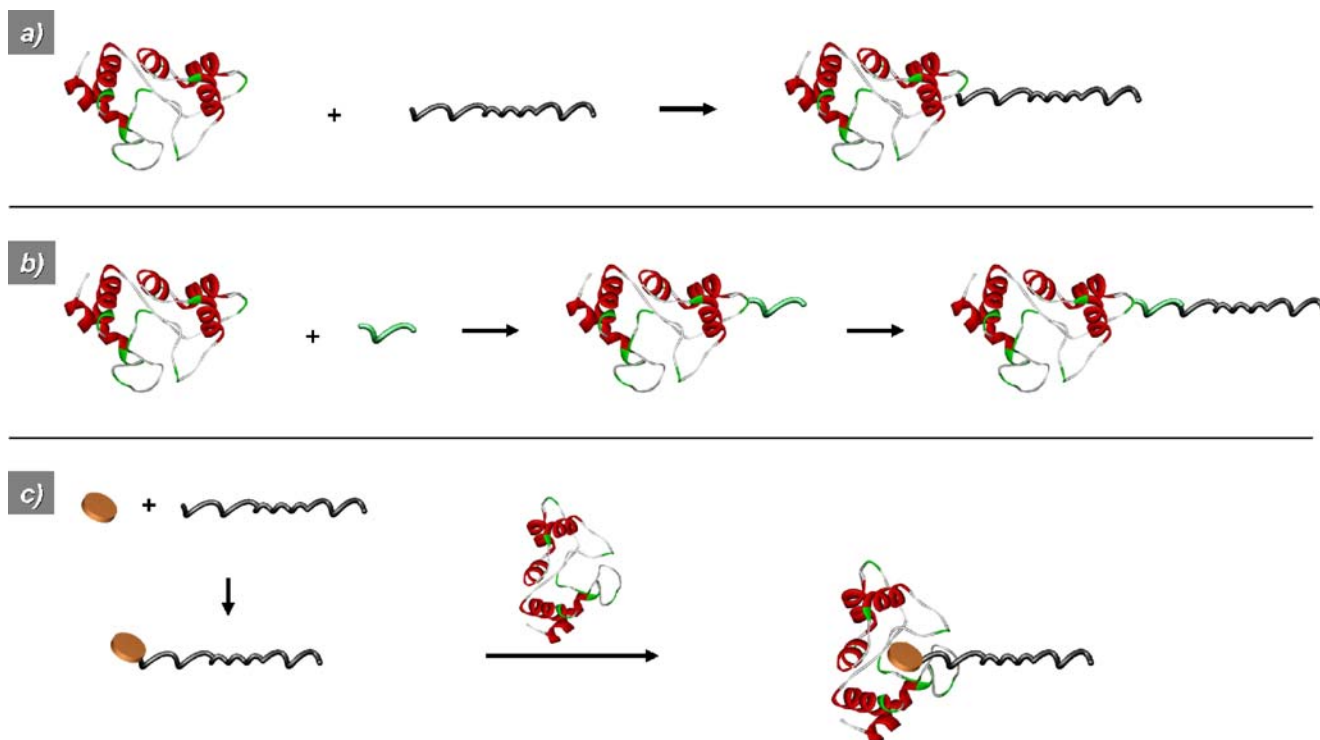


Fig. 1 Methods for specific protein functionalization: **a** direct functionalization, **b** indirect functionalization, and **c** functionalization via cofactors/ligands

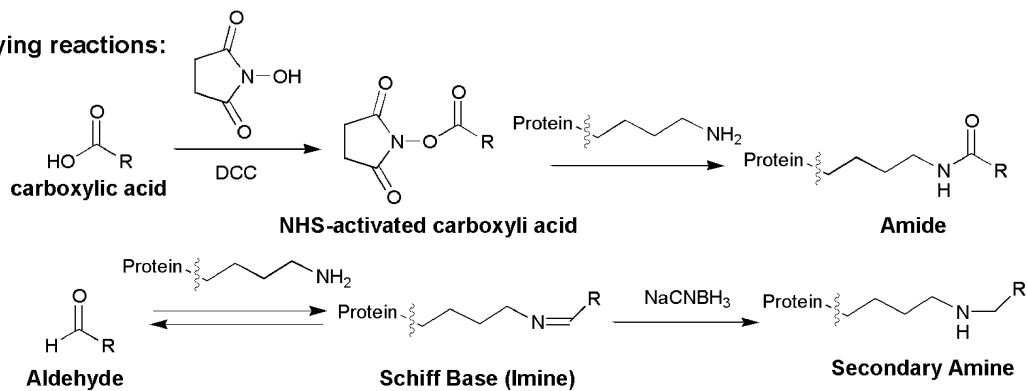
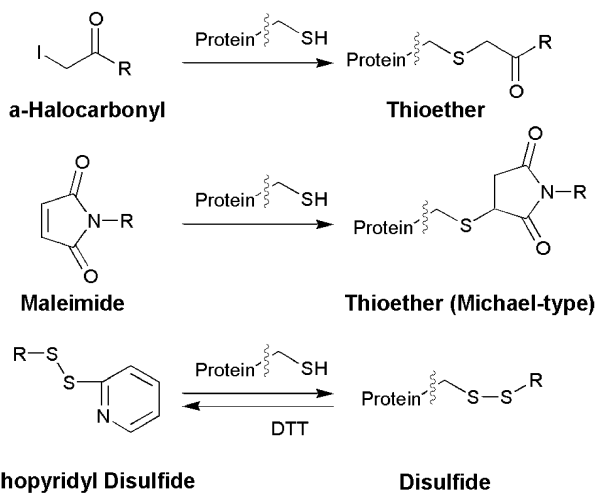
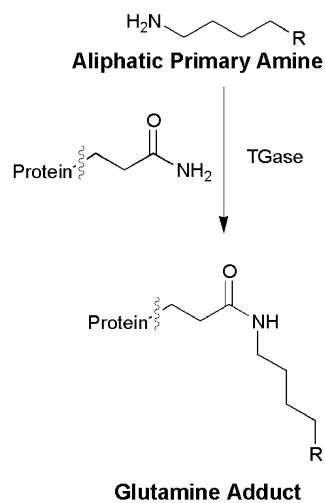
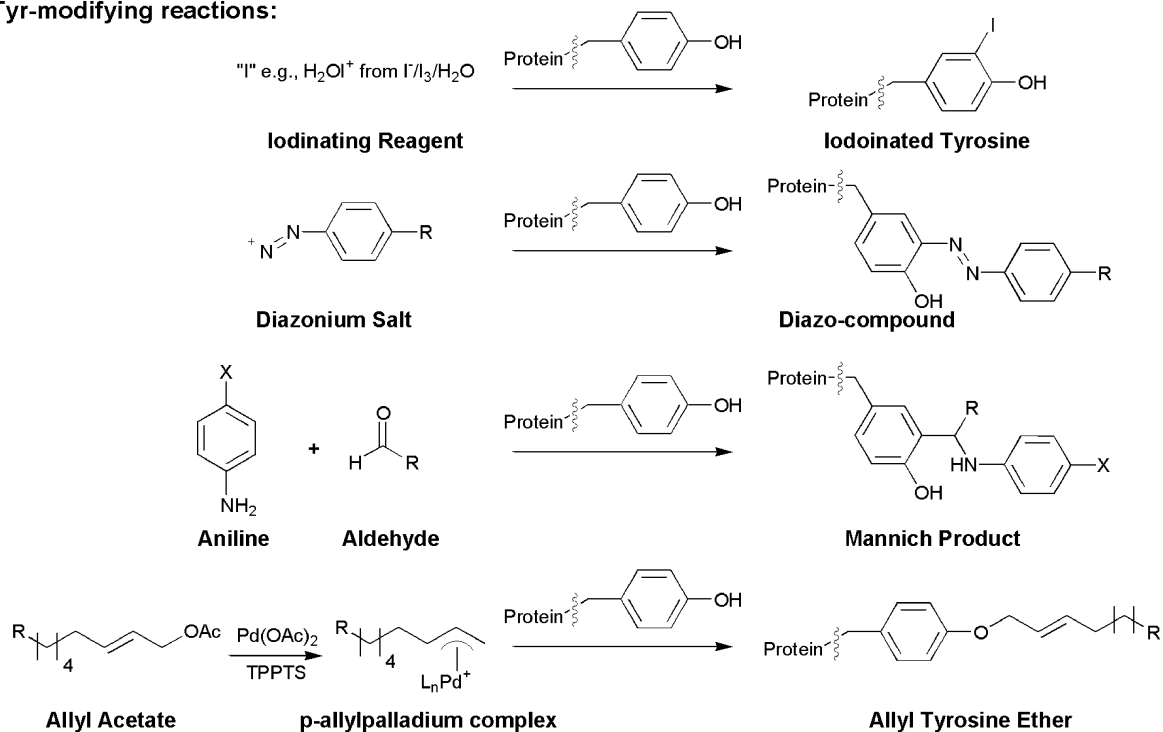
Lys and/or the α -N terminus with aldehydes results in the reversible formation of a Schiff base (imide), which can in turn be reduced with NaCNBH_3 to form a secondary amine linker. Numerous methods are available to activate carboxylic acids for the reaction with Lys and α -N terminus, among which *N*-hydroxysuccinimide (NHS) activation is usually the method of choice (Veronese and Pasut 2005). Both of these amine-modifying methods have advantages and disadvantages. Using NHS-activated carboxylic-acid-terminated polymers or spacers is straightforward, but the selectivity of the reaction is low. This problem is augmented by the fact that many proteins have several Lys on their surface, in addition to the often exposed α -N terminus. As the ϵ -amine group on Lys and α -N terminus have different $\text{p}K_{\text{a}}$, (about 10 and 7.8, respectively; Klok 2005), their difference in reactivity towards activated carboxylic acids could be utilized for the selective functionalization of the α -N-terminus. In practice, however, this is somewhat difficult (Wetzel et al. 1990; Gaertner and Offord 1996).

