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CHEMICAL REVIEWS

Review

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Achieving Controlled Biomolecule–Biomaterial Conjugation

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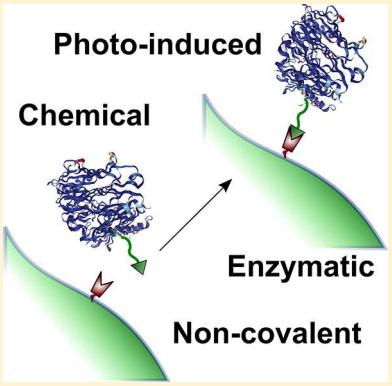
ABSTRACT: The conjugation of biomolecules can impart materials with the bioactivity necessary to modulate specific cell behaviors. While the biological roles of particular polypeptide, oligonucleotide, and glycan structures have been extensively reviewed, along with the influence of attachment on material structure and function, the key role played by the conjugation strategy in determining activity is often overlooked. In this review, we focus on the chemistry of biomolecule conjugation and provide a comprehensive overview of the key strategies for achieving controlled biomaterial functionalization. No universal method exists to provide optimal attachment, and here we will discuss both the relative advantages and disadvantages of each technique. In doing so, we highlight the importance of carefully considering the impact and suitability of a particular technique during biomaterial design.



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1. INTRODUCTION

The use of biomaterials to stimulate the repair, regrowth, or replacement of damaged biological tissue has emerged as a vital tool in the treatment and prevention of disease.[1−6] Biomaterial-based technologies typically take the form of cellular or acellular scaffolds for implantation in vivo, or alternatively platforms to support the in vitro growth of tissue for subsequent grafting.[6] A key challenge in biomaterial design is the creation of structures able to mimic or supplement the

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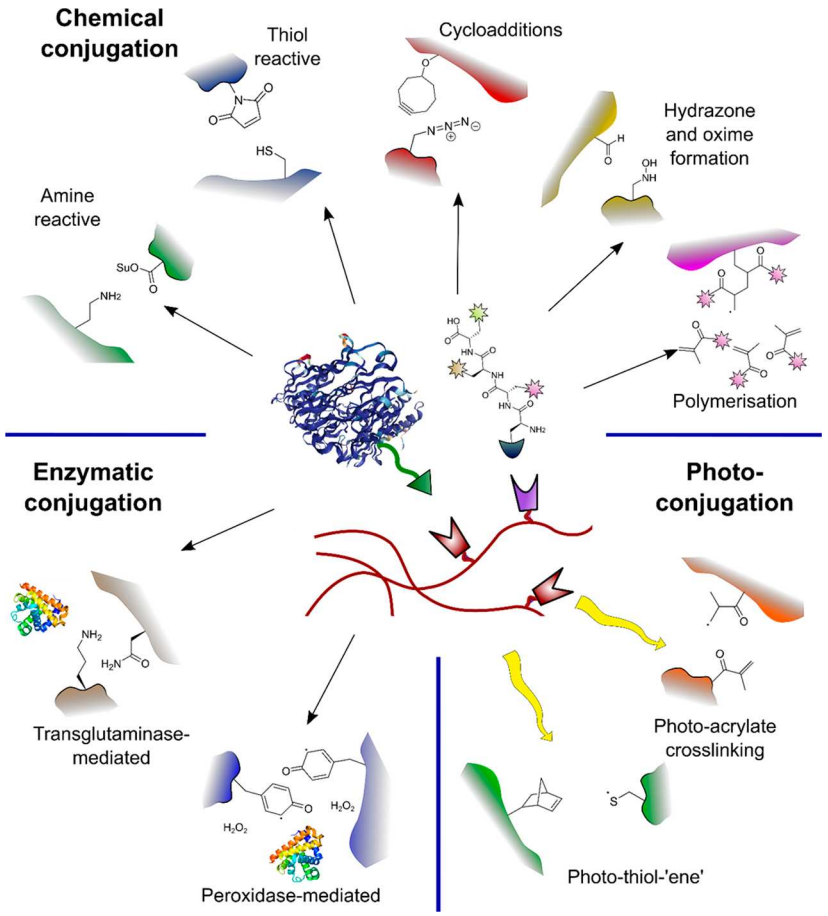

Figure 1. Key covalent methods by which biomolecule-material conjugation can be achieved. Each shall be discussed in detail during this review.

cues provided by the native extracellular matrix (ECM).[7−9] By doing so, cellular growth, infiltration, differentiation, and signaling can be controlled in order to aid tissue development and generate a successful clinical outcome. The processing of scaffolds to recreate the physical or topological features of native tissue,[8,10] the synthesis of polymers which mimic the chemical characteristics of ECM,[11,12] and the incorporation of functional components which bind and manipulate minerals and biomolecules in vivo[13,14] can all be used to direct cell behavior. However, the installation of motifs able to provide biochemical stimuli remains the most powerful means to influence growing tissue. The functionalization of scaffolds with active biomolecules is therefore of particular interest to generate effective materials for regenerative medicine.

Proteins and signaling peptides present within the extracellular environment play a vital role in determining and regulating a wide range of cellular behaviors. The tethering of peptides and proteins to a core structural component has therefore found widespread use as a method for endowing both synthetic and naturally derived materials with the bioactive properties required for successful clinical translation.[15−17] Similarly, glycans within the extracellular environment are influential in controlling cellular processes, acting to mediate signaling, adhesion, and trafficking.[18,19] To date, the formation of glyco-conjugates has been an underutilized tool in the field of biomaterial design. However, with rapidly improving appreciation of the importance of glycobiology and increasing progress in the synthesis of complex oligosaccharides, carbohydrate-functionalized materials are becoming more prominent.[20,21] Finally, the use of oligonucleotide-conjugates as a means to impart bioinstructive properties on a material has emerged as a significant area of research in recent years.[22,23] While the natural roles of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) sequences are difficult to exploit when tethered in the extracellular environment, the structural and functional capabilities of non-natural and evolved sequences are increasingly being employed in biomaterial design. In particular, aptamer sequences able to bind strongly to a target of interest have been used as a means to control the deposition of regenerative proteins, recruit endogenous growth factors, and control cell-selective adhesion.[24−26]

Biomolecule-material conjugates are able to play a diverse role in recreating the native extracellular niche, promoting cellular adhesion, providing degradable linkers for scaffold remodeling, controlling cell signaling for tissue growth or differentiation, or acting to sequester and provide structure to deposited matrix.[27,28] The biological roles of polypeptide, glycan, and oligonucleotide conjugates and the influence of particular sequences and structures on biological behavior have been reviewed extensively.[18,19,23,27−30] Similarly the choice of core scaffold material, topological and physical effects on cell behavior, and the influence of biomaterial degradability and plasticity are equally well documented.[7,8,31−35] Although briefly covered here as an introduction to the field, the reader is therefore directed to these excellent and comprehensive reviews for further information.

In this review we will focus on the chemistry used to produce biomolecule-biomaterial conjugates (Figure 1). This

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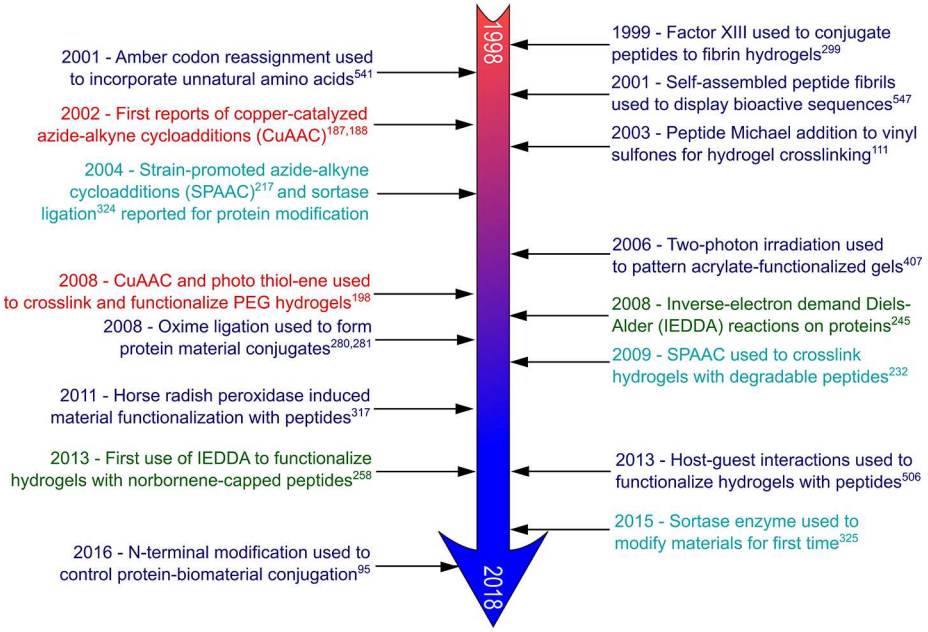

Figure 2. Major developments in biomaterial functionalization over the past 20 years. Many conjugation techniques have first been reported for the functionalization of proteins with small molecules, before later being translated to biomaterial derivatization.

important aspect of biomaterial design is often overlooked, despite playing a key role in influencing bioactivity and construct performance. There is no single optimal method for conjugation, and it is important to consider carefully the impact and suitability of a particular technique before embarking on the production of a functionalized device.[17] Key factors including the site-selectivity of conjugation, efficiency and rate of reaction, orthogonality to other modifications, ability to provide biomolecule patterning, and accessibility of reaction partners must all be considered.[36] The concept of selective-conjugation will be a common theme throughout the discussion, particularly in the context of peptide and protein conjugation. It has been widely shown that favorable surface orientation is essential to maximize bioactivity.[37−39] Modification strategies that are able to target a uniquely reactive amino acid are thus particularly powerful.[39−43]

It is important that a particular conjugation method is carefully matched to the core material and end application, and what is best in one scenario may prove to be entirely inadequate for another. Here, we therefore aim to provide a comprehensive overview of the different reactions and conjugation techniques that may be utilized to tether polypeptides, glycans, and oligonucleotides to biomaterial scaffolds. We will focus on the advantages and disadvantages of each technique, including the selectivity and sequence specificity provided by each method and their ease of use. We envisage that this work will provide both a useful guide for those looking to enter the field, as well as a detailed resource for experienced researchers. By doing so, we hope to stimulate the design and production of new biomaterial systems with enhanced bioactivity and efficacy, as well as encouraging the use of new or overlooked conjugation techniques in the tissue engineering field which may overcome the shortcomings of existing methods.

2. LAYOUT OF THE REVIEW

This review will be split broadly into four sections: chemical-, enzymatic-, photo-, and noncovalent conjugations (Figure 1). Although all of the techniques discussed in this review could be considered "chemical" in nature, here we utilize the term more specifically to describe reactions that rely on the chemical reaction between the conjugation components to generate a covalent linkage, without the need for exogenous biological or physical stimuli (e.g., enzymes or light). Many of the techniques described take inspiration from more general reports in the absence of biomaterials or biomolecules. A timeline outlining these key developments, as well as their application for biomaterial functionalization over the last 20 years is provided in Figure 2. We will briefly discuss this key preceding literature, setting the scene for a detailed discussion of the uses and limitations of biomolecule conjugation strategies for biomaterial functionalization. Finally, we will summarize the key findings enabled by each technology where relevant. For more detailed discussions on the applications of the developed materials in the tissue engineering field, as well as the wider impact of particular modification strategies outside of this area, the reader will be directed to key reviews throughout. At the end of each section we provide a table summarizing the major pros and cons of each conjugation strategy, as well as details of selectivity and orthogonality to other methods (Tables 1−4).

3. CHALLENGES IN SELECTIVITY

Biomolecule functionalization strategies can be broadly split into two categories: those which target a specific site for modification and those which modify multiple groups indiscriminately. This idea of "site-selectivity" is a key concept in biomolecule conjugation which will be considered throughout this review. It plays a crucial role in determining biological activity, conjugation efficiency, ease of use, and device reproducibility.[36,44,45] To demonstrate this, we can consider a hypothetical protein containing 10 surface-exposed

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Table 1. Advantages, Disadvantages, and Selectivity of the Chemical Conjugation Strategies Outlined in Sections 4 and 6

conjugation strategy	advantages	disadvantages	selectivity
amide formation	ease of use	site-selectivity very low	selective for amines and carboxylic acids
	high reagent availability	side-reactions affect yield	
	requires no prior engineering	poor tolerance of water	
	robust and reliable	leads to heterogeneous products	
reductive amination	good chemo-selectivity	slow kinetics	selective for amines and aldehydes/ketones
	ease of use	site-selectivity very low	tolerant of other groups
	aldehydes often easily introduced into materials	aldehydes potentially toxic	
thiol Michael addition	rapid conjugation	potentially reversible	side-reactions with amines necessitates careful control
	tunable reaction kinetics	maleimides undergo hydrolysis	
	low occurrence of cysteine enhances selectivity	thiols often key for bioactivity	
	ease of introduction	disulfides form over time	
	robust protocols for use		
disulfide formation	ease of use	very slow	high selectivity for thiols
	easy to trigger cleavage	form mixed or homodisulfides	prone to formation of mixed or homodisulfides
CuAAC	high chemo-selectivity	copper potentially toxic	very high selectivity for azides and alkynes
	reactive groups absent from native biomolecules	azide/alkynes must be introduced into biomolecule	copper chelated by many functional groups
	functional groups readily accessible	high catalyst loadings often needed	activated alkynes prone to thiol−yne reaction
SPAAC	requires no added catalyst	alkynes hard to synthesize/expensive	generally high selectivity for azides and strained alkynes
	ease of use	alkynes are highly hydrophobic	cyclooctynes undergo side reaction with thiols
	kinetics can be tuned by alkyne structure	kinetics slow compared to IEDDA	
	reactive groups absent from native biomolecules	azide/alkynes must be introduced into biomolecule	
	very low toxicity		
IEDDA	very fast kinetics	some tetrazines unstable	high selectivity for strained alkenes and tetrazines
	reactive groups absent from native biomolecules	fastest alkenes difficult to access and hydrophobic	cyclooctenes can be isomerized by presence of thiols
	high selectivity	alkene/tetrazine must be introduced into biomolecule	
oxime/hydrazone formation	dynamic conjugation	low stability if reversibility not desired	equilibrium with imine formation is dominated by hydrazones/oximes.
	hydrazines and hydroxylamines absent from biomolecules	catalysts needed to boost kinetics	tolerant of other groups
	aldehydes often easily introduced	aldehydes potentially toxic	
		reactive groups must be introduced	
Free-radical polymerization	high tolerance of functional groups	deoxygenation necessary	tolerant of most functional groups
	widely used in biomaterial field	polymerizations and excess monomer often toxic	cross reactivity with Michael addition and IEDDA reactive handles
	allows conjugation during material formation	radicals damaging to biomolecules	
	excess reactive handles can be used for further conjugation		

lysines and a single cysteine. Methods to functionalize a material via these lysine residues are typically straightforward; however, they will also result in a heterogeneous mixture of at least 10 different protein orientations, before we begin to consider more complex scenarios in which multiple lysines are modified within a single protein. While some of these conformations might maintain activity, others may possess hindered or blocked active sites or deformed protein structures. The importance of favorable orientation and geometry following material tethering has been widely reported, particularly for protein and glycans, and is a crucial factor affecting biological efficacy.[37−39,46,47]

In contrast, if we instead target the single cysteine via a site-selective reaction homogeneous constructs will be produced. If the site of modification tolerates alteration and is located in a way to expose the key active regions of the protein, then we can expect to maintain activity in a way that is unlikely to be achieved via nonselective strategies. However, it is important to note that the opposite can also prove true; if the cysteine is crucial to activity, our attempts to achieve selectivity may instead lead to drastic loss of activity. Selective modification strategies are therefore an important means through which to maximize device performance but require careful planning and design to implement.[36] Whether the increased complexity provides sufficient strategic benefit is something that must be considered on a case by case basis.

Difficulties in achieving selectivity originate from the limited diversity of chemical functionality that exists in native biomolecules. Methods exist to functionalize the amines, alcohols, and carboxylic acids that make up the bulk of functional groups in amino acids, monosaccharides, and nucleotides, but typically a large number of each reactive handle are present in any given biomolecule.[48] The rarity of the thiol side group of cysteine in polypeptides partially

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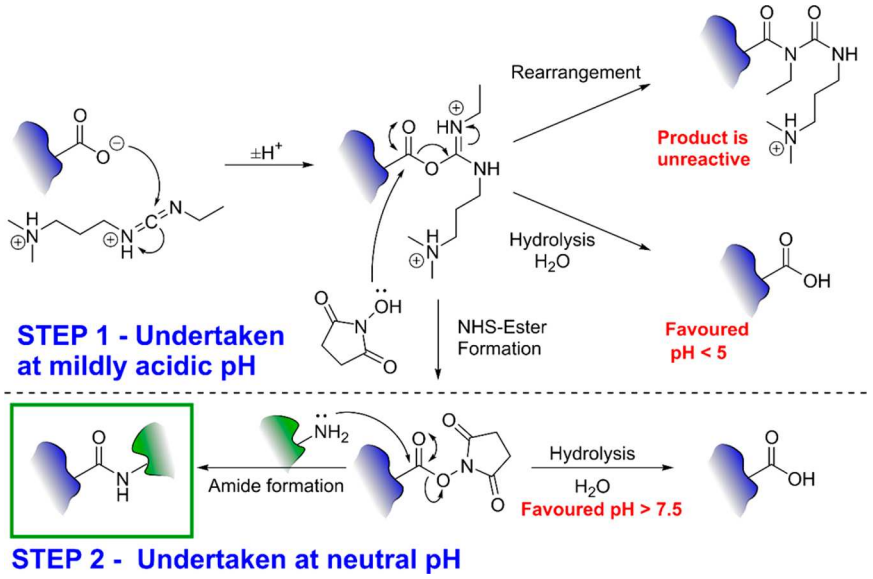

Figure 3. EDC/NHS couplings are most commonly used to undertake amide couplings via a two-step process, first activating the carboxylic acid at pH 5, and subsequently undertaking conjugation at pH 8. However, the reaction is associated with side reactions which limit conjugation efficiency.

mitigates this problem but comes with its own limitations that will be discussed in section 4.2.[49] Three approaches are therefore primarily used by researchers to undertake site-selective bioconjugation:

(i) Modification strategies that can select a single motif among many, rather than targeting a generic reactive handle. This is often determined by surrounding sequence, local environment, or subtle differences in reactivity. The ability of enzymes to modify a specific amino acid within a protein sequence or a glycan at a single position are particularly prominent examples and will be the focus of section 5. Reactions that display exquisite chemo-selectivity also fall within this category, such as those that target the unique reactivity of the protein N-terminus or the anomeric position of glycans.

