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Copper-catalyzed 'Click' Chemistry for Surface Engineering

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12.1 Introduction

In the past few decades, there has been rapid development in the field of surface engineering, relating to the control of structure and properties of surfaces, which is of utmost importance for applications such as cell biology, tissue engineering, microfluidics, optics and electronics.[1,2] Surface attributes (such as wettability, charge and surface reactivity) depend on the chemical and physical details of the molecular structure at the interface. At the same time, the ability to modify inorganic surfaces with organic molecules or biological ligands is also a common requirement for a host of applications.

Surface modification or functionalization via covalent coupling is one of the major strategies being explored by surface scientists. Surface functionalization reaction involves a solid surface interacting with the reactant in liquid or vapor phase and may involve complicated steric and kinetic effects. At the same time, most of the coupling reactions available for surface chemistry are limited by incomplete conversions, nonspecificity, harsh reaction conditions and side reactions. In this respect, copper-catalyzed Huisgen's 1,3-dipolar cycloaddition between terminal alkyne and azide groups has proven to be an excellent choice due to its superior properties such as mild reaction conditions, high conversions, selectivity and reproducibility.[3–8] In general, this so-called click reaction is a reaction between terminal alkyne and azide groups to yield 1,4-disubstituted 1,2,3-triazoles. It is compatible with a wide range of functional groups except for groups which disrupt the catalytic activity of copper.[9] Alkyne–azide click reaction demonstrates high reactivity in heterogeneous

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reaction systems, so it is useful for surface reactions. This also implies that the solvent and catalyst system utilized during click reaction is quite important.[10]

This chapter focuses on strategies being employed to fabricate alkyne and azide functionalized surfaces and also highlights the applicability of click reaction for surface reactions with a specific focus on conjugation of biological ligands such as saccharides, oligonucleotides, proteins and peptide sequences.

12.2 Synthesis of Alkyne or Azide-functionalized Surfaces

The first step is to create surfaces amenable to copper-catalyzed Huisgen's 1,3-dipolar cycloaddition, i.e., surfaces containing alkyne or azide groups. This has been achieved by utilizing several methods such as self-assembled monolayers (SAMs), spin-coating, layer-by-layer assembly (LbL), lithography and polymeric methods.

12.2.1 Self-assembled Monolayers of Alkanethiolates

Self-assembled monolayers are monomolecular films, which are formed by spontaneous organization of active surfactant molecules onto specific solid substrates.[11] The most commonly used SAM system is the gold–alkanethiolate system due its ease of formation and the ability to use gold as an electrode. Typically the self-assembling molecules are modified and then assembled; however this procedure is often cumbersome and may not form the desired monolayer. Hence various surface coupling reactions have been used to modify SAMs after assembly. Modification of SAMs is limited by the thermal stability of the gold–thiol bond, because thiols desorb at higher temperatures, typically above 100 °C. These monolayers are also sensitive to pH conditions and solvents. Therefore Huisgen's 1,3-dipolar cycloaddition is an attractive option for surface reactions on monolayers. Copper-catalyzed click reaction was first applied to mixed monolayers of azidoundecanethiol and decanethiol on gold by Collman *et al*.[12] These monolayers were subsequently reacted with alkyne-functionalized ferrocene compounds (Figure 12.1). Collman *et al*. also assembled several other azide-terminated monolayers and the reactive azide surface coverage and rate of click reaction were determined via electrochemical and spectroscopic techniques.[13] Other researchers have also used gold-alkanethiolate SAMs in conjunction with alkyne–azide click chemistry for the introduction of several ligands to the monolayer surface under mild conditions (discussed later).[14–20]

12.2.2 Self-assembled Monolayers of Silanes and Siloxanes

Another self-assembling system that has been studied extensively is alkylsiloxane [CH_3-$(CH_2)_n$-SiO_x] monolayers on silica. The high reactivity of the surface anchor group makes it difficult to functionalize the opposite end of the monolayer. Lummerstorfer and Hoffmann created azide-containing siloxane monolayers on silica by nucleophillic substitution of bromine-terminated monolayers and subsequently reacted the monolayers with substituted acetylenes.[21] Several attempts have also been made to functionalize the silane monolayers using click chemistry.[22–24] Organic silanes are of special interest since they form stable monolayer films and can be attached to substrates with hydroxyl or oxide groups such as glass and silica. Ostaci *et al*. synthesized alkyne-containing silane monolayers on silicon