This difference in reactivity is more useful in reactions involving aldehyde-functionalized polymers and heterofunctional crosslinkers as only the α -N terminus will react with these at $\text{pH}=5$ (Kinstler et al. 1996; Veronese and Pasut 2005; Klok 2005). Another advantage of using this method is that it will maintain the overall charge of the protein, while the use of activated (NHS) carboxylic acids

results in the loss of one positive charge when the new amide bond is formed (Veronese and Pasut 2005). The main disadvantages of the aldehyde method are that it is a two-step procedure and that aldehyde synthesis is often even more challenging than that of carboxylic-acid-terminated polymers or heterofunctional spacers. Recently, a variation of this method has been developed using an iridium catalyst to carry out this reduction in situ and sodium formate as the hydride source (McFarland and Francis 2005).

The thiol side chain in cysteine (Cys) is a mild nucleophile—a fact that has been extensively utilized for the specific chemical modification of proteins. Under the appropriate conditions, Cys can be modified selectively, rapidly, and in a quantitative fashion. Another advantage of targeting the Cys residue is that it is rare in proteins while most of them contain only one accessible Cys. Broadly speaking, the Cys modification reactions can be divided into alkylation and disulfide formation. In both cases, the overall charge of the protein is maintained, adding to the advantages of targeting Cys.

Alkylation of Cys can be achieved using either α -halo (usually iodine) carbonyl compounds or maleimides. Partial deprotonation of the thiol in Cys at pH 9–10 allows the formation of thioethers using α -halo carbonyl compounds, while Cys can also be modified at neutral pH (6.8–7.0) using maleimide-functionalized groups as Michael acceptors (Hodgson and Sanderson 2004). Of the two methods,

Lys-modifying reactions:**Cys-modifying Reactions:****Gln-modifying Reactions:****Tyr-modifying reactions:**

◀ **Fig. 2** Methods for the direct specific protein functionalization on the natural amino acids side chains of cysteine (Cys), lysine (Lys), tyrosine (Tyr), and glutamine (Gln) together with the α -N terminus (α -N) of the peptide backbone

the latter prevails for the modification of proteins with polymers (Veronese and Pasut 2005) given the milder conditions, greater specificity (both can side-react with other amino acids, but maleimides will only react with amines at pH >8), but, first and foremost, the better reactivity of maleimide. This is quite important, as Cys is rather hydrophobic and is often found partially or fully buried within the protein structure.

Disulfide formation, especially with polymers and heterofunctional spacers terminated with the *o*-pyridyldisulfide group, is the most specific method for functionalization of Cys. It is also a fully reversible reaction through the use of standard reducing agents such as dithiothreitol (DTT), which can be regarded as an advantage or disadvantage depending on the application. DTT can also be used to expose more Cys in the protein by cleaving disulfide bridges, although the structural and functional integrity of the proteins needs to be checked carefully after this treatment, given the important role that disulfide bridges play in protein structures (Velonia et al. 2002; Heredia et al. 2005).

The third most important amino acid for specific protein functionalization is tyrosine (Tyr). The nucleophilic character of Tyr is greatly enhanced under mildly basic conditions (pH >8.5) that deprotonate the Tyr phenol side chain. The most commonly used methods for modifying Tyr include iodination and reactions with diazonium salts (Hermanson 1996). A number of alkylation and acylation reactions for modifying Tyr can also be carried out, but these have not gained momentum due to cross-reactivity towards Cys and/or Lys often combined with the need for quite harsh conditions. Significant advantages have been recently achieved in this area, mostly due to the work of Francis and co-workers (Joshi et al. 2004; Schlick et al. 2005; Tilley and Francis 2006). This group has developed several mild and highly selective methods for the modification of Tyr. One of these involves a three-component Mannich-type reaction between Tyr, an aliphatic aldehyde and an aniline derivative (Joshi et al. 2004). This reaction proceeds smoothly and at nearly neutral pH (6.5), provided a large ($\times 1,000$) excess of the synthetic aldehyde and aniline is used. Another method requires only a moderate excess ($\times 5$) of the synthetic partner when a palladium catalyst is used to couple a π -allyl species to Tyr under mildly basic conditions (Tilley and Francis 2006).