(ii) The site-specific incorporation of unnatural functionalities, by hijacking native biosynthetic pathways.[50] Developments over the past 20 years have led to technologies through which uniquely reactive residues can be selectively installed. Such systems will be described in more detail in section 10.

(iii) The installation of unique reactivity via chemical synthesis. The complete or partial synthesis of peptides and oligonucleotides is widespread, particularly using solid-phase approaches. These techniques allow access to sequences of up to 100 amino acids or 200 nucleotides, with the ability to install a wide variety of functionalized monomers with precise positional control.[22,51,52] Indeed, examples of oligonucleotide-material conjugation rely almost exclusively on chemically synthesized sequences. While the routine synthesis of glycan sequences in an analogous manner remains a pipedream, recent developments in glyco-chemistry are beginning to make solid-phase oligosaccharide synthesis a realistic prospect.[53−55]

4. CHEMICAL CONJUGATION

Chemical conjugation techniques are the most widely studied and applied method for creating biomaterial−biomolecule conjugates. This is partly due to the ease and generality with which they can be undertaken, without the requirement for specialist techniques or equipment. Here, we will split the discussion by the key reactive handles or reaction mechanism involved. Many of the bioconjugation strategies were first developed within the context of protein-small molecule modification. The reader is directed to a number of excellent reviews on this topic, which forms the basis for much of the work to be described in this section.[36,48,56−58]

4.1. Amine Reactive

Amine groups are widespread in native biomolecules, both on the side chain of lysine residues and at the N-terminus of polypeptides, and in amino-sugar glycan building blocks. Similarly, both 3′- and 5′-amino modification of oligonucleotides is common. As such, amines provide perhaps the simplest reactive handle for undertaking conjugation. This is reflected by the extensive literature on their use, a wealth of detailed protocols for their modification, and the widespread commercial availability of reagents and kits that allow conjugation with minimal difficulty.[59−61] However, the common occurrence of amines which makes them so attractive for achieving conjugation is also their most severe drawback: modification of amine residues is typically associated with low site selectivity and the generation of heterogeneous product isoforms, as discussed in section 3. For example, a typical protein will possess numerous surface exposed lysine residues, each of which will display similar reactivity. Efforts to modify amines therefore typically lead to biomaterial attachment at a variety of sites, with a mixture of valencies. This poor site-selectivity often leads to dramatic drops in bioactivity or function. This is true even for short peptide substrates, where competition between pendant lysines and the N-terminus can occur. As a result, both the stoichiometry and position of conjugation varies greatly during amine modification and is difficult to control.[36] Despite this major disadvantage, if only a single amine motif is present in a peptide or glycan sequence, or if the researcher is unconcerned about selectivity, amine modification may still be a viable method for conjugation. In a particularly exciting recent development, Matos et al. have

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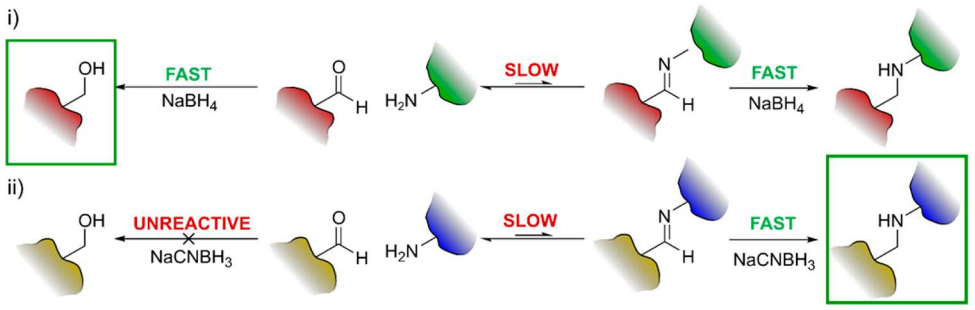

Figure 4. Imine formation is the rate-limiting step during reductive amination. Sodium borohydride is able to reduce the aldehydes which dominate and therefore fails to generate the desired conjugate. In contrast, sodium cyanoborohydride selectively reduces imines, leading to the formation of stable amine-conjugates.

demonstrated that careful tuning of reactivity and control of stoichiometry can lead to single lysine modification, even on challenging protein substrates.[62]

4.1.1. Amide Formation. Amide formation is among the most widely studied and used transformations in synthetic chemistry. A vast range of coupling agents and carboxyl-activating species therefore exist for undertaking such reactions.[63] When focusing on the aqueous conditions often required for biomolecule conjugation the options are greatly reduced. A small number of key methods have therefore commonly been applied and will be discussed here.

The use of reactive *N*-hydroxysuccinimide (NHS) esters is particularly widespread. While NHS-esters can be preformed, often they are instead generated in situ through the use of *N*-(3-(dimethylamino)propyl)-*N*′-ethylcarbodiimide (EDC) coupling chemistry and coupled directly to the species of interest.[60,64] Although formation of the activated NHS-ester is favored under mildly acidic conditions (pH ∼5), subsequent amide coupling is accelerated at higher pHs at which the amine coupling partner is not protonated. One-step modification at an intermediate pH of ∼6.5 is possible, but this method is characterized by low coupling efficiency.[65] Conjugation is therefore typically undertaken by first forming the active NHS-ester at pH 5, before raising the pH to ∼8 and adding the amine coupling partner in a two-step procedure (Figure 3).[60] Selectivity for amines is generally high. Although side reactions with alcohols and thiols are known at basic pH, they occur at a greatly reduced rate and are outcompeted in the presence of amines. However, ester hydrolysis can be problematic in aqueous conditions due to the high effective concentration of water.[66]

Despite the widespread use of EDC/NHS coupling for biomolecule conjugation, a number of key limitations hinder utility. In addition to the pH dependency described above, side reactions are known to significantly reduce reaction conjugation efficiency, including rearrangement of intermediate EDC-esters to a stable and unreactive *N*-acylurea (Figure 3).[67,68] Furthermore, the hydrophobicity of NHS-esters may limit water solubility. To overcome this problem, the more water-soluble derivative sulfo-NHS is often utilized as an alternative, offering a similar activation and coupling profile.[67,69,70]

EDC/NHS amide coupling chemistry has been widely used in the biomaterial community despite these disadvantages, with a vast body of literature highlighting the ease with which such reactions can be applied to biomolecule conjugations. However, the limitations of amine targeted ligations noted above often make such reactions ill-suited to maximizing biological efficacy and construct performance.

It is important to note that peptide/protein conjugations are poorly compatible with the functionalization of common amine-containing scaffolds, such as collagen and chitosan, due to competing cross-linking and intramolecular reactions.[71,72] In contrast, the oxidation of reducing oligo-saccharides to generate terminal carboxylates enables glycan functionalization of amino-scaffolds, albeit with concurrent loss of functionality from the terminal monosaccharide.[73,74] Similarly, the ease with which terminal-amines can be introduced during oligonucleotide synthesis has led to a number of reports on EDC/NHS-mediated biomaterial-aptamer functionalization.[75,76]

A number of alternative coupling systems have been used for biomaterial functionalization, though they continue to suffer from the same limitations. These include the use of *O*-nitrophenyl esters (which possess reduced stability in aqueous conditions)[77,78] or 1,1′-carbonyldiimidazole (CDI) to form amine-bridging carbamate linkages rather than amides.[79,80] Hydrazines can also be used in place of amines during EDC/NHS mediated couplings.[68,81] Although not as widely available or synthetically facile as the corresponding amine, the lower pK_a of hydrazines makes them less liable to protonation under the mildly acidic conditions used for ester activation. As a result, hydrazine-functionalized peptides can be coupled to biomaterials in a single step at pH 5−6. In doing so, a degree of site-selectivity can be achieved over lysine residues present. This approach has been successfully implemented by Madl and co-workers to conjugate reactive groups to alginate hydrogels, enabling indirect functionalization with growth factors and adhesion peptides.[81]

4.1.2. Reductive Amination. Amines can react reversibly with aldehydes to form a transient imine moiety, with accompanying elimination of water. This reaction takes place in rapid equilibrium, with the unconjugated starting materials being strongly favored in aqueous conditions due to the high concentration of water. However, in a second step the unstable imine can be irreversibly reduced to the corresponding amine via treatment with sodium cyanoborohydride. This mild reducing reagent enables the selective reduction of imines even in the presence of unreacted aldehydes (Figure 4, reaction ii).[82] As a result, irreversible conjugation of a biomolecule can gradually occur to a biomaterial of interest. However, rates of reaction are often very sluggish due to the unfavorable formation of the intermediate imine in aqueous conditions.[69,83−85] In contrast, stronger reducing agents such as sodium borohydride are also able to reduce aldehydes.

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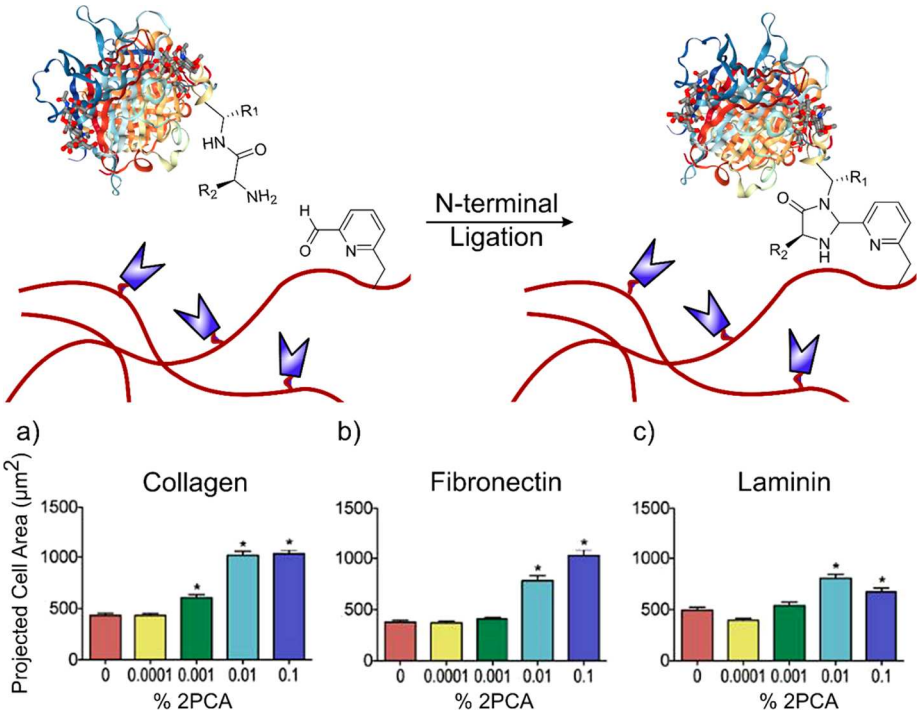

Figure 5. N-terminal specific conjugation can be used to functionalize 2-pyridinecarboxaldheyde (2PCA) hydrogels with native ECM proteins. Increased cell spreading is observed with increased scaffold functionalization with (a) collagen, (b) fibronectin, and (c) laminin. Adapted from ref 95. Copyright 2016 with permission from Elsevier.

Conjugation is therefore ineffective with alcohol formation dominating (Figure 4, reaction i).

This two-step reductive amination process can also be utilized for the modification of ketones. However, such reactions are rarely used for biomaterial conjugation due to even slower reaction kinetics and the ease with which aldehydes can be introduced into polysaccharide based scaffolds through the oxidation of primary alcohols.[86] Reductive amination has therefore been primarily used for the modification of sodium periodate-treated alginate and chitosan scaffolds, though care must be taken not to disrupt the physical and chemical properties of the core material through overoxidation.[86] The order of reactivity can also be reversed for the attachment of reducing sugars, by exploiting the terminal aldehyde/ketone generated in the open-chain form. This strategy has been commonly exploited to mimic the glucosylation and galactosylation patterns of native collagen in ECM, via reductive amination of maltose[87] and lactose[88−90] respectively, as well as more recently by Sgamboto et al. to install sialoside epitopes able to drive stem cell fate.[91]

4.1.3. N-Terminal Modification. Although amine modifications typically proceed with low site-selectivity, the N-terminus of polypeptides has subtly altered reactivity when compared to the side-chains of lysine residues and offers opportunities for selective conjugation. This unique reactivity results from the slightly lowered pK_a of the N-terminal amine, and the structural environment imparted by the adjacent amide group of the peptide backbone.[44] A number of methods for achieving site-selective N-terminal modification have been reported in the context of protein−small molecule conjugation.[92−94] However, to the best of our knowledge, such methods have only been utilized on a single occasion to directly conjugate a polypeptide to a biomaterial scaffold. Lee et al. recently demonstrated that 2-pyridinecarboxaldehyde modified acrylamide hydrogels could react specifically with the N-terminus of ECM proteins, forming a cyclic imidazolidinone product with the adjacent amide bond and enabling the orientated display of these key bioinstructive motifs (Figure 5).[95] Although imine formation with lysine residues is also possible, this reversible reaction is in thermodynamic equilibrium and the added stability afforded by formation of the imidazolidinone drives N-terminal specificity.

This method is highly attractive as a means to form biomaterial-conjugates, due to its ability to site-specifically conjugate any peptide or protein that possesses a solvent exposed free N-terminus. It is important to note that effective conjugation relies on an equilibrium-driven process, albeit one that favors product formation. The stability of the resultant construct is thus a vital consideration and merits further investigation.[93] If this potential shortcoming can be overcome, such reactions will be applicable to a wide range of commercially available, recombinant, or synthetic substrates and can be expected to find increasing utility in the near future. However, substrate scope may be partially limited by the extensive occurrence of natural N-terminal post-translational modifications in eukaryotic proteins.[96]

Although the C-termini of proteins also have the potential for unique reactivity, the targeting of such groups remains challenging. A recent report of selective C-terminal decarboxylation by the MacMillan group highlights the possibility of targeting such residues in the future.[97] However, the poor functional group tolerance of this reaction is currently limiting. Further developments, able to offer exquisite chemo-selectivity over other carboxylic acids with only subtly shifted reactivity, would represent a major advance.

4.2. Thiol Reactive

The thiol group of cysteine is the most nucleophilic functional group found among the 20 proteinogenic amino acids.

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Through careful control of pH, selective modification over other nucleophilic residues such as lysine can be readily achieved.[49] The redox sensitivity of thiols further diversifies the chemical modifications they may undergo. When coupled with the low natural occurrence of cysteine residues (<2%, typically lower among surface exposed residues) and the ease with which they can be engineered into recombinant proteins, thiol groups emerge as particularly attractive targets for undertaking site-selective polypeptide conjugation (see section 10 for a discussion on cysteine introduction).[98] A wide-range of powerful modification strategies have been developed for tethering thiol-containing peptides and proteins to biomaterial substrates, and such methods are among the most widely used in the field of tissue engineering.[99] Thiol modification of oligonucleotides has also been commonly used to enable derivatization, though the ease with which alternative reactive handles with enhanced chemical orthogonality can be installed has limited use for biomaterial-conjugation.

Despite the advantages noted above, the modification of thiols is not without its drawbacks. While it may at first seem counterintuitive, the rarity of cysteine in native proteins highlights their vital importance in controlling activity. Many cysteine residues are therefore found within enzymatic active sites or else form key structural disulfide bridges.[100] As a result, often wild-type cysteine residues are either not accessible for modification or at best can be altered only at the drawback of greatly decreased protein activity. Although additional thiols can be introduced onto the surface of proteins through the modification of lysine residues with Traut's reagent, the resultant conjugates suffer from the same lack of amine-selectivity described above and thus possess low strategic value.[60] Even within short synthetic peptides, cysteine residues are often key to activity, while disulfide bridges are commonly used to induce cyclization and boost biological efficacy.[101] This propensity of cysteine residues to oxidation can also prove problematic when precise modification stoichiometry is required, necessitating prior disulfide reduction before conjugation is undertaken.[49,102]

4.2.1. Michael Addition. The conjugate addition of thiols to α,β-unsaturated carbonyls, also known as Michael addition, is perhaps the most widely used method to form polypeptide conjugates in the fields of tissue engineering, functional materials, and protein modification. A number of detailed reviews focusing solely on this reaction class have been published, and the reader is directed to these for a comprehensive overview of reaction mechanism, kinetics, reagent choice, and applications across a broad range of topics.[103,104] Herein, we will focus on the key factors that determine conjugation efficiency in the context of biomaterial−biomolecule ligation.

The Michael addition of thiol groups proceeds in a highly specific manner under mild conditions, with almost quantitative conversion even in dilute solutions. Furthermore, the reactions are largely insensitive to the presence of water, oxygen, and salts, greatly facilitating their implementation in tissue engineering and biomaterial applications.[105]

In general, reaction rates and conjugation efficiencies are primarily controlled by three factors:

(i) The pK_a of the thiol. The thiolate anion is the active nucleophile during Michael addition, and the propensity of the thiol to undergo deprotonation therefore determines thiolate concentration and thus reaction rates. For example, the lower pK_a of aromatic thiols, when compared to their aliphatic counterparts, leads to a higher rate of reaction rate a weak base is used to catalyze the reaction (see below).[103,106] As a result, local structure can significantly alter conjugation efficiency, particularly for polypeptide substrates. The pK_a and reactivity of cysteine containing peptides can be altered significantly through rational choice of surrounding amino acids. Specifically, the presence of positively charged amino acids, such as lysine and arginine, acts to lower the thiol pK_a and thus enhance reactivity. In contrast, aspartic or glutamic acid residues retard reaction.[107] Careful peptide design is therefore a key consideration when designing a biomaterial conjugation strategy.

(ii) The electrophilicity of the Michael-acceptor. In general, as the Michael-acceptor becomes more electron deficient it becomes more activated toward nucleophilic attack, and thus reaction rates increase. Within the most widely utilized acceptors in the biomaterial field, a trend of reactivity can be generalized as maleimides > vinyl sulfones > acrylates > acrylamides > methacrylates (Figure 6).[103] Each of these acceptor classes will be discussed in turn below.

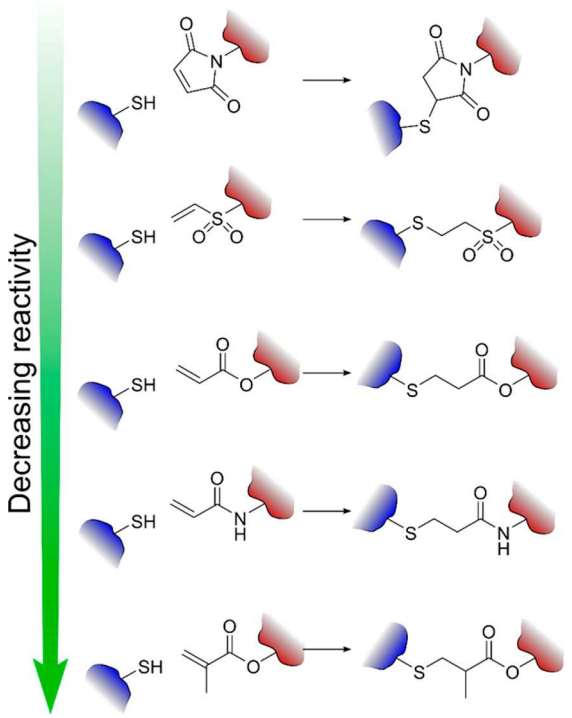

Figure 6. The rate of thiol Michael addition is dependent on the electrophilicity of the acceptor. The more electron-deficient an alkene, the more susceptible it is to nucleophilic attack.