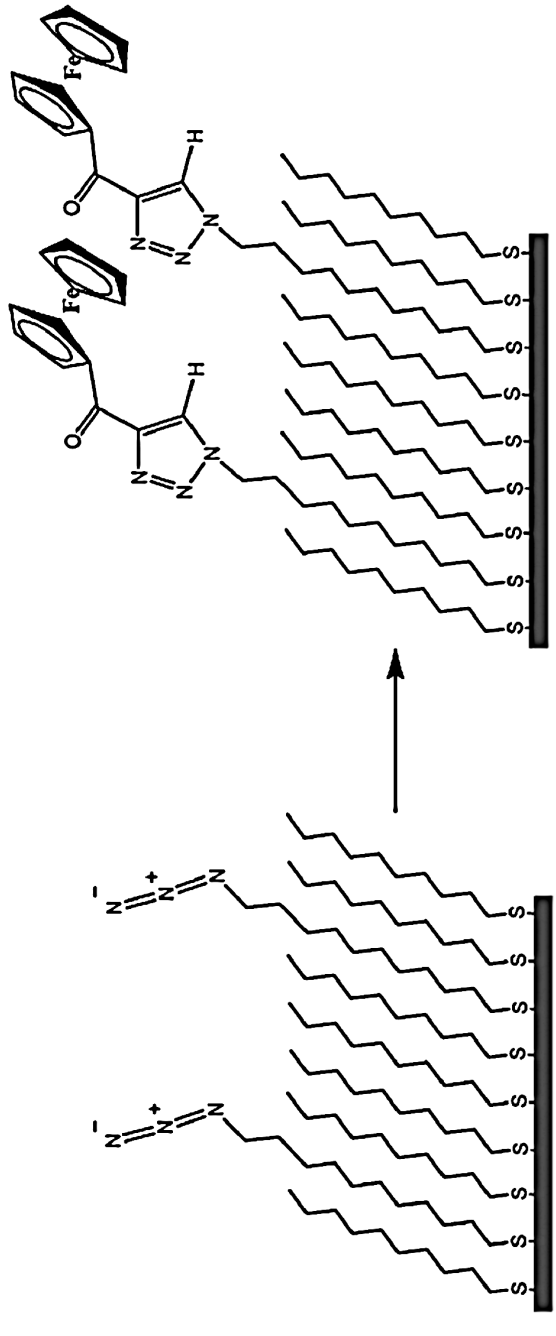

Figure 12.1 *Schematic showing the immobilization of ferrocenepropynone onto mixed monolayers of azidoundecanethiol and decanethiol via click reaction. Reprinted from ref.[12]. Copyright 2004 American Chemical Society.*

substrates and grafted ω-azido-modified linear polymer brushes [such as poly(ethylene glycol), polystyrene and poly(methylmethacrylate)] onto the monolayer-coated substrates (Figure 12.2).[23] It was further observed that the silane monolayer also acted as a passivation layer preventing nonspecific adsorption of the polymers. A combination of silanization and click chemistry was also used to modify glass microfluidic channels with preformed polymers by Prakash *et al*.[25] They covalently attached alkyne-terminated linear and dendritic polymers to glass substrates and conducted electroosmotic flow measurements in these channels. Covalent attachment of the polymers was seen to alter the surface charge in the channel (zeta potential) and in turn affect the fluid transport properties.

Haensch *et al*. used click chemistry to immobilize alkyne functionalized supramolecular terpyridine units on to azide-terminated silane monolayers.[26] Terpyridine moieties typically form complexes with a range of metal ions, which influences the physiochemical properties of the surface. In this case, the terpyridine ligand, 4′-(4-ethynylphenyl)-2,2′:6′,2″-terpyridine was synthesized and conjugated with Fe(II), which prevented complexation between the copper catalyst and terpyridine. The Fe(II) protected supramolecular ligand was immobilized onto 11-bromoundecyltrichlorosilane monolayers and deprotected under suitable conditions to obtain free terpyridine ligands on the substrate.

To address the important issue of passivation of native silicon surfaces, Rohde *et al*. acetylated silicon surfaces in a two-step process and coupled azide-functionalized benzoquinone using click chemistry.[27] Benzoquinone was further electrochemically activated to yield an amine-terminus, which was used for subsequent functionalization with ligands such as ferrocene carboxylic acid and biotin. During this procedure, minimal oxidation of silicon was observed, so it can be used in sensor applications, which are highly sensitive to the presence of a SiO_2 layer. Interestingly, Evrard *et al*. functionalized carbon electrodes using the electrochemical reduction of diazonium salts of phenylazide or phenylacetylene, which resulted in a grafted layer containing azide or acetylene groups respectively.[28] This method provided higher control over the density of the functional group and the surface retained its reactivity.

Click chemistry has been utilized to PEGylate porous silicon (pSi) substrates, which are of significance in biomedical applications as drug delivery vehicles and for biosensing.[29] Britchet and coworkers functionalized porous Si surfaces with acetylene groups via hydrosilation with 1,6-heptadiyne and then reacted with PEG–azide. The PEGylated pSi surface demonstrated a dramatic improvement in wettability. On the other hand, Schlossbauer and coworkers incorporated azide groups on mesoporous silica by simply treating it with sodium azide and then immobilized alkyne-containing trypsin.[30] They further demonstrated that the surface immobilized enzyme trypsin retained its activity.

12.2.3 Block Copolymers

Another common technique for the functionalization of surfaces, such as glass and silicon oxide is spin-coating.[31–33] The spin-coating technique is preferred for a variety of applications, because the thickness of the film can be precisely controlled. Rengifo *et al*. reported the use of spin-coating for the self-assembly of alkyne-functionalized diblock copolymers on various substrates, which was probed by subsequent click reaction with fluorescently-labeled azide ligands.[31] The diblock copolymer sequence, α-alkyne-ω-Br-poly(*tert*-butylacrylate-*b*-methylmethacrylate) [poly(^{t}BA-MMA)] was selected such that