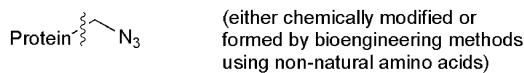
Glutamine (Gln) is also a unique type of target for selective functionalization of proteins, which proceeds using the enzyme transglutaminase (TGase) to mediate the

reaction. This enzyme catalyzes the cross-linking reaction between Gln and various primary amines, including the Lys side chain (Sato 2002). TGases are highly selective towards the target Lys and will only catalyze this reaction if the primary amine is highly exposed. This biosynthetic approach can be utilized to introduce PEG-polymers and other synthetic targets, provided they contain an exposed long aliphatic primary amine such as the aminobutane functionality.

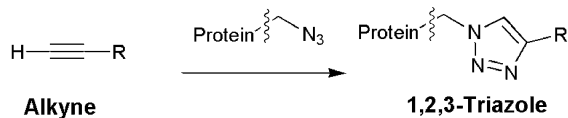
All the methods discussed above for the direct specific functionalization of proteins (Fig. 1a) can also be used for the indirect specific functionalization of proteins (Fig. 1b). It should be noted that “indirect” in this context refers to the target group (e.g., polymer) not reacting directly with the protein but rather being linked via a secondary non-natural functionality that is initially introduced to the protein structure by a specific functionalization. A simple example would include heterobifunctional spacers such as an NHS-activated carboxyl acid spacer terminated with a maleimide group. This spacer could be used to functionalize the ϵ -amine group of Lys or the α -N terminus of a protein and would be followed by a second reaction with a thiol-terminated target compound. When linking polymers to proteins, these simple heterofunctional groups are usually used to avoid the problems associated with lack of reactivity when two large molecules are brought together (the protein and the polymer). Heterofunctional spacers designed for protein cross-linking (e.g., the NHS-activated maleimide above) are often used to circumvent this problem; however, these have to be chosen with care to avoid intra- or intermolecular cross-linking in the protein of interest.

Recent heterofunctional spacers that bear functionalities such as azides and alkynes (in position 1), which do not react with natural amino acids, have also been used to couple a secondary target with a high degree of selectivity through *click-chemistry* techniques (Fig. 3; Kolb et al. 2001; Köhn and Breinbauer 2004). The latter include the aqueous version of the [3+2] Huisgen cycloaddition (Lewis et al. 2002) and the modified (non) traceless Staudinger ligation (Saxon et al. 2000; Kiick et al. 2002; Köhn and Breinbauer 2004). Methods that allow the expansion of the genetic code to include non-natural amino acids, including those bearing azides or alkynes in the expressed proteins (Budisa 2004; Wang and Schultz 2005), have allowed the use of *click chemistry* techniques for the subsequent direct functionalization of these genetically modified proteins. In another approach for indirectly modifying proteins, reported by Francis et al., Tyr was initially modified with a *p*-diazonium salt derived from *p*-aminoacetophenone. The ketone-modified bioconjugate was then converted to a range of bioconjugate oximes by reaction with a series of alkoxyamines, including polymeric oximes (Schlick et al.

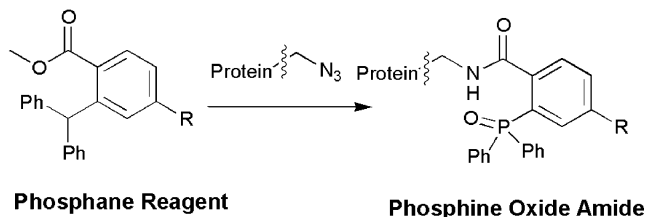
Protein "Click Chemistry" Reactions



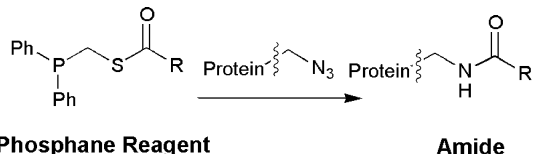
Huisgen:



Nontraceless Staudinger Ligation:



Traceless Staudinger Ligation:



Phosphane Reagent

Amide

Fig. 3 Click-chemistry direct specific protein functionalization approach

2005). Finally, proteins have also been specifically modified with an initiator suitable for atom transfer radical polymerization (ATRP), to create a protein-polymer conjugate, which was then used to achieve ATRP polymerization reactions (Heredia et al. 2005; Lele et al. 2005).

Protein cofactors and ligands (Fig. 4) such as the heme (porphyrin), FAD, NAD(P)⁺, and biotin (vitamin B₇) are also attractive targets for specifically modifying protein structures (Fig. 1c). The main advantage of modifying low-molecular weight cofactors and ligands is that this can be done using traditional synthetic and purification techniques, including organic solvents and silica gel chromatography. After purification, these modified cofactors and ligands are simply mixed with the apo-protein and the resulting reconstituted bioconjugate purified by dialysis or size-exclusion chromatography. This approach is efficient, provided the cofactor/ligand modification does not impede the reconstitution of protein or the binding to it. Biotin has proved to be the simplest and easiest ligand to work with, as its carboxylic acid terminus can be modified at will without having much effect on binding to the avidin family of proteins. The heme group can also be modified via its two propionic acid side chains, and modified hemes have been successfully introduced into myoglobin, hemoglobin, cytochrome *b*₅₆₂, and related proteins (Hamachi and Shinkai

1999). The adenine family of cofactors, including NAD(P)⁺ (Bückmann and Carrea 1989) and FAD (Bückmann et al. 1997), can be functionalized on the adenine ring, leaving intact the active nicotinamide or flavin part of these cofactors. Aminoethyl-appended NAD, for instance, has been successfully modified with both PEG and dextran without significant loss of its cofactor activity with yeast alcohol dehydrogenase, compared with that of the actual NAD (Bückmann et al. 1981).

Well-defined protein-polymer biohybrids

PEGylated proteins

Protein PEGylation is undeniably the most extensively studied area of specific polymer conjugation, as it has been

Co-factors and ligands used for protein modification

Arrows point to modification sites

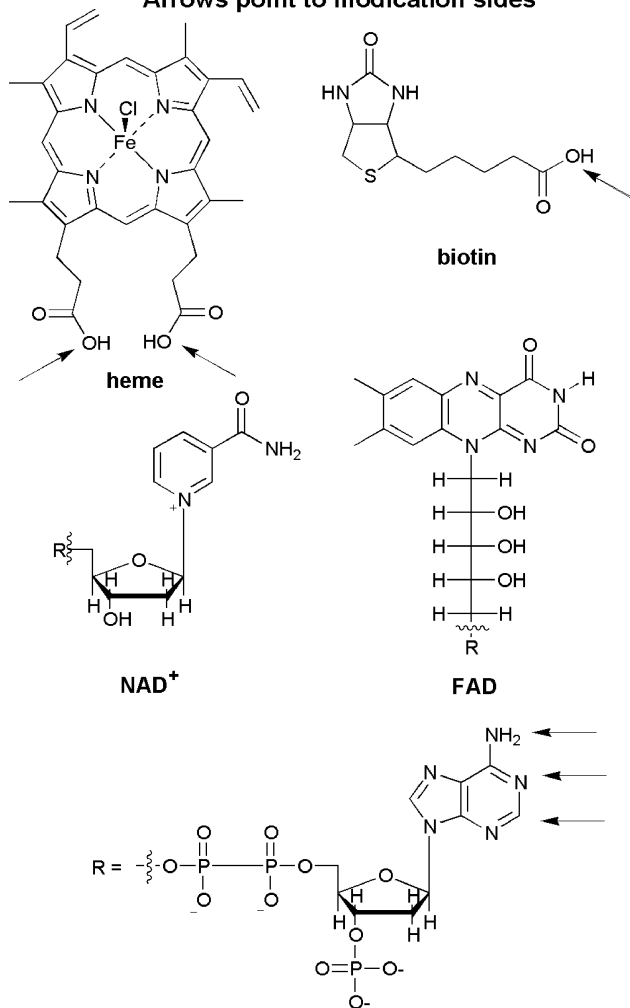


Fig. 4 Cofactors and ligands used for protein modification. The arrows point to common modification sites

