4

Protein and Peptide Conjugation to Polymers and Surfaces Using Oxime Chemistry

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4.1 Introduction

Aldehydes and ketones react with alkoxyamines to form a stable oxime species (Figure 4.1). The process occurs in aqueous solution in the presence of a wide variety of functional groups without the addition of other reagents. Oxime chemistry is compatible with biomolecules. As a result, there has been increasing interest in employing this bond formation in conjugation reactions. This chapter focuses on studies that use oxime chemistry to conjugate proteins and peptides to polymers and surfaces.

Oxime bond formation provides a convenient method to produce site-specific conjugates.[1,2] Proteins and peptides often contain multiple amine groups, which also form Schiff bases with aldehydes and ketones. Typically these are unstable in aqueous solution because the equilibrium favors the oxo version. For *O*-hydroxylamine compounds, the equilibrium favors the oxime. This is significant because site-specific conjugation is important for retention of bioactivity, a required feature for most applications.

Aldehyde, ketone and aminooxy groups are not among the side-chains of naturally occurring amino acids. Because these functionalities may be easily incorporated into peptides and proteins, conjugation can be restricted to only the desired site on the protein or peptide.[3–5] Peptides are readily synthesized with either aminooxy groups or other oxo moieties using solid-phase methods.[6] A facile method to install α-oxoamides at the *N*-termini of proteins was reported by Dixon.[7–11] A transamination reaction in the presence of sodium

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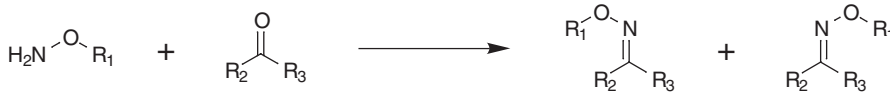

Figure 4.1 *Oxime bond formation.*

glyoxylate and copper sulfate forms the desired group (Figure 4.2), which, in turn, can be used to form conjugates with amiooxy-functionalized polymers. More recently Francis and coworkers reported that most *N*-terminal amino acids can be subjected to a transamination reaction using pyridoxal-5-phosphate (PLP).[12] They did note that the reaction was not successful for *N*-terminal serine, cysteine, proline, tryptophan or threonine residues. An artificial amino acid containing the appropriate moiety can also be introduced through recombinant methods or by enzymatic *N*-terminal addition.[13] Introduction of N^{ε}-levulinyl lysine residues site-specifically in proteins and peptides can also be achieved by solid-phase peptide synthesis or native chemical ligation.[3,4] Clearly, all of these strategies can be used to form biomolecules for reaction with functionalized polymers or surfaces.

4.2 Protein/Peptide–Polymer Conjugates

Covalent attachment of polymers to proteins and peptides has also been shown to improve properties such as stability, biocompatibility and performance compared with the naturally derived materials.[14,15] Moreover, polymers can confer new properties such as externally switchable phase behavior or self-assembly properties. Indeed, peptide–polymer and protein–polymer bioconjugates are hybrid materials that make up a large class of biopharmaceuticals.[16–18] A second class of conjugates is made from 'smart' polymers. 'Smart' polymers respond to external stimuli, such as changes in pH, temperature and light, and can impart this response to a conjugated peptide or protein.[19]

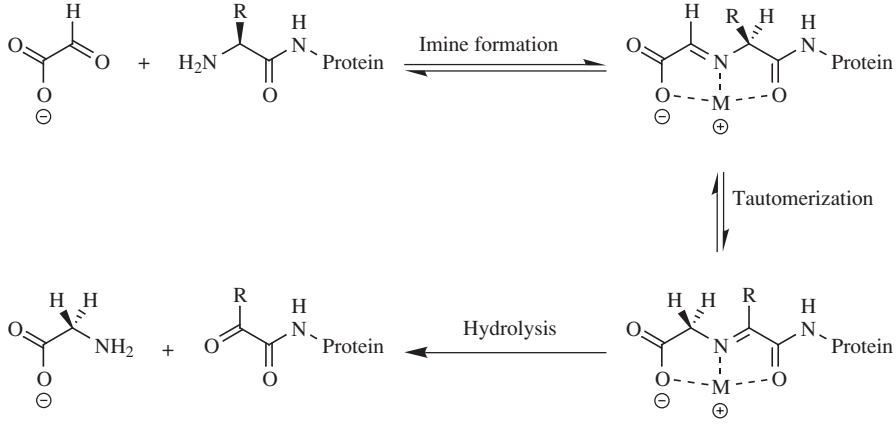

Figure 4.2 *N-terminal oxidation via transamination with sodium glyoxylate.*

There are a number of different synthetic strategies available to prepare these hybrids.[20–22] The more traditional approach involves synthesis of protein-reactive polymers, and covalent attachment of the polymer to the side chains of the proteins post-polymerization. Another strategy that we recently developed involves the synthesis of polymers directly from proteins that have been modified with polymerization initiators. The final method involves conjugation of proteins or peptides to side chain-reactive polymers.

The first reported use of oxime bond formation to achieve protein–drug conjugates was by Webb and Kaneko in 1990.[23] Their approach was to conjugate a drug molecule to monoclonal antibodies (MAb) via a bifunctional linker containing a hydroxylamine and pyridyl disulfide. The pyridyl disulfide allowed for conjugation to the free cysteine of the MAb, while the aminooxy allowed for formation of an oxime bond with a model drug. The linkage was found to be highly stable, requiring exposure to pH ~2 to hydrolyze (Figure 4.3).

