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# Designing Peptide and Protein Modified Hydrogels: Selecting the Optimal Conjugation Strategy

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**ABSTRACT:** Hydrogels are used in a wide variety of biomedical applications including tissue engineering, biomolecule delivery, cell delivery, and cell culture. These hydrogels are often designed with a specific biological function in mind, requiring the chemical incorporation of bioactive factors to either mimic extracellular matrix or to deliver a payload to diseased tissue. Appropriate synthetic techniques to ligate bioactive factors, such as peptides and proteins, onto hydrogels are critical in designing materials with biological function. Here, we outline strategies for peptide and protein immobilization. We specifically focus on click chemistry, enzymatic ligation, and affinity binding for transient immobilization. Protein modification strategies have shifted toward site-specific modification using unnatural amino acids and engineered site-selective amino acid sequences to preserve both activity and structure. The selection of appropriate protein immobilization strategies is vital to engineering functional hydrogels. We provide insight into chemistry that balances the need for facile reactions while maintaining protein bioactivity or desired release.

## 1. INTRODUCTION

A constant challenge in the design of biomimetic materials for tissue engineering and drug delivery, is finding the material with the appropriate chemical and mechanical properties to mimic the native environment. Hydrogels are particularly compelling materials to mimic soft tissue as their mechanical properties can be manipulated through chemical and physical cross-linking. Proteins play a significant role in the extracellular matrix and thus they, and their peptide analogues, are often incorporated into hydrogels, either by stable covalent bonds or through transient, noncovalent interactions. Yet, finding the appropriate chemistry with which to incorporate the proteins/peptides into hydrogels is nontrivial. Here, we explore a series of conjugation strategies, with special attention to maintaining protein activity and using aqueous-based reactions at physiologically relevant pH and temperatures. Although there are reviews that describe protein and peptide modification strategies,[1−3] we emphasize strategies for peptide and protein immobilization to hydrogels, highlighting the challenges faced with each technique and the circumstances under which each is favored. We specifically focus on click chemistry, enzymatic ligation strategies, and affinity binding for transient immobilization because these aqueous-based systems allow for site directed modifications and maintain protein activity (Figure 1).

## 2. CLICK CHEMISTRY

In order to achieve bioactive materials, peptides and proteins are conjugated using bioorthogonal chemical reactions. Click chemistry is particularly attractive as it results in high yields, with limited (if any) byproducts, and can be conducted under ambient conditions (aqueous, near physiological pH and temperature). Click reactions often take advantage of thiol chemistry of the lowly abundant natural amino acid cysteine (~2.3% of human proteome) to immobilize peptides and proteins to polymer backbones modified with thiophilic functional groups.[4] While the use of cysteine does not require the addition of unnatural amino acids, thiols can be problematic for conjugation chemistry because of their propensity to oxidize to form unreactive disulfide bridges or sulfones/sulfoxides. Additionally, protein conjugation strategies have also taken advantage of the facile modification of nucleophilic lysine residues. For example, iodoacetamide can be used to non-specifically modify nucleophilic amino acids such as histidine, cysteine and lysine. Additionally, activated carboxylic acid derivatives, such as *N*-hydroxysuccinimidyl (NHS) esters, have been extensively used to modify the $\varepsilon$-amine of lysine residues in peptides and proteins; yet, when more than one of the targeted amino acids exists in a protein, the resultant products will frequently be heterogeneous. Moreover, the presence of intramolecular carboxylic acids, such as the C-terminus and asparate/glutamate residues, can result in undesired intramolecular amidation. If the modification perturbs the active site there is a risk of compromising protein bioactivity. Site-specific protein modification using unnatural amino acid handles has gained popularity to preserve both bioactivity and structure.[5,6] However, the synthesis and incorporation of unnatural amino acids into a protein or peptide can be challenging. Table 1 summarizes the most widely used click reactions for protein and peptide conjugation to hydrogels. Table 2 summarizes reactivity between click functional groups. In cases where two click reactions are required in a system, such as independent reactions for hydrogel cross-linking and protein conjugation, the functional groups participating in the cross-linking reaction should not cross-react.

**2.1. Thiol−Ene(yne) Reaction.** The thiol−ene reaction involves the addition of a thiol to an alkene to form an alkyl sulfide, which proceeds through either a base/nucleophile

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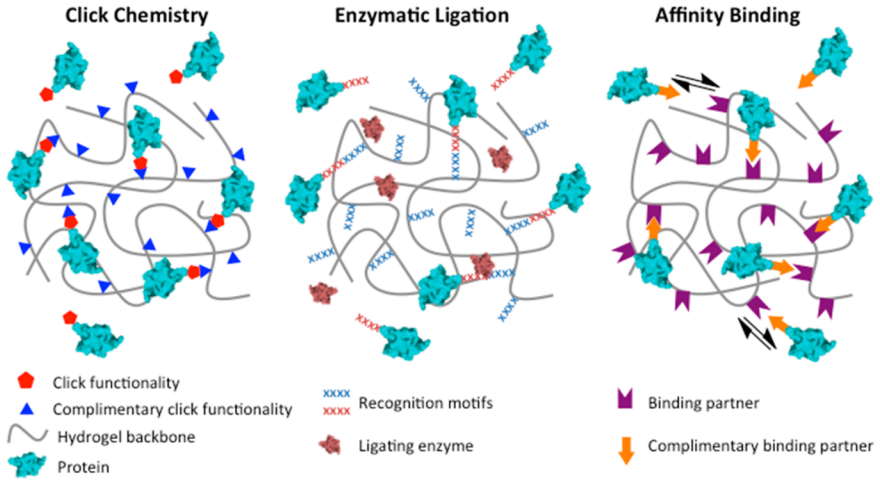


**Figure 1.** Schematic depicting click chemistry, enzymatic ligation, and affinity binding strategies to immobilize proteins and peptides to hydrogels.

catalyzed mechanism (i.e., thiol-Michael addition discussed in the following section) or a free-radical induced mechanism, herein referred to as the thiol−ene reaction. The free-radical induced thiol−ene and thiol−yne reactions are initiated by heat or light sources, typically in the presence of a photoinitiators for photoclick reactions.[52] We focus on the thiol−ene reaction, as the thiol−yne reaction is less commonly used in protein conjugation strategies. Thiol−ene chemistry has been widely employed for protein conjugation to hydrogels, taking advantage of thiol-containing cysteine amino acids. Alkene functionalities can also be incorporated chemically or recombinantly into proteins; however, this strategy requires additional synthesis or chemical modification. The thiol−ene reaction has also been used both to generate hydrogels via step-growth photopolymerization and to immobilize proteins in defined volumes using photomasks or two-photon excitation strategies.[53−55] The reaction can proceed in a mild aqueous environment, under atmospheric conditions, with the greatest efficiency between pH 4 and 7.[7] At higher pH values, the reaction efficiency decreases due to an increase in the formation of thiolates, which limit thiyl radical formation.[7] The kinetics and efficiency of the reaction vary greatly depending on the structure of the alkene moiety, with strained and electron rich alkenes having the greatest reactivity.[56−58]