proven to be one of the most straightforward procedures for enhancing the therapeutic and biotechnological potential of peptides and proteins (Hooftman et al. 1996; Kodera et al. 1998; Veronese 2001; Roberts et al. 2002; Caliceti and Veronese 2003; Veronese and Pasut 2005; Haag and Kratz 2006; Fee and Van Alstineb 2006). Many therapeutic proteins have been conjugated with poly(ethylene glycol) (PEG) to enhance their pharmacological properties both in vivo and in vitro, since its applicability was first demonstrated in 1977 (Abuchowski et al. 1977). The exhaustive list of methods and applications of protein PEGylation is beyond the scope of this review and already well documented elsewhere. With the established methodology and the numerous applications of PEGylated proteins such as PEG-Intron for Hepatitis C and the FDA filing of Pegasys (PEG-IFN-alpha 2a) and PEG-Neupogen (PEG-G-CSF) already in the market, the effect of mono- vs poly-substitution on the properties of the biohybrids is even more pronounced.

Several examples demonstrate the importance of mono-PEGylation in the properties of the resulting hybrids; perhaps the most pronounced of which is the PEG-Intron, which results from the monoPEGylation of an interferon by a 12-kDa PEG (Wang et al. 2002). In this case, the modification of the interferon with only one polymer chain was found to be responsible for its successful pharmacological action. It is also worth mentioning the example of mono-PEGylation of a lysine-deficient tumor necrosis factor (TNF), which was performed at the N terminus to avoid PEGylation of lysine residues close to the active site in the natural form (Yamamoto et al. 2003). Site-directed mutagenesis was exploited to control PEGylation and the resulting activity of the protein interleukin (Pettit et al. 1996) as well as to insert a free cysteine group into human Fv fragments and further couple the resulting free thiol with a maleimide-activated PEG (Yang et al. 2003). Finally, in a more exotic approach, Sato and coworkers used enzymatic catalysis with microbial transglutaminase for site-specific PEGylation of recombinant interleukin-2 (Sato 2002). There have also been reported cases, however, where the multi-functionalization of a protein was responsible for attaining the required action (Heathcote et al. 1999). The main benefit for the latter was that multi-PEGylation allowed the use of PEGs with lower molecular weights, which may be more rapidly cleared from the body.

Proteins specifically linked to stimuli-responsive polymers

The conjugation of proteins with stimuli responsive, or “smart” polymers, as named by R. Dagani (Dagani 1995) due to their ability to mimic the nonlinear response of biopolymers as a result of the cooperative interaction between monomers, has been thoroughly investigated by

several groups. These stimuli-responsive polymers undergo a reversible change in size and hydrophobicity in response to external stimuli such as temperature and/or pH. The coupling of *smart* polymers to various proteins has been performed both randomly (Cole et al. 1987; Nguyen and Luong 1989; Chen and Hoffman 1993; Galaev and Mattiasson 1993; Ding et al. 1998; Mantovani et al. 2005) and at specific sites of the protein structure (Ding et al. 1999; Bulmus et al. 2000).

Hoffman and his collaborators mainly employed the tetrameric protein streptavidin and genetically engineered mutants of this protein in their studies and specifically coupled it with a biotinylated PNIPAAm at temperatures below the lower critical solution temperature, LCST. Upon the formation of the biohybrid macromolecules, the authors demonstrated that a change in the physical properties of the polymer through small external changes in temperature or pH could be used to reversibly control biotin and biotinylated macromolecule access to, and/or release from, smart polymer–streptavidin bioconjugates (Stayton et al. 1995; Ding et al. 1999; Bulmus et al. 2000; Ding et al. 2001; Shimoboji et al. 2001). It was for the first time also demonstrated that *smart* polymers could act as molecular switches (gates) in an attempt to control protein activity. By using a site-specific poly(*N,N*-diethylacrylamide)/streptavidin conjugate, it was further shown that the blocking of biotinylated proteins was size-selective. Gating was found to be sensitive to the size of the protein, e.g., the immunoglobulin, IgG (150 kDa) was unable to bind below and above LCST, the protein G (6.2 kDa) was found to bind at all temperatures, but bovine serum albumin, BSA (67 kDa), could only bind at temperatures above LCST, where the polymer was collapsed. In summary, the authors proved that below the LCST, the polymer sterically interferes with the access to the adjacent binding site acting as a “polymer shield”, whereas above the LCST, polymer collapse exposes the adjacent site (Ding et al. 2001).

Extremely interesting behavior was observed by biohybrids resulting from the specific coupling of the temperature-responsive and photoresponsive DMA-co-4-phenylazophenyl acrylate (DMAA) and DMA-co-*N*-4-phenylazophenyl acrylamide (DMAAm), to an N55C mutant of the commercially important endoglucanase 12A (EG 12A) from *Trichoderma reesei* (Shimoboji et al. 2002). The above-mentioned copolymers display inverse phase transitions in response to UV and VIS light irradiation together with temperature-responsive LCST behavior. Interestingly, their LCSTs are shifted in opposite directions under UV vs VIS illumination. These opposite photo-induced phase transitions of DMAA and DMAAm were used to engineer molecular switches that turn “on” EG 12A as DMAA becomes hydrated and expands with VIS light, and “off” with UV light as the DMAA coil becomes

hydrophobic and collapses, with the opposite responses for DMAAm. The conjugates both displayed almost a complete lack of activity when the polymers were in their collapsed state, and when the UV or VIS light stimulated the rehydration and expansion of the polymer coil, the conjugates displayed approximately 60% of the activity of the unconjugated enzyme. Furthermore, the photoswitching was found to be reversible and could be cycled between the active *on*- and the inactive *off*-state and proved to be efficient both when the conjugate was free in solution or immobilized on magnetic beads. In such a system, the photo-responsive polymers serve jointly as antennae and actuators that reversibly respond to distinct optical signals to switch the polymer–enzyme conjugates *on* and *off*. The ability to photo-regulate enzyme activities provides exciting opportunities in the fields of bioprocessing, diagnostics, and microfluidics.