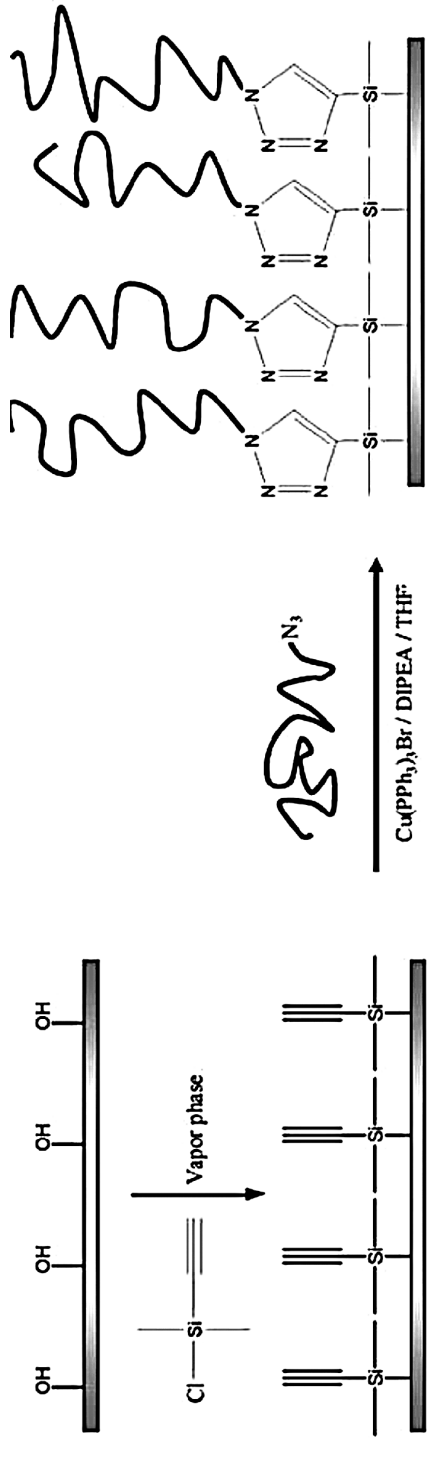

Figure 12.2 *Grafting of linear polymer chains onto alkyne-functionalized silane monolayers.*[23]

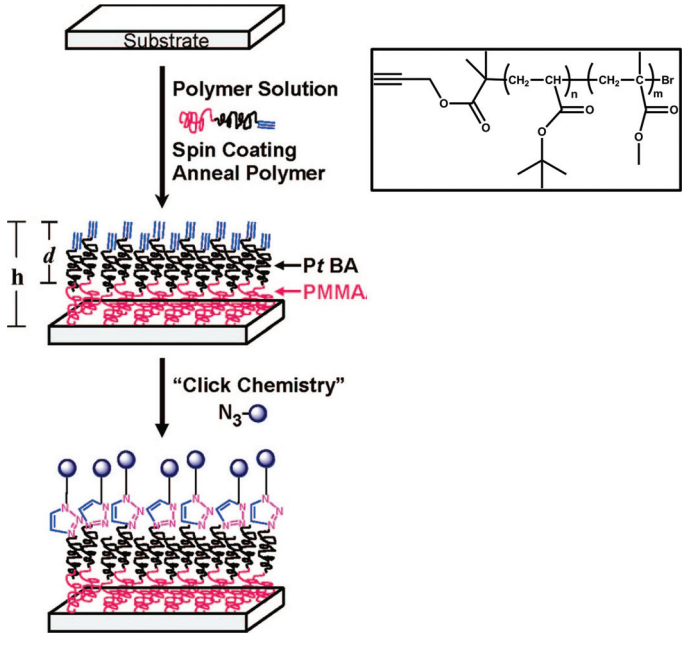

Figure 12.3 *Click reaction on films of spin-coated diblock copolymer, α-alkyne-ω-Br-poly(*tert*-butylacrylate-*b*-methylmethacrylate) [poly(*t*BA-MMA)]. Inset: chemical structure of the copolymer. Reprinted with permission from ref.*[31]*. Copyright 2008 American Chemical Society.*

the first block (MMA) physisorbed strongly onto the substrate and the second sequence (*t*BA) possessed a low surface tension, such that it remained on the surface, thus imparting a layered structure (Figure 12.3). The alkyne groups were attached to the second block and thus were presented at the surface for further reaction. The density of reactive groups on the film surface was controlled by the thickness of the film, which in turn was dependent on the molecular weight of the copolymer, concentration of the spin-coating solution and the spinning speed. This unique copolymer system was applied for the quantitative immobilization of azide-terminated 20-mer DNA molecules.[32]

Fleischmann *et al*. synthesized a terpolymer containing styrene (base of the polymer film), 4-(ethynyl)styrene (part containing the alkyne groups) and glycidyl methacrylate (anchor to the silicon substrate) using nitroxide mediated radical polymerization (NMRP).[33] This terpolymer was spin-coated on silicon substrates and the reactivity was probed with fluorescently-labeled azides.

12.2.4 Layer-by-layer Films

Layer-by-layer (LbL) assembly of composite films is generally based on charge interactions or hydrogen bonding interactions between sequential polymer layers. Crosslinking of the films using covalent reactions between layers can increase the stability of the films. The other advantage of incorporating covalent reactions between layers is that it enables the assembly of polymers with similar electrostatic properties (noncharged or nonhydrogen

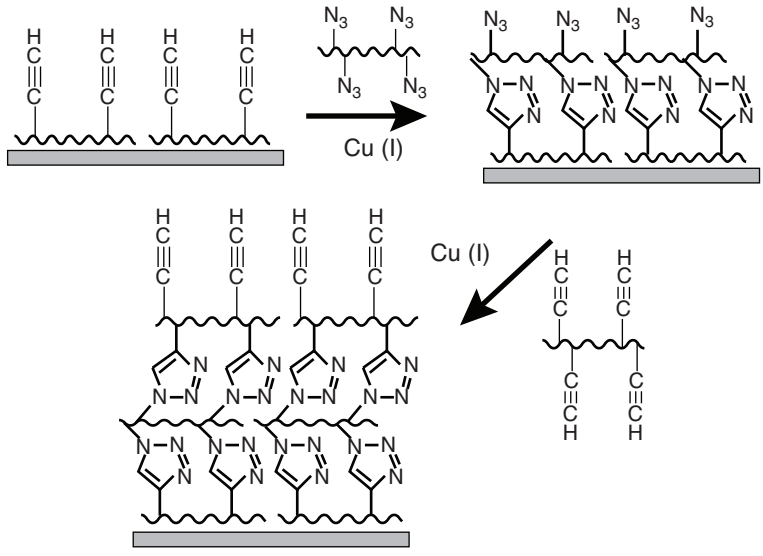

Figure 12.4 *Utilization of click reaction for the layer-by-layer assembly of thin polymer films with alkyne and azide functional groups. Reprinted with permission from ref.[34]. Copyright 2006 American Chemical Society.*

bonding), albeit with different reactive groups, thus creating single-component films. Taking advantage of these aspects, Such *et al*. synthesized multilayer films via LbL assembly of alkyne and azide functionalized versions of the same polyelectrolyte (polyacrylic acid), using copper-catalyzed click reaction as the crosslinking reaction (Figure 12.4).[34] Similarly, LbL assembly of alkyne- and azide-functionalized poly(n-isopropylacrylamide) was achieved by click chemistry.[35] The entire assembled film was subsequently covalently attached to an alkyne-functionalized polyethylene surface.