The molecular environment has been shown to impact the kinetics of thiol−ene reactions. The position of cysteine within the sequence, as well as neighboring amino acids have been shown to impact the reactivity of the thiol. Peptide sequences with cysteine in the terminal N-position (**C**GGSF) were found to be less reactive than cysteine in the second position (G**C**GSF). This was hypothesized to be a result of the thiol being two carbons away from the N-terminal primary amine in the **C**GGSF peptide, lowering the p$K_a$ of the thiol and decreasing reactivity just above neutral pH. However, the efficiency of terminal cysteines was increased with a neighboring negatively charged aspartic acid (**C**DGSF).[7]

The addition of an external stimulus such as heat or light to initiate the radical mediated thiol−ene(yne) reaction may be damaging to cells or proteins within the hydrogel. Heat-initiated reactions may be particularly challenging as temperatures above 37 °C may denature proteins and cause cell death. For light-mediated thiol−ene(yne) reactions, care must be taken to select cytocompatible wavelengths, exposure time, and the type and concentration of initiator when this chemistry is used in the presence of cells. Reactive free radicals may also denature proteins or reduce cell viability. It is challenging to select thiol−ene(yne) reaction conditions that are both cytocompatible and rapid enough to allow for cell encapsulation. Additionally, thiol-containing membrane proteins on cells may participate in reactions; consequently altering cell behavior through the unintentional membrane incorporation or uptake of polymers or proteins containing thiol-reactive groups.[59,60]

One particular challenge with thiol−ene chemistry is the sensitivity of thiol groups to oxidation. Oxidative stability and reaction efficiency must be monitored via associated thiol-detection colormetric assays, such as the Ellman's assay and biotin-iodoacetamide assays.[61] Additionally, the compatibility of a protein sequence to terminal modification must be evaluated through studies to ensure bioactivity is preserved. The thiol−ene reaction for peptide and protein conjugation is not compatible with a number of other click chemistries highlighted in Table 2.

The Anseth group has taken advantage of the photoinitated thiol−ene reaction to immobilize peptides in poly(ethylene glycol) (PEG) based hydrogels containing allyloxycarbonyl (alloc)-protected (photoreactive alkene) polypeptides. Upon exposure to visible light, cell adhesive RGD peptides containing C-terminus cysteine are immobilized to the alloc-containing hydrogel in a spatially defined region within the gel. NIH 3T3 cells were shown to preferentially adhere to areas on the hydrogel containing the RGD peptide.[62,54]

**2.2. Thiol-Michael Addition.** The thiol-Michael addition reaction is a subset of the thiol−ene reaction that has been widely implemented in protein conjugation. Although thiols react slowly with electon-poor alkenes such as maleimides in radical mediated thiol−ene chemistry, the base-catalyzed Michael addition of thiols to conjugated enones (e.g., maleimides), occurs quite rapidly. Thiol-Michael addition is often favored over thiol−ene reactions in situations where UV irradiation or photoinitiators may be damaging to cells or materials.

Thiol-Michael addition has been harnessed for immobilizing pendant proteins and peptides to hydrogels as well as cross-linking hydrogels with peptide sequences. The Shoichet group has demonstrated spatiotemporal photopatterning of proteins via thiol-Michael addition where thiolated agarose or hyaluronic acid hydrogels are protected with the photocleavable group bromo-hydroxycoumarin (Bhc).[12,63] Exposure to two-photon


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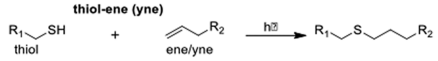
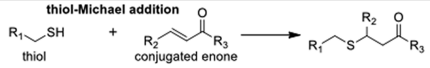
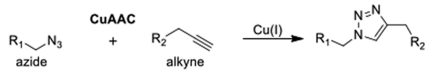
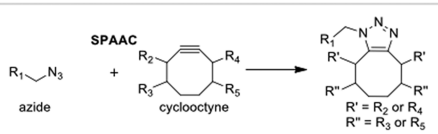
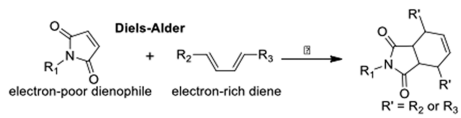
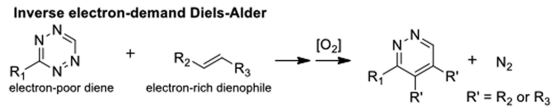
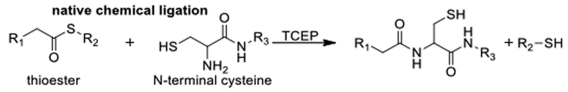
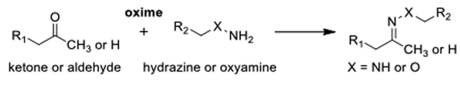
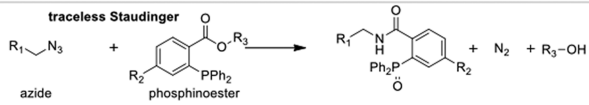
**Table 1. Summary of Rate Kinetics, Advantages, and Considerations of Commonly Used Click Reactions for Protein and Peptide Conjugation[7−39]**