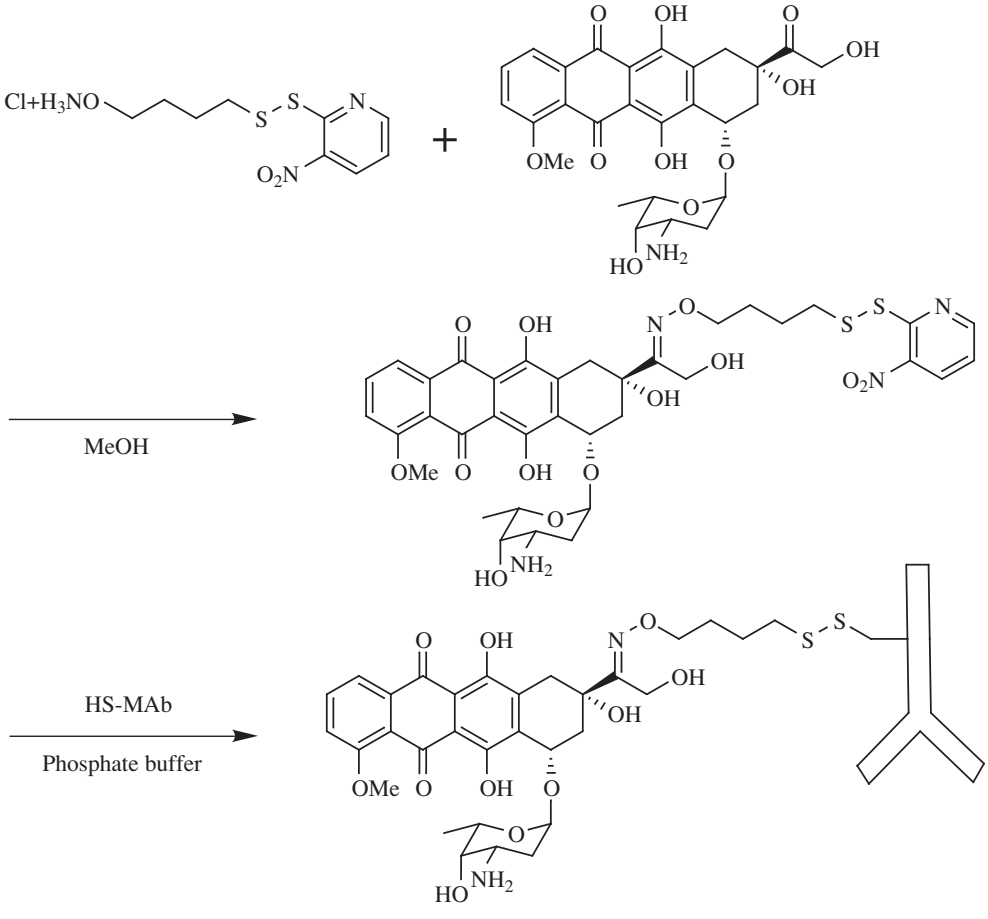

Figure 4.3 *Conjugation of adriamycin to monoclonal antibody via oxime bond formation with a bifunctional linker. Reprinted with permission from Webb, R. R., Kaneko, T., (1990),* Bioconjugate Chemistry, ***1****, 96–99. Copyright 1990 American Chemical Society.*

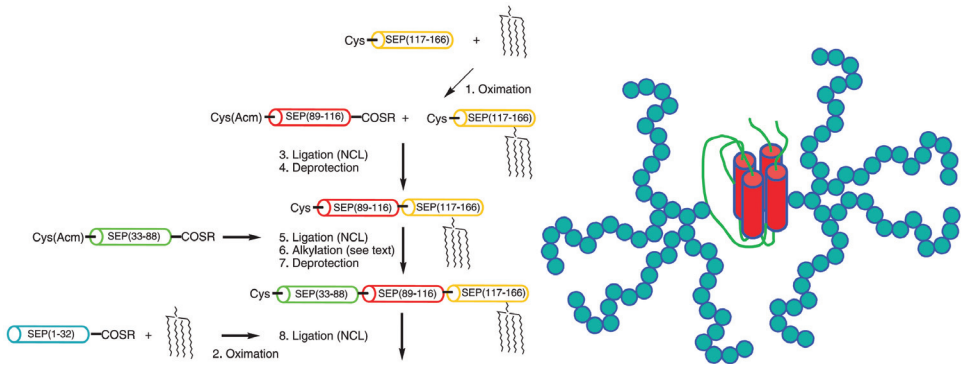

Figure 4.4 *Selective conjugation of branched PEG to synthetic erythropoietin. Reprinted with permission from Kochendoerfer, G. G.* et al.*, (2003),* Science*, **299**, 884–887. Copyright 2003 AAAS.*

The utility of oxime chemistry in preparing protein–polymer conjugate drugs was first demonstrated in 2003 in the synthesis of PEGylated erythropoietin (Epo).[3] Kochendoerfer and coworkers prepared a synthetic Epo that incorporated two N^{ε}-levulinyl-modified lysine residues (Figure 4.4). These ketone-functionalized residues provided specific sites for polymer attachment. Aminooxy-functionalized four-arm PEG was conjugated to the ketone-bearing subunits, which were then assembled to form the final drug via native chemical ligation. The results demonstrated the orthogonality of oxime chemistry to other functionalities in proteins and peptides. The pharmacokinetics of the conjugate was also studied, and found to be superior to those of the native protein. Remarkably, at the same time the conjugate displayed equivalent bioactivity. This suggested that the oxime bond and polymer chains did not interfere with protein function.

The same group further demonstrated with the use of oxime chemistry by preparing PEGylated CCL-5 (RANTES).[4] Lys-45 of the CCL-5 subunit (34–67) was modified with isopropylidine-protected aminooxyacetic acid. Following native chemical ligation with the second subunit, the aminooxy functionality was revealed through oxime exchange with methoxyamine (Figure 4.5). Linear PEG–aldehyde was then conjugated to the protein. This result showed that either aminooxy or aldehyde functionality can be incorporated into proteins for conjugation. The conjugate was found to be more active against HIV, a property attributed to the polymer blocking aggregation through the GAG binding site.

CCL-5 has also been modified with branched PEG via oxime bond formation.[24] Tumelty and coworkers altered Lys-67 to contain a 1,3-dithiolane-protected N^{ε}-levulinyl lysine. Removal of the dithiolane protecting group was accomplished in the presence of free cysteines by treatment with silver triflate. Following deprotection, the ketone-modified protein was conjugated to an aminooxy-functionalized four-arm star PEG (Figure 4.6).