12.2.5 Chemical Vapor Deposition Polymerization

Surface reaction on thin polymer films requires robust attachment of the films to the flat substrate. In this respect chemical vapor deposition (CVD) has been used to fabricate functionalized coatings with good adhesion towards a wide variety of substrates.[36] This vapor-based process provides a solvent-free environment, good film adhesion and generates conformal coatings. More recently CVD has been extended to create alkyne-derivatized polymer coatings.[37,38] Nandivada *et al*. synthesized a reactive coating containing alkyne functional groups, poly(4-ethynyl-*p*-xylylene-co-*p*-xylylene), via CVD polymerization of 4-ethynyl-[2.2]paracyclophane.[37] Azide-bearing biotinylated ligands were conjugated to this reactive coating using microcontact printing and the reaction was probed with fluorescently-labeled streptavidin (Figure 12.5). In a different study, Im *et al*. synthesized an alkyne-functionalized polymer coating using an initiated-CVD (iCVD) process.[38] Using a single-step approach, a commercially available monomer, propargyl methacrylate, was polymerized to form poly(propargyl methacrylate). This polymer was also patterned using e-beam lithography to form nanometer patterns and the reactivity of the polymer was demonstrated by click reaction with azide-functionalized biotin.

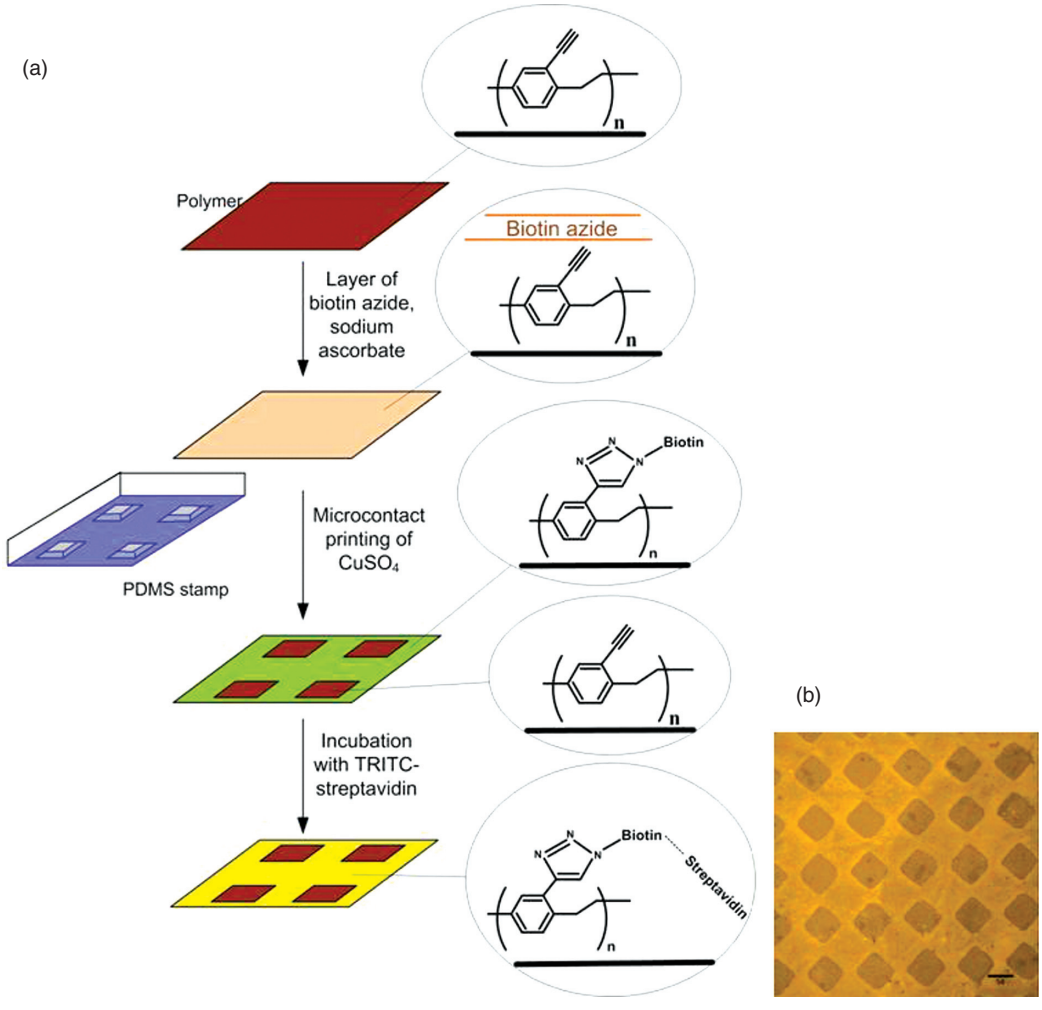

Figure 12.5 *(a) Schematic showing alkyne-functionalized polymer created using CVD and patterned via microcontact printing of biotin azide. (b) A fluorescence micrograph demonstrating the binding of TRITC-streptavidin on biotin azide patterns. Reprinted with permission from ref.[37]. Copyright 2006 Wiley-VCH.*