| Reaction | Rate ($M^{-1}s^{-1}$) | Advantages | Considerations | Ref |
|---|---|---|---|---|
|  thiol-ene (yne) | - | • Stimulus controllable | • Sensitive to pKa of thiol<br>• Sequence sensitive<br>• Thiol oxidation | 7 |
|  thiol-Michael addition | $2.8\times10^{-6}$-$7.34\times10^{2}$ | • Low cost | • Thiol oxidation<br>• Base sensitivity of Michael acceptor | 8-12 |
|  CuAAC | $1.0\times10^{1}$-$2.0\times10^{2}$ | • Low cost | • Met, Cys and His sensitive to oxidation<br>• Stoichiometric Cu<br>• Difficult to remove cytotoxic Cu | 13-16 |
|  SPAAC | $5.4\times10^{-2}$-$4.73\times10^{1}$ | • Rapid reaction kinetics | • Ring-strained substrates may be sensitive to hydrolysis<br>• Cyclooctynes are expensive | 8, 17-19 |
|  Diels-Alder | $2.6\times10^{-1}$-8.95 | • Can be photocatalyzed | • Dienophile may be sensitive to hydrolysis<br>• Reversible | 20-27 |
|  Inverse electron-demand Diels-Alder | $1.6\text{-}2.8\times10^{6}$ | • Irreversible product | • May release by-product by retro-Diels-Alder<br>• Tetrazine synthesis is expensive and scale limited | 28-31 |
|  native chemical ligation | $6.7\times10^{-2}$-$2.6\times10^{-1}$ | • Results in physiological amide bond | • Sequence sensitive<br>• Requires a reducing agent and often thiophenol<br>• Thioester hydrolysis<br>• Thiol oxidation | 8, 32, 33 |
|  oxime | $1.3\times10^{-3}$-$2.7\times10^{1}$ | • Oxime is hydrolyzed at a slower rate than hydrazone<br>• Controllable kinetics aldehyde > ketone | • May require nucleophilic catalyst which can promote dimerization<br>• Reaction rate of aldehyde > ketone<br>• Oxyamine is reactive and potentially unstable | 8, 34-36 |
|  traceless Staudinger | $7.7\times10^{-3}$-$1.0\times10^{-2}$ | • Results in physiological amide bond | • Phosphine sensitive to oxidation<br>• Possible side reaction of | 37-39 |

irradiation cleaves Bhc, liberating free thiols that can react with maleimide-streptavidin or maleimide-barnase creating streptavidin or barnase patterns, respectively. Biotin-ciliary neurotrophic factor (CNTF) and barstar-sonic hedgehog (SHH) selectively bind to their complementary partners, streptavidin and barnase, respectively, resulting in 3-dimensional, spatially defined patterns of CNTF and SHH.[12] By keeping the thiols caged with a Bhc-protecting group, the issue of thiol oxidation is prevented. Additionally, the Shoichet group modified thiolated methylcellulose with maleimide-RGD and maleimide streptavidin. Further conjugation with biotin-PDGF showed improved differentiation of NSPCs into oligodendrocytes compared to control unmodified hydrogels and hydrogels with only RGD, thereby demonstrating the maintenance of bioactivity using this modification strategy.[64]

Thiol-Michael addition allows for facile cell encapsulation without worrying about the effects of potentially cytotoxic initiators typically used in radical mediated thiol−ene(yne) chemistry. Therefore, thiol-Michael addition may be favored over radical mediated chemistry when external spatiotemporal



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Table 2. Reactivity between Click Chemistry Functional Groups: High (Green), Low (Blue), Unreactive (Grey)[40−51a]

| | Thiol-ene/yne or Thiol-Michael addition | Thiol-ene | Thiol-yne or CuAAC | Thiol-Michael addition | Diels-Alder or Thiol-Michael addition | Diels-Alder | Inverse Electron-demand Diels-Alder | Inverse Electron-demand Diels-Alder | SPAAC | Native chemical ligation | Native chemical ligation or Thiol-ene/yne or Thiol-Michael addition | Hydrazone or Oxime | Hydrazone or Oxime | CuAAC or SPAAC or Traceless-Staudinger ligation | Traceless-Staudinger ligation |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| |  thiol |  ene |  yne |  conjugated enone |  electron-poor dienophile |  electron-rich diene |  electron-poor diene |  electron-rich dienophile |  cyclooctyne |  thioester |  N-terminal cysteine |  ketone or aldehyde |  X = NH or O hydrazine or oxyamine |  azide |  phosphinoester |
|  thiol | | *hv/cat* | *hv/cat* | | | | | *hv/cat* | *hv/cat* | | | | | | |
|  ene | *hv/cat* | *hv/cat* | *hv/cat* | *hv* | | | | | | | *hv/cat* | | | *cat* | |
|  yne | *hv/cat* | *hv/cat* | *hv/cat* | *hv* | | | | | | | *hv/cat* | | | *cat* | |
|  conjugated enone | | *hv* | *hv* | | | | | *hv/cat* | | | | | | | |
|  electron-poor dienophile | | | | | | | | | | | | | | | |
|  electron-rich diene | | | | | | | | | | | | | | | |
|  electron-poor diene | | | | | | | | | | | | | | | |
|  electron-rich dienophile | *hv/cat* | | | *hv/cat* | | | | | | | *hv/cat* | | | | |
|  cyclooctyne | *hv/cat* | | | | | | | | | | | | | | |
|  thioester | | | | | | | | | | | | | | | |
|  N-terminal cysteine | | *hv/cat* | *hv/cat* | | | | | *hv/cat* | | | | | | | |
|  ketone or aldehyde | | | | | | | | | | | | | | | |
|  X = NH or O hydrazine or oxyamine | | | | | | | | | | | | | | | |
|  azide | | *cat* | *cat* | | | | | | | | | | | | |
|  phosphinoester | | | | | | | | | | | | | | | |

[a]Entries with *hv* indicate that additional photo or thermal energy is required, while *cat* indicates a catalyst is required, such as a photo- or thermoinitiator, or ligand.

control of hydrogel modification is not required or the system is sensitive to damage from free radicals, light exposure, or cytotoxic reagents. The Burdick group took advantage of the benefits of both thiol-Michael addition and radical mediated chemistry to create a hydrogel with two sequential cross-linking steps that allowed temporal control over the degree of hydrogel cross-linking. Hyaluronic acid (HA), modified with both maleimide and methacrylate moieties, was first cross-linked via Michael addition between the maleimide present on the HA and the cysteines present on the matrix metalloproteinase (MMP) cleavable peptide GCRD**VPMS↓MRGG**DRCG. Human mesenchymal stem cells, which secrete MMPs, were encapsulated in the hydrogels during this initial cross-linking step. A secondary cross-linking step was triggered after 7 days by exposing the hydrogels to UV light in the presence of a photoinitiator to photopolymerize the methacrylate groups.



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The secondary cross-linking step restricted cell degradation of the hydrogel and switched cell differentiation from an osteogenic to an adipogenic fate.[65] This system allowed for temporal control over hydrogel mechanics. However, it should be noted that if thiols on the peptide cross-linker were not fully consumed during the initial cross-linking reaction, cross-reaction could occur between the methacrylate groups and the thiols in the peptide cross-linker.