Francis and coworkers exploited their reported PLP method to specifically modify proteins at the *N*-terminus to introduce aminooxy-PEG onto proteins.[12] The method was also used with expressed protein ligation (EPL) to create a protein with reactive groups at both termini.[25] eGFP was expressed in *Escherichia coli* as an intein–chitin fusion and a cysteine piperidone amide was attached by EPL.[26] The protein was then subjected to PLP to create a

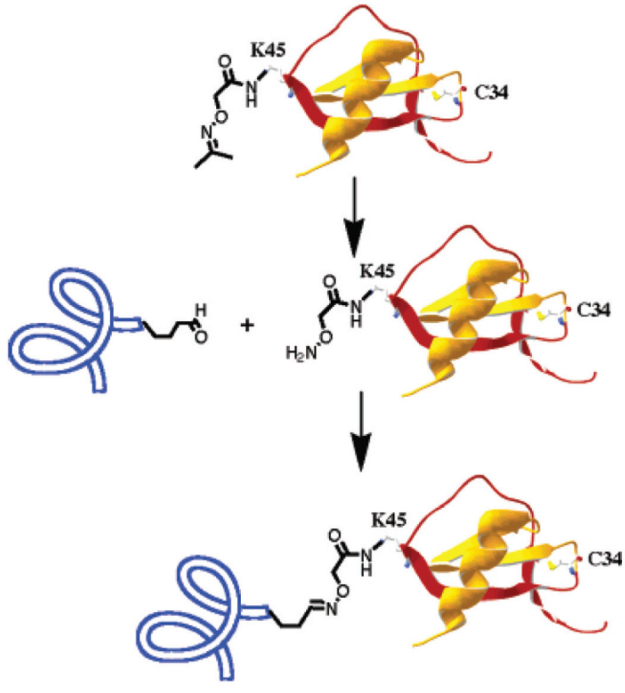

Figure 4.5 *Conjugation of PEG to aminooxy-modified CCL-5. Reprinted with permission from Shao, H.,* et al.*, (2005),* Journal of the American Chemical Society, ***127****, 1350–1351. Copyright 2005 American Chemical Society.*

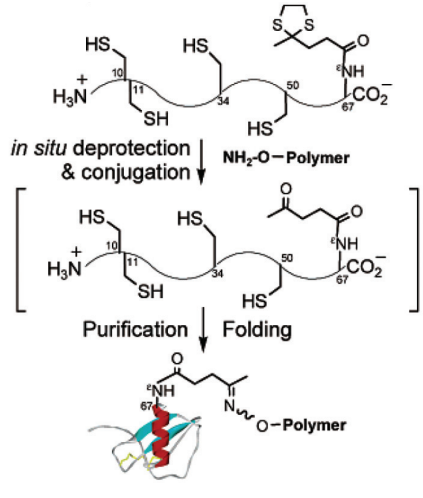

Figure 4.6 *Conjugation of PEG to ketone-modified CCL-5. Reprinted with permission from Tumelty, D.* et al.*, (2003),* Journal of the American Chemical Society, ***125****, 14238–14239. Copyright 2003 American Chemical Society.*

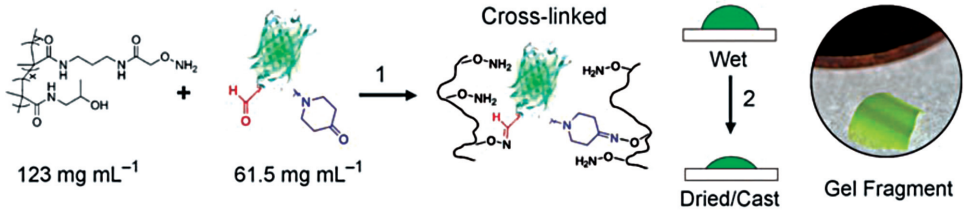

Figure 4.7 *Preparation of protein–polymer hydrogels.* N-*Oxo*-C-*keto GFP forms oxime crosslinks with a polymer bearing aminooxy side-chains. Reprinted with permission from A. P. Esser-Kahn, M. B. Francis, (2008),* Angewandte Chemie – International Edition in English*, **47**, 3751–3754. Copyright 2008 Wiley-VCH.*

protein modified for conjugation to aminooxy functionalities at both termini. In this work, the authors synthesized an alkoxyamine-*co*-hydroxypropyl methacrylate for conjugation to the protein. Hydrogels were formed by mixing the activated protein with the polymer (Figure 4.7). Furthermore, the hydrogel was found to undergo a structural denaturation from 60 to 80 °C, which was consistent with the properties of eGFP. This work demonstrated the ability to create a bulk material with properties of both the protein and polymer and should be useful to create a number of active biomaterials.

Peschke and coworkers developed a complementary method for modifying proteins for oxime bond formation utilizing a transpeptidation reaction to incorporate ketone functionality at the *C*-terminus.[27] An hGH subunit (178–191) was modified at the *C*-terminus with an N^{ε}-ketone-modified lysine derivative. The ketone-modified peptide was then conjugated to an aminooxy-functionalized two-arm PEG (Figure 4.8). The one disadvantage of this method was that the oxime formation required 10 days. However, this appeared to be unique to this system.

We have focused on developing straightforward synthetic methods to produce aminooxy-end functionalized polymers in a single step. For this purpose, controlled radical polymerization was used to prepare aminooxy end-functionalized polymers suitable for conjugation to ketone- or aldehyde-functionalized proteins.[28] Two initiators for atom transfer radical polymerization (ATRP) were prepared via reaction of Boc-aminooxyacetic acid. One, a bromoisobutyrate, was efficient for ATRP of methacrylate monomers, and the other, a chloropropionate, was suitable for ATRP of acrylamide or styrenyl monomers. Poly (*N*-isopropylacrylamide), prepared by ATRP from the chloropropionate initiator, was conjugated to bovine serum albumin (BSA) modified with N^{ε}-levulinyl lysine residues (Figure 4.9). This method has advantages over previous methods to produce the polymers, which required installing the aminooxy moiety post-polymerization.