(iii) The choice of catalyst. Michael additions can be accelerated by either basic or nucleophilic catalysis (although both act by increasing the concentration of the active thiolate[108,109]). During basic catalysis, the pK_a of the thiol becomes less significant for reaction rate with increasing base strength. As biomolecule conjugations are typically undertaken in buffered aqueous solution, it can be summarized that with increasing buffer pH reaction rates will also increase. However, it is important to note that above pH 8 competing amine Michael addition and thiol oxidation reactions may become significant, leading to a drop in conjugation efficiency and

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specificity which can be highly detrimental to the end application.[110,111]

These complementary factors must all be carefully considered when preparing a biomolecule−material conjugate, with applicability often determined by the end function of the scaffold.[112,113] For example, a rapid rate of reaction may be required when utilizing a peptide to induce hydrogelation, to minimize gel defects and ensure rapid gelation at the site of administration, or for the efficient attachment of proteins or oligonucleotides present at dilute concentrations.[114−116] However, if reactivity is too high, off-target conjugation (particularly with amines) or inadequate mixing of reaction components may occur, which in turn leads to heterogeneous devices with poor performance.[117,118]

4.2.1.1. Maleimides. Maleimides are the most widely used Michael-acceptors within the biomaterial community. The high reactivity of the maleimide group originates from a combination of the 2 electrophilic carbonyl groups, arranged in a reactive cis conformation, and the high bond strain induced by the unsaturated 5-membered ring.[105] As a result, thiol conjugation occurs rapidly even at neutral pH. The kinetics of the thiol-maleimide reaction are particularly useful for undertaking conjugation at low concentrations or when requiring extremely high efficiencies due to the value of the biomolecule substrate. Maleimides are therefore often seen as superior to alternative Michael-acceptors in many applications.[115] The widespread use of maleimides is further enhanced by the ease with which they can be introduced into a wide range of scaffold materials, through the modification of amines with the difunctional reagent succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate, more commonly referred to by its abbreviation SMCC. This reagent has been widely used to first introduce a maleimide reactive handle on a biomaterial of choice and then to enable the attachment of both peptides[119−123] and growth factors[124−128] to produce bioactive scaffolds. Importantly, although selectivity is high at neutral pH (the thiol-maleimide reaction rate is >1000 times faster than reaction with amines), because maleimides are so reactive the rates of side reactions are not inconsequential. At high amine or low thiol concentrations the contribution of undesired conjugations can become an important consideration. Furthermore, selectivity is lost under weakly basic conditions, and careful control of pH is therefore essential to prevent side-reaction at lysine residues.[129]

Although maleimides are generally perceived to form stable conjugates, following a simple Michael addition mechanism, many recent reports have highlighted the reversibility of thiol conjugation via a retro-Michael reaction (Figure 7).[131−133] In the absence of exogenous thiols, the effect of reversibility is negligible, with the biomaterial-conjugate being rapidly reformed. However, in many biological environments an excess of thiols exist, for example on cell surface proteins, or in the form of small molecule metabolites such as glutathione.[134,135] Thiol-exchange can then result, through the equilibration of forward- and retro-Michael additions, leading to a gradual loss of conjugation and a drop in device performance. This situation is complicated further by the competitive formation of stable conjugates by ring-opening hydrolysis of maleimide−thiol adducts under basic conditions. Recent literature has exploited this side reaction and highlighted the ability of adjacent amines,[136,137] ortho-amino aromatics,[138] and electron withdrawing phenyl groups[139] to accelerate the rate of hydrolysis, minimizing the effects of

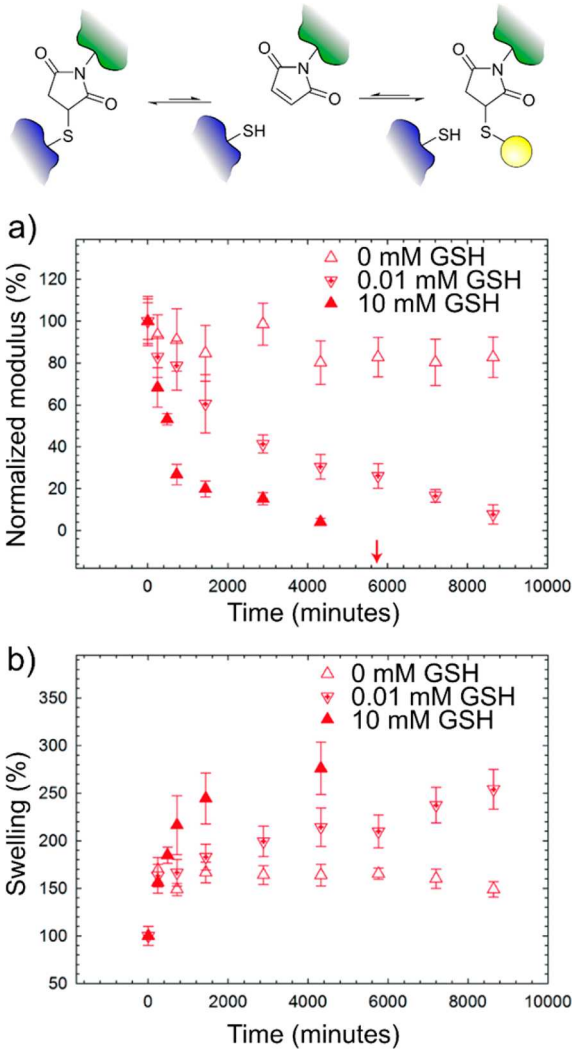

Figure 7. Maleimide adducts can undergo retro-Michael addition and thiol-exchange in the presence of exogenous thiols. (a) Decrease in storage modulus of maleimide−thiol cross-linked hydrogels following incubation with different concentrations of glutathione (GSH); (b) increased hydrogel swelling as a result of decreased cross-linking density. Adapted from ref 130 with permission of The Royal Society of Chemistry.

retro-Michael addition and allowing the production of stable bioconjugates. Kalia et al. have further demonstrated that exocyclic maleimides are less prone to retro-addition.[140] Exploiting these recent developments for biomaterial functionalization may thus overcome some of the limitations of current maleimide modification strategies.

4.2.1.2. Vinyl Sulfones. The Michael addition of thiols to activated vinyl sulfones to form biomolecule−material conjugates was first reported by Lutolf and Hubbell (Figure 2).[111] In a series of papers, they demonstrated that cysteine capped peptides could cross-link vinyl-sulfone functionalized multiarm PEGs to form protease responsive hydrogels, enabling cell invasion during tissue growth.[111,141−143] The vinyl sulfone double bond is less electrophilic than analogous maleimide substrates. Michael additions therefore occur at a slower rate, yet still proceed rapidly to high conjugation efficiencies sufficient for most applications. In contrast to maleimide adducts, the resultant thioether bond is resistant to hydrolysis and is therefore particularly attractive when

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construct stability is key to device performance. Although cross-reactivity with amines and alcohols has been reported in the absence of suitable thiol nucleophiles, these side reactions are significantly slower than those observed with maleimides. Selectivity is therefore generally very high, particularly with careful control over reaction pH.[144−146] Vinyl-sulfone additions have therefore been widely utilized for the conjugation of peptide[147−150] and protein substrates[144,151] to a variety of biomaterial scaffolds.

4.2.1.3. Acrylates, Acrylamides, and Methacrylates. Acrylates and methacrylates are versatile reactive handles which can be used to ligate biomolecules via a number of different reaction modes. As well as their propensity to undergo Michael addition, as described here, they may also undergo radical mediated polymerization, cross-linking, and cross-conjugation as discussed in sections 6 and 7.1. This diverse reactivity can in itself be problematic. Acrylate substrates in particular must be carefully stored cold and away from light to prevent unwanted polymerization.[152]

Acrylates have been widely used as Michael acceptors for conjugation due to the ease with which they can be introduced into both material and biomolecule coupling partners.[151,153−158] Although reaction rates are inferior to maleimides and vinyl sulfones they are fast enough for many applications, making acrylates an efficient, facile, and reasonably fast handle for undertaking conjugation. Indeed, the reactivity of acrylates remains high enough for side-reactions with amines to occur when reaction conditions are not carefully controlled.[159,160] The lower electrophilicity of methacrylates provides higher thiol selectivity, though at the cost of drastically reduced reaction rates and efficiency.[107,160,161]

For both acrylate and methacrylate handles, the presence of a hydrolytically labile ester linkage is an important and often overlooked factor. Although susceptibility to gradual cleavage may be advantageous in certain circumstances, often the loss of conjugated biomolecule is highly detrimental to device performance. While methacrylate hydrolysis is slower, cleavage can still occur quickly under physiological conditions.[151] As an alternative, amide-linked acrylamides provide improved stability for biomaterial conjugation. However, this comes at the cost of a dramatic reduction in reactivity, with the reduced electrophilicity imparted by the amide bond leading to a further drop in reaction kinetics.[153]

4.2.1.4. Thiol−yne. The most common examples of thiol-Michael additions occur at electron-deficient alkenes, so-called "thiol−ene" reactions. Although less commonly exploited, "thiol−yne" additions to activated alkynes, such as propiolic acid esters, have also been reported for biomaterial functionalization. Under weakly basic conditions a mixture of alkene isomers are generated following addition. Unlike the radical thiol−yne reactions to be discussed in section 7.3, these adducts do not typically undergo further conjugation and therefore form a monoaddition product.[162−164] The nucleophilic thiol−yne reaction has been shown to be a cell-compatible method for hydrogel formation and peptide functionalization, although a degree of cross-reactivity with amines may still hinder complete selectivity.[163]

4.2.2. Disulfide Formation. Thiols undergo spontaneous oxidation to form disulfide bonds under ambient conditions. This redox sensitivity is widely exploited in nature to induce and maintain protein and peptide stability. Although disulfide formation can be promoted by added oxidant and utilized as a method for polypeptide conjugation,[128,165,166] spontaneous oxidation is typically slow and proceeds with low selectivity due to the formation of either mixed disulfides or homodimers.[36,49] The use of disulfide exchange reactions is therefore favored for introducing peptides or proteins of interest. The most commonly used reagents in tissue engineering are based upon reactive pyridylthio-disulfides, which undergo rapid thiol-exchange to release the poorly nucleophilic and spectroscopically active 2-mercaptopyridine.[167−170] The resultant conjugation products are largely stable under ambient conditions. However, due to the reversible nature of disulfide bond formation, cleavage can be controlled with temporal precision by the addition of reducing agents such as dithiothreitol (DTT) or glutathione.[130,170,171] While this sensitivity to thiol exchange may be advantageous for certain applications, it can also prove problematic when irreversible modification is desired, particularly in vivo where native thiols can lower stability within the extracellular environment.[109]

4.2.3. Other Thiol Modifications. The unique nucleophilicity of thiols can be exploited for selective reaction with a number of alternative electrophiles, which, while less commonly used for biomaterial conjugation than Michael acceptors, still allow efficient and selective biomolecule attachment to be achieved. One such group are α-halocarbonyls, with iodoacetamide based reagents finding particular utility.[167,172] Higher thiol selectivity can be achieved using less electrophilic bromo and even chloro derivatives, though reactivity is also drastically reduced.[49] More recently, methylsulfonyl heteroaromatic derivatives have emerged as promising reagents for thiol-specific conjugation. These reagents rely on nucleophilic aromatic substitution to efficiently and selectively introduce stable thiol modifications under ambient conditions.[173] The group of del Campo have shown that these reagents can effectively functionalize hydrogels with both peptide and protein substrates.[174,175]

There is much interest in the identification of alternative thiol-reactive handles, such as disulfide-bridging pyridazinediones,[176,177] carbonylacrylic reagents,[178] and cyclopropenyl ketones[179]. These reactive handles possess fast reaction kinetics, yet overcome the instability of maleimide derivatives. Though currently limited to the formation of small molecule conjugates, such developments are at the cutting edge of bioconjugation strategies. It is critical that further investigations are undertaken to comprehensively determine the thiol-selectivity of these motifs under diverse and challenging conditions, to enable future advances in biomaterial functionalization.

In a final example of thiol-reactive conjugation, native chemical ligation (NCL) can be utilized to attach peptides and proteins to biomaterial scaffolds via peptide bond formation. NCL relies on the reversibility of thioester formation, enabling the exchange of a reactive thioester present on one reacting species, with an N-terminal cysteine on the other.[180−182] Due to the favorable steric arrangement of the resultant complex, a chemo- and regioselective *S*,*N*-acyl shift can occur, leading to peptide bond formation between the two reaction components. The application of NCL in the field of biomaterial conjugation is limited by difficulties in producing the thioester reaction partner, and the slow reaction kinetics which necessitate extended conjugation periods.[183] However, NCL has been successfully applied to the formation of peptide cross-

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linked hydrogels, via the reaction of cysteine end-caps with thioester functionalized hydrogel building blocks.[183−185]

4.3. Cycloaddition Reactions

The past 20 years has seen an explosion in the popularity of cycloaddition reactions for undertaking selective, fast, and often mild biomolecule−material conjugation. Although many cycloadditions were first identified in the middle part of the 20th century, it is only more recently that a number of key developments have enabled their routine use in tissue engineering. During cycloaddition, two or more unsaturated molecules are brought together to form a cyclic product with a reduction in the degree of unsaturation (Figure 8). The reaction partners required are typically absent from natural systems, and so the use of cycloadditions for conjugation requires the introduction of unnatural functionality within the biomolecule coupling partner. This requirement may limit application and ease of implementation, though, as described in section 10, a wide range of methods for installing the required functional groups are now available and are routinely utilized in molecular biology and tissue engineering.

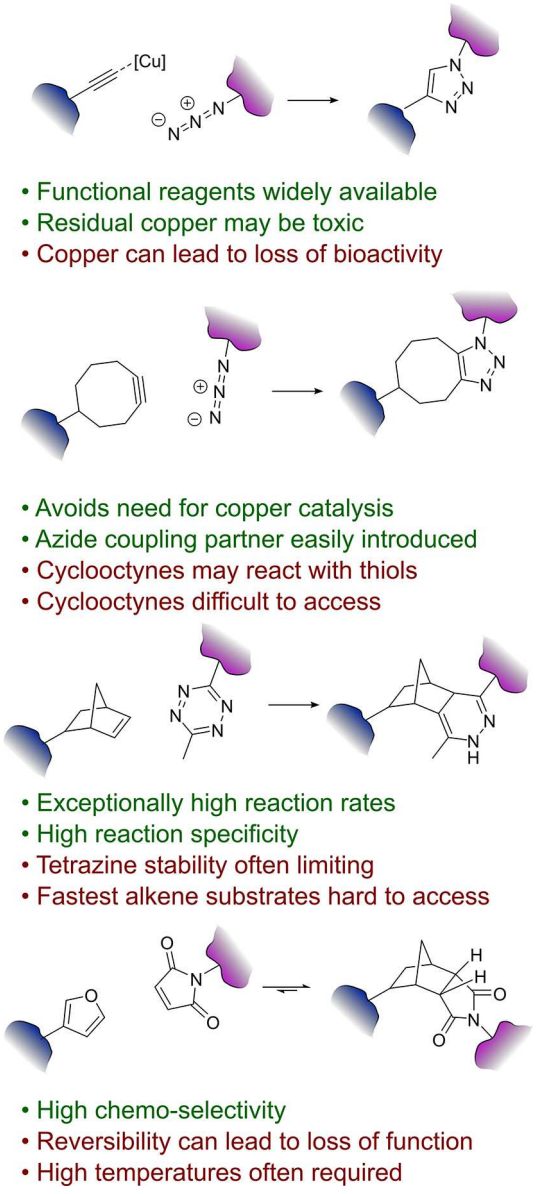

Figure 8. Key advantages and limitations of cycloaddition reactions used for biomolecule-material conjugation. (a) Copper-catalyzed azide−alkyne cycloaddition (CuAAC); (b) strain-promoted azide−alkyne cycloaddition (SPAAC); (c) inverse-electron demand Diels−Alder reaction (IEDDA); and (d) furan-maleimide Diels−Alder reaction.

4.3.1. Copper-Catalyzed Azide−Alkyne Cycloadditions (CuAAC). The (3 + 2) cycloaddition between an azide and alkyne proceeds spontaneously at high temperatures (>90 °C), producing a mixture of two triazole isomers.[186] However, these high temperatures are prohibitive for most applications, and the thermally induced reaction has found little use. A major breakthrough was achieved in 2002, independently by the groups of Sharpless[187] and Meldal[188], with the demonstration that copper(I) catalysis could dramatically promote and accelerate triazole formation (in exclusively the 1,4-conformation). This reaction was found to proceed at room temperature, even in ambient, oxygenated, and aqueous environments. These mild reaction conditions, together with the relative inertness of azides and alkynes and their absence from biological systems, has led to the copper-catalyzed azide−alkyne cycloaddition (CuAAC, often referred to as the "copper-click" reaction, Figure 8a) finding widespread use across the biological, chemical, and material sciences.[189−195]

Following on from the early work of the Hilborn[196] and Hawker[197] groups, Polizzotti et al. reported the formation of peptide−material conjugates by CuAAC, using alkyne-capped peptides to form hydrogels with azide-functionalized PEG (Figure 2).[198] Since this early work, the CuAAC has been widely used to functionalize scaffolds with alkyne and azide functionalized peptides and carbohydrates, in part due to the ease with which the amino acids azidolysine and homopropargylglycine can be introduced by solid-phase peptide synthesis.[193,199−203]

To achieve biomaterial conjugation via CuAAC, the required copper(I) catalyst can either be added directly, or generated in situ by reduction of an initial copper(II) complex, most commonly using ascorbic acid. The addition of a reducing agent further reduces the sensitivity of the CuAAC ligation to oxygen, which may otherwise detrimentally oxidize copper(I) to an inactive copper(II) species. Although no additional ligand is necessary for triazole formation, the addition of tertiary amine based ligands has been shown to stabilize the copper(I) catalytic complex and to significantly improve reaction kinetics and efficiency. A wide range of ligand and catalyst combinations have been reported for undertaking CuAAC reactions and reviewed elsewhere.[204] Importantly, the choice of a specific system is often dependent on the particular application in mind, and the interaction of the copper catalyst with the core scaffold structure, the ease of copper removal following conjugation, the concentration of the biomolecule partner, and the cost of catalyst−ligand systems must all be considered.