Giant amphiphiles

The innovative class of bio-surfactants consisting of a protein or an enzyme linked to a hydrophobic polymer, i.e., the *giant amphiphiles*, was extensively studied by Nolte and collaborators following different approaches (Fig. 5, Velonia et al. 2002; Boerakker et al. 2002; Hannink et al. 2001). Using the well-established streptavidin-biotin approach, monolayers of giant amphiphiles were constructed by the association of biotinylated polystyrene with streptavidin (Fig. 5c, Hannink et al. 2001). The manner in which these giant amphiphiles were synthesized left two of the streptavidin binding sites unoccupied. These sites were

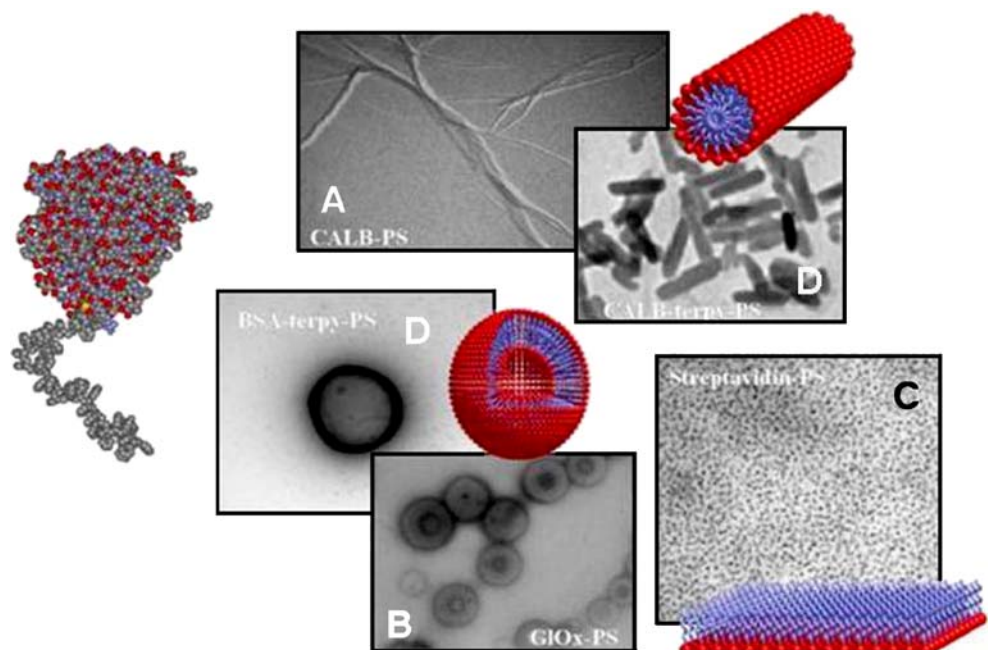
subsequently used to create functionalized amphiphiles by the binding of a biotinylated form of the iron storage protein ferritin or horseradish peroxidase.

Nolte and coworkers also reported on the synthesis and characterization of well-defined biohybrids through covalent coupling of a polymer directly to the enzyme (Velonia et al. 2002). To achieve the covalent coupling in a predefined position, a disulfide bridge exposed in the surface of the lipase B from *Candida antarctica* (CALB) was specifically reduced to provide two readily functionalizable thiol groups. A maleimide capped polystyrene was attached to the enzyme (in a THF/water mixture) to afford micrometer-long fibers that retained part of the catalytic activity of the enzyme head group (Fig. 5a). This was the first example reported in bibliography, of a *giant amphiphile* exhibiting aggregation behavior similar to that of the traditional amphiphiles and the block copolymers.

Responsive polymers, were also employed for the formation of *giant amphiphiles*. Uludag et al. have synthesized NIPAAm polymers that contain protein-reactive *N*-acryloxysuccinimide and LCST-altering, hydrophobic alkylmethacrylates to obtain thermo-responsive, protein-conjugating polymers. The thermo-sensitive polymers were capable of coupling with a co-injected therapeutic protein and retain it at an application site where tissue regeneration was required. Such bioconjugates might, therefore, be applied for drug delivery (Uludag et al. 2001).

An alternative approach to the synthesis of giant amphiphiles makes use of the cofactor/enzyme self-assembly found in Nature. In this case, the *giant amphiphile* construction was accomplished through the direct coupling

Fig. 5 Morphologies reported for giant amphiphiles: **a** CALB-polystyrene micellar rods (Reprinted with permission from Velonia et al. 2002; Copyright 2002 ACS Publications), **b** horseradish peroxidase polystyrene vesicles (Reprinted with permission from Boerraker et al. 2002; Copyright 2002 Wiley-VCH), **c** streptavidin monolayers, (Reprinted with permission from Hannink et al. 2001; Copyright 2002 Wiley-VCH), **d** BSA-polystyrene vesicles and CALB-polystyrene tubular structures



of a polymer to the cofactor of an enzyme followed by the subsequent reconstitution of the apoenzyme around the functionalized cofactor. This method was employed by Nolte and coworkers to create and study polystyrene horseradish peroxidase biohybrids (Boerakker et al. 2002). The desired biohybrid amphiphiles were prepared by adding a THF solution of the functionalized polymer to an aqueous solution of the apoenzyme. Electron microscopy (Fig. 5b) revealed the formation of vesicular aggregates with diameters of 80–400 nm. In most cases, these aggregates enclosed spherical objects, often located away from the center of the aggregates. To explain these structures, the authors assumed that the hemin-functionalized polymer initially forms aggregates on to which the apo-HRP can be reconstituted in the form of the biohybrid. More specifically, these structures arise from vesicles growing from the polystyrene aggregates in such a way that they enclose the initial aggregate. No activity could be observed when HRP was reconstituted at 4°C to form the bioassemblies. However, when the reconstitution of apo-HRP was carried out at 22°C, the enzyme–polymer hybrid surprisingly regained activity.

One newly reported category of biohybrid amphiphiles is the metal-to-ligand coordinated giant amphiphiles that combine the properties of a functional biocopolymer, with the versatility of a metallo-supramolecular copolymer (Fig. 5d, Velonia et al. 2003, 2004). In this modular approach the proteins/enzymes (BSA/CALB) were initially functionalized with a 2,2':6', 2"-terpyridine moiety to afford terpyridine functionalized biohybrids, which were characterized using MALDI. The formation of protein-polymer giant amphiphiles as asymmetric Ruthenium(II) functionalized metal complexes was in turn performed by adding a THF solution of mono-metallated-terpyridine-appended polystyrene. As in the case of previously reported biohybrids, these novel giant soaps were found to aggregate in manners similar to that of molecular amphiphiles. Interestingly, the superstructures formed by this modular approach significantly differed from those in the analogous amphiphiles formed by the direct coupling of the polymer to the protein surface. This new synthetic approach paved the way to the alternative pathway of constructing a plethora of biohybrids through the initial functionalized protein and coordination chemistry.

Furthermore, in very recent communications, Nolte and coworkers have reported that *click* chemistry, i.e., the Cu^(I) catalyzed [3+2] *Huisgen cycloaddition*, can also be used for the synthesis of a biohybrid BSA-PS giant amphiphile (Dirks et al. 2005) and a BSA-lipase biohybrid (Hatzakis et al. 2006).

Interestingly, in all the above-mentioned pilot studies, the *giant amphiphiles* were found to exhibit assembling properties analogous to those of synthetic copolymers and

molecular amphiphiles and to retain part of the catalytic activity of the enzyme head group. While the methodology for their synthesis and characterization has been developed, the influence of the composition of the different components to the overall architecture of the assemblies and to the catalytic properties of the head group remains to be studied.