Oxime chemistry can also be used to prepare side-chain-functionalized polymers. We polymerized 3,3′-diethyoxypropyl methacrylate (DEPMA) by reversible addition–fragmentation chain transfer (RAFT) polymerization.[29] Following acid deprotection, the resulting polymer with aldehyde-functionalized side chains was conjugated to a mixture of aminooxyacetic acid and *N*-terminal aminooxy-RGD (Figure 4.10). The latter is a ligand for cell surface integrins. The polymer composition was found to be identical to that of the feed solution, indicating that the oxime formation was efficient and that copolymers can be readily prepared utilizing this technique.

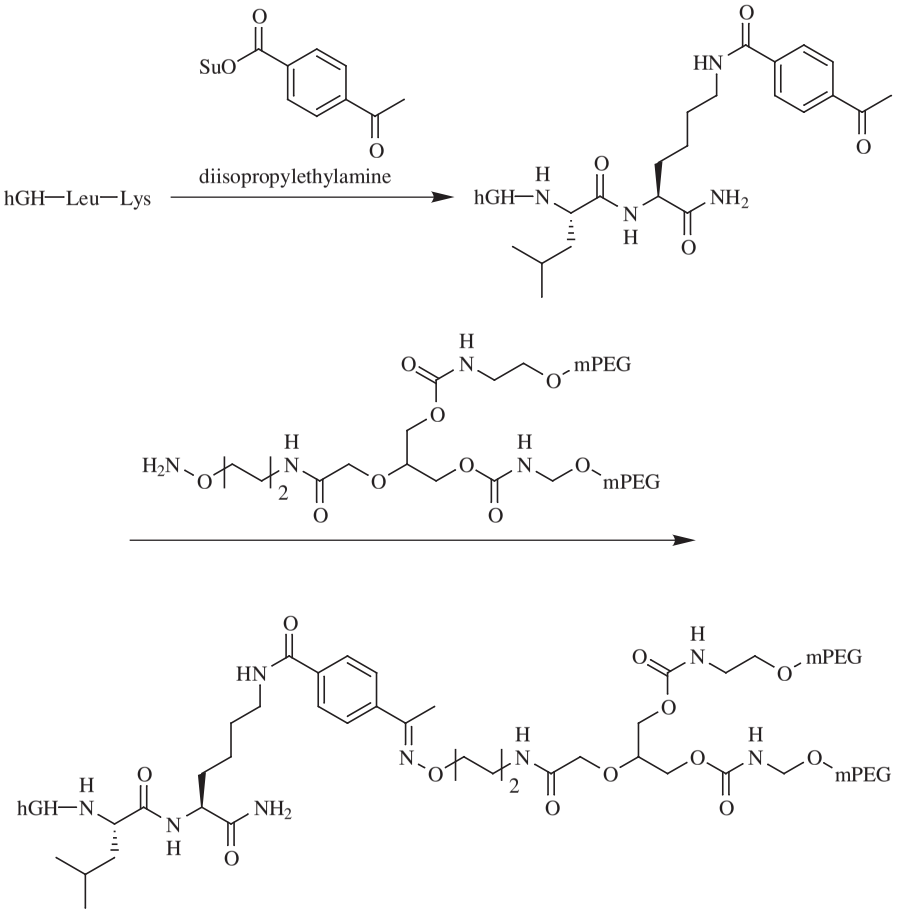

Figure 4.8 *C-terminal ketone modification of hGH, and subsequent conjugation to a branched aminooxy-PEG. Reprinted with permission from B. Peschke* et al., *(2007),* Bioorganic and Medicinal Chemistry, ***15****, 4382–4395. Copyright 2007 Elsevier.*

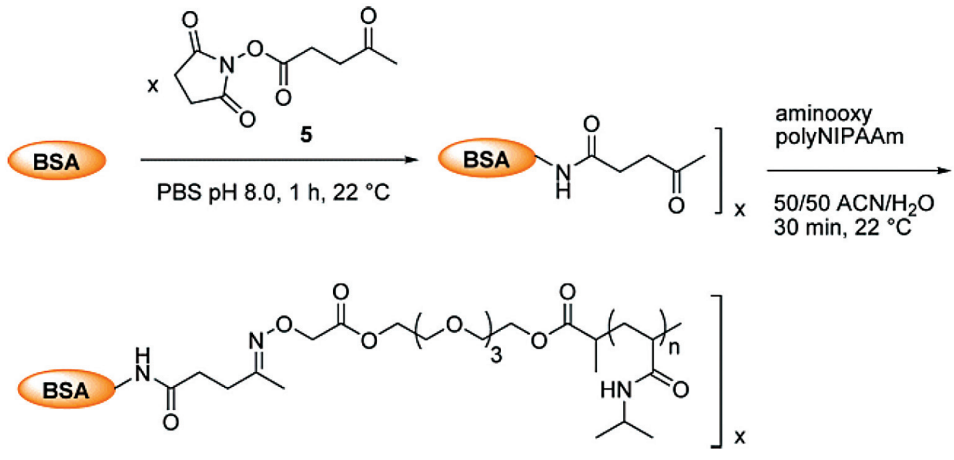

Figure 4.9 *Ketone modification of BSA and subsequent conjugation to aminooxy-pNIPAAm. Reprinted with permission from Heredia, K. L.* et al.*, (2007),* Macromolecules*,* ***40****, 4772–4779. Copyright 2007 American Chemical Society.*

4.3 Immobilization of Proteins and Peptides on Surfaces

Peptide and protein arrays are widely used in the fields of biomaterials, medicine and biotechnology for applications that include diagnostics and microarray technology, as well as cell and tissue engineering.[19,22] Thus, there is a growing need for versatile synthetic strategies to immobilize the biomolecules on surfaces.[30–33] A number of different chemistries have been demonstrated.[34] This section focuses on the use of oxime chemistry to chemically attach proteins and peptides to surfaces. In the beginning, oligonucleotide and carbohydrate conjugations are also described for introductory purposes.