Azide and alkynes are generally stable under standard conditions, though a couple of important considerations should be taken into account when designing a CuAAC conjugation strategy. The alkyne reactive partner in particular is susceptible to side reactions. Perhaps most significantly, activated alkynes such as propiolic esters can react with thiols via thiol−yne addition, as described in section 4.2.1.4, making this substrate class poorly compatible with selective modification.[205,206] Similarly, propargyl esters and carbamates

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should be avoided, due to their susceptibility to copper-catalyzed cleavage.[205]

Despite the prevalence of CuAAC reactions in the biomedical field, the need for copper catalysis comes with severe drawbacks. The most prominent lies in the toxicity of residual metal, which is known to bind nonspecifically to peptide and protein substrates and is often difficult to remove.[207−210] Furthermore, it has been shown that copper may disrupt peptide and protein structure, leading to a loss of function following conjugation.[211] The effects of copper are even more deleterious for oligonucleotide sequences, with the copper-induced generation of reactive oxygen species (ROS) resulting in base modifications and backbone scission.[158] These problems are exacerbated by the need for high catalyst loadings to maintain conjugation efficiency, as a result of the high affinity of copper for both the triazole reaction product and many biologically relevant functional groups, effectively sequestering the active catalyst from the conjugation mixture.[200] These limitations can be partially mitigated by ligands able to strongly stabilize the active copper(I) catalyst or chelate azides to enhance reaction rates,[212−214] though copper induced damage to biomolecules is still problematic.[209]

Interestingly, the generation of active copper(I) via photo-initiated reduction of a copper(II) precursor has recently been reported by Adzima et al.[215] This allows the induction of CuAAC reactions with spatial and temporal control, with patterning of features as small as 5 μm being reported. This resolution is dictated by the speed at which the catalyst is deactivated by oxygen and is increased by the aforementioned affinity of the copper for the triazole reaction product which significantly slows catalyst diffusion.[215,216] Although the photoinitiated CuAAC has not yet been exploited for biomolecule conjugation, these early reports of spatial hydrogel patterning offer intriguing possibilities to do so in the near future.

4.3.2. Strain-Promoted Azide−Alkyne Cycloadditions (SPAAC). In 2004 the Bertozzi group published a seminal report on the use of strain, rather than metal catalysis, to promote the azide−alkyne cycloaddition (SPAAC, or often the "copper-free click reaction", Figure 8b).[217] Building upon the work of Wittig and Krebs in the 1960s,[218] they showed that highly strained cyclooctynes react readily with azides to form triazoles under physiological conditions, without the need for any added catalyst. Without the need for a transition metal species, the azide−alkyne cycloaddition is essentially nontoxic, with the high bioorthogonality of the reaction allowing it be applied to live cells and even inside animals.[219−223]

Since the original reports on SPAAC using simple cyclooctynes, a series of increasingly strained, and thus increasingly reactive alkynes have been reported, with corresponding increases in reaction rates and efficiencies (from an original reaction rate $k \approx 0.002$ M^{-1} s^{-1}, up to $k \approx 1$ M^{-1} s^{-1} in the most recent incarnations).[224] These moderate rates may prove high enough for efficient conjugation under standard conditions. However, even the most reactive cyclooctyne substrates may still result in low conjugation efficiencies at low concentrations of, for example, a biologically active protein.[225] Furthermore, increased rates of reaction come at the cost of decreased stability and therefore increased chance of cross-reactivity. In particular, cyclooctynes have been shown to react at non-negligible levels with thiol groups, reducing the bioorthogonality of SPAAC reactions in the presence of cysteine containing proteins and peptides.[221,226,227]

The low water-solubility of the cyclooctyne group may also prove problematic. In addition to the problems this creates in solubilizing the conjugation-partners and the decreased availability of the reactive handle, increased lipophilicity may also lead to increased nonspecific biomaterial binding and a loss of strategic value to pursuing site-selective conjugation.[224,228−230] Finally, the accessibility and availability of SPAAC reagents must also be carefully considered. The laborious multistep synthetic routes required for cyclooctyne construction often rely on specialist techniques, which in turn prevent their widespread production in biomedical and tissue engineering laboratories.[224,231] Although suitably functionalized derivatives are becoming increasingly commercially available, high costs partially limit their further application.

In spite of these disadvantages, the low toxicity and high selectivity under controlled conditions provided by SPAAC have made it a popular emerging method for conjugation. DeForest and Anseth were the first to demonstrate the use of SPAAC to cross-link PEG-azide based hydrogels, using cyclooctyne-functionalized, MMP-cleavable peptides (Figure 2).[232] A series of papers have subsequently developed this experimental setup,[233−235] with biomaterial functionalization in the presence of cells of particular note. In contrast to the analogous CuAAC modification strategy, little to no cellular toxicity has been reported in a variety of systems, even at high reagent concentrations.[230,234,236,237] This cytocompatibility of SPAAC reactions is particularly attractive, as is the lack of ROS generation, enabling the efficient functionalization and cross-linking of hydrogels with complementary DNA strands.[238]

In addition to the use of SPAAC for peptide conjugation, a number of prominent reports have used SPAAC to conjugate protein substrates to cyclooctyne functionalized biomaterials. Key to these reports is the introduction of an unnatural azide motif into the protein coupling partner. This has been achieved via a number of routes, including maleimide functionalization of native cysteines present in bone morphogenetic protein-2 (BMP-2),[239] via enzyme-mediated N-terminal modification of IFN-γ,[240] or via codon reassignment with the unnatural amino acid 4-azidophenylalanine in a number of protein substrates,[225] as discussed in section 10.

As an alternative to strain-promotion, the Francis group has recently reported that supramolecular host−guest interactions can also be used to promote azide−alkyne cycloaddition.[241] By bringing the two reactive partners into close proximity within the cavity of a cucurbit[6]uril host, efficient cycloaddition could be achieved on the surface of proteins. Although proximal tertiary amines were necessary on both the azide and alkyne coupling partner to promote formation of the necessary host−guest complex, such reactions offer the potential to overcome the limitations of both CuAAC and SPAAC noted above, and are of merit for further investigation in the field of biomaterial functionalization.

4.3.3. Inverse-Electron Demand Diels−Alder Reactions (IEDDA). In recent years, the inverse-electron demand Diels−Alder (IEDDA) reaction between 1,2,4,5-tetrazines and strained alkenes or alkynes has garnered significant interest (Figure 8c). IEDDA reactions offer high bioorthogonality and compatibility, along with very fast reaction rates.[242] Unlike standard Diels−Alder reactions, which often require elevated temperatures and utilize reactive groups which are susceptible to nucleophilic attack, IEDDA reactions proceed spontaneously to irreversibly release nitrogen gas and form a stable dihydropyridazine product. Through suitable choice of

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reaction partners, kinetics can be tuned over 10 orders of magnitude up to impressive rates of 10^5 M^{-1} s^{-1} (the reader is directed to an excellent recent review by Mayer and Lang for a detailed description of the factors influencing IEDDA reactions).[243]

Although tetrazine stability decreases with increasing reactivity, a wide range of suitable derivatives for undertaking biomolecule conjugation have been reported. For example, 4-hydro-tetrazines have been shown to degrade rapidly in buffered solution over a matter of hours, limiting their utility for reproducible conjugation. However, 4-methyl-derivatization greatly improves stability, while maintaining sufficient reactivity for effective conjugation.[228] Similarly, triazines have recently been shown to provide a compromise between high reactivity and effective conjugation.[244]

A variety of strained alkenes/alkynes are also suitable for IEDDA reactions. The most popular belong to a series of increasingly strained (and thus reactive) *trans*-cyclooctenes first introduced by the group of Fox.[245−248] Although cyclooctenes offer the highest reactivity, as for the cyclooctynes utilized for SPAAC their synthetic and commercial accessibility partially hinders their application. Furthermore, their ability to undergo isomerization to the significantly less reactive *cis*-alkene in the presence of thiols may prove limiting.[249] In contrast, suitably functionalized norbornene derivatives are widely available and thus provide a facile handle for undertaking IEDDA reactions. Although reaction rates are greatly reduced (typically 1−10 M^{-1} s^{-1}),[243] they remain high enough to undertake conjugation in most cases and compare favorably with those possible through CuAAC and SPAAC ligations.[250−252] Recently developed spirohexene reactive handles offer a potential alternative, combining sufficient stability with fast reaction rates.[253]

IEDDA reactions have been implemented in a number of instances for the formation of functional materials.[162,243,254−257] However, despite its attractive features the use of IEDDA for biofunctionalization has to date been limited. In the most prominent example, Alge et al. used norbornene-functionalized peptides to induce hydrogelation of tetrazine-capped PEGs (Figure 2).[258] In this case, the reactivity of the norbornene group was sufficiently high to allow rapid gelation, while still enabling thorough mixing and the minimization of gel defects. Furthermore, residual norbornene residues could be used for subsequent photoinduced thiol−ene reactions (see section 7.2), allowing further material derivatization and the incorporation of additional bioinstructive peptides and proteins. Importantly, Truong et al. have noted that the generation of nitrogen as a reaction side-product may be potentially detrimental to the use of IEDDA cycloadditions for hydrogel formation.[162] Particularly in dense networks, this gas can be trapped, forming bubbles which act as gel defects and disrupting the desired homogeneity of ideal gels.

4.3.4. Furan-Maleimide Diels−Alder Reactions. The hetero-Diels−Alder cycloaddition of maleimides and furans proceeds with good chemo-selectivity and is greatly accelerated in aqueous conditions, lending itself to the formation of biomaterial conjugates.[259−261] Although conjugation is possible at neutral pH, reaction rates are accelerated under slightly acidic conditions. Furan-maleimide additions have predominantly found use as a method to cross-link synthetic or natural polymers for the construction of core biomaterial scaffolds (Figure 8d).[172,262−265] Examples of such reactions being used for biomolecule conjugation are rare,[266,267] in part due to the rapid and well-established reaction of maleimides with thiols, as described above in section 4.2.1. Diels−Alder conjugations are therefore not suitable for functionalization in the presence of thiolated biomolecules or materials.

The desired Diels−Alder process is in equilibrium with the corresponding reverse retro-Diels−Alder reaction, regenerating the starting maleimide and furan. High temperatures are typically required in order to shift the equilibrium in favor of the starting materials; however, at 37 °C the contribution of the retro-reaction is non-negligible. While in many cases this may be detrimental to the final outcome, it can also be exploited for applications in which controlled release is desired. Koehler et al. have described the coupling of furan-functionalized RGDS peptides to maleimide-functionalized PEG-hydrogels.[268] By controlling the ratio of free maleimides in the system, which act to effectively trap the furan-peptide by promoting the forward Diels−Alder reaction, the rate of peptide release could be tuned. Alternatively, the effects of the retro-Diels−Alder reaction can be mitigated by utilizing difunctional peptides for cross-linking.[269] In such scenarios, although retro-reaction may still occur, the peptide is not released as it remains tethered at the other end, and the forward Diels−Alder reaction is allowed to proceed once again (Figure 9). It should be noted that the coupling of furans and maleimides can generate two stereoisomers, and thus a mixture of endo and exo products exists at ratios dependent on the precise structures and coupling conditions.[260] Although the impact of this is rarely discussed in the context of biomaterial conjugation, it is known that endo isomers undergo retro-Diels−Alder reactions at a lower temperature than the analogous exo structure.[270] In systems where reversibility is desired, this isomer-dependency is therefore an important design consideration.

4.4. Oxime and Hydrazone Formation

Aldehydes and ketones are absent from proteinogenic amino acids and typically only found at the open-form termini of reducing sugars. When combined with their propensity to react with a wide range of nucleophiles, this makes them attractive handles for undertaking polypeptide and glycan conjugation. As already described in section 4.1.2, aldehydes undergo reversible reactions with amines to form transient imines, which can then be trapped by reductive amination. However, under aqueous conditions hydrolysis by water dominates and the imine intermediate is highly disfavored and unstable. In contrast, the increased nucleophilicity of hydrazines and hydroxylamines allows them to form hydrazone and oxime bonds respectively, with greatly increased stability (Figure 10). These reactions are commonly used as a means to modify biomolecule substrates across the biological and physical sciences.[271]

Hydrazines react to form hydrazone linkages readily under mildly acidic or neutral conditions, with elimination of water. Although hydrazone bonds are more stable than the analogous imine, with an equilibrium favoring product formation, they remain prone to hydrolysis and their formation is thus reversible.[272] While this may be damaging if conjugate stability is required, the dynamic nature of the hydrazone bond can also be exploited to create self-healing or degradable materials (Figure 11).[69,273−275] Indeed, it has been shown that in vitro cytocompatibility and cell growth can be enhanced within structurally dynamic biomaterial scaffolds. The beneficial

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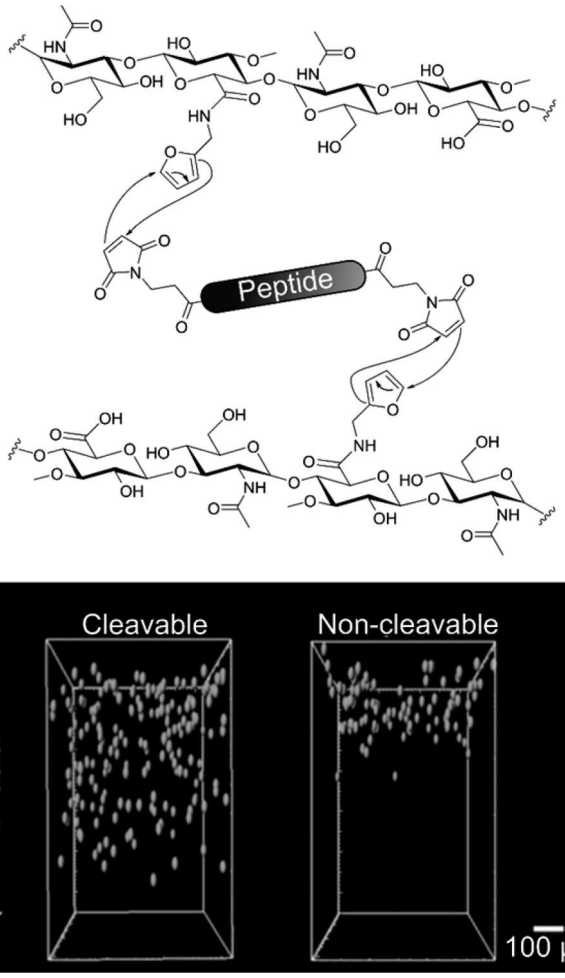

Figure 9. Furan-functionalized hyaluronic acid hydrogels can be cross-linked with a dimaleimide-functionalized peptide via Diels–Alder cycloaddition. MMP-cleavable peptides enable the migration of seeded breast cancer cells through the gel. Adapted with permission from ref 269. Copyright 2015 John Wiley and Sons.

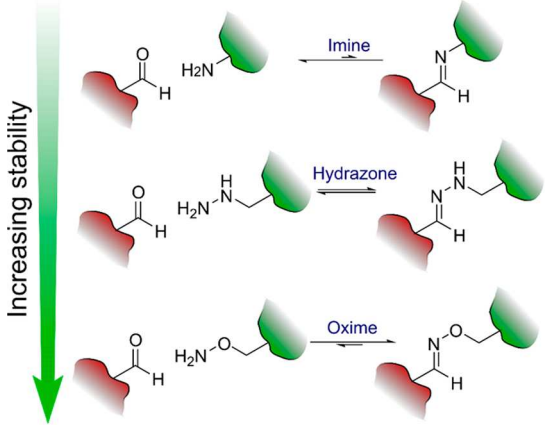

Figure 10. Addition of amines to aldehydes to form imines is a reversible process, with equilibrium strongly favoring the starting materials in aqueous solution. In contrast, hydrazones and oximes are less susceptible to hydrolysis, providing the possibility for stable, but reversible conjugation.

plasticity imparted by these structures is believed to allow cells to manipulate and model their local environment.[274]

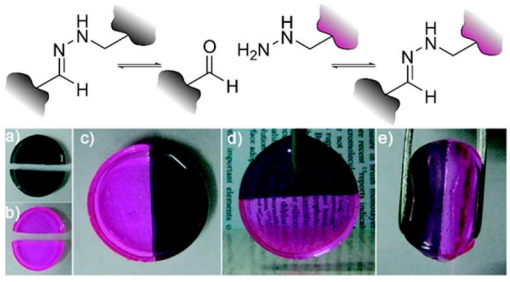

Figure 11. Reversibility of hydrazone formation enables materials to possess self-healing properties. (a and b) Dye-loaded, hydrazone cross-linked PEG-hydrogels are cut in half; (c–e) incubation for 7 h is able to merge two halves placed together via reformation of hydrazone linkages at the gel interface. Adapted with permission from ref 273. Copyright 2010 American Chemical Society.

The rate of hydrolysis is closely linked to hydrazone structure, and in particular basicity. Both bond formation and hydrolysis are promoted under mildly acidic conditions, following protonation of the imine nitrogen. Oommen et al. have demonstrated that hydrazino-ureas allow a greater than 15× increase in stability to be achieved under mildly acidic conditions, and with negligible hydrolysis at neutral pH as a result of the electron withdrawing urea motif.[276] Similarly, equilibrium can be shifted strongly toward product formation, through the use of aliphatic rather than aromatic hydrazines.[274,277] The rate of hydrolysis can therefore be tuned over several orders of magnitude, from minutes to months, depending on the desired application.[272] The stable attachment of peptides and DNA to biomaterials via hydrazone formation can be achieved via difunctional cross-linking. In such a scenario, even with reversible hydrolysis at one end of the biomolecule, the stable attachment at the other prevents release of the conjugate.[278,279] However, as described below oximes often provide a more suitable linkage for such applications.

In contrast to hydrazones, oximes possess greatly improved hydrolytic stability with the reaction equilibrium lying far toward product formation (Figure 10). Even under acidic conditions which promote hydrolysis, stable constructs can be formed, although again stability is highly dependent on the exact structure and local environment.[272] As a result, oximes have been more commonly utilized for the formation of biomolecule-material conjugates. Esser-Kahn and Francis first reported the attachment of ketone or aldehyde modified green fluorescent protein (GFP)[280] or metallothionein[281] to hydroxylamine-functionalized synthetic polymers (Figure 2). Protein cross-linked hydrogels were produced through oxime modification at both the protein N- and C-termini. Oxime formation has subsequently been utilized for the modification of hydroxylamine functionalized hydrogels with ketone-modified adhesion peptides,[282] the formation of cell supporting glycopolymer films,[283] and the light-induced attachment of aldehyde-functionalized proteins to photocaged hydroxylamine gels[235] (see section 8 for further details).