Applications

Most of the applications arising from the creation of polymer–protein hybrids are found in pharmaceuticals and medicine and mainly concern PEGylated proteins. As mentioned above, PEGylated proteins usually display increased plasma half-life, due to the increase of both their stability and solubility caused by the polymer. The latter, termed as enhanced permeability and retention (EPR) effect, has already been used since the 1980s to create passive anticancer agents (Maeda et al. 1992, Maeda 2001a) and has been the theme of intense studies in the last decade leading to several novel PEGylated anticancer agents that are currently in the stage of clinical trials (Duncan 2003).

The stimuli responsive polymers have also already found interesting applications in the areas of protein isolation/separation. The extensively studied thermo-responsive protein conjugates are used in selectively precipitating/solubilizing proteins, a procedure that is used to facilitate protein purification or its easy recovery from a reaction mixture (Hoffman et al. 2000). In the category of the protein-responsive polymer biohybrids, one of the most exciting applications derives the controlled release of biotin from a poly(*N,N*-diethylacrylamide)/streptavidin conjugate leading to responsive biomolecular switches (Ding et al. 2001). Similar results were observed when the photo-responsive DMA-AZAAM copolymer collapsed and blocked substrate access upon UV irradiation. (Shimoboji et al. 2002).

PEGylation has also led to the synthesis of protein hybrids with interesting biotechnological applications through the increase of their solubility and stability in organic solvents (Kodera et al. 1998). Several PEGylated proteins and enzymes have been studied and found to be catalytically active in organic solvents such as benzene or chloroform (Takahashi et al. 1984) or possess an increased stability that is a prerequisite for the formation of bioreactors and biosensors (Ohno and Yamaguchi 1994).

Conclusions

Research into polymer–protein conjugation has come a long way since the first papers on PEGylated proteins in the 1970s (Abuchowski et al. 1977; Kodera et al. 1998). Early

works in this field focused on improving the pharmacological properties of proteins for their use in medicine. The unique material and catalytic properties of proteins and enzymes were also recognized very early in the fields of organic/bioorganic chemistry, where their modification was initially aimed at stabilization and improvement of their catalytic action, including their use in organic solvents. In these early attempts, the site and degree of polymer modification on the target protein was poorly controlled. Nowadays, to obtain an FDA approval, the composition of the synthetic protein–polymer hybrids must be clearly defined (Veronese and Pasut 2005), thus increasing the need for highly specific modifications, especially in the fields of medicine and pharmaceuticals.

During the last decades, the advances in molecular biology, biotechnology (protein expression, site-directed mutagenesis), polymer chemistry, physicochemical, bioimaging and characterization (especially mass spectrometry) techniques, have led to an impressive development of methods for the specific modification of proteins and the characterization of the resulting chimeric macromolecular structures. Several well-designed protein pharmaceuticals are already in the market, while rapid growth is also observed in the area of the development of polymer–protein conjugates with preprogrammed functions. This includes both “smart” polymer–protein conjugates and polymer–protein giant amphiphiles that are preprogrammed to self-assemble into well-defined nanostructures.

Nanoscale self-assembly and response to external stimuli are hallmarks of natural systems, and modifying proteins to achieve such properties has enormous potential. Nature provides a wide variety of proteins and enzymes possessing a continuum of material and functional properties. The programmed utilization of their chemical and topographical properties through specific modification will allow building novel nanometer- and micrometer-sized devices, as well as developing biomaterials that mimic various natural processes or that can be used as components for lab-on-a-chip applications (Niemeyer 2001; Seeman and Belcher 2002; Sarikaya et al. 2003; Zhang 2003; Velonia et al. 2005). These would include bio-mimetic sensors, environmentally responsive biomaterials for applications in microfluidics and tissue engineering together with bio-mimetic energy conversion, light-harvesting, signal transduction, and pollutant degradation. Clearly, we are still far from biotechnological building devices with the efficiency of Nature, but systematic fundamental and interdisciplinary research using polymer-modified proteins appears an obvious choice towards achieving these targets.

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References

- Abuchowski A, Van Es T, Palczuk NC, Davis FF (1977) Alteration of immunological properties of bovine serum albumin by covalent attachment of polyethylene glycol. *J Biol Chem* 252:3578–3581
- Boerakker MJ, Hannink JM, Bomans PHH, Frederik PM, Nolte RJM, Meijer EM, Sommerdijk NAJM (2002) Giant amphiphiles by cofactor reconstitution. *Angew Chem Int Ed Engl* 41:4239–4241
- Budisa N (2004) Prolegomena to future experimental efforts on genetic code engineering by expanding its amino acid repertoire. *Angew Chem Int Ed Engl* 43:6426–6463
- Bulmus V, Ding Z, Long CJ, Stayton PS, Hoffman AS (2000) Site-specific polymer–streptavidin bioconjugate for pH-controlled binding and triggered release of biotin. *Bioconjug Chem* 11:78–83
- Bückmann AF, Carrea G (1989) Synthesis and application of water-soluble macromolecular derivatives of the coenzymes NAD(H), NADP(H). *Adv Biochem Eng Biotechnol* 39:97–152
- Bückmann AF, Kula M-R, Wichmann R, Wandrey C (1981) An efficient synthesis of high-molecular-weight NAD(H) derivatives suitable for continuous operation with coenzyme-dependent enzyme systems. *J Appl Biochem* 3:301–315
- Bückmann AF, Wray V, Stocker A (1997) Synthesis of N^6 -(2-aminoethyl)-FAD, N^6 -(6-carboxyhexyl)-FAD, and related compounds. *Methods Enzymol* 280:360–374
- Caliceti P, Veronese FM (2003) Pharmacokinetic and biodistribution properties of poly(ethylene glycol)-protein conjugates. *Adv Drug Deliv Rev* 55:1261–1277
- Chen G, Hoffman AS (1993) Preparation and properties of thermoreversible, phase-separating enzyme-oligo(*N*-isopropylacrylamide) conjugates. *Bioconjug Chem* 4:509–514
- Cole C-A, Schreiner SM, Priest JH, Monji N, Hoffman AS (1987) *N*-isopropylacrylamide and *N*-acryloxysuccinimide copolymer—a thermally reversible, water-soluble, activated polymer for protein conjugation. *ACS Symp Ser* 350:245–254
- Dagani R (1995) Polymeric smart materials respond to changes in their environment. *Chem Eng News* 73(38):30–33
- DeSantis G, Jones JB (1999) Chemical modification of enzymes for enhanced functionality. *Curr Opin Biotechnol* 10:324–330
- Ding Z, Chen G, Hoffman AS (1998) Unusual properties of thermally sensitive oligomer-enzyme conjugates of poly(*N*-isopropylacrylamide)-trypsin. *J Biomed Mater Res* 39:498–505
- Ding Z, Long CJ, Hayashi Y, Bulmus EV, Hoffman AS, Stayton PS (1999) Thermoprecipitation of streptavidin via oligonucleotide-mediated self-assembly with poly(*N*-isopropylacrylamide). *Bioconjug Chem* 10:395–400
- Ding Z, Fong RB, Long CJ, Stayton PS, Hoffman AS (2001) Size-dependent control of the binding of biotinylated proteins to streptavidin using a polymer shield. *Nature* 411:59–62
- Dirks AJ, van Berkel SS, Hatzakis NS, Opsteen JA, van Delft FL, Cornelissen JJLM, Rowan AE, van Hest JCM, Rutjes FPJT, Nolte RJM (2005) Preparation of biohybrid amphiphiles via the copper catalysed Huisgen [3 + 2] dipolar cycloaddition reaction. *Chem Commun (Camb)* 33:4172–4174
- Duncan R (2003) The dawning era of polymer therapeutics. *Nat Rev Drug Discov* 2:347–360
- Fee CJ, Van Alstineb JM (2006) PEG-proteins: reaction engineering and separation issues. *Chem Eng Sci* 61:924–939
- Gaertner HF, Offord RE (1996) Site-specific attachment of functionalized poly(ethylene glycol) to the amino terminus of proteins. *Bioconjug Chem* 7:38–44
- Galaev IY, Mattiasson B (1993) Affinity thermoprecipitation: contribution of the efficiency of ligand–protein interaction and access of the ligand. *Biotechnol Bioeng* 41:1101–1106
- Haag R, Kratz F (2006) Polymer therapeutics: concepts and applications. *Angew Chem Int Ed Engl* 45:1198–1215