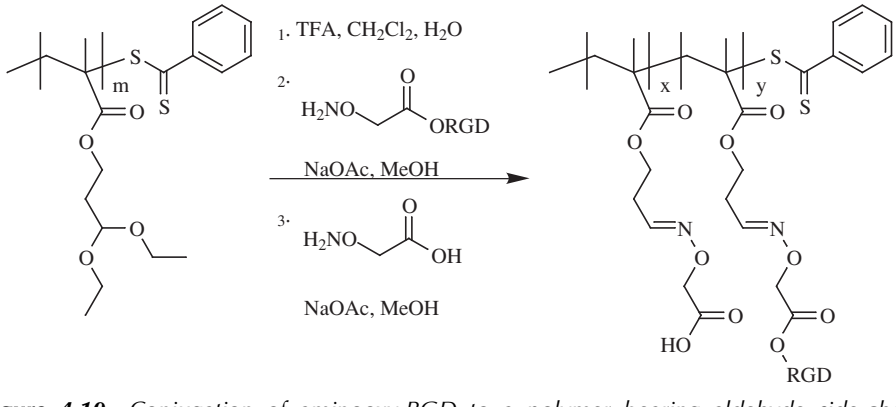

Figure 4.10 *Conjugation of aminooxy-RGD to a polymer bearing aldehyde side-chains. Reprinted with permission from Hwang, J. Y.* et al.*, (2007),* Journal of Controlled Release*,* ***122****, 279–286. Copyright 2007 Elsevier.*

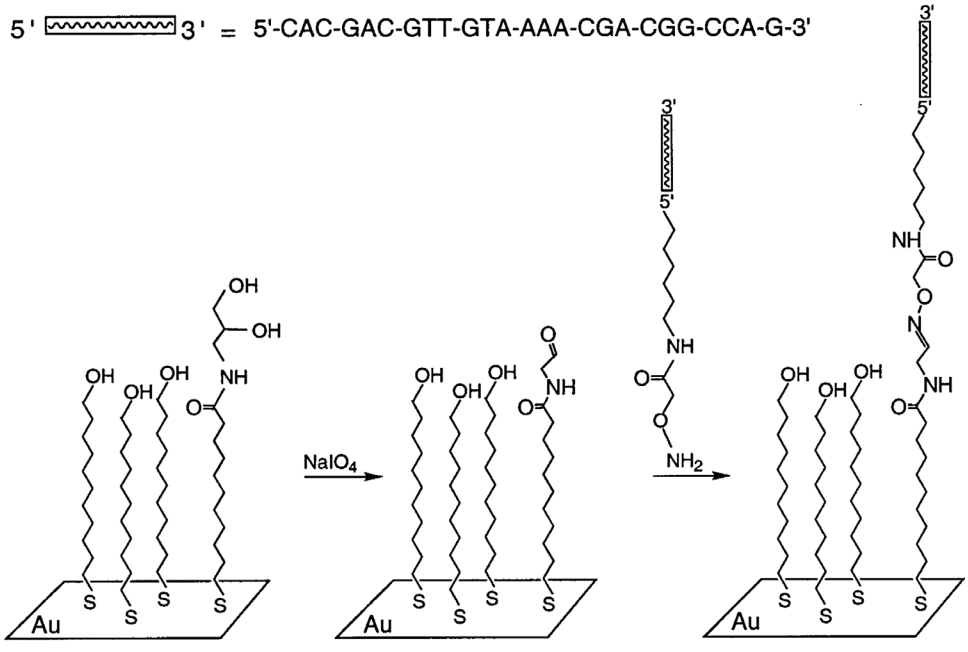

Figure 4.11 *Immobilization of oligonucleotides via oxime bond formation. Reprinted with permission from Boncheva, M.,* et al., *(1999),* Langmuir, ***15****, 4317–4320. Copyright 1999 American Chemical Society.*

The first example of oxime chemistry for surface immobilization of biomolecules was demonstrated by Boncheva *et al*. in 1999.[35] In this work, oligonucleotides were coupled to mixed alkanethiol-on-gold self-assembled monolayers (SAMs) to achieve arrays (Figure 4.11). The surface density of the DNA arrays was optimized to allow for high hybridization efficiency. The materials were characterized by surface plasmon resonance (SPR) and attenuated total reflectance-FTIR spectroscopy. This first example nicely demonstrated the utility of oxime chemistry for immobilization of biomolecules on surfaces.

This chemoselective immobilization technique was later used by a number of other groups to create oligonucleotide arrays[36–38] as well as immobilized microarrays of carbohydrates.[39,40] Oxime chemistry has also been used to immobilize oligonucleotides on micropatterned glass substrates[36,37] and inside glass capillaries.[38] Carbohydrates immobilized on polymer films using oxime bonds were demonstrated by Onodera and coworkers.[40] Specifically, a Boc-protected aminooxy monomer was copolymerized with methyl methacrylate and the polymer was cast onto a 96-well plate. The hydroxylamines were deprotected using 20% TFA and the carbohydrates were immobilized on the film in aqueous HCl (pH 2). The sugar trapping efficiencies in this work were reported to be about 80%. This was attributed to the fact that the pH required for glycoblotting was lower than the optimal pH for oxime bond formation. Tully *et al*. demonstrated synthetic chrondroitin sulfate (CS) gylcosaminoglycan microarrays utilizing oxime chemistry.[39] A series of synthetic CS molecules were synthesized with allyl end groups. Upon ozonolysis and subsequent