12.2.6 Fiber Networks

Apart from planar or flat substrates, click chemistry has also been employed to functionalize fiber networks. Shi *et al*. demonstrated the immobilization of a testis-specific protease (TSP50) on biodegradable polymer fibers.[39] Ultrathin biodegradable lactide fibers containing propargyl groups were created using electrospinning and azide-functionalized TSP50 molecules were conjugated to this fiber via click chemistry. Another example of fiber modification was demonstrated by Bhaskar *et al*., where biphasic fibers containing alkyne groups in only one phase were fabricated using electrohydrodynamic co-jetting of alkyne-modified poly(lactide-co-glycolide) (PLGA) with unmodified PLGA.[40] The

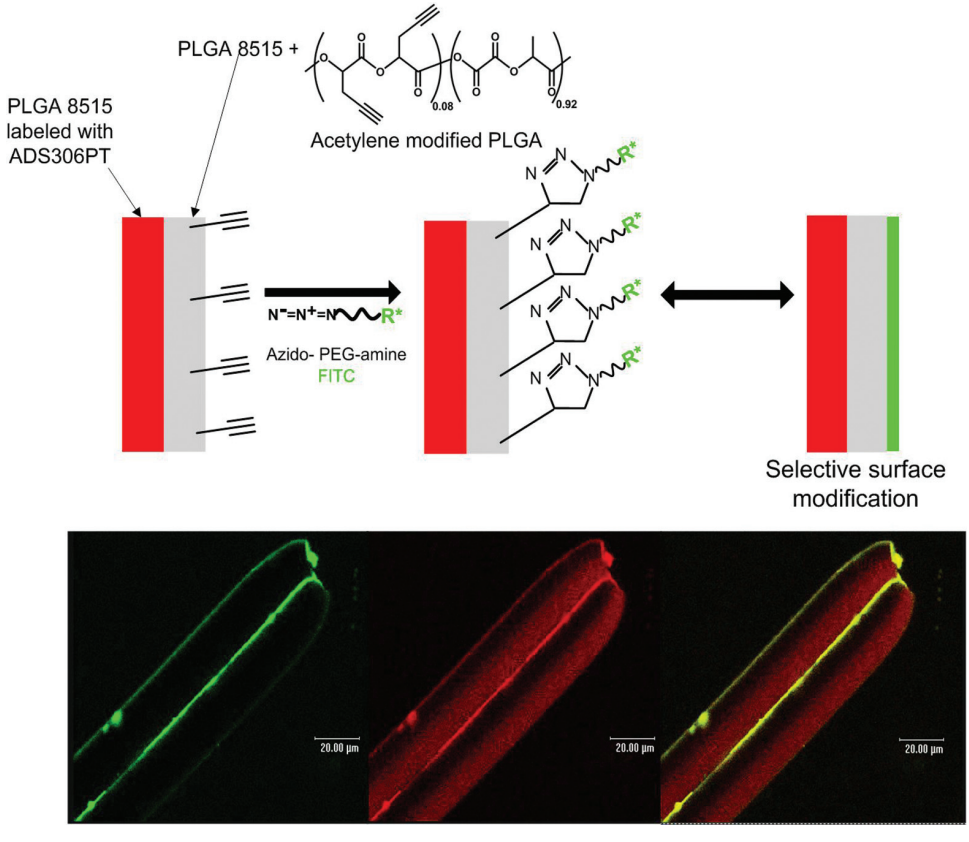

Figure 12.6 *Schematic and fluorescence micrographs showing the selective click modification of the biphasic fibers containing alkyne-functionalized PLGA in only one phase. Reprinted with permission from ref.[40]. Copyright 2008 Wiley-VCH.*

fibers were further selectively modified with an azide-functionalized fluorescent dye (Figure 12.6).

The use of copper-catalyzed 1,3-dipolar cycloaddition reaction has not been limited to biodegradable fiber surfaces. Krouit *et al*. employed click reaction to graft polycaprolactone macromolecular chains to the surface of cellulose fibers.[41] Cotton fibers have also been modified by Chen *et al*. to incorporate alkyne groups and further reacted with azide-functionalized methyl methacrylate polymers.[42]

12.3 Spatially Controlled Click Chemistry

The ability to spatially pattern a surface is quite important for electronics as well as biotechnological applications. The versatile click reaction is compatible with microcontact printing, which is a soft-lithographic process frequently employed to create micro or nanoscale patterns by depositing molecules on surfaces using a patterned stamp.[37,43–45] For instance, Nandivada *et al*. microcontact printed the copper catalyst onto a thin layer of

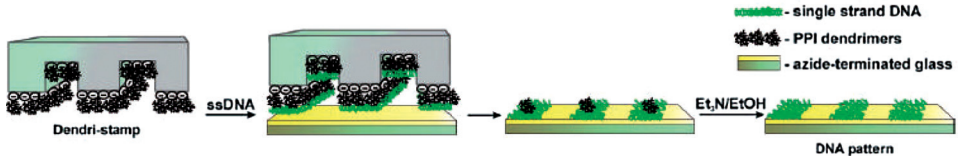

Figure 12.7 *Schematic description of catalyst-free click reaction via microcontact printing of alkyne-modified ss-DNA onto azide-functionalized substrate. The oxidized PDMS stamp is inked with dendrimers, incubated with ss-DNA and stamped onto azide functionalized surfaces. Reprinted with permission from ref.[46]. Copyright 2007 Wiley-VCH.*

biotinylated azide ligands adsorbed on vapor-based alkyne-functionalized polymer coatings.[37] By decoupling the catalyst and the reactants, the reaction was catalyzed only in the regions where the copper catalyst was deposited, thus creating patterns of covalently bound biotin. On the other hand, Rozkiewicz *et al*. performed the reaction without the copper catalyst, solely relying on the high concentration of reactants during microcontact printing as a driving force for the reaction.[43] Briefly, bromo-terminated SAMs were treated with sodium azide to create azide-terminated monolayers and alkyne-functionalized ligands were directly microcontact printed onto this monolayer. In the past, proximity of the ligands on the surface has been shown to lead to an enhancement of the reaction rate thus improving the efficiency of surface functionalization reactions.[9] This catalyst-free microcontact printing technique was further extended to pattern alkyne-containing oligonucleotides onto azide-modified glass slides.[46] To create surface patterns, a layer of positively-charged dendrimers was first inked onto the PDMS stamp, which promoted the binding of single-stranded DNA (ss-DNA) 'ink' to the stamp surface. This stamp was then brought into contact with the azido-substrate without the presence of the catalyst and click reaction was initiated (Figure 12.7). Furthermore, the covalently immobilized oligonucleotides were hybridized with their complementary strands.