- Hamachi I, Shinkai S (1999) Chemical modification of the structures and functions of proteins by the cofactor reconstitution method. *Eur J Org Chem* 539–549
- Hannink JM, Cornelissen JJLM, Farrera JA, Foubert P, De Schryver FC, Sommerdijk NAJM, Nolte RJM (2001) Protein–polymer hybrid amphiphiles. *Angew Chem Int Ed Engl* 40:4732–4734
- Hatzakis NS, Engelkamp H, Velonia K, Hofkens J, Christianen PCM, Svendsen A, Patkar SA, Vind J, Maan JC, Rowan AE, Nolte RJM (2006) Synthesis and single enzyme activity of a clicked lipase-BSA hetero-dimer. *Chem Commun (Camb)* 19:2012–2014
- Heathcote EJ, Pockros PJ, Fried MW, Bain MA, DePamphilis J, Modi M (1999) The pharmacokinetics of PEGylated-40 k interferon alfa-2a (PEG-IFN) in chronic hepatitis C (CHC) patients with cirrhosis. *Gastroenterology* 116:A735
- Heredia KL, Bontempo D, Ly T, Byers, JT, Halstenberg S, Maynard HD (2005) In situ preparation of protein-“smart” polymer conjugates with retention of bioactivity. *J Am Chem Soc* 127:16955–16960
- Hermanson GT (1996) *Bioconjugate techniques*. Academic, San Diego, CA
- Hodgson DRW, Sanderson JM (2004) The synthesis of peptides and proteins containing non-natural amino acids. *Chem Soc Rev* 33:422–430
- Hoffman AS, Stayton PS, Bulmus V, Chen G, Chen J, Cheung C, Chilkoti A, Ding Z, Dong L, Fong R, Lackey C A, Long CJ, Miura M, Morris JE, Murthy N, Nabeshima Y, Park TG, Press OW, Shimoboji T, Shoemaker S, Yang HJ, Monji N, Nowinski RC, Cole CA, Priest JH, Harris MJ, Nakamae K, Nishino T, Miyata T (2000) Really smart bioconjugates of smart polymers and receptor proteins. *J Biomed Mater Res* 52:577–586
- Hooftman G, Herman S, Schacht E (1996) Poly(ethylene glycol)s with reactive endgroups. II. Practical consideration for the preparation of protein-PEG conjugates. *J Bioact Compat Polym* 11:135–159
- Joshi NS, Whitake LR, Francis MB (2004) A three-component Mannich-type reaction for selective tyrosine bioconjugation. *J Am Chem Soc* 126:15942–15943
- Kiick KL, Saxon E, Tirrell DA, Bertozzi CR (2002) Incorporation of azides into recombinant proteins for chemoselective modification by the Staudinger ligation. *Proc Natl Acad Sci U S A* 99:19–24
- Kinstler OB, Brems DN, Lauren SL, Paige AG, Hamburger JB, Treuheit MJ (1996) Characterization and stability of *N*-terminal PEGylated rhG-CSF. *Pharm Res* 13:996–1002
- Klok H-A (2005) Biological-synthetic hybrid block copolymers: combining the best from two worlds. *J Polym Sci A Polym Chem* 43:1–17
- Kodera Y, Matsushima A, Hiroto M, Nishimura H, Ishii A, Ueno T, Inada Y (1998) PEGylation of proteins and bioactive substances for medical and technical applications. *Prog Polym Sci* 23:1233–1271
- Köhn M, Breinbauer R (2004) The Staudinger ligation—a gift to chemical biology. *Angew Chem Int Ed Engl* 43:3106–3116
- Kolb HC, Finn MG, Sharpless KB (2001) Click chemistry: diverse chemical function from a few good reactions. *Angew Chem Int Ed Engl* 40:2004–2021
- Lele BS, Murata H, Matyjaszewski K, Russell AJ (2005) Synthesis of uniform protein–polymer conjugates. *Biomacromolecules* 6:3380–3387
- Lewis WG, Greem LG, Grynszpan F, Radia Z, Carlier PR, Taylor P, Finn MG, Sharpless KB (2002) Click chemistry in situ: acetylcholinesterase as a reaction vessel for the selective assembly of a femtomolar inhibitor from an array of building blocks. *Angew Chem Int Ed Engl* 41:1053–1057
- Maeda H (2001a) The enhanced permeability and retention (EPR) effect in tumor vasculature: the key role of tumor-selective macromolecular drug targeting. *Adv Enzyme Regul* 41:189–207
- Maeda H (2001b) SMANCS and polymer-conjugated macromolecular drugs: advantages in cancer chemotherapy. *Adv Drug Deliv Rev* 46:169–185
- Maeda H, Seymour L, Miyamoto Y (1992) Conjugation of anticancer agents and polymers: advantages of macromolecular therapeutics in vivo. *Bioconjug Chem* 3:351–362
- Mantovani G, Lecolley F, Tao L, Haddleton DM, Clerx J, Cornelissen JJLM, Velonia K (2005) Design and synthesis of *N*-maleimido-functionalized hydrophilic polymers via copper-mediated living radical polymerization: a suitable alternative to PEGylation chemistry. *J Am Chem Soc* 127:2966–2973
- Matthews KS, Chakerian AE, Gardner JA (1991) Protein chemical modification as probe of structure-function relationships. *Methods Enzymol* 208:468–496
- McFarland JM, Francis MB (2005) Reductive alkylation of proteins using Iridium catalyzed transfer hydrogenation. *J Am Chem Soc* 127:13490–13491
- Meredith GD, Wu HY, Allbritton NL (2004) Targeted protein functionalization using His-tags. *Bioconjug Chem* 15:969–982
- Nguyen AL, Luong JHT (1989) Synthesis and applications of water-soluble reactive polymers for purification and immobilization of biomolecules. *Biotechnol Bioeng* 34:1186–1190
- Niemeyer CM (2001) Nanoparticles, proteins, and nucleic acids: biotechnology meets materials science. *Angew Chem Int Ed Engl* 40:4128–4158
- Ohno H, Yamaguchi N (1994) Redox reaction of poly(ethylene oxide)-modified hemoglobin in poly(ethylene oxide) oligomers at 120 degrees C. *Bioconjug Chem* 5:379–381
- Omelyanenko V, Kopeckova P, Gentry C, Shiah JG, Kopecek J (1996) HPMA copolymer-anticancer drug-OV-TL16 antibody conjugates. 1. Influence of the method of synthesis on the binding affinity to ovcar-3 ovarian carcinoma cells in vitro. *J Drug Target* 3:357–373
- Pennadam SS, Firman K, Alexander C, Górecki DC (2004) Protein–polymer nano-machines. Towards synthetic control of biological processes. *J Nanobiotechnology* 2:8
- Pettit DK, Bonnert TP, Eisenman J, Srinivasan S, Paxton R, Beers C, Lynch D, Miller B, Yost J, Grabstein KH, Gombotz WR (1996) Structure-function studies of interleukin 15 using site-specific mutagenesis, polyethylene glycol conjugation and homology modelling. *J Biol Chem* 272:2312–2318
- Qi D, Tann C-M, Haring D, Distefano MD (2001) Generation of new enzymes via covalent modification of existing proteins. *Chem Rev* 101:3081–3111
- Roberts MJ, Bentley MD, Harris JM (2002) Chemistry for peptide and protein PEGylation. *Adv Drug Deliv Rev* 54:459–476
- Sarikaya M, Tamerler C, Jen AK-Y, Schulten K, Babeyx F (2003) Molecular biomimetics: nanotechnology through biology. *Nat Mater* 2:577–585
- Sato H (2002) Enzymatic procedure for site-specific pegylation of proteins. *Adv Drug Deliv Rev* 54:487–504
- Saxon E, Armstrong JI, Bertozzi CR (2000) A “traceless” Staudinger ligation for the chemoselective synthesis of amide bonds. *Org Lett* 2:2141–2143
- Schlick TF, Ding Z, Kovacs EW, Francis MB (2005) Dual-surface modification of the tobacco mosaic virus. *J Am Chem Soc* 127:3718–3723
- Seeman NC, Belcher AM (2002) Emulating biology: building nanostructures from the bottom up. *Proc Natl Acad Sci U S A* 99:6451–6455
- Shimoboji T, Ding Z, Stayton PS, Hoffman AS (2001) Mechanistic investigation of smart polymer–protein conjugates. *Bioconjug Chem* 12:314–319
- Shimoboji T, Larenas E, Fowler T, Kulkarni S, Hoffman AS, Stayton PS (2002) Photoresponsive polymer-enzyme switches. *Proc Natl Acad Sci U S A* 99:16592–16596