Although the thioether product of thiol-Michael addition between thiols and maleimides is relatively stable, one consideration is the first order retro-Michael addition reaction. Thiol-containing compounds, such as glutathione can undergo a retro-exchange and reverse the previously formed *N*-ethylmaleimide−thiol adducts under physiological conditions, and may be employed as a release system.[66] Because Michael addition can be reversed under certain conditions, such as in the presence of other thiol containing compounds, complex biological environments may impact the stability of hydrogels modified with this chemistry. However, this retro thiol-Michael addition can be blocked via hydrolysis of the thiol-maleimide adduct.[67] It is beneficial to prevent the retro reaction in cases where a stable hydrogel system is required over longer periods of time. Depending on the Michael acceptor and the reaction reversibility, thiol-Michael addition may be limited by side reactions, such as the hydrolysis of maleimides prior to conjugation. Maleimides are susceptible to ring opening hydrolysis under slightly basic conditions (i.e., pH > 7.4).[68,69] To limit hydrolysis of maleimides, the forward thiol-Michael addition takes place under slightly acidic conditions. Additionally, maleimides can also react with amino groups. While the reaction between maleimides and thiols is 1000 times faster than that with amines at pH 7, under alkaline conditions cross reactivity with amino groups becomes significant.[70−72] This may lead to cross reactions in the presence of additional proteins or cells.

A method to dynamically "click" and "unclick" two peptides/proteins together or conjugate them to polymers has recently been reported, which uses a derivative of Meldrum's acid to reversibly couple amines and thiols.[73] This reaction can be stopped in basic pH (>8) and reversed with the common reducing agent 1,4-dithiothreitol.[73] This method is useful in either clicking two peptides/proteins together or conjugating proteins and peptides to polymers.

**2.3. Cu(I) Catalyzed Azide−Alkyne Cycloaddition (CuAAC).** The modified Hüisgen cycloaddition, referred to as the Cu(I) catalyzed azide−alkyne 1,3-dipolar clycloaddition (CuAAC), is a commonly used click reaction for peptide and protein conjugation.[74] Despite being widely used in protein and peptide modification, CuAAC has many limitations as a result of the Cu(I) catalyst. Due to the oxidation of Cu(I) to Cu(II), CuAAC often requires degassing, preforming the reaction under inert gas, or the use of reducing agents, such as ascorbic acid and/or sodium ascorbate.[75] Additionally, cytotoxic Cu(I) must be removed before the hydrogel can be used for biological applications.[76] Removing trace Cu(I) from polymers is difficult, requiring purification strategies such as extensive dialysis, high performance liquid chromatography (HPLC), or extensive washing.[77] Several ligands have been utilized to form activated Cu(I) complexes, which prevent oxidation and sequester copper for reduced toxicity, allowing the reaction to proceed at low Cu(I) concentrations.,[15,78−82]

Even if copper toxicity can be mitigated, reaction conditions may be too harsh for the protein. For example, the modification of horse spleen apoferritin with copper sulfate in combination with ascorbic acid or tris(2-carboxyethyl)phosphine (TCEP) resulted in structural damage to the protein. This was avoided by using copper(I) bromide and oxygen sensitive bathophenanthroline disulfonate disodium salt.[83] The Bertozzi lab also observed a decrease in the immunoreactivity of GlyCAM-Ig following a similar modification.[84] This raises concerns about using CuAAC for protein conjugation and highlights the need to perform bioactivity assays following protein or peptide conjugation.

**2.4. Strain-Promoted Azide−Alkyne Cycloaddition (SPAAC).** Strain-promoted azide−alkyne cycloaddition (SPAAC) overcomes the limitations of CuAAC associated with the use of metal ions, by eliminating the need for a metal catalyst. The conformationally unfavorable geometry of the strained cyclooctyne is inherently more reactive than cyclooctynes. The SPAAC reaction can be carried out under ambient conditions in neutral aqueous conditions.

The reaction rate of SPAAC is largely dependent on the molecular structure of cyclooctyne. Difluorinated cyclooctyne (DIFO), which contains electron withdrawing fluorines to lower the LUMO, has been widely used for SPAAC because its reaction kinetics are similar to CuAAC.[85] Boon and co-workers found that the click reaction between a 4-dibenzocyclooctynol and benzyl azide resulted in the second order rate constant as high as 2.3 $M^{-1}$ $s^{-1}$.[86] The additional aromatic ring strain of 4-dibenzocyclooctynols increases the reactivity of the alkyne toward azides, while the ortho-hydrogens limit nucleophilic attack of the alkyne.[86] Although SPAAC has been effectively used to conjugate proteins and peptides, the high reactivity of the strained cyclooctyne results in poor stability[18] and a challenging synthesis. Free thiols can also react with the strained cyclooctyne via the thiol−yne reaction. Therefore, SPAAC may result in nonspecific protein labeling or additional polymer network cross-linking if any accessible thiols are present in the biomaterial system. For example, DIFO has been found to bind mouse serum albumin, likely through the two free cysteine residues present on the protein.[87]

The use of DIFO and other strained cyclooctynes is challenging due to their large size and hydrophobic nature. In order to address issues of solubility and facilitate synthesis, other derivatives have been synthesized; however, these can reduce ligation efficiency. The synthesis of the second generation DIFO reagent (Figure 2, **1**), first reported by

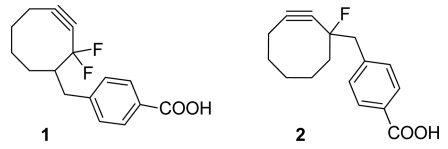


**Figure 2.** Second generation difluorocycloocytne reagent (**1**) has a faster reaction rate whereas the monofluorocyclooctyne (**2**) has a simpler synthesis.

Bertozzi and co-workers, was simplified by producing a derivative bearing one fluoro group in the $\alpha$ position (**2**).[88,89] However, the reactivity of **2** is approximately 10x lower than **1** ($4.3 \times 10^{-3}$ $M^{-1}$ $s^{-1}$ vs $4.2 \times 10^{-2}$ $M^{-1}$ $s^{-1}$).[89,90]

The Anseth group has adopted SPAAC for the formation of peptide cross-linked PEG hydrogels. In their approach, a bis-DIFO modified degradable peptide cross-linker reacts with a four-arm star PEG, forming a cross-linked hydrogel network.[62]


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Dibenzo-aza-cyclooctyne (DIBAC), also referred to as aza-dibenzocyclooctyne (ADIBO) or dibenzocyclooctyne (DBCO), is the most widely used cyclooctyne for SPAAC reactions in part due to its commercial availability. The Kloxin group immobilized azide containing fluorescent proteins to a DBCO modified PEG hydrogel. Azide groups were genetically incorporated into cyan fluorescent protein, mCherry fluorescent protein, and mCherry fluorescent protein containing a thrombin-cut site. The mCherry fluorescent protein containing the thrombin-cut site could then be selectively released from the hydrogel following the addition of thrombin.[91] Bicyclononyne (BCN), another commercially available cyclooctyne, is also frequently used in SPAAC reactions. DeForest and Tirrell designed a hydrogel for spatiotemporal protein immobilization using three bioorthogonal chemistries: SPAAC for cross-linking tetraBCN four-arm PEG with azide functionalized peptides to form a hydrogel, photomediated oxime-ligation to spatially control protein immobilization, and ortho-nitrobenzyl ester photocleavage to subsequently release those proteins.[92]

