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Bioconjugation Using Thiols: Old Chemistry Rediscovered to Connect Polymers with Nature's Building Blocks

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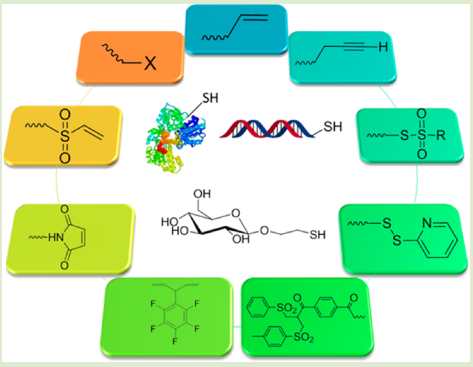
ABSTRACT: Various pathways to bioconjugates based on thiol chemistry are discussed. Thiol-halogeno, thiol-parafluoro, thiol-ene, thiol-yne, thiol-vinylsulfone and thiol−vinyl sulfone, thiol−maleimide, thiol−bisulfone, and thiol−pyridyl disulfide are well-established synthetic routes discovered in recent years as tools to marry polymers with biomolecules such as carbohydrates, proteins, peptide, DNA, antibodies, or other building blocks from nature.

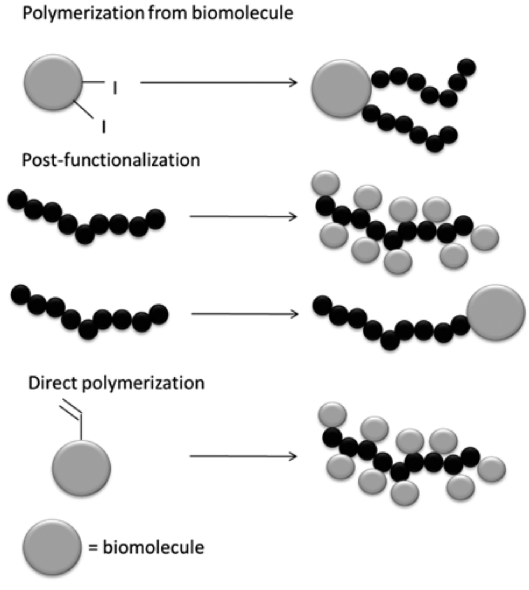


Bioconjugates, the marriage of synthetic polymers with nature's building blocks such as carbohydrates and proteins, have been the center of attention for many years now. The underpinning idea is to combine the properties of both worlds to create new advanced materials. Polymers are well-known for their versatility. Polymers can be water- or oil-soluble, stimuli-responsive, brittle, elastomeric, conductive, and much more. However, missing features are the precise structure known to many biopolymers and the ability to be biologically active, often with high selectivity. Nature's building blocks such as proteins, polynucleotides, polysaccharides, or smaller naturally occurring building blocks such as amino acids and sugars are now increasingly conjugated to polymers to create materials with unique properties, combining the best of both worlds.

The types of bioconjugate that draw the most attention are combinations of synthetic polymers with carbohydrates (glycopolymers)[1] or polysaccharides[2] and the union of polymers with peptides[3] or proteins.[4] Cautious attempts have been made to conjugate polymers to antibodies or DNA.[5] Theoretically, there are several pathways to achieving this aim including the immobilization of the initiator or the controlling agent onto the biomolecule, a route which is commonly used for polysaccharides[2] and proteins.[6] Alternatively, functional polymers are postmodified with the desired biomolecules. Almost unique to glycopolymers is the functionalization of the underlying carbohydrate with a polymerizable group, viz., vinyl groups, and direct polymerization (Scheme 1).