Despite these promising reports, the improved properties of oximes over hydrazones, and the precedent for oxime ligation in the field of site-selective protein modification,[36,271] oxime formation has yet to be fully embraced by the biomaterial community as a means to undertake biomolecule conjugation. Instead, the field has primarily focused on the use of oximes to

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Table 2. Advantages, Disadvantages, and Selectivity of the Enzymatic Conjugation Strategies Outlined in Section 5

enzyme class	advantages	disadvantages	selectivity
transglutaminases	broad substrate scope	promiscuity can limit site-selectivity	sequence selectivity dependent on isoform
	good availability		conjugation between lysines and glutamine residues
	tolerant of sequence substitution		often highly promiscuous with low selectivity
	can be used with a wide variety of protein substrates		
peroxidases	high availability	requires added hydrogen peroxide	oxidative conjugation between phenols, such as tyrosine
	broad substrate scope	low selectivity	sequence independent
	small molecule and synthetic mimics available		leads to thiol oxidation
	reaction rate easily tuned		
sortase	high sequence specificity	enzyme is expensive, limiting scale	N-terminal "LPXTG" tag to a C-terminal "GGG" tag
	efficient conjugation	"tag" must be introduced	very high specificity
	peptide "tag" is short and minimally disruptive		
SpyTag-SpyCatcher	inherently reactive peptide−protein pair	SpyCatcher is a large and potentially disruptive "tag"	very high specificity
	potential for orthogonal pairs	requires recombinant engineering of biomolecule	affinity can be tuned by structure alteration

form injectable, biocompatible, and mechanically tunable materials for biomedical applications.[275,282,284]

In addition to the resultant stability and function of hydrazone/oxime linkages, a number of other key considerations should be taken into account during material design. Although the kinetics of hydrazone/oxime formation are often satisfactory for conjugation, in a dilute regime they may limit product formation. Dirksen and Dawson reported that the addition of a nucleophilic catalyst, such as aniline, can promote hydrazone/oxime formation via an intermediate aniline-imine. As a result, dramatic rate enhancements can be achieved in the range of 10−1000 M^{-1} s^{-1}.[116,285,286] However, the high toxicity of aniline prevents the benefits of this adaptation being exploited during conjugation in the presence of cells. Finally, the potential toxicity of aldehyde reactive handles must be considered. The propensity of aldehydes to transiently form imines with amines present in biological systems may induce cell death, particularly at high concentrations.[278,287,288] Ketones offer an alternative reactive handle for both hydrazone and oxime formation in such scenarios. Although they are less electrophilic, and thus form adducts at a reduced rate, the lowered toxicity of ketones may prove beneficial for conjugation.[282,287]

5. ENZYME-MEDIATED CONJUGATION

Enzymatic methods for polypeptide conjugation have a number of key advantages that set them apart from their synthetic counterparts. Among these, the high sequence-specificity, the ease of introducing reactive sequences via mutagenesis, the mild conditions required for conjugation, the tolerance of complex biological milieu, and the lack of detrimental effects on other biomolecules and cells are of particular importance. Enzymatic-modification of carbohydrates has typically been restricted to the extension, remodelling, or trimming of preinstalled oligosaccharides and thus falls outside the scope of this reviews. Readers are instead directed to comprehensive reviews on this topic.[289−291] Similarly, examples of enzymatic oligonucleotide conjugation to biomaterial scaffolds are rare. In this section, we will therefore focus on the key enzymes that have found use in peptide and protein material conjugation (see Table 2).

5.1. Transglutaminase and Factor XIII

The transglutaminase enzyme family catalyzes the formation of isopeptide bonds between the primary amine of lysine side chains and the amide bonds of a complementary glutamine residue. The resultant bonds are highly resistant to degradation and are used in nature to form cross-linked networks of insoluble protein. For example, the transglutaminase Factor XIII plays an important role in triggering the coagulation of blood.[292,293] The substrate scopes of transglutaminases are often broad, although preferred conjugation sequences have been identified for specific enzymes based on their natural cross-linking partners.

The first example of transglutaminase-mediated hydrogel formation was reported by Sperinde et al. (Figure 2). Tissue transglutaminase (tTG) was used to induce the cross-linking of a glutaminamide terminated PEG and a random copolymer of lysine and phenylalanine residues.[294] Although tTG favors hydrophobic residues proximal to the lysine coupling partner and the presence of glutamine repeats, it possesses low sequence specificity and will cross-link a wide range of lysine/glutamine functionalized sequences.[295] This high promiscuity limits the use of tTG for achieving site-specific conjugation. As a result, tTG has been most commonly utilized for the cross-linking of protein based materials.[296,297]

In contrast to the promiscuity of tTG, the transglutaminase Factor XIII possesses improved sequence selectivity, though over 150 Factor XIII substrates have still been identified from natural cross-linking sites in human plasma alone.[298] Schense and Hubbell demonstrated that Factor XIII could be used to conjugate RGD and DGEA peptides to fibrin matrices by appending native Factor XIII substrates prior to the bioactive sequence, imparting nonfibrin properties.[299] Although peptide sequences functionalized with lysine reactive handles were found to be more tolerant of substitution, reversing the coupling partners and exploiting glutamine-containing substrates resulted in higher conjugation efficiencies and a higher density of bioactive sequence display.[299,300] Since this initial work, the use of Factor XIII to functionalize materials with

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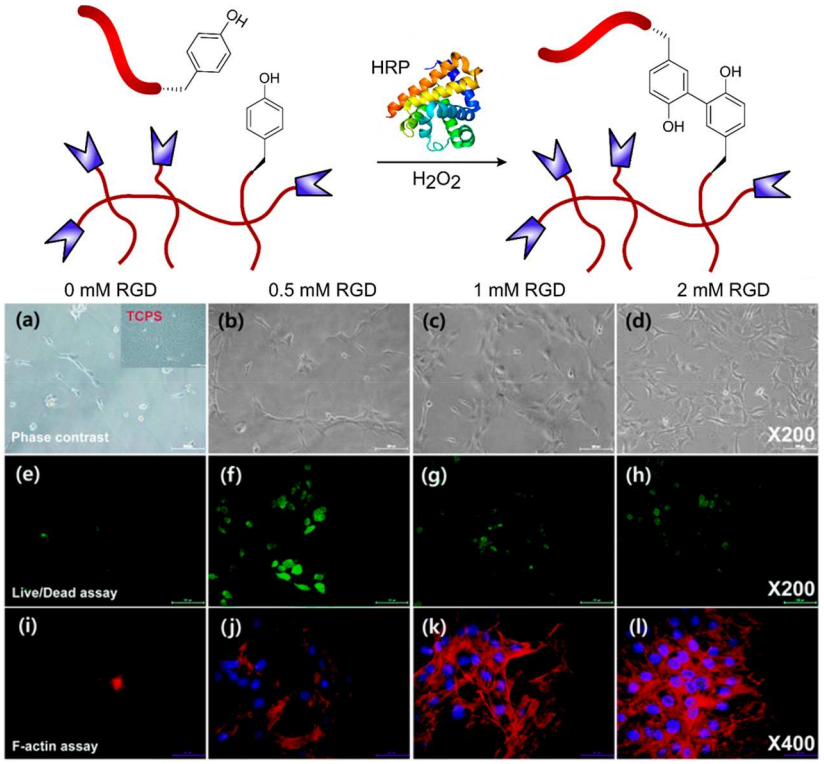

Figure 12. HRP/H_2O_2 treatment can lead to the formation of diphenol linkages between tyrosine containing peptides and phenol-capped synthetic polymers. In doing so, hydrogels bearing different levels of pendant RGD peptides can be produced. (a–d) Phase contrast microscopy images of seeded MC3T3-E1 cells at different RGD grafting densities; (e–h) live/dead assay, green, living cells and red, dead cells; (i–l) F-actin (red, phalloidin) and nuclei staining (blue, Hoechst 33258). Adapted from ref 317 with permission of The Royal Society of Chemistry.

both peptide[149,300−302] and protein[149,303,304] based substrates has become widespread, primarily due to the facile nature of conjugation and the ease with which coupling sequences can be incorporated into the polypeptide of choice. However, while Factor XIII may be more selective than many other transglutaminases, it is still able to catalyze the cross-linking of a diverse range of substrates. Although this may be advantageous in some scenarios, it may also limit selectivity and lead to problems with cross-reactivity.

5.2. Peroxidase-Mediated Conjugation

Peroxidases catalyze oxidation of their substrates, most commonly converting hydrogen peroxide to water in the process. Horse radish peroxidase (HRP) is the most widely utilized member of the peroxidase family, due to its ability to oxidize a wide range of organic substrates. In the presence of hydrogen peroxide, HRP can oxidize the phenol group of tyrosine, as well as other electron-rich aromatic groups, generating a highly reactive radical or quinone intermediate.[305] Oxidised species then undergo spontaneous dimerization, resulting in the formation of an ortho carbon−carbon bond between two tyrosine residues.

During HRP-mediated conjugations the reaction kinetics and efficiency are highly dependent on the precise experimental conditions. Factors such as the enzyme and hydrogen peroxide concentration, reaction temperature, pH, and reagent structure can play a major role.[306−308] Indeed, the flexibility this offers is a generally beneficial property of enzyme-mediated conjugations over their chemical counterparts. During HRP catalysis an increase in hydrogen peroxide concentration is typically associated with an increase in reaction rate. However, above a critical peroxide concentration enzyme inhibition begins to occur and a rapid drop-off in oxidation activity is observed.[309,310]

HRP-mediated oxidation has been widely applied to cross-link protein based materials, as well to nonspecifically attach peptides and proteins to oxidation sensitive substrates.[310−316] In contrast, there are relatively few reports on controlled HRP-mediated conjugation. This is largely due to the need to exclude exposed tyrosine residues from the surface of the polypeptide of interest in order to ensure selectivity, as well as the susceptibility of cysteine residues to HRP-mediated oxidation. The Park group have reported the attachment of pendant tyrosine-capped peptides to phenol-functionalized tetronic and gelatin polymers in the presence of HRP and hydrogen peroxide (Figures 12 and 2).[317,318] A similar strategy has been utilized by Menzies et al. to conjugate fibronectin derived peptides to PEG-based scaffolds.[319] In these reports, conjugation and scaffold cross-linking occur in parallel, with fast reaction kinetics.

Singh et al. have demonstrated that, even in the absence of added peroxide, the background auto-oxidation of thiol groups is able to generate sufficient stoichiometric concentrations of hydrogen peroxide to participate in HRP-mediated hydrogelation via disulfide formation.[165] While this mechanism of HRP action has not yet been utilized for peptide/protein conjugation, it offers intriguing possibilities for controlled disulfide formation, as described in section 4.2.2. Alternatively, the oxidizing enzyme laccase offers possibilities for peptide and protein conjugation in the absence of added peroxide, due to its ability to utilize atmospheric oxygen to undertake phenol oxidation, generating water as the only byproduct.[320] Finally, a large number of reagents able to mimic the activity of HRP have been developed, which avoid the potential limitations of

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enzyme instability and environmental sensitivity. Many such systems exploit metal complexes able to oxidize organic substrates in the presence of hydrogen peroxide. For example, Kakinoki and Yamaoka recently demonstrated the use of copper-mediated tyrosine oxidation as a means to attach cell-adhesive peptides to polymers, via the formation of an intermediate reactive catechol motif.[321]

5.3. Other Enzymatic Methods

Over the last 15 years a number of short peptide "tags" have been identified which are able to undergo efficient enzymatic modification with high sequence specificity. These "tags", as short as 5 amino acids long, can be appended to a peptide or protein substrate in order to allow their subsequent modification.[322] Despite the interest in using these powerful enzymatic strategies to undertake site-selective protein modification, particularly for cellular labeling, they have been more rarely used for the formation of peptide/protein-biomaterial conjugates. In an early example, Mosiewicz et al. exploited the ability of phosphopatetheinyl transferases to conjugate coenzyme A (CoA)-functionalized molecules to carrier proteins to form peptide functionalized hydrogels.[323] Although the carrier protein can be condensed down to a peptide "tag" as short as 11 amino acids, the authors instead used dimers of the full length protein to first cross-link CoA-functionalized PEG. The hydrogels were then further derivatized using CoA-functionalized RGDS peptides. Although conjugation was found to be slow compared to alternative enzymatic methods for polypeptide conjugation, functionalized materials could be produced in a highly specific manner.

More recently, Cambria et al. reported the ligation of human epidermal growth factor (hEGF) to PEG hydrogels, via the action of the prokaryotic enzyme sortase (Figure 2).[324,325] The five amino acid long sortase "tag" LPXTG was first introduced into the hydrogel structure via Michael addition, as discussed in section 4.2.1. Recombinantly expressed hEGF bearing the C-terminal GGG motif required for sortase-mediated ligation was then conjugated in the presence of an engineered sortase derivative with enhanced reaction kinetics.[326] Gao et al. subsequently demonstrated that sortase could be used to functionalize stimuli-responsive microgels with recombinant proteins.[327] Given the increasing prominence of sortase-mediated protein modification and reports on the optimization of enzymatic activity and specificity, it is likely that such reactions will find increasing utility in biomaterial functionalization in the near future.[328,329]

Despite these important demonstrations of peptide "tag" modification for biomaterial functionalization, a number of alternative prominent enzymatic modification systems have so far been largely overlooked by the biomaterials community, including biotin ligase (discussed in further detail in section 9.4),[330] formylglycine-generating enzyme,[331] and lipoic acid ligase[332]. This may be due in part to the large quantities of enzyme required for efficient biomaterial modification, in contrast to the small volumes required for traditional applications such as intracellular labeling or the formation of small molecule conjugates. This is particularly true when considering the high cost of many of the enzymes noted in this section, which severely limits the widespread application of such techniques.

The need to introduce the "tag" into a protein target prior to labeling may also prove limiting. However, in an exciting recent development, Weeks and Wells have demonstrated that mutant libraries of the peptide ligase subtiligase can be generated with specificity for a wide range of N-terminal dipeptide sequences. In doing so, the enzyme-mediated modification of native protein substrates will likely be enabled without the need for prior engineering, and it is anticipated that this work will have great impact on the biomaterial field in the coming years.[333]

Inherently reactive peptide sequences, which do not require enzymes to induce conjugation, are an attractive alternative to the systems described above. For example, peptides derived from naturally occurring isopeptide linkages found in Gram-positive bacteria have been shown to spontaneously cross-link under mild conditions. The most prominent sequences are the SpyTag-SpyCatcher peptide pair, derived from the fibronectin-binding protein of *Streptococcus pyogenes*.[334,335] Sun et al. demonstrated that this system could be used to cross-link and functionalize suitably derivatized elastin-like protein hydrogel networks with complementarily functionalized cytokines.[336] Gao et al. subsequently reported the tethering of SpyTag-modified proteins to SpyCatcher-functionalized hydrogels.[337] Recent developments identifying orthogonal SpyTag-SpyCatcher pairs offer intriguing possibilities for the generation of multifunctional biomaterials.[338]

6. POLYMERIZATIONS OF LOW MOLECULAR WEIGHT MONOMERS

Polymerization can be classified as proceeding via one of two mechanisms, either chain-growth or step-growth. During chain-growth polymerization, monomers are added at the "active" end of a growing polymer chain, resulting in the formation of high molecular weight materials even at low conversions.[339] In stark contrast, during step-growth polymerizations short oligomer chains couple to form polymeric species, requiring high conversions in order to reach high molecular weights. Both techniques can be used to form biomolecule−polymer conjugates, though the free radical-mediated chain-growth polymerization of vinyl monomers has been by far the most popular method. The polymerization of acrylate and methacrylate monomers has proven particularly fruitful and has found widespread utility throughout material science.[340,341]

In this section we will focus on the key aspects that make chain-growth polymerizations suitable for the formation of biomolecule−material conjugates. In particular, we will discuss systems in which this is achieved through a monomer prefunctionalized with the biomolecule of choice, commonly referred to as a "grafting-through" approach.[342] This is in contrast to systems which rely on a biomolecule initiator ("grafting-from") or conjugation to a preformed polymer ("grafting-to"). For a discussion of these techniques, as well as a comprehensive overview of the field of free-radical polymerization, the reader is directed to a number of excellent reviews on these topics.[342−347]

Among the most attractive features of free-radical polymerizations are their robustness, synthetic ease, and tolerance of water, as well as the diversity of functionalized monomers that can be utilized.[341] Acrylate and methacrylate modified peptides and glycans can be readily polymerized, representing one of the oldest methods to produce materials bearing a high density of pendant functionalities.[199,348−351] Similarly, due to the widespread availability of the synthetic oligonucleotide phosphoramidite building block "Acrydite", free-radical poly-

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Table 3. Advantages, Disadvantages, and Selectivity of the Photo-Conjugation Strategies Outlined in Section 7

photoconjugation strategy	advantages	disadvantages	selectivity
acrylate-cross-linking	versatile	requires high radical concentrations	thiols will also react with acrylates
	high functional group tolerance	highly damaging to biomolecules	
		acrylates can be toxic	
thiol−ene	greatly reduced radical concentration required	even low radical concentrations potentially damaging	alkene reactivity must be matched to thiol to prevent chain transfer
	stable radicals generated	thiols often key for bioactivity	
	rapid reaction kinetics		
	allows 1-photon and 2-photon patterning		
	low occurrence of cysteine enhances selectivity		
	ease of introduction		
thiol−yne	greatly reduced radical concentration required	even low radical concentrations potentially damaging	alkyne reactivity must be matched to thiol to prevent chain transfer
	stable radicals generated	thiols often key for bioactivity	
	more efficient than corresponding thiol−ene	slower than corresponding thiol−ene reactions	
	results in bis-addition		
	allows 1-photon and 2-photon patterning		
	low occurrence of cysteine enhances selectivity		
	ease of introduction		

merization remains one of the most common methods through which to form DNA and RNA functionalized biomaterials.[76,352−356] By undertaking polymerization in the presence of a comonomer, the density of biomolecule presentation can be easily tuned, allowing potential difficulties from steric hindrance to be overcome.[342] Initiation of polymerization can be triggered by a number of means, including heat, UV and visible light (as discussed in section 7.1), redox reactions, and electrochemistry.[357] Radical polymerizations have therefore been widely adopted by the field to produce functional biomaterials.[199,339,349−351,358,359] Indeed, acrylate modified proteins can also undergo polymerization to produce functional materials, while retaining biological activity.[360,361]

Although free-radical chain-growth polymerizations are attractive for the formation of biomolecule conjugates, a number of concerns with their use remain. Monomer toxicity can be limiting when polymerization is undertaken in the presence of cells. This is enhanced by the slow rate with which chain-growth polymerizations approach complete monomer conversion. Acrylates are particularly toxic, and as such methacrylate based monomers are often used in cellularised systems, albeit at the cost of reduced polymerization kinetics.[362] However, more significantly the intrinsic radical nature of the reactive species is in itself highly limiting. In addition to imparting high sensitivity to oxygen,[363] the reactivity of the "active" chain-end can potentially damage proteins, DNA/RNA, and cells via chain transfer.[339] Although damage can be partially mitigated in the presence of an excess of reactive monomer, the danger of unforeseen drops in conjugate activity still exist.