**2.5. Diels–Alder Cycloaddition.** The Diels–Alder reaction is a [4 + 2] cycloaddition that involves an electron-rich diene and an electron-poor dienophile. It can occur at ambient temperatures without any byproducts or toxic catalyst and is accelerated by water up to a factor of $10^4$ compared to organic solvents.[93−95] While the Diels–Alder reaction is typically quite slow, the reaction rate can be increased at higher temperatures; however, temperatures higher than 37 °C should be avoided due to the possibility of protein denaturation and at higher temperatures the Diels–Alder reaction is reversible.[96]

The Shoichet group has successfully used the Diels–Alder click reaction to form hydrogels from bis-maleimide modified degradable peptide cross-linkers and furan modified HA.[97] A limitation of using furan-maleimide chemistry is that the reaction rate is slow, taking several hours to form hydrogels. This slower reaction rate and the need for acidic pH to prevent hydrolysis of the maleimide make these hydrogels incompatible with cell encapsulation. Additionally, maleimides can react with thiols via Michael addition and may not be compatible with systems that contain free thiols.

Diels–Alder cycloaddition adducts can be destabilized over time due to the retro Diels–Alder reaction, although it is significantly slower than the forward reaction. For example, Koehler et al. demonstrated the release of furan-RGDS from a PEG-maleimide hydrogel at varying temperatures. The release of furan-RGDS was tuned by varying temperatures between 37 and 80 °C, with greater release occurring at higher temperatures.[96] However, such high temperatures will denature most proteins, limiting the utility of this reaction for biomedical applications.

In contrast to Diels–Alder cycloaddition, inverse electron demand Diels–Alder involves an electron-rich dienophile, such as an alkyne or a ring strained alkene, reacting with an electron-poor diene, such as tetrazine derivatives. These reactions have much faster reaction kinetics than Diels–Alder reactions ($k_2$ 2 × $10^3$ $M^{-1}$ $s^{-1}$ vs $k_2$ 0.26 $M^{-1}$ $s^{-1}$) and have the fastest reaction rate out of all the click reactions.[26,28] Seitchik et al. developed a tetrazine-containing amino acid that can be genetically incorporated into any protein in a site-specific manner. The tetrazine-derived amino acid was stable under cell culture conditions, but still readily reacted with a strained trans-cyclooctene.[98] This chemistry allows for site-specific and fast protein conjugation in complex biological mixtures that could be used to immobilize tetrazine-containing proteins/peptides to strained alkene-modified hydrogels.

**2.6. Thioester-Amine (Native Chemical Ligation).** Native chemical ligation (NCL) involves the formation of an amide bond through the ligation of a C-terminal thioester with an N-terminal cysteine residue.[33] NCL overcomes the limitations of solid phase peptide synthesis by allowing peptides larger than 50 amino acids to be constructed by synthesizing the peptide in fragments that are then ligated together.[99] This ligation produces high yield without the need for protection of amino acid side chains.[99] NCL is carried out in aqueous solutions at neutral pH in the presence of denaturing agents, such as guanidine hydrochloride to prevent protein aggregation. Control of proper pH in this reaction is important as high pH values can hydrolyze thioesters while lower pH values reduce the cysteine thiol amine reactivity, slowing the reaction rate. Although NCL achieves high yields, it is relatively slow, taking approximately a day for complete conversion.[33] The reaction kinetics are largely governed by the structure of the thioester, the amino acid residue near the thioester, and the reaction buffer.[100]

Although NCL has been used to covalently immobilize peptides containing an N-terminal cysteine to hydrogel backbones, the use of NCL as a conjugation method between proteins/peptides to polymers is challenging due to extended reaction times, possible cross-reactivity with hydrogel functionalities, thioester hydrolysis, potential change in protein structure, and the need to use TCEP.[8,101]

Recently, a novel method to selectively immobilize proteins by their N-termini was developed. Collagen, fibronectin, and laminin were immobilized to 2-pyridinecarboxaldehyde modified polyacrylamide gels in a single step reaction, resulting in surfaces that promoted cell adhesion and spreading. Because this reaction cannot occur with lysine side chain amines, 2-pyridinecarboxaldehyde exclusively reacts with N-terminal amines. This chemistry offers many advantages over NCL for immobilization of proteins through their N-termini, including faster reaction times.[102]

**2.7. Phosphine-Azide (Staudinger Ligation).** The reaction between an azide and triarylphosphine derivatives to form an amide bond, also known as Staudinger Ligation, was developed by the Bertozzi group for cell surface modification, where cells displaying azides are reacted with phospines.[103] This biorthogonal reaction is carried out at ambient temperature in an aqueous environment.[104] Generally, the reaction is quite slow, taking 1 to 2 days for completion with second order reaction constants of 7.7 × $10^{-3}$ $M^{-1}$ $s^{-1}$ reported using 14% DMF.[37]

Although Staudinger ligation has been used to form protein-carbohydrate, and protein–polymer conjugates, and immobilize proteins on glass and gold surfaces, few have used Staudinger ligation to conjugate peptides and proteins to hydrogels.[105−109] This is likely due to the slow kinetics of the reaction and the poor stability of phosphine, which oxidizes over time and further reduces the ligation yield. The formation of an amide bond in both Staudinger ligation and NCL is advantageous when ligating peptides/proteins together or forming glycopeptides; however, an amide bond is typically not an essential design consideration when conjugating proteins to hydrogels.