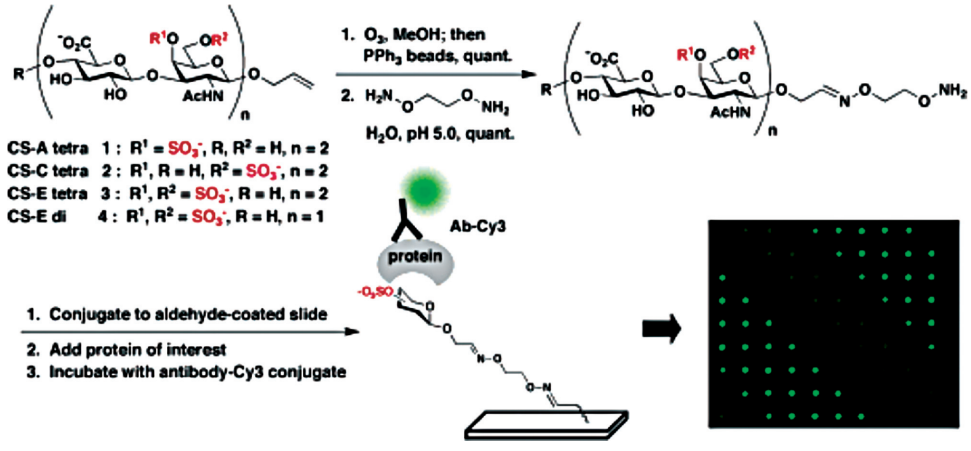

Figure 4.12 *Formation of carbohydrate arrays via oxime bond formation. Reprinted with permission from Tully, S. E.* et al.*, (2006),* Journal of the American Chemical Society, ***128****, 7740–7741. Copyright 2006 American Chemical Society.*

treatment with 1,2-(bisaminooxy)ethane, aminooxy groups were created for conjugation to aldehyde functionalized glass slides (Figure 4.12).

The use of peptides in synthetic chemistry has increased significantly since the introduction of solid-phase peptide synthesis in the 1960s. Oxime bond formation was first used to immobilize peptides by Falsey and coworkers in 2001.[41] In this work, peptide microarrays were fabricated (Figure 4.13) for cell adhesion and functional assays. Commercially

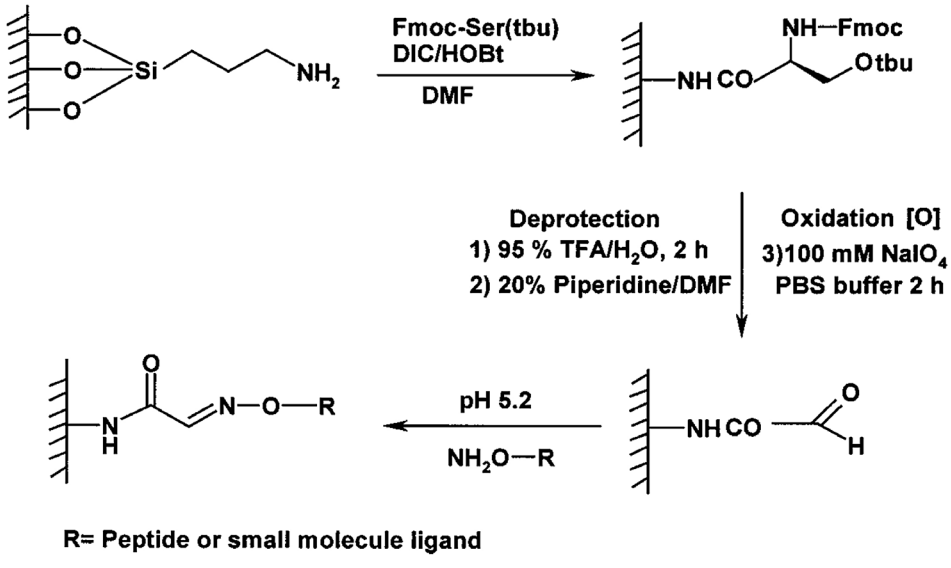

Figure 4.13 *Aldehyde functionalization of glass substrates, and subsequent immobilization of aminooxy-peptides. Reprinted with permission from Falsey, J. R.* et al.*, (2001),* Bioconjugate Chemistry, ***12****, 346–353. Copyright 2001 American Chemical Society.*

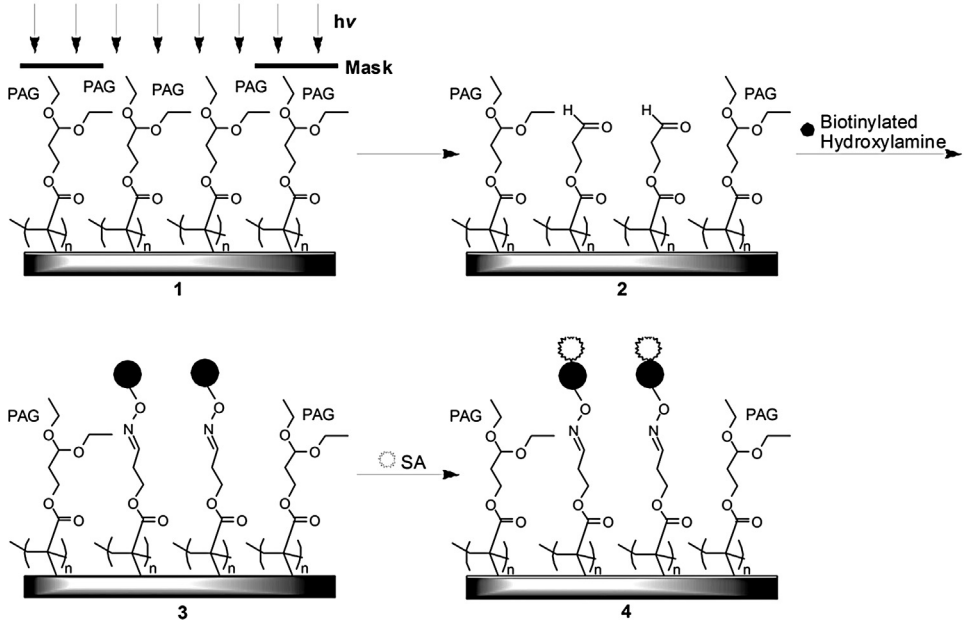

Figure 4.14 *Fabrication of streptavidin micropatterns via binding to a biotinylated surface prepared by oxime bond formation with an aldehyde-patterned polymer. Reprinted with permission from Christman, K. L., Maynard, H. D., (2005),* Langmuir, ***21****, 8389–8393. Copyright 2005 American Chemical Society.*

available microscope slides were first incubated with (3-aminopropyl)triethoxysilane to create amine-functionalized glass slides. The slides were subsequently converted to glyoxyl derivates by two different routes: coupling with Fmoc-protected serine, followed by subsequent deprotection and oxidation to the aldehydes or coupling with protected glyoxylic acid and subsequent deprotection using HCl. The surfaces were treated to remove nonspecific binding by co-spotting with steric acid in a ratio of 1:4 glyoxylic:steric acid.