The scope of copper-catalyzed click reactions has been broadened to create nanoscale patterns on solid surfaces via dip-pen nanolithography.[47] AFM tips were used to deliver Cu(I) catalyst and azide-functionalized dendrons to alkyne-functionalized surfaces. This technique takes advantage of the fact that shorter distances between the reactants may lead to an enhancement of the reaction rate. The main advantage of this approach is that multiple azides can be delivered sequentially or using different AFM tips to the same alkynated surface.

Furthermore, fluorescent molecules were patterned using a technique called scanning electrochemical microscopy (SECM), where a gold microelectrode was used to generate Cu(I) ions locally to catalyze the click reaction between alkyne-functionalized fluorophores and azide groups on a nonconductive glass substrate.[48]

12.4 Copper-catalyzed Click Chemistry for Bioimmobilization

The immobilization of biomolecules on surfaces is of tremendous interest for a wide variety of applications such as biosensors, microarrays, bioactive implant surfaces and tissue engineering. Preservation of the biomolecular activity after the reaction is a key attribute

for a successful bioconjugation reaction. This typically requires mild reaction conditions and absence of cross-reactivity between the functional groups present. By definition, click chemistry represents a collection of reactions with mild operating conditions, high yields and nonreactivity towards other functional groups. Therefore, in this respect, alkyne–azide click reaction is attracting a lot of attention from material and surface scientists for bioconjugation, specifically due to the inactivity of alkyne and azide groups towards other functional groups present in biomolecules. Conjugation of molecules like carbohydrates, oligonucleotide probes, proteins and peptide sequences has been successfully demonstrated using click reaction on surfaces and will be discussed in further detail.

Several reports have described the immobilization of biotin on flat surfaces via click chemistry and have taken advantage of the highly specific albeit noncovalent biotin–streptavidin binding.[37,49,50] For example, Lee *et al*. used click chemistry to functionalize polymeric nanobrushes with azide end-groups.[50] Ethylene glycol-based polymer films were synthesized using surface-initiated ATRP onto initiator-containing SAMs. Subsequently the bromide-presenting polymer was reacted with sodium azide to introduce azide groups on the surface, which were then reacted with alkyne-containing biotin. This ethylene glycol-based polymer film demonstrated nonbiofouling characteristics combined with specific reactivity towards alkyne-functionalized ligands.

Glycan arrays provide an opportunity to study the complex protein–sugar interactions and enhance our understanding of role of glycans present on the cell surface.[51] Microarrays also allow the screening of multiple ligands simultaneously with minimal use of material. Copper-catalyzed click chemistry presents a robust strategy to covalently link saccharide molecules to a flat surface, thus mimicking the cell surface expressing these glycans.[52] Sugar-modified SAMs or glyco-SAMs have been used as glycan arrays because they provide better control over the density and orientation of the saccharide molecules and can be characterized after immobilization via surface analysis techniques. For example, Zhang *et al*. employed alkyne–azide click reaction to immobilize azide-functionalized sugars (mannose, lactose, α-galactose) on to alkyne-containing SAMs.[20] This method is much simpler than the direct assembly of pre-synthesized thiol-terminated sugar molecules, which require complex synthesis procedures. The unique platform displaying sugar-functionalized SAMs was further used to study binding interactions between sugars and lectins by employing electrochemical characterization and surface plasmon resonance spectroscopy. This approach represents a label-free technique to elucidate real-time structure–activity information. Similarly, Kleinart *et al*. extended this study by synthesizing a series of functionalized thiol molecules and comparing the assembly of pre-formed glyco molecules with the previously described 'click on SAM' approach.[19] Miura *et al*. studied the interactions between pathogenic protein Alzheimer amyloid-β (Aβ) and monosaccharide displaying silane-based monolayers which were created using Huisgen's 1,3-cycloaddition.[53] This study enabled the estimation of the core saccharide interacting structure of the Aβ protein.

Click conjugation has also been used to reversibly capture azide-modified saccharides on an alkyne-functionalized microtiter plate.[54,55] A disulfide bridge was included in the linker to enable the cleavage and release of the captured oligosaccharide molecule for further characterization utilizing a reductive treatment with a thiol (dithiothreitol, DTT) (Figure 12.8). Using this technique, a breast cancer antigen, Globo-H, was captured and analyzed after cleavage, thus demonstrating the utility of this method for biosensor applications.[55]