More recently "living" radical polymerizations (LRPs) have emerged as attractive alternatives to standard free-radical polymerizations. LRPs are controlled by a dynamic equilibrium between a dormant chain and the active radical, minimizing chain termination.[346] The most commonly used LRPs for the formation of bioconjugates include atom-transfer radical polymerization (ATRP),[364−367] reversible addition−fragmentation chain transfer (RAFT) polymerization,[365,368,369] and nitroxide-mediated polymerization (NMP)[370]. Toxicity resulting from residual catalysts and metals, or the products of RAFT agent cleavage, may be a potential drawback of LRP techniques but is offset by a greatly reduced active radical concentration. This not only limits potential biomolecule damage but also provides high control over molecular structure, material architecture, and biomolecule distribution.[46,342] Many exciting developments in the field of LRP are continuing to push forward our ability to synthesize polymer−bioconjugates. While many cutting-edge techniques are yet to find application for biomaterial functionalization, they provide exciting opportunities for future advances. Readers are directed to a number of excellent recent reviews which highlight the advantages provided by new LRP methodologies and their potential advantages over currently employed techniques in the biomaterial community.[345,347,371−373] For example, recent reports on cytocompatible radical polymerizations using photoinduced electron transfer (PET)-RAFT polymerization and oxygen tolerant LRPs may overcome the limitations of traditional methods.[373−375]

In contrast to chain-growth polymerizations, step-growth processes must reach high conversions to produce high molecular weight materials. As a consequence, high molecular weight "macromers" are often used to drive scaffold formation. Most commonly this is achieved via the "polymerization" of a suitably functionalized natural or synthetic macromer with a multifunctional complementary cross-linker.[339] Many examples of such chemistries are discussed throughout the course of this review, in the context of chemical or photoinduced biomolecule conjugation techniques, and so will not be discussed here. However, it should be noted that polymer cross-linking reactions using radical or nucleophilic thiol−ene reactions, CuAAC reactions, or hydrazone/oxime formation as discussed above, all in effect proceed via a step-growth mechanism.

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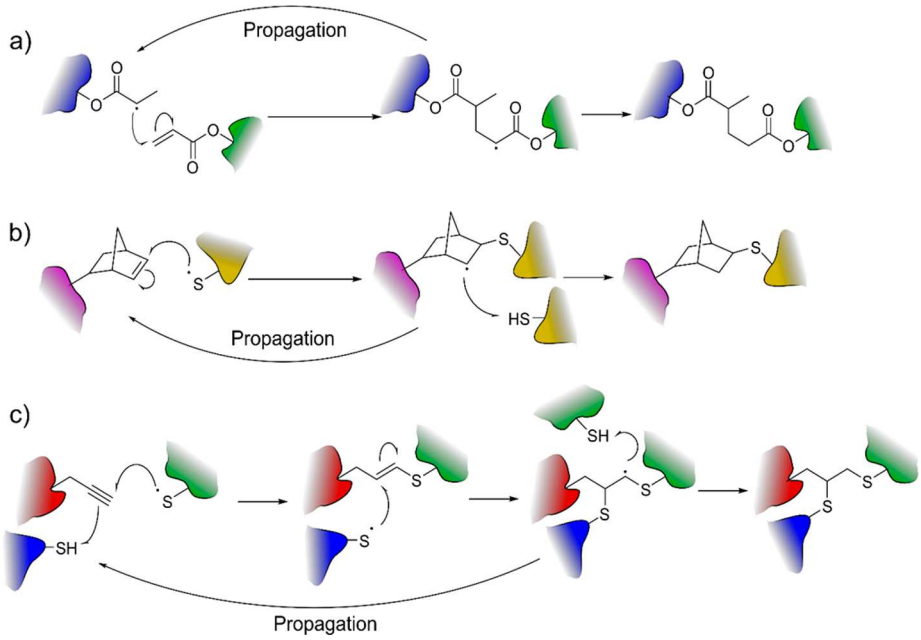

Figure 13. Conjugation mechanism of (a) photoacrylate cross-linking; (b) photo thiol−ene reaction; and (c) photo thiol−yne diaddition.

The step-growth polymerization of low-molecular weight monomers has been far less commonly utilized. One such system has been recently reported by the groups of Grubbs and Tirrell, using the ring-opening metathesis polymerization (ROMP) of peptide-functionalized strained norbornene monomers, to produce functionalized films that support cell growth and adhesion.[376] Building upon the work of Maynard et al. in the early 2000s,[377,378] efficient ruthenium catalysts were used to access high molecular weight polymers which could be subsequently cross-linked to form free-standing films. Although these reports demonstrate the utility of peptide monomers in producing functional biomaterials, they also highlight the inherent limitations of using step-growth polymerizations as a method for producing polypeptide conjugates.

7. PHOTOCONJUGATION AND ACTIVATION

The use of light to instigate conjugation chemistry has emerged over the last 25 years as a powerful technique in the biomaterial field. In particular, it offers the opportunity to control the spatial and temporal patterning and activity of biomolecules, as well as to manipulate the physical structure of a core scaffold.[379] These photomediated techniques typically generate a reactive radical species or cause bond scission to generate a reactive intermediate which can subsequently undergo bond formation. Ultraviolet (UV) irradiation has long been used as an initiator for the polymerization and conjugation of vinyl based monomers and functional materials.[380] However, recent trends toward less-cytotoxic visible-light initiation systems,[381] functionally selective conjugations which minimize biomolecule damage,[105,382] and two-photon excitation to allow precise 3D-patterning have revolutionized the field.[383] Here, we will focus on the key light-mediated techniques that have been used for direct biomolecule-material conjugation (see Table 3). For a detailed overview of the use of photosystems for controlling material properties and prepatterning of reactive handles, as well as the key technological developments which have enabled the impressive control offered by cutting-edge techniques, the reader is referred to a number of comprehensive reviews in the field.[383−386]

7.1. Photoacrylate Cross-Linking

In section 6 we discussed the free-radical polymerization of vinyl monomers, particularly acrylates and methacrylates bearing pendant biomolecules. Polymerization is initiated by the production of a radical species, which then propagates through bond formation to create an "active" polymer chain (Figure 13a). This key initiation step can be induced via a number of stimuli, with thermal decomposition, redox activation, and electrochemical ionization of an initiating species being among the most common.[357,387] Alternatively, many initiators can be activated via light-induced photolytic bond breakage (type I) or photoactivated abstraction of protons from a co-initiator (type II).[388] Photoinitiation offers the benefits of being applicable across a wide temperature range, using narrow and tunable activation wavelengths dependent on the initiator used, rapidly generating radicals, and the ability to control polymerization by removing the light source.[357,389] Importantly, the tolerance of polymerizations to oxygen is greatly enhanced, enabling polymerization in the presence of cells and tissues.[390,391] The incorporation of acrylate-functionalized peptides[154,392−399] and proteins[394,400−406] during photopolymerization has therefore been widely used as a method for producing biomaterial conjugates. Alternatively, the photoinitiated attachment of polypeptides to pendant vinyl groups on preformed materials has also been widely reported and more recently used for 3D patterning via two-photon excitation.[121,398,403,407,408]

A wide range of photoinitiators have found use in photoacrylate conjugations. The ideal choice of initiator is dependent on a number of factors, including the activation wavelength, water solubility, and, perhaps most importantly in the context of this review, biocompatibility of activation. Many early reports of biomaterial formation and peptide functionalization focused on the use of Eosin Y[154,390] and 2,2-dimethoxy-2-phenyl-acetophenone[394,396,400] (commonly re-

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ferred to by its trade name Igracure 651). In subsequent years, other initiators with improved cytocompatibility, such as Igracure D2959, have come to prominence.[399,402,404,406,409] Most recently, initiators with increased water solubility, such as lithium phenyl-2,4,6-trimethylbenzoylphosphinate,[410] or visible light activatable systems such as riboflavin[411,412] have become more common.

Although acrylate and methacrylate based systems are most prevalent, alternative activated alkenes can also undergo polymerization, with vinyl sulfones displaying particularly rapid cross-linking due to destabilization of the intermediate radical species during propagation. Day et al. recently showed that vinyl sulfone cross-linked hydrogels elicited a reduced immune response in vivo when compared to the equivalent acrylate-linked structure, though further investigation is essential to assess the generality of this observation.[413]

Photoacrylate cross-linking has been widely used in the fields of biomaterials and tissue engineering due to its ease of use. However, such techniques are severely limited by the damage caused to proteins, oligonucleotides, and cells by a combination of UV light initiation, highly reactive acrylates, and the propagating radical species generated during conjugation. On its own, low wavelength UV light (<350 nm) can cause detrimental localized heating, protein degradation, and DNA/RNA scission.[414,415] When biomolecule-functionalized monomers are polymerized this problem is exacerbated by the need for long UV exposure times, in order to maintain the propagating radical species for long periods. Protein activity can also be lost following the light-independent modification of lysine residues by reactive acrylates via Michael addition.[160,416] However, the most important limitation is the damage caused by radicals following initiator activation. As early as 2000, Bryant et al. highlighted the toxicity of a number of UV photoinitiators including Igracures I184, I907, I651, and D2959.[417] The authors hypothesized that the nature of the generated radical (methyl vs benzoyl) played an important role in determining the level of toxicity observed. Of the initiators investigated, Igracure D2959 was found to cause the lowest levels of damage. These results were supported by Williams et al., who also highlighted that different cell types have altered susceptibility to radical induced toxicity, with cells with high proliferation rates most significantly affected.[418] A number of subsequent papers have discussed the origins of the observed toxicity, with the persistence of radicals on growing polymer chains,[419] the generation of radicals following monomer consumption,[420] and the difference in initiator hydrophobicity[421] all being used to explain differences between initiators.

In addition to the toxicity caused to cells, the generation and persistence of radicals is also highly detrimental to the activity of tethered peptides, proteins, and oligonucleotides during acellular biomaterial conjugation. Although there have been conflicting reports on the degree and nature of the damage caused, it is clear that even in cases where UV irradiation alone does not cause significant problems the generation of reactive radicals can be highly damaging.[160,420−422] In particular, the generation of ROS as a result of the radical activation of triplet oxygen has been shown to lead to protein cleavage and a multitude of detrimental alterations to oligonucleotide structure and function.[423−426] Although drops in protein activity can be partly mitigated by careful control of irradiation strength, duration of application, and initiator choice, even under such conditions the drop in bioactivity is still an important consideration. As a result, although acrylate-cross-linking is still widely used in the biomedical community it is becoming increasingly unpopular for the conjugation of biomolecule substrates to biomaterial scaffolds. Instead, methods which require far lower radical concentrations, such as those discussed in the following sections, have become more favored.

7.2. Photo Thiol−ene Reactions

In section 4.2.1, we discussed the Michael addition of thiols to electron-deficient alkenes, so-called nucleophilic thiol−ene reactions. Thiols can also react with alkenes via a free-radical mechanism. A thiol radical first reacts with an alkene to generate a carbon-centered radical, which can then abstract a proton from another thiol and thus propagate the reaction (Figure 13b).[427] This photo thiol−ene reaction offers several benefits over photoacrylate cross-linking methods, including rapid reaction kinetics, procedural simplicity and robustness, lowered risk of nonproductive side reactions, and importantly an even further reduced sensitivity to oxygen as a result of the increased stability of thiol radicals.[424] This radical stability also leads to a significant reduction in the number of radicals which need to be generated for high conjugation efficiencies to be achieved, with a concomitant reduction in damage to the cells and biomolecules present.[420] Many of the severe drawbacks of photo-cross-linking reactions are therefore largely mitigated, and photo thiol−ene reactions have emerged as the reaction of choice for photopatterned conjugation, particularly of peptides and proteins.[105,130]

A major advantage of the photo thiol−ene reaction is its broad substrate scope. In order to achieve efficient conjugation, it is essential that the intermediate carbocation induces propagation by regenerating a thiol radical, rather than reacting with another "ene" to cause alkene-cross-linking and chain transfer.[427] Alkene choice is therefore largely determined by the rate of conjugation, the ability to limit this damaging chain transfer process, and importantly synthetic accessibility. In contrast to their nucleophilic analogues, photo thiol−ene reactions are generally accelerated by electron-rich alkenes, which generate unstable carbon-radical intermediates able to rapidly abstract thiol-hydrogens. Exceptions to this rule are norbornene derivatives, in which reactivity is driven instead by the release of ring strain upon thiol addition. This leads to a general trend in reactivity of norbornene > vinyl ether > propenyl > allyl ether > acrylate > maleimide.[428] Norbornenes and allyloxycarbonyls (alloc groups) have been particularly widely used for peptide/protein-biomaterial functionalization, due to the almost negligible contribution of chain transfer and their ease of introduction during peptide synthesis, respectively. In contrast to acrylate photo-cross-linking, the reactive handles utilized in both cases are essentially inert to protein or cellular substrates prior to irradiation, providing significantly improved specificity and temporal and spatial control.

In an early example, Polizzoti et al. demonstrated that the alloc group, typically used as an orthogonal lysine protecting group during solid-phase peptide synthesis, is an efficient photo thiol−ene reactive handle (Figure 14a). Alloc-functionalized PEG hydrogels could therefore be photopatterned with cysteine-capped RGDS peptides following irradiation in the presence of Igracure D2959.[198] Alloc groups have been predominantly utilized for the patterning of pendant peptides within PEG hydrogels, including through the use of visible light and highly water-soluble photoinitiators (Figure 15).[232−234,429,430] In a series of papers, the Anseth group

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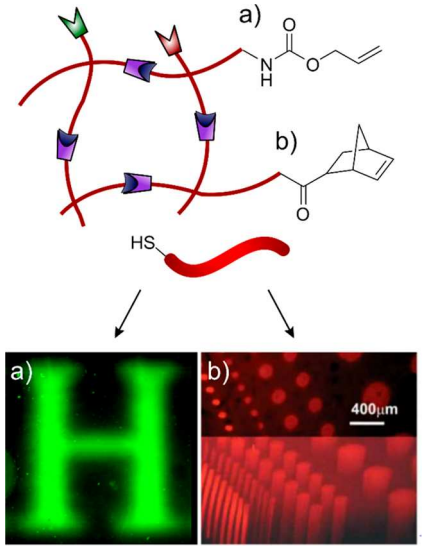

Figure 14. (a) Alloc- and (b) norbornene-handles are most widely used for photo thiol−ene reactions and can be used to create 3D patterns of pendant fluorescently labeled peptides. Panel a is adapted with permission from ref 198. Copyright 2010 American Chemical Society. Panel b is adapted with permission from ref 117. Copyright 2009 John Wiley and Sons.

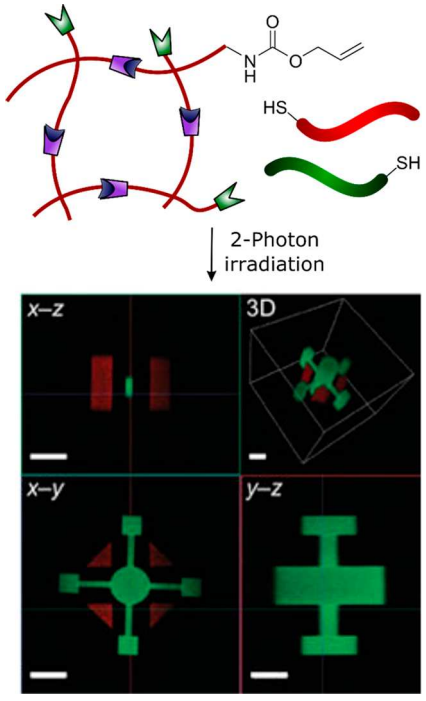

Figure 15. Two-photon activation can be used to induce photo thiol−ene reactions with precise 3D resolution. In doing so, intricate patterns of multiple fluorescently labeled peptides can be sequentially introduced into an alloc functionalized hydrogel. Scale bar = 100 μm. Adapted by permission from Springer Nature: ref 234. Copyright 2011.

subsequently demonstrated the power of norbornene reactive handles for both the cross-linking and pendant functionalization of synthetic polymers with cysteine capped peptides, in the presence of Igracure D2959 (Figure 14b).[117,258,431−435] Following these initial reports, a number of groups have gone on to demonstrate the use of norbornene photo thiol−ene reactions for the tethering and spatial patterning of bioactive peptides and growth factor proteins.[436−440]

In addition to the most commonly used alloc and norbornene reactive groups, other alkenes have also been used for biomaterial functionalization. For example, codon reassignment has been used to site-specifically incorporate allyl-cysteine residues into proteins, which can subsequently undergo conjugation through the use of photo thiol−ene reactions.[441] Alternatively, acrylates can undergo mixed-mode photopolymerizations in the presence of cysteine capped peptides,[442,443] while allyl disulfide structures have recently been shown to undergo reversible and controlled exchange of conjugated thiols.[444]

As discussed in section 7.1, the generation of radicals upon irradiation of photoinitiators can be highly detrimental to cell viability and biomolecule activity.[417,418,421] Although the initiation of photo thiol−ene reactions proceeds via similar mechanisms to those used for photoacrylate cross-linking, it has been demonstrated that the damage caused is greatly reduced. This can be attributed to a number of factors. First and foremost, the rapid reaction kinetics and insensitivity to oxygen of photo thiol−ene reactions enables the use of greatly reduced irradiation times and initiator loadings. This in turn limits radical concentrations, the generation of ROS, and ultimately damage to cells, proteins, and oligonucleotides (Figure 16).[420,424] Indeed, thiol−ene reactions are able to