**2.8. Oxime Ligation.** Oxime ligation involves nucleophilic oxyamine attack at the electron deficient aldehyde or ketone, generating an oxime bond and producing water as a byproduct. Oxime ligation does not require any metal catalyst, is highly


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selective, typically bioorthogonal, has almost quantitative conversion, and can be performed under mildly acidic aqueous conditions. It has been under utilized for protein and peptide immobilization due to difficulties in synthesizing and storing proteins containing either aldehyde/ketone functionalities or oxyamine groups.[110] The oxime bond is stable at relatively neutral pH; however above pH 9 and below pH 3 hydrolysis can occur.[111] Thus, oxime ligation is often used for pH sensitive materials.[112] However, oximes have greater stability to hydrolysis than imines and hydrazones, making oxime ligation better suited to systems requiring stable bioconjugation at neutral pH.[36]

Oxime ligation was first used to modify proteins in 1990, with the formation of a protein-drug conjugate.[113] Since then, oxime ligation has been used to form a number of protein−polymer conjugates, immobilize proteins on surfaces, and modify cell surfaces.[114−117] Grover et al. was the first to apply oxime ligation to form peptide functionalized hydrogels, which supported the growth of encapsulated mesenchymal stem cells (MSCs). Ketone functionalities were added to the N-terminus of the cell adhesive peptide GRGDSP via solid phase peptide synthesis with Fmoc-5-aminolevulinic acid. Aminooxy-modified PEG was then functionalized with ketone-GRGDSP, mixed with MSCs, and then cross-linked with gluteraldehyde to produce PEG hydrogels containing encapsulated MSCs.[118] In another study by the same group, patterned surfaces of PEG microgels containing aminooxy groups where formed via electron beam lithography. In this process, the PEG is cross-linked when exposed to the electron beam and unreacted PEG is washed away leaving a wafer with topographical hydrogel features. The hydrogels were subsequently reacted with ketone functionalized GRGDSPG, creating patterns of adhesive peptides for use in cell culture.[119] DeForest and Tirrell used photomediated oxime ligation for protein immobilization in PEG based hydrogels. Hydrogels were functionalized with 2-(2-nitrophenyl)propyloxycarbonyl caged alkoxyamine. Exposure to UV light caused uncaging of alkoxyamine and subsequent reaction with aldehyde functionalized proteins, creating patterns of immobilized protein in the hydrogel.[92]

## 3. ENZYMATIC LIGATION

Enzyme-mediated protein ligation is particularly compelling as it takes advantage of the inherent enzyme activity and obviates the need for complex chemistry. There are numerous enzymes that are useful for protein conjugation, including sortase A, transglutaminase, glutathione *S*-transferase and SpyTag, tyrosinase, peroxidases, among others. Enzymatic ligation can be used to conjugate a reactive moiety, which can then participate in additional bioorthogonal reactions to cross-link or immobilize proteins within a hydrogel network. Enzymatic ligation typically occurs in aqueous solutions under physiological pH, temperatures ranging from 4 to 37 °C, and atmospheric conditions. Table 3 summarizes the advantages and considerations of commonly used enzymatic ligation strategies for protein and peptide conjugation.

**3.1. Sortase A.** Sortase A is a prokaryotic transpeptidase typically used to covalently anchor proteins containing a short C-terminal recognition motif (LPXTG) to N-terminal $(Gly)_n$ present on the cell walls of Gram-positive bacteria.[120] The catalytic cysteine residue on the enzyme cleaves the peptide bond between Thr and Gly, resulting in a thioacyl-enzyme intermediate, which is then displaced by N-terminus glycine on another peptide/protein, linking the proteins with a new amide

**Table 3. Advantages and Considerations of Common Enzymatic Ligation Strategies for Peptide and Protein Conjugation**

| enzymatic ligation strategy | advantages | considerations | ref |
|---|---|---|---|
| Sortase A | Short recognition motifs for easy incorporation into proteins or polymers; Sortase A is stable; Commercially available; Site-selective | Product contains sortase A recognition motif, leading to reverse reaction; Reduced yield due to reverse reaction; Large excess of enzyme and one of the substrates required; Removal of released Gly to prevent reverse reaction; Calcium dependent | 120, 121, 122, 123 |
| Transglutaminase | Known transglutaminase substrate peptides can be appended to polymers and proteins; Commercially available | Challenging to identify substrates on proteins whose transglutaminase reactivity has not been characterized; Has Gln residue, but not sequence specificity; Calcium dependent | 124, 125 |
| Glutathione *S*-transferase (GST) | Affinity between GST and glutathione (GSH) substrate can be developed for affinity immobilization and release systems; Commercially available | Promiscuous nature of GST may lead to undesired side reactions | 126 |
| SpyTag-SpyCatcher | No exogenous enzymes need to be added or removed; Site selective | Spycatcher is large and may be difficult to append to polymers and proteins; Final product contains large SpyCatcher protein sequence | 127 |
| SpyTag-SpyLigase-KTag | Large SpyCatcher protein split into smaller peptides; Easier to append shorter peptide motifs to proteins and polymers; Final product contains a smaller peptide fragment than SpyTag-SpyCatcher; Site selective | Slower and lower yielding than SpyTag-SpyCatcher | 128, 129 |
| Peroxidases | Horseradish peroxidase (HRP) is well characterized and widely used; HRP is very stable; Commercially available | Reactions with phenol containing molecules, such as tyrosine residues may cause undesired side reactions; Requires $H_2O_2$ | 130 |



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bond,[121] as shown in Figure 3. A limitation of sortase A ligation is that the Thr-Gly peptide linkage in the protein product

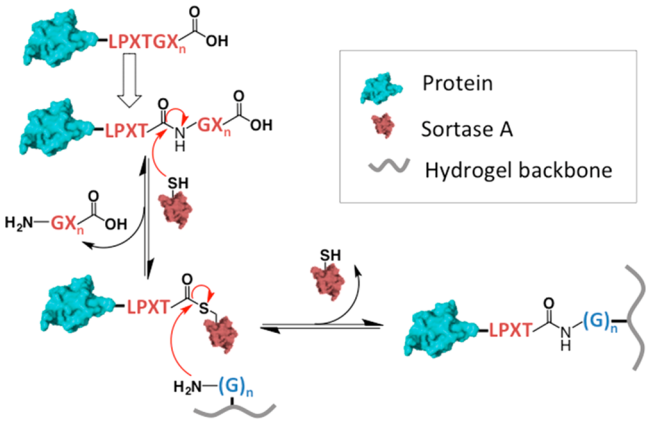


**Figure 3.** Schematic showing the immobilization of a protein to the backbone of a hydrogel via Sortase A ligation. Sortase A ligates a recognition motif (LPXTGX$_n$), where X is any amino acid, on a protein to a polyglycine, (G)$_n$, containing hydrogel. A new amide bond is formed between LPXT and polyglycine.

regenerates the sortase A recognition motif, which can participate in the reverse reaction, reducing conversion. To achieve higher conversion rates, a large excess of one of the substrates and the enzyme is required, along with removal of released Gly to prevent degradation of the product.[123,131] To overcome the limitations associated with the reverse reaction, Turnbull and co-workers identified depsipeptide substrates (containing ester linkages) that prevent the reverse reaction: the amide bond between T and G in the LPXTG motif is replaced by an ester linkage, which releases an alcohol during ligation that cannot participate in the reverse reaction.[132] Cambria et al. exploited sortase A ligation to modify PEG hydrogels with human epidermal growth factor (EGF), using an EGF containing the GGG recognition motif with an LPRTG modified PEG.[133] This system is advantageous as it is simple and site specific, resulting in bioactive, immobilized EGF.