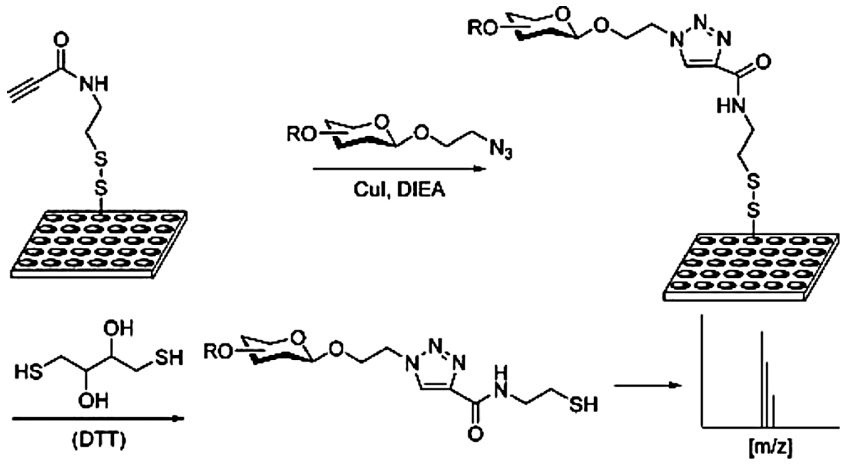

Figure 12.8 *Alkyne-functionalized microtiter plates which captured azide-modified saccharides. Saccharides were cleaved by DTT for further MS analyses. Reprinted with permission from ref.[55]. Copyright 2004 American Chemical Society.*

Copper-catalyzed Huisgen's 1,3-dipolar cycloaddition has also been used in conjunction with another reaction from the click family, namely Diels–Alder reaction, for the immobilization of carbohydrates on solid surfaces.[49] Sun *et al*. synthesized a short heterobifunctional PEG linker with alkyne and cyclodiene terminal groups on either side. This linker was conjugated to maleimidocaproyl-functionalized substrates via Diels–Alder reaction leaving the alkyne-terminal end for subsequent alkyne–azide click reaction with azide-functionalized ligands. This 'dual-click' approach was used for the successful immobilization of biomolecules such as biotin, lactose and *r*-thrombomodulin. Success of the immobilization step was further confirmed using antibody-binding via surface plasmon resonance (SPR) spectroscopy.

Immobilization of saccharide molecules has also been achieved via microcontact printing of alkyne-functionalized carbohydrates onto azido SAMs.[44] Michel and Ravoo microcontact printed carbohydrate (mannose, glucose, galactose and maltose) conjugates with alkyne functionality and used the corresponding lectins to probe the sugars. These arrays provide important information regarding structure–function relationships, which may ultimately lead to better understanding of the immune responses.

On the other hand, DNA arrays are generally created using nucleophilic–electrophilic reactions, which are hampered by the lack of efficiency, chemoselectivity and reproducibility. Immobilization of DNA further requires an aqueous reaction environment. An elegant strategy utilizing copper-catalyzed click chemistry for the fabrication of oligonucleotide arrays was demonstrated by Devaraj *et al*.[18] Alkyne-functionalized oligodeoxyribonucleotides were synthesized and conjugated with azide-terminated monolayers in the presence of a triazolylamine copper ligand, tris(bezyltriazolylmethyl)amine [Cu(I)TBTA] as the catalyst. Cu(I)TBTA accelerates the cycloaddition reaction without damaging the structure of DNA as opposed to free Cu(I), which disrupts the oligonucleotide structure in the presence of reactive oxygen species. On the other hand, Seo *et al*. created a photocleavable DNA array using click chemistry where azido-labeled DNA was attached to alkyne-modified glass surfaces (Figure 12.9).[56] Furthermore, DNA–polymerase-extension

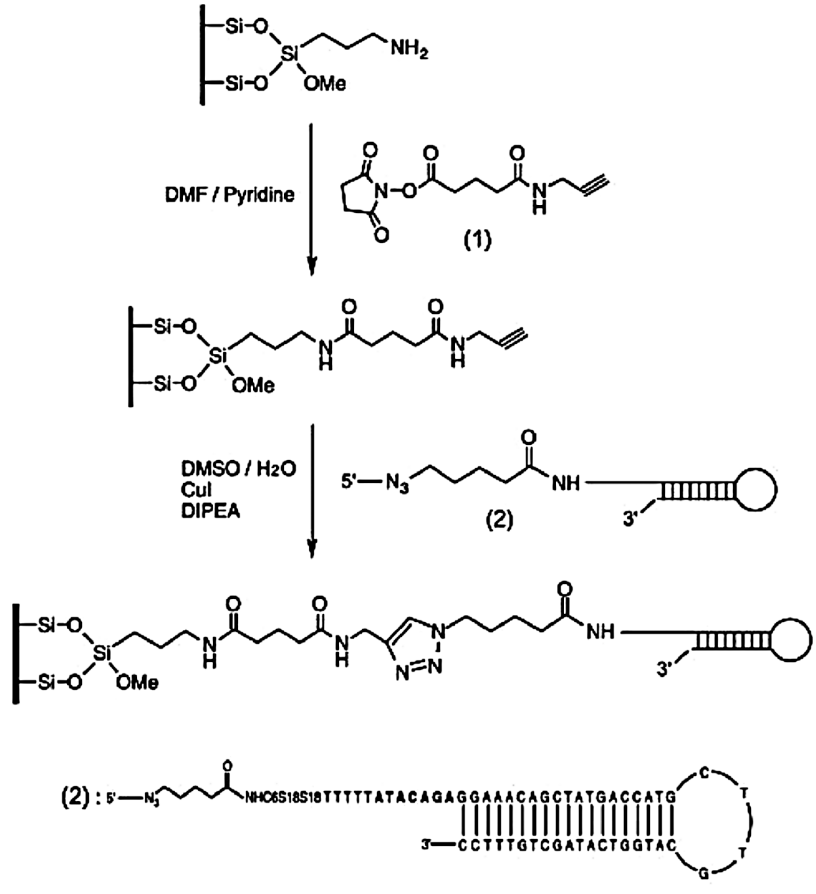

Figure 12.9 *Schematic demonstrating the construction of a DNA chip using copper catalyzed click reaction. Alkyne-functionalized glass slides form a covalent bond with 5′-azide-modified DNA. Reprinted with permission from ref.*[56]*. Copyright 2004 National Academy of Sciences, USA.*

reaction was accurately performed on this array to incorporate fluorescent nucleoside analogs.