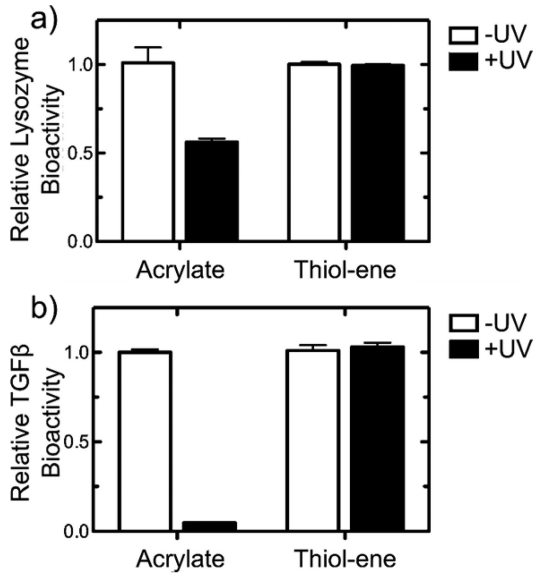

Figure 16. Comparison of (a) lysozyme and (b) TGF-β; bioactivity following UV-irradiation in the presence of either acrylate-cross-linking or thiol−ene photopolymerization reactive species. Irradiation was continued until gelation occurred −180 s for acrylate cross-linking, compared to just 10 s for thiol−ene reaction. Reproduced with permission from ref 420. Copyright 2012 American Chemical Society.

proceed efficiently with type II initiators even in the absence of co-initiators, conditions which lead to highly inefficient radical formation.[445] Second, it has been hypothesized that thiyl radicals are less destructive to biomolecules than the carbon-centered radicals generated during acrylate cross-linking.[419,420,446] Finally, the ability of thiol−ene reactions to be propagated by ROS leads to the consumption of these highly

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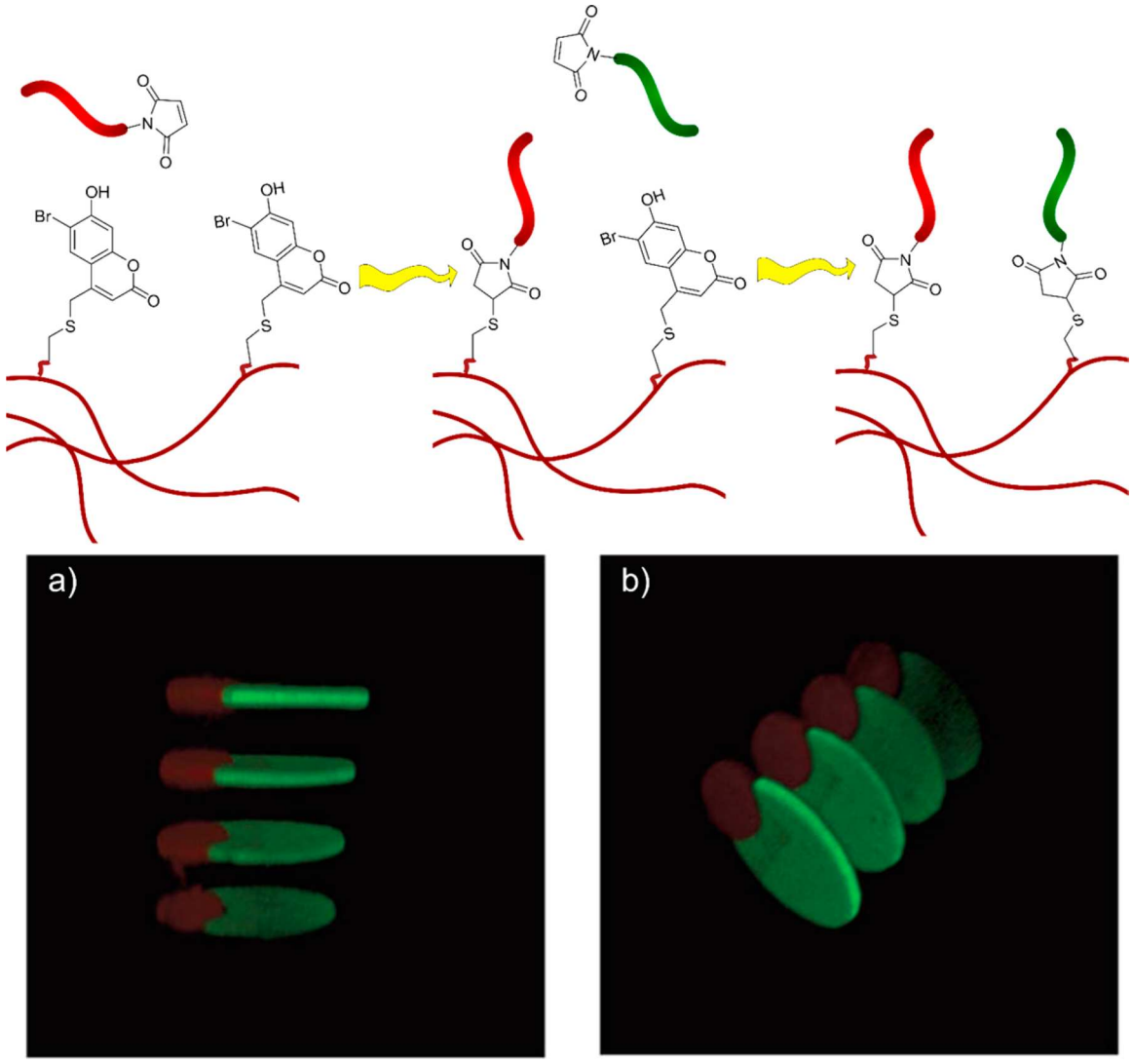

Figure 17. Two-photon induced decaging of thiol-bromocoumarins enables photopatterning of agarose hydrogels. Through sequential deprotection-conjugation steps, multiple peptides/proteins can be patterned with high 3D resolution. (a and b) 3D projections of patterned hydrogels from different angles, labeled with fluorescent barstar (green) and streptavidin (red). Adapted by permission from Springer Nature: ref 463. Copyright 2011.

reactive species, thus reducing the chance of associated damage and toxicity.[424,447]

7.3. Photo Thiol−yne Reactions

Just as thiol−ene reactions can proceed via both nucleophilic and radical mechanisms, so too can the related thiol−yne reactions. In contrast to the products generated from Michael addition, photo thiol−yne reactions undergo double thiol addition to produce 1,2-bisaddition products. This is formally achieved through an initial thiol−yne addition, followed by a subsequent second thiol−ene step (Figure 13c).[382] To date, most examples of photo thiol−yne reactions have exploited simple propargyl-ether or -amine reactive handles. Although strained species such as cyclooctynes are also known to undergo reaction, in an analogous fashion to the reaction of thiols and strained norbornenes, the instability and high cost of cyclooctyne derivatives may limit their usefulness as discussed in section 4.3.2.[448]

Even though thiol−ene reactions are generally faster, thiol−yne conjugations have been shown to take place with higher efficiency, enabling the use of lower radical concentrations.[449,450] Despite this advantage, to date there are only a few examples of the use of photo thiol−yne reactions for the production of biomolecule−material conjugates. Costa et al. demonstrated the functionalization of polymer brush surfaces with adhesive RGD motifs.[450] Indeed, cell adhesion to surfaces modified by thiol−yne modification was found to be greater than those modified via a thiol−ene mechanism. Similarly, Pedron et al. demonstrated the simultaneous photomediated modification of both alkene and alkyne groups for the spatial patterning of polyglycerol hydrogels with adhesive peptides.[451]

8. PHOTOCAGING AND ACTIVATION OF REACTIVE FUNCTIONALITIES

In the previous section, we focused on the use of light to induce biomolecule conjugation. This discussion was framed around the concept of generating a transient reactive species, whether an acrylate or thiol derived radical. In addition to these key reactions, light can also be used to treat photoreactive handles which subsequently expose functionality following irradiation. These so-called "photocaged" groups

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Table 4. Advantages, Disadvantages, and Selectivity of the Noncovalent Conjugation Strategies Outlined in Section 9

conjugation strategy	advantages	disadvantages	selectivity
binding sequences	can be evolved to target of interest	affinity of short sequences is often low	dependent on target and sequence. Often high
	enable recruitment of biomolecules in vivo	aptamers prone to degradation	difficult to distinguish similar biomolecules
	allow postfabrication modification		
self-assembling peptides	high density of display readily achieved	large ligands may disrupt assembly	N/A, synthesized sequence
	properties easily tuned		
host−guest chemistry	often high stability	guest must be introduced into biomolecule	very high, dependent on system
	reversible display can be triggered		
	orthogonal to native systems		
biotin−(strept)avidin	very high stability and rapid conjugation kinetics	streptavidin introduces large bulk into constructs	very high. Biotin and biotin-binding proteins are very rare in native systems
	mostly orthogonal to native systems	biotinylation requires low specificity or recombinant synthesis	
	easy to undertake and robust		
nucleic acids	multiplexed conjugation possible	DNA/RNA low stability	very high; determined by sequence complementarity
	high specificity and robust display	high cost, even for PNAs	
	forms stable conjugates		

have found widespread utility as a means to photopattern biomaterial substrates. In contrast to the highly reactive intermediates produced in situ during photoconjugation, photocaging is more commonly used to mask or protect a functional group until it is desirable for it to be exposed.[452−454]

Photocaging in the field of biomaterials has most commonly focused on the capping of thiol groups. The most widely utilized "cages" are based around *o*-nitrobenzyl and coumarin chromophores. In a pivotal paper, Luo and Shoichet demonstrated that nitrobenzyl-capped cysteine residues could be decaged by irradiation with 325 nm UV light. The released thiol could then react with maleimide-functionalized peptides via Michael addition, to generate a patterned hydrogel able to guide cell migration.[455] In these early reports, the limitations of using single-photon irradiation restricted patterning to two dimensions.[455,456] With the advent of two-photon patterning techniques, it was observed that *o*-nitrobenzyl groups were poorly cleaved due to their poor two-photon absorption cross-section. As a result, 6-bromo-hydroxycoumarins have become the favored thiol-"caging" group within the biomaterials community (Figure 17).[457,458] The Shoichet group has been particularly prominent in demonstrating the power of thiol-"caging" for the patterning of growth factors with high spatial precision, including VEGF,[459] FGF,[128] and EGF.[172] Recent reports on the increased decaging efficiency of methyl-coumarin capping groups, as a result of reduced chromophore isomerization upon irradiation, is likely to lead to a further increase in conjugation efficiency.[460] Although less commonly investigated, reports on the nitrobenzyl-capping of amines[461] for the "decaging" of Factor XIII substrates and hydroxyl-amines[235,462] for the spatially defined patterning of proteins via oxime ligation are also of note.

Although reactive promiscuity is a drawback for achieving site-specific conjugation, the generation of a highly reactive functionality in situ as a result of UV light irradiation enables conjugation to take place based on proximity, rather than chemical selectivity. This strategy has found widespread use in the fields of chemical and cell biology, as a means to label a biomolecule of interest with a probe brought into proximity due to target binding. So called "photoaffinity probes" generate a highly reactive intermediate upon irradiation, which then reacts rapidly with the nearest accessible functional group with high spatial precision.[464] In the context of biomolecule conjugation, such methods enable accurate photopatterning to be achieved, albeit while sacrificing the choice of conjugation site.[80,465−467] Although a number of photoreactive groups are available, the most commonly used are phenyl-azides, benzophenones, and phenyl-diazirines. Each has advantages depending on the labeling strategy being pursued, and the reader is referred to the excellent review of Smith and Collins for further details.[464]

More recently, photocaged cycloadditions have emerged as a potential means to achieve spatially defined biomaterial functionalization. In 2009, the Popik group demonstrated that cyclopropenones could be used to mask latent cyclo-octynes for SPAAC reactions and then decaged under UV irradiation.[468] These functional groups have subsequently been used for the 2D patterning of flat surfaces[469,470] and more recently for the generation of 3D patterns using two- and even three-photon activation.[471] Similarly, the UV irradiation of tetrazoles has been shown to generate a reactive nitrile-imine intermediate which can undergo rapid cycloaddition with electron-deficient alkenes such as acrylates or acrylamides.[472] However, recent reports suggest that the selectivity and orthogonality of such reactions may be low, with nitrile-imines being found to react with a range of other biologically relevant nucleophiles.[473] Indeed, Feng et al. have exploited nitrile-imine side-reactivity with thiols to site-specifically conjugate cysteine containing proteins to tetrazole functionalized surfaces.[474] Interestingly, reactions with carboxylic acid groups, widespread on biomaterial surfaces, are most efficient, though reaction with alcohols and amines is also possible.[473] Finally, the visible light-activation of IEDDA reactions has recently been reported by Truong et al. By conversion of an unreactive dehydro-tetrazine to its active oxidized form, latent functionality can be exploited to overcome the low stability of reactive functional handles.[475] These transformations are likely to come to prominence in biomaterial functionalization in the coming years, combining the high specificity and fast reaction kinetics of cycloadditions, with the spatial precision associated with photoactivation.

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9. NONCOVALENT SYSTEMS

While the conjugation techniques discussed so far rely on forming covalent bonds between the molecules of interest and the biomaterial, there is a growing set of tools which utilize noncovalent interactions to achieve similar goals. Noncovalent binding plays a vital role in cells, controlling biomolecular interfaces and influencing protein−protein interactions, DNA−DNA complexation, DNA−protein interfaces, protein localization, and more.[476] These motifs, along with unnatural functional groups, have been used for both the modification and engineering of biomolecules of interest. Here, we provide a brief summary of the most commonly applied noncovalent systems utilized by researchers for the functionalization of biomaterials (see Table 4).

9.1. Binding Sequences

A significant challenge in incorporating bioactive factors into biomaterials is that the conjugation chemistry often dictates that additional functional groups must be added to the molecules of interest, or else functional groups present on the native biomolecule must be used for covalent coupling. Both of these methods can lead to a reduction in bioactivity. Many groups have therefore been interested in utilizing noncovalent sequences which display a binding affinity for the biomolecule of interest, allowing for postfabrication modification or for native biomolecules to be simply sequestered from the surroundings within biological samples.

The most commonly used binding sequences are short peptides between 7 and 20 amino acids in length, derived from a variety of sources, including known protein binding domains present in vivo[477] or determined through techniques such as phage display[478]. Though peptide sequences have the advantage of being easily synthesized and modified with functional groups for conjugation, their binding affinities toward biomolecules tend to be significantly weaker than those of full length proteins. Short oligonucleotides known as aptamers can also be used to bind a variety of protein substrates, including the cytokines vascular endothelial growth factor (VEGF)[479] and platelet derived growth factor (PDGF),[480] as well as cell surface proteins such as epidermal growth factor receptor (EGFR)[481]. Indeed, many of the examples of biomaterial−oligonucleotide conjugation described in this review act as a means to subsequently bind and control the release of growth factors, cytokines, and ECM proteins.[76,158,352,353,355,356,482] DNA and RNA are inherently less stable than peptides and often more expensive to access synthetically. However, the ability to access long aptamer sequences is attractive, allowing the production of constructs with increased binding affinity. It is also possible to incorporate multiple binding motifs on a single DNA strand, allowing the colocalization of two separate proteins or biomolecules.[483]

An emerging technology in material binding is that of molecular imprinting.[484,485] In such systems, recognition domains are imparted into a polymerized surface via templating, subsequently allowing the display of bioactive sequences. Pan et al. recently showed that such a technique could be used to reversibly control cell adhesion and spreading, via the attachment of RGD.[486]

Adding an extra layer of complexity, binding sequences can also be introduced into a biomaterial with affinity for native biopolymers, such as heparin. The ability of such polymers to bind and activate secreted proteins is a key and often overlooked step in many cell signaling processes. As such, by first inducing biopolymer binding, the adsorption of an added or endogenous growth factor or signaling protein to a biomaterial scaffold can then be controlled.[9,487,488]

Finally, binding affinity at the amino acid level can also be exploited to enable peptide and protein conjugation to certain biomaterial substrates. In particular, the binding of unnatural catechol-based amino acids can be used to induce binding to metal oxide containing bioglasses[489,490] and metallic implants,[491] enabling the bioactivity of these important technologies to be enhanced.

9.2. Self-Assembling Peptides

Native peptides and proteins adopt a series of secondary structures, including β-sheets and α-helices, which can both stabilize individual sequences and control interprotein aggregation. One of the simplest methods to noncovalently incorporate biological motifs into a biomaterial scaffold is to exploit these self-assembly processes to form the material itself. Self-assembling peptides have been used extensively to assemble hydrogels and fibrous materials, and the reviewer is referred to a number of comprehensive reviews for an overview of the field.[492−495] In many of these structures, biological epitopes or functional groups can be appended to some or all of the peptide building blocks during peptide synthesis, to add the desired bioactivity into the system. Peptide-ligands ranging from simple adhesion motifs, to laminin derived epitopes,[496] and growth factor mimetics[497] have all been displayed on the surface of self-assembled fibrils (Figures 18 and 2). Alternatively, glycopeptides can be assembled in order to recruit extracellular signaling proteins and growth fac-

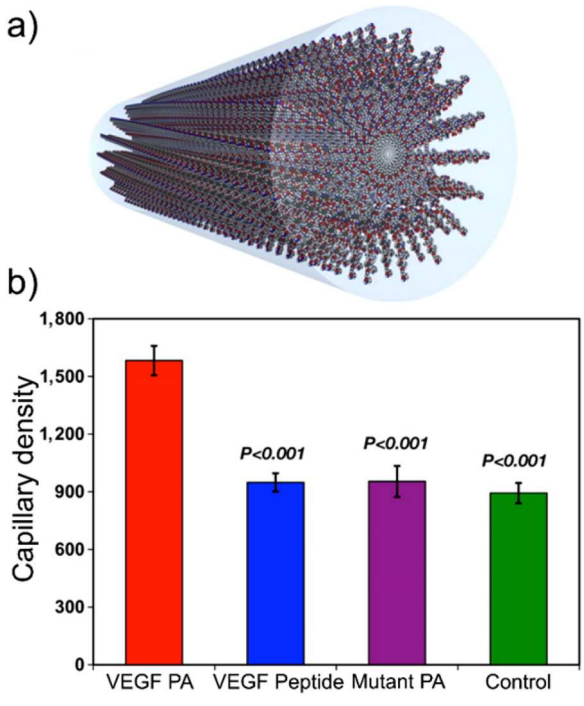

Figure 18. (a) Illustration of a VEGF-mimicking peptide amphiphile, self-assembling to form nanofibres; (b) quantification of muscle capillary density within a murine hind-leg ischemia model following the administration of peptide amphiphile (VEGF PA), VEGF mimicking peptide, a mutant amphiphile, or control treatment, based on the staining of CD31 positive capillaries; Reproduced with permission from ref 497. Copyright 2011 National Academy of Sciences.