We reported a method of creating oxime micropatterns using a pH-responsive polymer, poly(3,3′-diethoxypropyl methacrylate) (PDEPMA).[42] The polymer was spin-coated onto Si–SiO_2 substrates. Upon exposure to acid, the acetal groups of PDEPMA were converted aldehydes for site-specific conjugation of proteins. In this work, chemical deprotection was achieved in two ways: incubation with aqueous HCl or photochemical deprotection using the photoacid generator (PAG) triphenylsulfonium triflate and deep UV light. Micropatterns of selectively deprotected acetals were achieved by exposing PDEMPMA and PAG to deep UV light through a 1000-mesh Ni TEM grid (Figure 4.14). In order to demonstrate that proteins and other biomolecules could also be immobilized on these micropatterns, the surface was incubated with biotinylated hydroxylamine, thus forming streptavidin (SA)-reactive micropatterns. Because streptavidin has four binding sites for biotin, the array may be used as a platform to immobilize other biotinylated proteins or antibodies.

In an extension of this work, we showed that submicron patterns of streptavidin could be produced for protein assembly.[43] In this study, the photoacid generator diphenyliodonium-9, 10-dimethoxyanthracene-2-sulfonate (DIAS), which is excited at 365 nm, was spin-coated

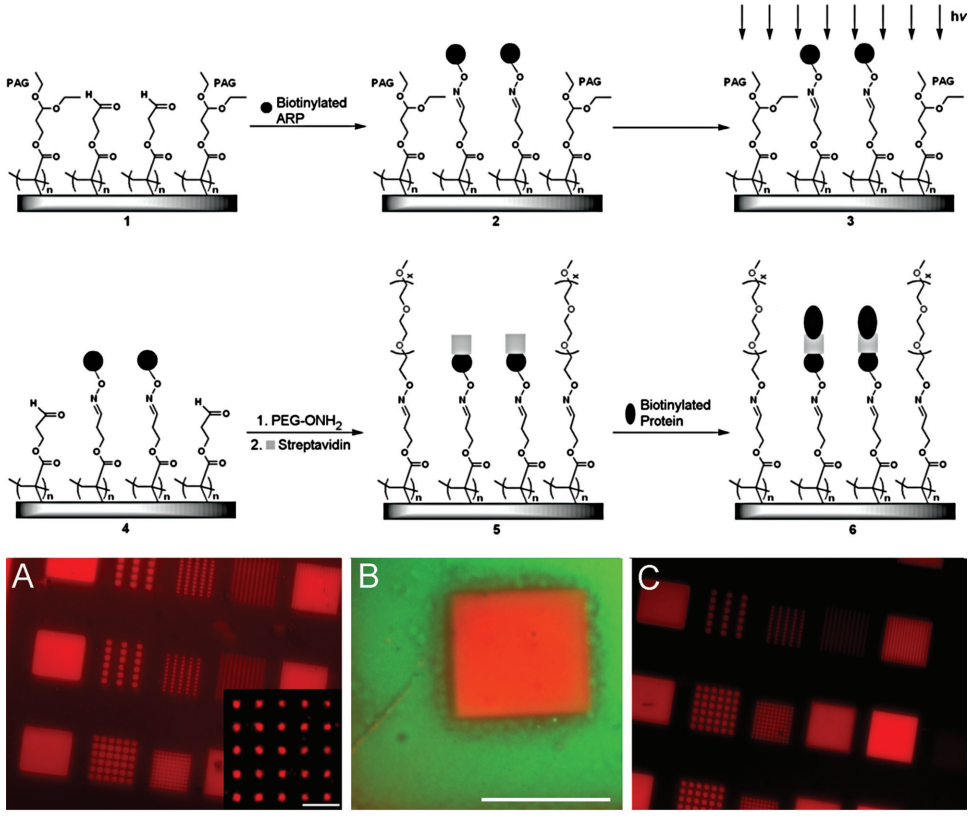

Figure 4.15 *Fabrication of micropatterned protein assemblies using streptavidin as a general linker. Reprinted with permission from Christman, K. L.* et al.*, (2006),* Langmuir*,* ***22****, 7444–7450. Copyright 2006 American Chemical Society.*

with the PDEPMA polymer. An i-line wafer stepper and a chrome-on-quartz mask were used to produce aldehyde features ranging from 500 nm to 40 μm. These aldehydes were then conjugated to SA via a biotinylated hydroxylamine, in a similar fashion to the previous report.[42] In an effort to reduce nonspecific binding of proteins, after biotinylation the background was hydrolyzed to aldehydes and conjugated to an aminooxy-terminated PEG (Figure 4.15). The PEG background was found to reduce the nonspecific absorption of SA by approximately 98%. To demonstrate the stepwise assembly of proteins, biotinylated anthrax toxin receptor-1 (ANTXR-1) and the green fluorescent protective antigen component of anthrax toxin were assembled on the patterns. This was the first example of submicron protein patterning by photolithography.