Protein microarrays can be used to study protein–protein and protein–ligand interactions. Immobilized proteins are more robust than noncovalently bound proteins and also an enhanced sensitivity can be achieved.[57] However it is challenging to maintain the activity and conformation of the proteins during immobilization reactions. Some research groups have demonstrated the immobilization of proteins using copper-catalyzed click chemistry.[58,59] Azide- or alkyne-modified proteins were covalently bound to alkynated or azidated glass slides via click chemistry.[59] Interestingly, it was observed that immobilization of the alkyne-functionalized protein onto an azide-presenting surface was more efficient than the other way around. This may indicate that alkyne groups were coupling with the copper ions during the reaction, thereby reducing the catalytic effects.

A fascinating approach for the fabrication of a density gradient of cell-adhesion peptides by 'clicking' RGD azide-peptides onto an alkyne-gradient substrate has been reported by

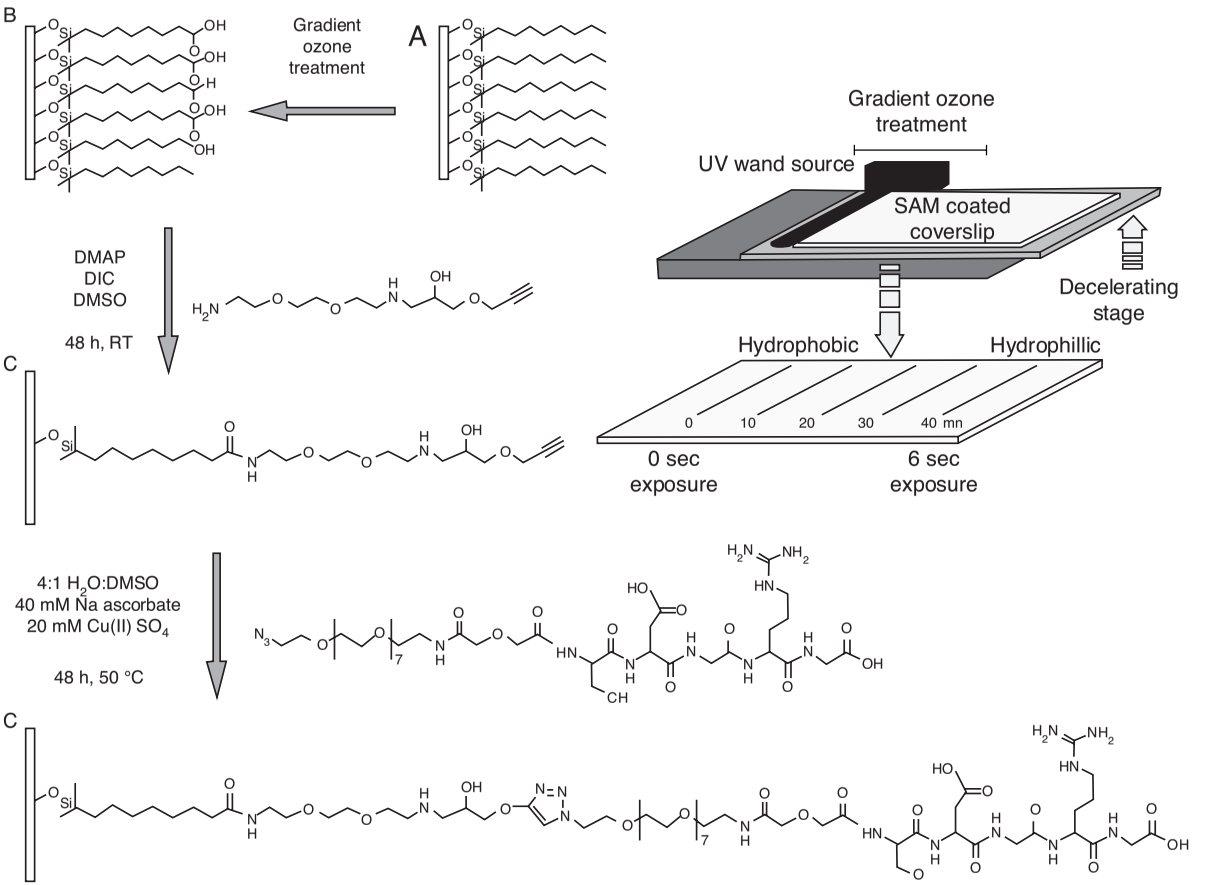

Figure 12.10 *Creation of a gradient substrate for RGD peptide immobilization by click chemistry. (A) Variable UV oxidation generated (B) a carboxyl gradient which was converted to (C) an alkyne gradient and (D) RGD azide–peptide was covalently immobilized by click cycloaddition reaction. Reprinted with permission from ref.*[60]*. Copyright 2007 Wiley-VCH.*

Gallant *et al*.[60] Briefly, variable UV oxidation followed by a bifunctional linker was used to synthesize an alkyne-functionalized gradient (Figure 12.10). Subsequent reaction with an RGD azide-modified peptide resulted in a gradient of peptides, which was able to modulate smooth muscle cell attachment. The density of the immobilized moiety depends on the density of the functional group and the efficiency of the coupling chemistry.

12.5 Summary

In this chapter, the use of copper-catalyzed click chemistry has been discussed in the context of surface engineering. Alkyne–azide click reactions were successfully used for coupling of different organic compounds and biomolecules to a wide variety of substrates. These generally robust reactions have gained immense popularity among surface scientists owing to their functional group tolerance, mild reaction conditions and high reactivity. Numerous strategies are being employed to incorporate the alkyne and azide functionalities onto the surface.

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