Postfunctionalization as a pathway to polymer bioconjugates has emerged as the main research activity in recent years and is dominated by the realm of click chemistries. The Cu(I)-catalyzed alkyne−azide Huisgen 1,3-dipolar cycloaddition was for many years the method of choice for efficient conjugation. Occasional problems with Cu(I) catalyst removal shifted the attention from this reaction to thiol-based reactions. The good nucleophilicity and the ability of thiols to take part in radical reactions make them an interesting choice as a reactive group. The types of reactions reported range from thiol−halogeno, thiol−parafluoro, thiol−ene, thiol−yne, thiol−vinylsulfone, thiol−vinyl sulfone, thiol−maleimide, thiol−bisulfone to thiol−pyridyl disulfide (Scheme 2). Some of these reactions

Scheme 1. Potential Pathways to Bioconjugates



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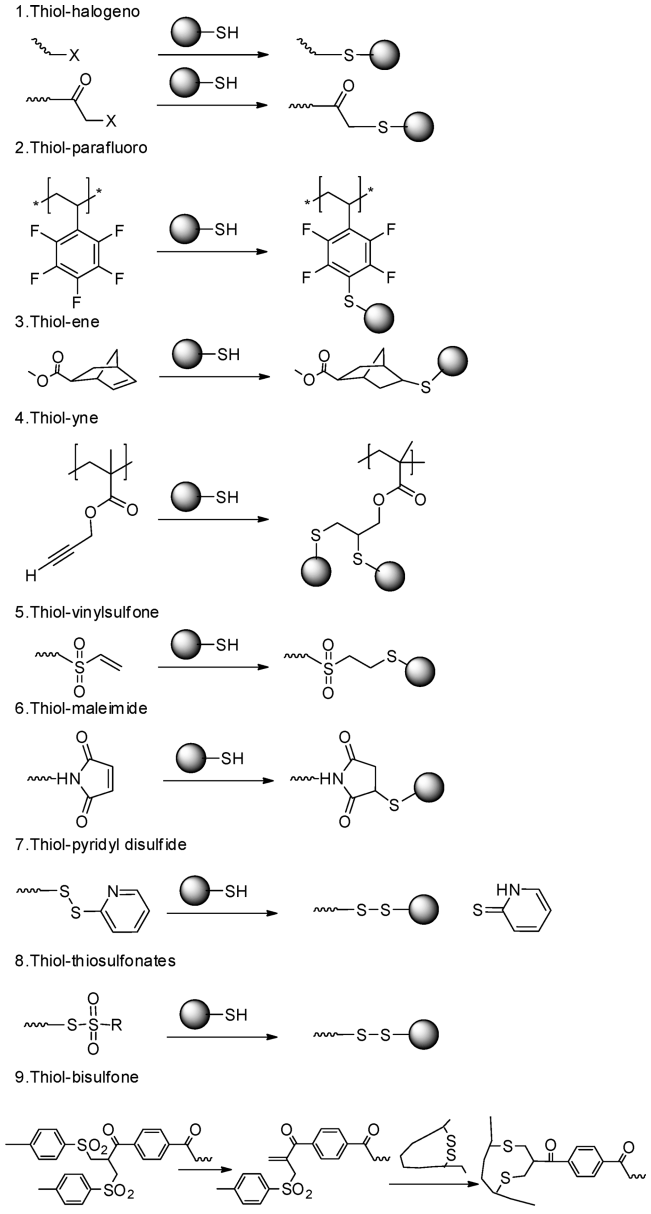
Scheme 2. Types of Thiol-Based Reactions



were coined *click* reactions in the literature, although the term *click* is only applicable to a few of them. The reader is referred to some excellent reviews on this topic.[7−9]

In the following, we are visiting the various types of reactions and discussing their ability to efficiently create bioconjugates. One focus will be on the synthesis of glycopolymers.[1] The aim of the attachment of many copies of sugar on a polymer is to display a multivalent effect where a collection of sugars binds disproportionally more to lectins than a single carbohydrate alone. Furthermore, the conjugation of polymers onto peptides or proteins deserves special attention. The natural abundance of the thiol-based amino acid cysteine leads unsurprisingly to the use of thiol-based chemistry circumventing the use of further modification of the biomolecule. The purpose of bioconjugation of polymers on the proteins and peptides is usually not to display a multivalent effect, but to provide protection,[4,6] increase water solubility, or create new materials based on the self-assembling power of peptides.[3]

Whatever the purpose, the polymer chemist can choose from a range of available reactions. In the following, the various types of thiol-based chemistries and their suitability for the preparation of bioconjugates are discussed.