Site-specific immobilization through oxime bond formation was also demonstrated utilizing a polymer with protected aminooxy groups.[44] A copolymer consisting of HEMA and Boc-protected aminooxy tetra(ethylene glycol) methacrylate was synthesized by free-radical polymerization. The polymer was designed with a PEG group in the monomer to provide protein resistance. The polymer was covalently attached to the native oxide of

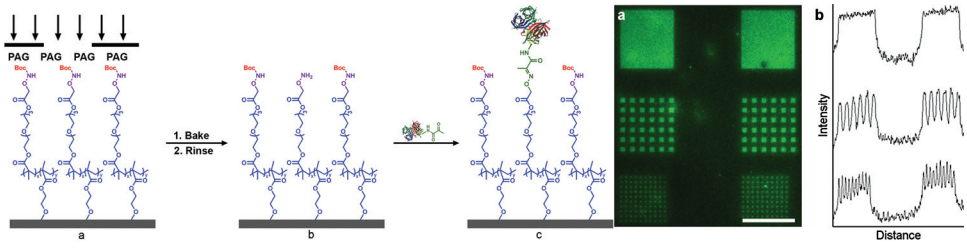

Figure 4.16 *Immobilization of* N*-terminal aldehyde-modified streptavidin on aminooxy micropatterns via oxime bond formation. A fluorescence image of the patterns (a), and intensity profile (b) are shown. Reprinted with permission from Christman, K. L.* et al.*, (2007),* Journal of Materials Chemistry, ***17****, 2021–2027. Copyright 2007 The Royal Society of Chemistry.*

silicon by spin-coating a film of the copolymer on the surface. After baking at 110 °C the hydroxyl groups were covalently linked to the surface. An i-line sensitive PAG was then used to create patterns of aminooxy groups (Figure 4.16). Finally, *N*-terminal α-ketoamide-modified SA was immobilized to the surface. This strategy could be used to attach numerous proteins via the *N*-termini to patterned surfaces.

Park and Yousaf demonstrated use of an interfacial oxime reaction to immobilize ligands and cells in patterns and gradients to photoactive surfaces.[45] In this work UV-active nitroveratryloxycarbonyl (NVOC)-protected aminooxy alkanethiol SAMs were prepared. Alkoxyamine micropatterns were formed via exposure to UV irradiation through a micropatterned mask (Figure 4.17). The micropatterns were incubated with ketone-functionalized RGD to form cell-adhesive micropatterned substrates. Cell adhesion was observed only in those areas which had been deprotected.

A complementary method has been reported by the same group, whereby an aldehyde-functionalized surface was employed to conjugate aminooxy-functionalized peptides.[46]

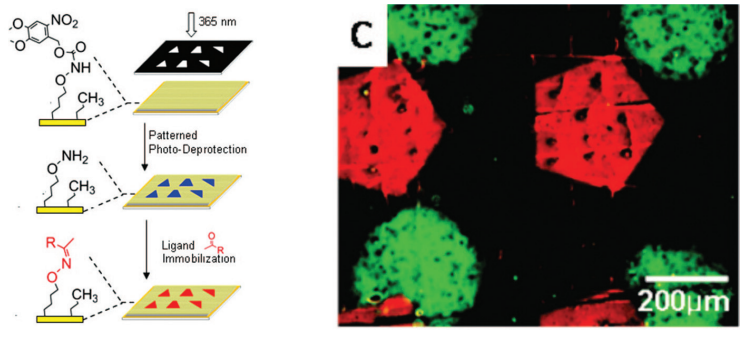

Figure 4.17 *Preparation of micropatterned aminooxy surfaces by photodeprotection, and subsequent ligand immobilization via oxime bond formation. Reprinted with permission from S. Park, M. N. Yousaf, (2008),* Langmuir, ***24****, 6201–6207. Copyright 2008 American Chemical Society.*

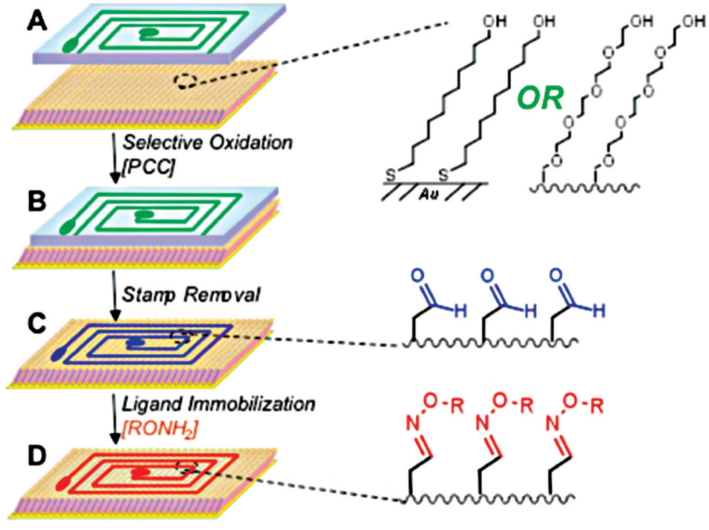

Figure 4.18 *Preparation of aldehyde micropatterns via selective oxidation within microfluidic channels and subsequent immobilization of aminooxy-functionalized ligands. Reprinted with permission from Westcott, N. P.* et al., *(2008),* Langmuir, ***24****, 9237–9240. Copyright 2008 American Chemical Society.*

PEGylated SAMs were prepared, and covered with a patterned poly(dimethylsiloxane) (PDMS) stamp to form microchannels. The SAM exposed to the microchannels was chemically oxidized to form micropatterned aldehydes (Figure 4.18). The PDMS was then removed, and the patterns were incubated with aminooxy-functionalized RGD to form cell-adhesive micropatterned substrates.

4.4 Conclusions

Protein– and peptide–polymer conjugates and surface hybrids are important in biomaterials and medicine. One crucial aspect to consider while preparing these materials is that the reaction site or sites be controlled. Oxime linkages provide a means to site-specific conjugations with retention of bioactivity. Moreover, installation of the reactive groups into the biomolecules is facile and can be achieved in aqueous solutions.

Acknowledgments

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