**3.2. Transglutaminase.** Transglutaminase catalyzes the post-translational modification of proteins through the formation of isopeptide bonds via the acyl transfer between the $\gamma$-carboxamide group of glutamine and primary amines, typically the $\varepsilon$-amino group of Lys residues. In vivo, transglutaminases polymerize proteins to form barriers, such as factor XIII (FXIII), which cross-links fibrin clots during blood coagulation. Transglutaminases have been widely utilized for the formation of antibody drug conjugates, PEGylation of proteins, and the gelation of hydrogels.[134−138] While Gln selectivity is not well-defined, chain mobility and local unfolding of the protein have been shown to direct site-specific modification of Gln,[139] resulting in only one or few Gln being modified and a homogeneous product. Additionally, it is challenging to identify natural transglutaminase substrates on proteins whose transglutaminase reactivity has not been characterized. However, transglutaminase substrates have been identified and engineered for enhanced specificity.[125,140] These substrates may be appended onto proteins and polymers to enable transglutaminase catalyzed conjugation. Ranga et al. adopted the transglutaminase catalyzed reaction to cross-link HA-peptide with PEG, demonstrating the first time that an HA-based hydrogel was formed via an enzymatic reaction. In this study, HA was modified with a transglutaminase substrate peptide (NQEQVSPL) that, in the presence of activated FXIII, cross-linked with an 8-arm star PEG containing eight terminal lysine substrate peptides.[141]

The Lutolf group employed transglutaminase-catalyzed ligation to spatially immobilize peptides and proteins in hydrogels. The active site (the $\varepsilon$-amine of Lys) of an FXIII substrate (AcFKG) was caged with a photosensitive nitroveratryloxycarbonyl molecule, and these inactive peptides substrates were covalently incorporated into PEG hydrogels. Exposing the hydrogel to UV light in spatially defined regions of interest, uncaged and therefore activated the Lys substrate. In the presence of transglutaminase, the activated Lys substrates could subsequently be ligated to proteins or fluorophores containing a FXIII Gln-substrate (NQEQVSPL), creating patterns of proteins or fluorophores in the hydrogel. Vascular endothelial growth factor (VEGF) was engineered with the NQEQVSPL substrate at the N-terminus and patterned into PEG hydrogels, demonstrating the use of this strategy for site-specific protein immobilization in spatially defined regions.[124]

**3.3. Glutathione *S*-Transferase.** The glutathione S-transferase (GST) family of proteins facilitates the nucleophilic addition of glutathione (GSH) via sulfhydryl groups to electrophilic moieties on a variety of substrates. GSH is a tripeptide ($\gamma$-ECG) with a gamma peptide bond between the carboxyl glutamate side chain and the amine of cysteine. In vivo, GSTs are a superfamily of enzymes that catalyze the conjugation of GSH to various electrophiles (exogenous toxins/xenobiotics), rendering nonpolar xenobiotics more water-soluble for detoxification of the cell, and preventing the interaction of the xenobiotics with cellular proteins.[142] A GST tag is a commonly used affinity system for the purification of proteins and can be adapted for the conjugation of proteins and peptides to hydrogels. A PEG diacrylate hydrogel was conjugated with GSH (PEGDA:GSH) via thiol−ene chemistry for purification of GST tagged proteins. Taking advantage of the affinity of GSH and GST, a green fluorescent protein (GFP) tagged with GST was successfully bound in PEG-diacrylate:GSH hydrogels.[143] This strategy could be used for controlled release of proteins where high intracellular concentrations of reduced GSH could elute GST tagged proteins from a GSH containing hydrogel. Lin et al. took advantage of the association between GSH and GST to create a UV triggered, covalent immobilization strategy. In this system, GSH was functionalized with PEG and a light-activated cross-linker benzophenone (GSBP-PEG). Following association between GSBP-PEG and GST, UV light covalently cross-linked GSH and GST, forming an irreversible bond.[144] GST can also catalyze the addition of an electrophile containing probe or biomolecule with a peptide or protein containing the N-terminus GSH sequence, offering selectivity over other cysteine residues present in the system.[145] However, due to the promiscuous nature of GST, where GSH can be conjugated to various electrophiles, complex biological mixtures may result in undesired side reactions with other electrophiles in the system. This could reduce the yield of proteins conjugated to hydrogels using this strategy.

**3.4. SpyTag-SpyCatcher.** Howard and co-workers developed SpyTag and SpyCatcher reactive protein partners based on isopeptide bonds found in Gram-positive bacteria. The second immunoglobulin-like collagen adhesion domain of the *Streptococcus pyogenes* fibronectin-binding protein was split into rationally modified protein and peptide fragments. The amine


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of Lys-31 on the 116-residue SpyCatcher protein attacks the γ-carbon of Asp-117 on the 13-residue SpyTag peptide, forming a spontaneous isopeptide bond.[127] The advantage of the SpyTag-SpyCatcher chemistry over other enzyme ligation systems is the SpyTag and SpyCatcher peptide and protein fragments are inherently present on the proteins or polymers being ligated. Therefore, no exogenous enzymes need to be added or removed from the system for ligation to occur and the reaction proceeds rapidly with ∼50% yield in 1 min.[127] However, this also means that the protein being modified will contain additional protein and peptide fragments. To avoid modifying proteins with a large SpyCatcher protein, researchers split SpyCatcher into a shorter KTag (10 residues) containing the reactive lysine and a 107 residue SpyLigase containing the catalytic glutamic acid. SpyLigase can dock with KTag and SpyTag (13 residues), linking the two peptide fragments. However, the SpyTag-SpyCatcher system is faster and higher yielding than ligation using the three-part SpyLigase strategy.[128,129]

Sun et al. applied the SpyTag-SpyCatcher system to form a recombinant elastin-like protein hydrogel network containing a variety of bioactive peptides and proteins including the cell adhesive sequence RGD, MMP-1 cleavable sequences, and leukemia inhibitory factor (LIF). These hydrogels supported the encapsulation and pluripotency of murine embryonic stem cells.[146]

**3.5. Peroxidases.** Peroxidases are a large family of enzymes responsible for consuming hydrogen peroxide ($H_2O_2$) via oxidization of a variety of organic or inorganic compounds. Horseradish peroxidase (HRP), found in the roots of horseradish, is a well-characterized peroxidase that has been widely used in bioanalytical applications, such as chemiluminescent and immuno assays.[130] Additionally, HRP has been utilized for biomedical applications, including forming hydrogels via HRP-mediated oxidative coupling of phenols in the presence of $H_2O_2$.[147−149] Wang et al. cross-linked tyramine modified HA using HRP and $H_2O_2$. The cell adhesive peptide RGD, modified with two phenol groups per peptide, was simultaneously cross-linked into the HA hydrogel, enhancing the cell adhesive properties of the hydrogel and supporting the growth of human umbilical vein endothelial (HUVEC) cells.[150]

HRP-mediated phenol coupling has been advantageous for in situ hydrogel cross-linking applications where tyramine-conjugated polymers also cross-linked with tyrosine residues in native ECM, integrating the hydrogel with the surrounding ECM.[148] However, this may be disadvantageous when conjugating proteins in complex solutions where unwanted cross-reactions would decrease conjugation efficiency.