First we discuss the thio−halogeno reaction (Scheme 2.1). The simple nucleophilic substitution of halogenides and thiols has been already discovered in the 1990s as a robust way to prepare glycopolymers. This pathway is often limited by the choice of polymerization techniques since halogenides can potentially act as chain transfer agents or interfere with initiators or catalysts. This requires the implementation of a two-step process where a functional polymer is modified, followed by the nucleophilic substitution with thiols.[10] Postmodification was often carried out to introduce α-halocarbonyls, which are more reactive than alkyl halides. A small selection of polymerization techniques such as Sonogashira[11] and Suzuki coupling[12] reactions as well as reversible addition−fragmentation chain transfer (RAFT) polymerization are suitable to create halogenated polymers directly.[13] Various thiolated sugars were then reacted with the polymer in the presence of triethyl amine (NEt_3), K_2CO_3, or 1,8-diazabicyclo[5.4.0]-undec-7-ene (DBU). In most cases, an excess of thiols was required to achieve full functionalization. Although equimolar ratios are possible, the reaction succumbs to a lengthy reaction time.[13] However, due to the robustness of this approach and the formation of a simple thioether bond, it is frequently applied to prepare glycopolymers.

This pathway was also employed to generate polymer−peptide conjugates,[14] but it is significantly less commonly used than in the synthesis of glycopolymers. To my knowledge, proteins have not yet been conjugated with polymers via this pathway, which is surprising considering that the functionalization of proteins with α-halocarbonyls is well-established.[15] The more reactive α-halocarbonyls compared to the linear alkyl halides may offer new opportunities ready to be explored.

Next we discuss the thiol−parafluoro reaction (Scheme 2.2). Pentafluorostyrenes can be polymerized using a wide range of techniques. The advantages of this route are not only easy access to various polymer architectures but also easy monitoring of reactions with thiols using ^{19}F NMR spectroscopy. Compared to the thiol−halogeno reaction, this approach seems to be faster, and full conversion was achieved at 40 °C within a few hours using approximately equimolar amounts of 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranose. The downside however is that an excess of NEt_3 is still necessary to yield complete conversion.[16] This efficient reaction has yet to be tested using peptides or proteins. One aspect that may deter the polymer chemist from using this approach is the presence of fluorine that might influence the materials' properties, although this can also potentially create interesting materials.

Thiol-based reactions are probably dominated by thiol−ene chemistry, especially in the synthesis of glycopolymers (Scheme 2.3). The most common pathway in creating glycopolymers with postfunctionalization is the reaction between thiols and pendant double bonds. Prerequisite is the synthesis of polymers with pendant double bonds, which limits the approach to polymerization techniques that are orthogonal to the presence of double bonds. Glycoconjugation using thiol−ene reactions to create low molecular weight compounds has been a well-established technique for many years,[17] but it took several years before polymer science discovered the simplicity of this pathway. Synthetic routes to generating reactive polymer backbones are limited since the direct radical polymerization

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of vinyl monomers with an additional pendant vinyl group is prone to cross-linking. Careful fine-tuning of the reactivity ratios of both vinyl functionalities to avoid cross-linking, and postfunctionalization of polymers with vinyl groups[18,19] are potential solutions that have been offered in literature. Cationic ring-opening polymerization,[20−22] living anionic ring-opening polymerization[23] and polyaddition,[24] in contrast, are techniques that provide direct access of polymers with vinyl functionalities for conjugation with carbohydrates[25] or peptides.[21]

Thiol−ene reactions are commonly initiated using a radical source, but depending on the structure of the vinyl functionality Michael addition reactions[21] or enzymatic synthesis[26] can offer an alternative pathway. However, most reports on bioconjugation focus on the radical approach. A small excess of the thiol such as 2,3,4,6-tetra-*O*-acetyl-1-thio-*β*-D-glucopyranose[25] or cysteine containing peptides[19,27] is usually irradiated with UV−vis light in a degassed solution for several hours.[20] To accelerate the reaction, photoinitiators such as 2,2-dimethoxy-2-phenylacetophenone (DMPA) can be added reducing the reaction time from a day to a few hours.[18,20] Alternatively, AIBN at elevated temperatures can act as the radical source.[19,23,27,28] Whatever the initiator, the product obtained is usually the anti-Markovnikov addition product although a small fraction of the Markovnikov product can occasionally be observed.[23]