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tors,[498,499] mimic glycosylation patterns within hyaluronic acid,[500] or investigate optimal sulfonation ratios in glycosaminoglycan scaffolds.[501] Increased activity can be achieved as a consequence of the ability to create high densities of epitope display on the surface of the nanofiber and to recreate the multivalent displays commonly present in native ECM. Self-assembling domains can also be added to full-length proteins, leading to the incorporation of pendant functionality during hydrogel formation.[502]

The propensity of peptides to form secondary structures has also been exploited within nonself-assembling scaffolds. This is most easily achieved by mixing a self-assembling peptide into a covalent hydrogel, composed of either a noninteracting polymer such as interpenetrating networks of PEG[503] or systems where additional charge interactions further stabilize the final construct, for example between positively charged peptides and negatively charged alginate gels.[504] As an alternative, pendant helical groups can be attached to a covalent material and used to drive the noncovalent attachment of bioactive groups such as growth factors via self-assembly into coiled-coil triple helices.[505]

9.3. Host–Guest Chemistry

Host–guest chemistry has recently had a surge in popularity in the biomaterials field, due to the ease of polymer modification, the relatively low costs of reagents, and the ability to tailor binding affinities to the desired application. Host–guest chemistry can be used to dynamically modify the bioactivity of hydrogels during cell culture. For example, Boekhoven et al. demonstrated that the adhesive properties of a β-cyclodextrin modified alginate scaffold could be controlled in situ through the addition of a guest naphthyl-functionalized RGDS peptide (Figures 19 and 2).[506] By subsequently introducing a non-cell adhesive adamantane-RGES peptide with a higher host binding constant, dynamic modulation of fibroblast cell attachment was enabled. Interestingly, at low RGD concentrations no guest-dependent effects were seen on cell spreading. However, at higher concentrations, significantly reduced cell spreading was induced by naphthyl-functionalized peptide when compared to the analogous adamantane system. The authors attributed this effect to the aggregation states of the unbound peptide. This hypothesis acts as an important reminder that noncovalent systems exist in a dynamic state, adding an additional layer of complexity during biomaterial design.

Host–guest chemistry has also been used as a supramolecular tool to build a system capable of binding growth factors. A guest molecule with a strong nickel-binding affinity was first bound to a material-tethered host molecule.[507] This nickel species was then used to induce the affinity-binding of polyhistidine-tagged proteins, such as anti-BMP antibodies, which in turn were capable of sequestering native BMP.

9.4. Biotin–(Strept)avidin

Avidin and streptavidin are homotetrameric proteins able to simultaneously bind up to four molecules of their small molecule binding partner biotin. The biotin–(strept)avidin affinities are among the strongest known noncovalent interactions, with dissociation constants in the range of 10^{-14}–10^{-15} M. The small size of biotin (with a mass of just 244 Da) and the ease with which it can be functionalized via its free carboxylic acid has led to biotin–(strept)avidin binding finding widespread use as a means to undertake biomaterial conjugation. Affinities are high enough to limit diffusion or loss of conjugate binding, providing largely stable devices under

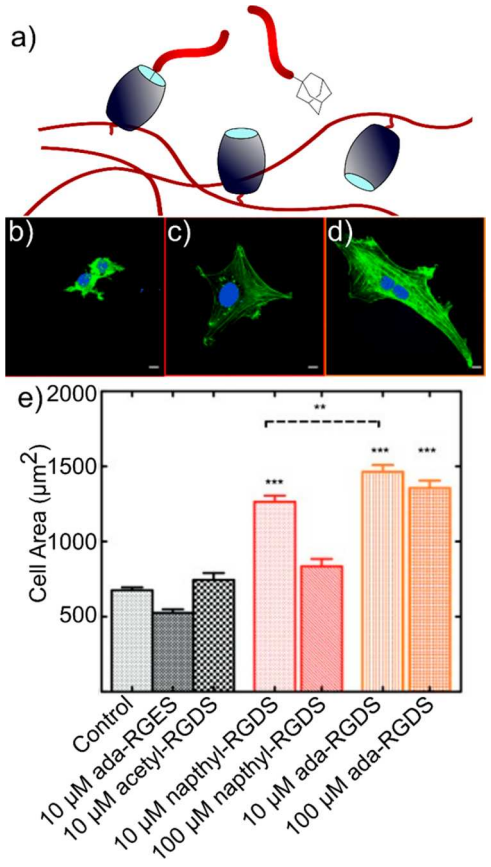

Figure 19. (a) Host–guest interactions between cyclodextrin and naphthyl- or adamantane-functionalized peptides allow alginate functionalization; Confocal microscope images of 3T3 fibroblasts on functionalized substrates: (b) without peptide, (c) 10 μM naphthyl-RGDS, and (d) 10 μM adamantane-RGDS. Scale bar = 10 μm. (e) Quantification of cell spreading on substrates in the presence of various guests. Adapted with permission from ref 506. Copyright 2013 John Wiley and Sons.

physiological conditions.[508,509] Streptavidin–protein fusions can be produced recombinantly and bound to suitably functionalized surfaces to achieve conjugation.[509,510] More commonly, biomolecule biotinylation is undertaken, and this construct is then bound to a (strept)avidin functionalized surface. This can either be achieved by a direct route, via chemical preconjugation of the material with (strept)-avidin,[463,508,511] or by exploiting the tetrameric binding of (strept)avidin to mediate indirect modification or cross-linking of biotin-functionalized scaffolds.[512,513] However, the use of large proteins to mediate conjugation (streptavidin has a mass of ~53 kDa and avidin of ~68 kDa) can prove problematic, introducing a large steric hindrance to normal material and cellular behavior.[509] Furthermore, the multimeric binding capabilities of a single (strept)avidin partially reduces control over the orientation of biomolecules following biomaterial conjugation.

The biotinylation of proteins and growth factors can be undertaken via nonspecific lysine modification, as discussed in section 4.1.[508] Alternatively, the protein-modifying enzyme biotin ligase, first introduced in section 5.3, can be used to induce site-specific biotinylation of a complementary peptide sequence, recombinantly combined with the protein of interest. Successive optimizations have led to the identification of a short 15-amino acid peptide "tag", commercially known as

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AviTag, that enables efficient biotinylation.[514−516] Recombinant expression has therefore been exploited to allow prebiotinylation and subsequent biomaterial conjugation with a wide range of cytokines and growth factors, including interferon-γ (IFN-γ), platelet-derived growth factor (PDGF), and BMP-2.[517−519]

Biotin−(strept)avidin binding is commonly viewed as being irreversible. Though this is not truly the case, with biotinylated biomolecules continuing to undergo reversible binding albeit at very slow rates, it is difficult to induce significant cleavage of biotin−biomaterial conjugates.[514] As an alternative, Lambert et al. recently utilized a biotin analogue, desthiobiotin, with significantly reduced affinity to provide reversible presentation.[509,520] Although binding was still strong (association constant of $\sim 10^{-11}$ M), streptavidin functionalized peptides could be cleaved from desthiobiotin modified surfaces by the addition of competitive biotin. The tolerance of biotin ligase for desthiobiotin substrates offers intriguing possibilities for this technology, though it is important to note that to achieve multiple cycles of reversibility, biomolecules must be functionalized with (strept)avidin, rather than biotin.[521]

9.5. Nucleic Acids

In this review we have discussed several methods through which biologically active oligonucleotides can be conjugated to a biomaterial scaffold. In an analogous fashion to self-assembling peptides, nucleic acids can also form assembled materials themselves, to generate tunable platforms for the display of biomolecules. While covalent conjugations and host−guest chemistries offer the possibility to add functionality to a biomaterial, both before and after cell seeding, combining multiple orthogonal reactions to display more than one motif within a single scaffold can be both synthetically and conceptually challenging. DNA and RNA offer a solution to this difficulty, as an arbitrary number of sequences can be made which can preferentially bind a complementary strand with high specificity. Nucleic acids have mostly been utilized to either modify biomaterial mechanical properties or to controllably deliver nucleic acid strands to cells. However, reports have demonstrated that DNA-tagged peptides and growth factors can be conjugated to a suitably functionalized biomaterial and used to elicit a desired biological effect on a localized cell population. Recently, synthetic techniques have been developed for the template-directed, site-selective DNA modification of proteins which offers great possibilities for this emerging technology.[507]

Despite the promise of nucleic acids as a means for biomaterial functionalization, their large size (a 10-mer typically has a mass >3 kDa) is potentially disruptive to protein activity and both DNA and RNA are inherently prone to degradation. Peptide nucleic acids (PNAs), featuring an unnatural backbone composed of amide bonds, have recently emerged as viable alternatives to combine the chemical stability of peptides with the specificity of DNA. The ability to synthesize PNA via peptide synthesis techniques enables the facile and automated fabrication of peptide−PNA conjugates.[522,523] These structures have been utilized in the biomaterial field for a variety of applications, including the conjugation of oligonucleotide sequences[524] and the cross-linking of hydrogels.[525] The PNAs themselves can have unusual properties, including fluorescence emission from structures containing as little as two bases.[526] In addition to their ability to be covalently coupled to polymers, PNAs have also been utilized as hydrophilic domains within self-assembling amphiphilic polymer systems, able to bind complementary DNA sequences and form nanostructures.[527,528] However, although the chemical stability and structural plasticity of PNAs is attractive for further applications in biomolecule-material conjugation, their high cost, at roughly 100× that of standard amino acids, is currently limiting to their widespread use.

10. INCORPORATING REACTIVE HANDLES

When designing a biomolecule-material composite, the choice of conjugation technique often requires a compromise between ease of use and site-specificity, as discussed in section 3. While lysine residues can be easily modified on virtually any synthetic or natural polypeptide substrate, their common occurrence often leads to heterogeneous mixtures of modified materials, typically at the detriment of biological activity. In contrast, though IEDDA reactions can be engineered to occur with exquisite precision and site-specificity, the synthesis of the required reactive handles and their subsequent incorporation into the reactive partners can prove challenging. In this section we will briefly discuss the key methods for introducing uniquely reactive motifs into biomolecule substrates, providing a chemical "tag" which allows single-site selectivity or specificity to be achieved.[529]

Short peptides and oligonucleotides can typically be produced via solid phase synthesis (SPS). The versatility of organic synthesis allows difficulties in reactive handle incorporation to be overcome, with a wide range of suitably functionalized amino acids and oligonucleotides available.[22,530,531] While some of the reactive handles described in this review may be more synthetically challenging than others, virtually all are now commercially available (though sometimes at high cost) in a form that is compatible with standard Fmoc- or phosporamidite-based SPS. In contrast, the synthesis of functionalized glycans is far more intensive. Analogous solid-phase techniques for carbohydrate synthesis remain limited to specialist laboratories, and often require significant optimization.[53,55] Solution-phase synthesis therefore remains the method of choice for glycan-derivatization, and the installation of reactive handles within substrates more complex than simple mono- or disaccharides often requires extensive synthetic efforts. Chemo-enzymatic methods for the functionalization of complex, naturally derived polysaccharides are therefore critical in order to allow selective biomaterial conjugation.[532]

The installation of unique reactive handles in larger protein substrates remains more challenging. Among the 21 natural amino acids, cysteine provides a unique opportunity to achieve site-selective modification due to its low natural abundance and high nucleophilicity, as described in section 4.2. The targeting of native cysteines on protein surfaces, or the introduction of a single residue into recombinantly expressed proteins by site-directed mutagenesis, allows selective thiol conjugation to be achieved.[49] While other natural amino acids can in principle be targeted, in practice this is complicated by their common occurrence on protein surfaces, and the resultant loss in selectivity this causes. However, there are many elegant examples in which impressive selectivity has been achieved by such means, and the reader is referred to a number of reviews that discuss such methods in detail.[36,60−62,533]

An alternative approach is to introduce unnatural amino acids (UAAs) bearing the desired reactive handles (Figure 20).

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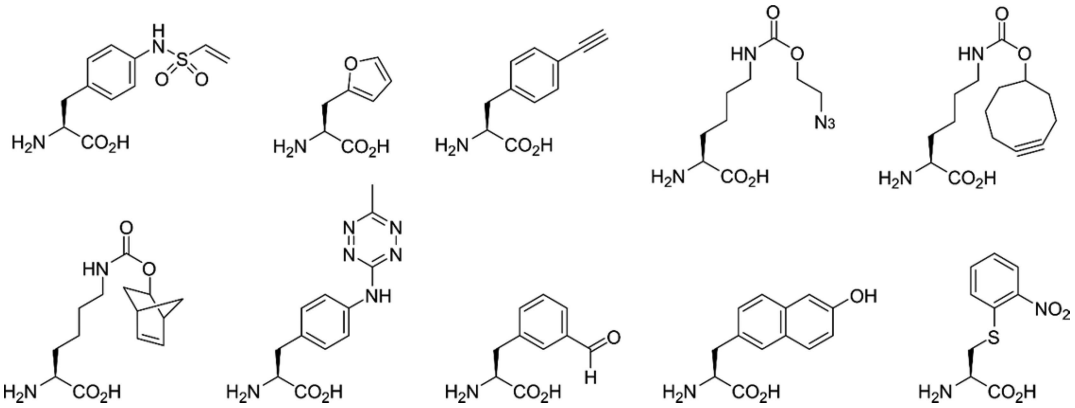

Figure 20. Examples of some of the conjugation-handles that can be site-selectively incorporated into proteins via codon reassignment. These include reactive motifs for nucleophilic and photo thiol−ene reactions, cycloadditions, aldehyde functionalization, host−guest chemistry, and photocaging.

This is often achieved via the modification of lysine residues with amine-reactive derivatives, largely negating any strategic advantage to the introduction of the unnatural structure. However, a number of powerful methods have been developed for the introduction of UAAs during the translation of recombinantly produced proteins. Among the earliest to be introduced was the use of auxotrophic bacterial strains, which are unable to biosynthesise a particular amino acid and thus require uptake from the growth media. By starving the bacteria of the native amino acid and supplementing it with a structurally related unnatural analogue, the bacterial cells can be "tricked" into incorporating the UAA during translation. This technique has been widely used to install azide- and alkyne-based mimics of methionine, leading to the introduction of reactive handles for undertaking CuAAC and SPAAC reactions.[534−537] Analogous strategies can be used for the incorporation of unnatural monosaccharides, enabling the remodelling of complex glycans. These technologies have most commonly been exploited for metabolic labeling.[538,539] However, in a notable example Iwasaki et al. demonstrated that the subsequent isolation of modified glycoproteins bearing methacrylated sialic acid motifs could be used to functionalize hydrogel scaffolds.[540]

Auxotrophic strains offer a useful means to introduce certain reactive structures. However, their use is limited in scope to structurally similar UAAs recognized by the natural cell translational machinery. A far more powerful technique is the use of codon reassignment using orthogonal tRNA and tRNA synthetase pairs that selectively recognize and charge an UAA during translation. This has been most commonly achieved by reassigning the amber "stop-codon", UAG, by incorporating a $tRNA_{CUA}$/tRNA synthetase pair from an alternative kingdom into the host cell. This pair is able to install the desired UAA, while being effectively invisible to the endogenous cell machinery. As a result, site-directed mutagenesis can be used to introduce a single TAG codon at the desired position of the coding DNA, leading to the singular introduction of the UAA with high specificity and selectivity. Since the pioneering work of the Schultz group and others in the late 20th century,[541] codon-reassignment has developed into a powerful technique at the cutting-edge of molecular biology and protein science. Recent examples of UAA incorporation in mammalian cells and even living animals are at the forefront of the field.[542] A large number of excellent reviews have been published on this topic, and the reader is directed toward these for a detailed overview of the key technologies and applications in which codon reassignment is finding use.[50,542−544] However, in the context of this review the key benefit of this technique is its ability to introduce a versatile range of unnatural reactive handles which cover virtually all of the reaction classes discussed here, as outlined in detail by Dumas et al.[50] Although to date codon reassignment has only rarely been used in the field of biomaterial conjugation, this is largely due to the far from widespread availability of the requisite plasmids.[225,366,441] As the tools and techniques required become more commonplace, these methods are likely to become more prominent in biomaterial−protein conjugate technologies.

11. OUTLOOK

The formation of biomolecule−material conjugates remains a major challenge in the biomedical field. While a vast amount of effort has been devoted to the development of generalized techniques to achieve controlled, reproducible, and effective conjugation, it is clear that no perfect conjugation technique is available. It is now common to talk of the "holy grail" of bioconjugation, a reaction that can combine ease of use, conjugation efficiency, chemo-selectivity, and substrate versatility. However, in truth achieving one global method is unrealistic.[36] The best technique available in a certain scenario is highly dependent on the precise construct and must be carefully considered during material design.[17] It is our opinion that researchers must carefully ask questions before embarking on the design and synthesis of a functionalized biomaterial. Perhaps most obviously, what is the nature of the scaffold, the biomolecule coupling substrate, and the end application? Is conjugation being undertaken under cellular or acellular conditions? Is spatial control or dynamic attachment required? Beyond these questions lay other less obvious considerations, particularly when moving toward clinical translation. Are the functional precursors stable to storage? How reproducibly and homogeneously can conjugation be achieved? Is production of the required reactive partners commercially viable? Are efficiencies maintained during scale-up?

On paper, modification strategies targeting amines or thiols can seem strategically clumsy, offering low selectivity and often diminished activity.[61] Yet these techniques persist across the academic literature and even in industry. This may in part be a case of habit, or it could be the consequence of a lack of expertise to enable the implementation of other more

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challenging conjugation strategies.[48] It may also be that, while a drop in bioactivity seems unpalatable, an end-device with acceptable performance can still be achieved, and so any downsides are greatly outweighed by the ease of implementation and cost. In contrast, the incorporation of an unnatural reactive handle into a bioactive motif may seem an overly challenging and time-consuming proposition, requiring significant optimization, cost, and heartache, merely to produce the coupling partner, before even considering the difficulties associated with conjugation.[545] However, if controlled orientation and a significant improvement in bioactivity can be achieved, potentially facilitating regulatory approval, then this initial effort may prove worthwhile.[546]

Although significant strides have been made over the past 20 years, there is still much scope for improving the effectiveness of biomaterial-based technologies. We believe that further innovation is required within the chemical biology and biomedical fields and, perhaps more importantly, greater dialogue and collaboration between researchers from the two communities. By doing so, elegant solutions to the problems which continue to dog biomaterial conjugates will be achieved. However, it is vital that developments are translatable to biomedical scenarios and offer real advantages over existing technologies. Potent peptides able to recapitulate the biological activities of proteins, improved and widely available methods for installing unnatural reactive handles, modular scaffolds that allow facile and versatile modification, highly selective conjugation handles that can be easily and cheaply introduced, and patterning techniques with improved biocompatibility are all highly desirable and can only be achieved through innovative interdisciplinary research.

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E. Thomas Pashuck received a B.S. from the University of Florida and a Ph.D. from Northwestern University, both in Materials Science and Engineering. He performed his doctoral work under the guidance of Prof. Samuel Stupp, in which he focused on tuning the properties of self-assembling peptides through molecular design. He moved to the Departments of Materials and Bioengineering and the Institute of Biomedical Engineering at Imperial College London in London, U.K. on a Marie Curie International Incoming Fellowship with Prof. Molly Stevens. He currently works at the New Jersey Center of Biomaterials at Rutgers University, and his research is focused on designing cell-responsive materials.

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