## 4. AFFINITY BINDING

A reversible approach to protein immobilization in hydrogels involves covalently immobilizing a binding ligand to the hydrogel backbone, which then reversibly binds a protein of interest via affinity interactions (hydrophobic, van der Waals, electrostatic). Affinity-based protein immobilization strategies are often used for controlled release of therapeutic proteins from hydrogels where reversible binding is required and the release can be tuned by selecting appropriate binding partners. Additionally, binding partners with high affinity, such as the interaction between streptavidin and biotin, can be used to form strong noncovalent interactions. Many proteins have natural binding partners. An example being the electrostatic interaction between negatively charged sulfate groups on heparin with proteins such as basic fibroblast growth factor (bFGF), transforming growth factor β (TGFβ), platelet-derived growth factor (PDGF), and VEGF. Sakiyama-Elbert and Hubbell used heparin-containing fibrin matrices to sequester and release heparin-binding proteins with affinities for heparin that are both high (bFGF) and low (beta-nerve growth factor, brain-derived neurotrophic factor, and neurotrophin-3).[138,151] In contrast, using an affinity binding strategy to immobilize proteins without natural binding partners requires the protein of interest to be either recombinantly or chemically modified with a binding ligand.

The interaction between avidin or (strept)avidin and biotin has been widely exploited for protein immobilization strategies. Peptides and proteins can be easily modified with biotin through many of the same protein modification strategies discussed in this perspective, as biotin is commercially available with several functionalities for protein labeling; however, chemical biotinylation produces a heterogeneous product. Enzymatic biotinylation produces a single product with high yield: a 15 amino acid AviTag is genetically added or ligated to the N-terminus, C-terminus, or exposed loops of the protein. The AviTag is recognized by *E. coli* biotin ligase (BirA), biotinylating the protein at a single, predefined site. Due to the site-specific control and physiological conditions of enzymatic biotinylation, protein activity is maintained better than with chemical modification strategies. Polymers modified with streptavidin will readily bind biotinylated peptides and proteins either pre- or post- hydrogel formation.[12,63]

A variety of binding partners have been employed to immobilize peptides and proteins to hydrogels using a similar strategy to the streptavidin−biotin binding system. However, most binding ligands are not commercially available with reactive functionalities, often requiring the binding ligand to be genetically inserted into the protein sequence. The Shoichet group has used affinity binding strategies to immobilize a variety of proteins in HA/methylcellulose hydrogels including chondroitinase ABC, human fibroblast growth factor (FGF2 or bFGF), and insulin-like growth factor-1 (IGF1).[152−154] In this system, thiol-modified methylcellulose was modified with Src homology 3 (SH-3)-binding peptides, while the proteins to be immobilized were recombinantly expressed with SH-3. This system allowed the controlled release of proteins from the hydrogel by varying the dissociation constants between SH-3 and its binding partner.[153] Similarly, Lin et al. used short affinity peptides to control the release of bFGF from UV cross-linked PEG diacrylate hydrogels over several weeks.[155] In this system, the peptide sequence (KRTGQYKL), known to bind bFGF was synthesized with an N-terminal cysteine residue to photo-cross-link PEG diacrylate. Release of bFGF from the hydrogels was tuned by varying the concentration of the affinity peptide. In this case, a binding partner for bFGF was available, but most of the time a direct binding partner for a protein is not known and the protein must be modified to contain a binding partner (as in the SH-3 fusion proteins). Systems that do not require modification of the protein sequence for controlled release are advantageous; however, in many systems, such as that of heparin and the heparin binding proteins, their release is limited by their inherent affinity. By designing the affinity, better control of release is usually obtained. While we briefly described some commonly used affinity-based systems for transient protein immobilization, Vulic and Shoichet, and Pakulska et al. provide more comprehensive reviews of this field.[156,157]



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## 5. CONCLUSIONS AND OUTLOOK

Bioactive hydrogels, where proteins or peptides are conjugated to a polymer backbone, are typically designed to mimic chemical signals of native ECM or deliver a therapeutic protein payload. The bioactivity of immobilized proteins must be both predictable and stable. Many of the first protein conjugation strategies took advantage of naturally occurring nucleophilic amino acid side chains such as the free amines of lysine or the thiols of cysteine. However, these strategies often resulted in a heterogeneous product mixture with multiple conjugation sites, thus decreasing bioactivity.

Site-selective protein and peptide modification has been achieved through the incorporation of unnatural amino acids into the protein sequence or altering amino acid reactivity via neighboring group effects. Unnatural amino acid handles selected to participate in bioorthogonal click reactions ensure that proteins are immobilized only at preselected regions that do not interfere with the protein's active site. Enzymatic ligation has emerged as an effective strategy for the site-specific conjugation of proteins with enzyme recognition motifs. Incorporation of recognition motifs to exposed loops or the termini of proteins ensures that only predefined regions of the protein or peptide will be conjugated, generating a homogeneous product and ensuring protein stability.

Affinity binding strategies can be used to transiently immobilize proteins and control their release via appropriate selection of binding pairs, creating hydrogels with dynamic bioactivity. Taking advantage of antibody engineering strategies to design specific affinity ligands for the protein of interest provides specificity without protein modification and hence an interesting outlook on the future of affinity release.[157]

Protein immobilization will continue to move toward site-selective, facile reactions that conserve protein activity and structure. Enzymatic ligation and affinity binding strategies will continue to gain momentum because they are inherently site-selective and orthogonal to most hydrogel cross-linking chemistries.

Tissue engineering began with the view of replacing lost tissues and organs with specifically designed scaffolds and cells for transplantation. These strategies have gained broader appeal and are now also being advanced for in vitro, 3D cell culture to provide insight into more predictive drug screening and drug toxicity. By combining 3D cell culture with microfluidic strategies, organ-on-a-chip technologies are emerging, as are methods to culture cancer cells in a more biomimetic environment. In these in vitro culture systems, the constraints on protein and peptide immobilization strategies remain the same, as the bioactivity of the immobilized protein is essential for functional utility.

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### Notes

The authors declare no competing financial interest.

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