Conjugation with thiolated sugars can be driven to completion and therefore satisfy most click criteria. However, more often than not, an excess of thiols had to be employed to reach full functionalization.[29] It seems to be occasionally more difficult to achieve the same with peptides. Conjugation efficiencies as low as 50% have been reported with peptides, which has been assigned to steric hindrance.[19,21,27]

Although the thiol−ene reaction has often been coined as click chemistry, side products such as dimers and disulfides were observed during the reaction.[27] In addition, long reaction times under the influence of UV−vis light limit this reaction to biomolecules that are stable under these conditions. Though, carefully fine-tuned reaction conditions allow the orthogonal thiol−ene photocoupling of peptides in a enzymatically degradable hydrogel matrix. Specific peptides could be placed in complex 3D structures by systematically scanning the focal point of a pulsed near-infrared laser.[30] While most thiol−ene reactions were carried out using linear vinyl groups, the recent body of work by Anseth and co-workers involved the use of polymers with pendant bicyclo[2.2.1]hept-2-ene.[31] The photoinitiated reaction is complete within a few minutes with a high fidelity in the presence of living cells.

Thiol−ene chemistry is making now a timid appearance in the synthesis of polymer−protein[32,33] and polymer−siRNA[34] conjugates, although other techniques are still favored when designing such bioconjugates. Although most approaches utilize the thiol functionality on the protein, vinyl groups can be generated on the polymer by the use of *O*-mesitylsulfonylhydroxylamine (MSH) resulting in proteins that are reactive toward thiols.[35]

The advantage of the thiol−yne reaction is the easy access of the reactive polymer (Scheme 2.4). In contrast to the thiol−ene reactions, which require the synthesis of polymers with vinyl pendant groups either by postfunctionalization or by careful adjustment of the reactivity of the two vinyl groups, the thiol−yne approach can utilize monomers such as 3-trimethylsilanyl-prop-2-ynyl(meth)acrylate, which can be easily polymerized in a controlled manner and deprotected using tetra-*n*-butylammonium fluoride (TBAF). Thiol−yne chemistry has however rarely been explored for bioconjugation, and only a few reports describing the synthesis of glycopolymers are known.[14,36] The reaction of two thiols with one alkyne group makes them a popular tool to design polymers with dendritic carbohydrate functionalities,[37] but this can also mean that steric hindrance may prevent full conjugation when a bulky thiol is employed.

A technique that does not require any added catalyst is the reaction between thiols and vinylsulfone groups (Scheme 2.5). Simple stirring in a preferably slightly alkaline aqueous solution or in organic solvent can lead to full conversion in less than a day. Polymers with pendant vinyl sulfone groups can be directly prepared for example by ring-opening polymerization resulting in polymers that can be subsequently reacted with peptides.[38,39] The thiol-vinyl sulfone route is particularly attractive when polymers are employed that have been generated using the RAFT process. A simple aminolysis step creates polymers with a thiol as their end functionality. Reactions with excess divinyl sulfone create reactive semitelechelic polymers, which can then be conjugated to BSA.[40,41]

Probably the most popular thiol-based route in the literature for conjugating polymers to peptides or proteins is the thiol−maleimide reaction (Scheme 2.6). The nucleophilic addition of thiols to maleimide does not require any heat or catalyst, and simple stirring of the two reactants at room temperature is often sufficient to achieve complete conversion. This avenue has been employed to conjugate polymers to peptides,[42] proteins,[43−46] DNA,[47] and even monoclonal antibodies.[48] The only limitation is the need to protect the maleimide functionality prior to radical polymerization. The reader is referred to recent review articles on this topic since this focus article cannot capture all the intricacies.[49,50]

An interesting recent development in this area is the use of (di)bromomaleimide for potentially reversible binding of proteins as well as targeting of the disulfide group. Although this has not yet been tested in combination with polymer, it may well be an interesting approach for polymer chemists.[51]