- Stayton PS, Shimoboji T, Long C, Chilkoti A, Chen G, Harris JM, Hoffman AS (1995) Control of protein-ligand recognition using a stimuli-responsive polymer. *Nature* 378:472–474
- Takahashi K, Nishimura H, Yoshimoto T, Saito Y, Inada Y (1984) A chemical modification to make horseradish peroxidase soluble and active in benzene. *Biochem Biophys Res Comm* 121:261–265
- Tilley SD, Francis MB (2006) Tyrosine-selective protein alkylation using π -allylpalladium complexes. *J Am Chem Soc* 128:1080–1081
- Ulbrich K, Strohalm J, Subr V, Plocova D, Duncan R, Rihova B (1996) Polymeric conjugates of drugs and antibodies for site-specific drug delivery. *Macromol Symp* 103:177–192
- Uludag H, Norrie B, Kousinioris N, Gao T (2001) Engineering temperature-sensitive poly(*N*-isopropylacrylamide) polymers as carriers of therapeutic proteins. *Biotechnol Bioeng* 73:510–521
- van Hest JCM, Tirrell DA (2001) Protein-based materials, toward a new level of structural control. *Chem Commun (Camb)* (19):1897–1904
- Velonia K, Rowan AE, Nolte RJM (2002) Lipase polystyrene giant amphiphiles. *J Am Chem Soc* 124:4224–4225
- Velonia K, Thordarson P, Andres PR, Schubert US, Rowan AE, Nolte RJM (2003) Polymer-protein giant amphiphiles by metal-to-ligand coordination. *Polymer Preprints* 44:648
- Velonia K, Thordarson P, Schubert US, Rowan AE, Nolte RJM (2004) Bio-hybrid giant amphiphiles by metal-to-ligand coordination. *Polymer Preprints* 45:401–402
- Velonia K, Cornelissen JJLM, Feiters MC, Rowan AE, Nolte RJM (2005) Aggregation of amphiphiles as a tool to create novel functional nano-objects. In: Huck WTS (ed) *Nanostructure assemblies*. Academic, New York
- Veronese FM (2001) Peptide and protein PEGylation: a review of problems and solutions. *Biomaterials* 22:405–417
- Veronese FM, Pasut G (2005) PEGylation, successful approach to drug delivery. *Drug Discov Today* 10:1451–1458
- Vriezema DM, Aragonès MC, Elemans JAAW, Cornelissen JJLM, Rowan AE, Nolte RJM (2005) Self-assembled nanoreactors. *Chem Rev* 105:1445–1489
- Wang L, Schultz PG (2005) Expanding the genetic code. *Angew Chem Int Ed Engl* 44:34–66
- Wang Y-S, Youngster S, Grace M, Bausch J, Bordens R, Wyss DF (2002) Structural and biological characterization of PEGylated recombinant interferon alpha-2b and its therapeutic implications. *Adv Drug Deliv Rev* 54:547–570
- Wetzel R, Halualani R, Stults JT, Quan C (1990) A general method for highly selective cross-linking of unprotected polypeptides via pH-controlled modification of N-terminal α -amino groups. *Bioconjug Chem* 1:114–122
- Yamamoto Y, Tsutsumi Y, Yoshioka Y, Nishibata T, Kobayashi K, Okamoto T, Mukai Y, Shimizu T, Nakagawa S, Nagata S, Mayumi T (2003) Site-specific PEGylation of a lysine-deficient TNF-alpha with full bioactivity. *Nat Biotechnol* 21:546–552
- Yang K, Basu A, Wang M, Chintala R, Hsieh M-C, Liu S, Hua J, Zhang Z, Zhou J, Li M, Phyu H, Petti G, Mendez M, Janjua H, Peng P, Longley C, Borowski V, Mehlig M, Filpula D (2003) Tailoring structure-function and pharmacokinetic properties of single-chain Fv proteins by site-specific PEGylation. *Protein Eng* 16:761–770
- Youngster S, Wang YS, Grace M, Bausch J, Bordens R, Wyss DF (2002) Structure, biology, and therapeutic implications of pegylated Interferon alpha-2b. *Curr Pharm Des* 8:2139–2157
- Zhang S (2003) Fabrication of novel biomaterials through molecular self-assembly. *Nat Biotechnol* 21:1171–1178