A very popular route to polymer−peptide conjugates is the fast reaction between thiols and pyridyl disulfides (PDS) (Scheme 2.7).[52] The pyridyl disulfide will undergo a fast exchange with free thiol groups resulting in mixed disulfides. The driving force of the reaction is the formation of the yellow pyridine-2-thione, which allows easy monitoring of the rate of the reaction. In contrast to the preceding reactions described above, the linkage formed between the polymer and biomolecule is based on a disulfide bridge, which can be cleaved again in the reductive cell environment allowing the release of the biomolecule. Polymers with PDS groups can be directly prepared, having either a pendant group[53,54] or a single reactive entity at the end of the polymer chain.[55−57] The bioconjugation with peptides[54,58,59] or proteins[60,61] is usually carried out at ambient temperature without the addition of any catalyst. Similar to other polymer−peptide conjugates, steric congestions are commonplace when attempting to attach multiple copies of a peptide along a polymer chain.[53] A detailed investigation revealed that an excess of thiol and a reaction time of around 2 days is required to achieve complete conjugation.[54] Conjugation to peptides,[62] proteins,[61,63,64] and siRNA[65−67] at the chain terminal is less prone to steric hindrance but may face other challenges.

Next we discuss thiol−thiosulfonates (Scheme 2.8). An emerging tool for bioconjugation is the use of thiosulfonates,[68] which can be easily accessed by nucleophilic substitution of

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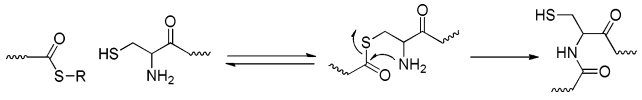
alkyl halides. Poly(ethylene)glycol modified with a terminal thiosulfonate was reacted with papain and recombinant human granulocyte colony-stimulating factor (rhG-CSF), respectively, at ambient temperature for one hour resulting in the formation of a disulfide bridge.[69]

The unique thiol−bisulfone reaction targets disulfide bridges, which are abundant in proteins and some cyclic peptides (Scheme 2.9). Instead of permanently cleaving the disulfide bridge, the conjugated polymer acts as a new junction.[70] While disulfide bridges have been targeted earlier such as during thiol−ene bioconjugation reactions,[32,33] the distinctive feature of the thiol−bisulfone reaction is the attachment of only one polymer chain.[70] Similar to the Michael addition with vinyl sulfone or maleimide, the reaction takes place initially between the thiol and the reactive vinyl functionality, which is originally protected by the sulfone functionality. After an elimination step, a consecutive Michael addition takes place.[71,72]

Although not strictly belonging to this group of thiol-chemistries, native chemical ligation should also be mentioned since the involvement of the cysteine thiol is vital to create a new peptide bond (Scheme 3). Polymer−petide conjugates have already successfully been prepared using this route.[73,74]

Scheme 3. Native Chemical Ligation



It becomes evident that the attractiveness of each of the various reactions is dependent on the type of bioconjugation sought. Thiol−halogenide reactions, which require long reaction times and often strong bases, are mainly applied in glycopolymer synthesis. Also, radical thiol−ene and thiol−yne reactions, which are subject to UV-irradiation or heat, are often not compatible with certain biomolecules, although this is not an issue when the reaction is fast enough.[31,75] Therefore, these are more commonly applied in polymer−peptide conjugations and glycopolymer synthesis. On the upside, the resulting thioether is stable under most conditions, and the group is not sterically demanding. Reactions 5−9 in Scheme 2 only require stirring at ambient temperature to proceed to completion and are therefore more germane for potentially fragile biomolecules such as certain proteins or antibodies. However, the resulting products have a possibly labile disulfide bridge at the nexus between both building blocks, which may or may not be an advantage (reactions 7 and 8 in Scheme 2). Some of these conjugation chemistries may lead to linkers between biomolecule and polymer that are sterically demanding, or they might affect the polarity of the final product. While this may not be of importance when a single polymer is attached to a large entity such as a protein, it is worth considering when several small molecules such as sugars or peptides are attached in multitudes to a polymer chain. This may explain why the thiol−halogeno and thiol−ene processes are the most attractive reactions for the synthesis of glycopolymers and polymers with pendant peptide groups.

Lastly, it needs to be mentioned that not all reactions presented here are click reactions. Many of the reactions presented here fulfill the criteria of having no side products or nonoffensive side products only. However, a common observation here is that, although most of the presented reactions are efficient, long reaction times or the excess of one component is commonly required to achieve high yields. Nevertheless, polymer chemists have found a versatile tool in thiol-based chemistry for creating polymer bioconjugates, allowing the design of a cornucopia of architectures.

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Notes

The authors declare no competing financial interest.

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