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2. Surface modification

Several surface modification techniques have been developed to improve wetting, adhesion, and printing of polymer surfaces by introducing a variety of polar groups, with little attention to functional group specificity. However, when surface modification is a precursor to attaching a bioactive compound, these techniques must be tailored to introduce a specific functional group. Techniques that modify surface properties by introducing random, non-specific groups or by coating the surface are less useful in bioconjugation to polymer surfaces.

2.1. Techniques in surface modification

2.1.1. Wet chemical

In wet chemical surface modification, a material is treated with liquid reagents to generate reactive functional groups on the surface. This classical approach to surface modification does not require specialized equipment and thus can be conducted in most laboratories. It is also more capable of penetrating porous three-dimensional substrates than plasma and other energy source surface modification techniques [9], and allows for *in situ* surface functionalization of microfluidic devices. Chromic acid and potassium permanganate in sulfuric acid have been used to introduce reactive oxygen-containing moieties to PE and PP [10–16]. For example, submersion in a 29:42:29 weight ratio solution of chromium trioxide, water, and sulfuric acid at 72 °C for 1 min resulted in 3.3 nmol/cm^2 carboxylic acid functionalities on PE [12]. Concentrated sodium hydroxide and sulfuric acid have been used to generate carboxylic acid groups by base and acid hydrolysis of PMMA [17–20]. Specifically, a 16 h treatment in 10 M sodium hydroxide at 40 °C was reported to produce 0.66 nmol/cm^2 carboxylic acids on PMMA [17]. Methyl ester side chains of PMMA also have been reduced to hydroxyls by treatment in lithium aluminum hydride in ether [21]. Primary amines have been introduced to PMMA, poly(urethane) (PU), PLA, and PLGA by aminolysis using various diamines including hydrazine hydrate, 1,6-hexanediamine, ethylenediamine, and *N*-aminoethyl-1,3-propanediamine, as well as lithiated diamines [7,19,22–28]. Lastly, PTFE surfaces have been modified by refluxing with elementary sodium in toluene to generate double bonds, followed by an 8 h oxidation at 120 °C in a 1:1 mixture of trifluoroacetic acid and hydrogen peroxide (38%) which improved membrane wetting and allowed immobilization of the enzyme alliinase [29].

Unfortunately, these wet chemical methods are non-specific, producing a range of oxygen-containing functional groups. In addition, those which target modification of polymer side chains (as in PMMA ester modification) depend on side chain surface orientation. The degree of surface functionalization may therefore not be repeatable between polymers of different molecular weight, crystallinity, or tacticity. These wet chemical methods also generate hazardous chemical waste and can lead to irregular surface etching [30]. Many of these techniques also require extended treatment in concentrated corrosive solutions. For these reasons, while useful in the laboratory environment, these wet chemical processes may not be suitable for larger scale, industrial applications.

2.1.2. Silane monolayers

The immobilization of organosilanes to surfaces was initially developed as a means to couple an organic polymer to an inorganic substrate, for example to promote adhesion between glass and polymers in the development of glass-reinforced polymers (fiberglass) [31]. The unique arrangement of silanes on a surface has been extensively studied by the Whitesides group, and has been dubbed a self-assembled monolayer (SAM) because of the ability to self-organize onto an appropriately functionalized surface as an ordered, single molecular layer [32–34]. This ordered, "quasi-crystalline" [34], structure has enabled silane monolayers to be widely investigated for the micro/nanofabrication of optics and electronics devices on inorganic substrates such as glass or silicon (via a siloxy linkage), and gold, copper, or silver (via a thiol linkage) [31]. While traditionally immobilized via wet chemical means, a technique called Molecular Vapor Deposition™ has been developed in which a vacuum evacuated chamber is used to first pretreat the surface with oxygen plasma, followed by chemical vapor deposition of the silane [35].

Organosilanes with different end functionalities, including poly(ethylene glycol) (PEG), bromine, and vinyl (–CH=CH$_2$), were applied to clean glass slides to observe differences in protein and fibroblast adhesion. Organosilanes were self-assembled by overnight submersion in a 1% (v/v) solution of silane in ethanol at room temperature, followed by

ethanol and distilled water rinses. Vinyl and bromine terminated silanes were then modified via wet chemical methods to generate carboxylic acids, amines, and hydroxyl functionalities, as confirmed by changes in water contact angle [36]. Because the target surface functional group in glass or silicon silanization is a hydroxyl, these same chemistries apply to hydroxylated polymer surfaces. Organosilanes have been immobilized onto H_2O plasma functionalized PMMA [37], lithium aluminum hydride reduced PMMA [21], and Ar plasma functionalized PTFE [38] to develop microelectromechanical systems (MEMS). Fluorinated silanes have been immobilized on PET for preparation of oil-repellant textiles [39], and silane coupling agents have been utilized to condition PET, ethylene vinyl alcohol (EVOH), and Nylon 6 surfaces with titanium oxide for the development of a bone tissue scaffold [40]. Because of their nearly crystalline organization, SAMs offer the potential for more defined surface functionalization than typical wet chemical or ionized gas functionalization techniques [33]. However, the siloxane linkage can be hydrolyzed at high temperatures or alkaline pH [34,41]. Although research on surface modification using silane monolayers continues to focus primarily on inorganic substrates, this is certain to be an emerging field for polymer surface modification.

2.1.3. Ionized gas treatments

2.1.3.1. Plasma. Plasma is a high energy state of matter, in which a gas is partially ionized into charged particles, electrons, and neutral molecules [42]. Plasma can provide modification of the top nanometer of a polymer surface without using solvents or generating chemical waste and with less degradation and roughening of the material than many wet chemical treatments [30,43–45]. The type of functionalization imparted can be varied by selection of plasma gas (Ar, N_2, O_2, H_2O, CO_2, NH_3) and operating parameters (pressure, power, time, gas flow rate) [44].

Oxygen plasma is often used to impart oxygen containing functional groups to polymer surfaces such as PCL [46], PE [11], and PET [47]. In addition to oxygen, carbon dioxide plasma has been used to introduce carboxyl groups on PP [48], PS [49], and PE [48,50–52], and air plasma has been used to oxidize PMMA [19]. Ammonia and nitrogen plasmas have been used to impart amine groups to the surface of PTFE [53–55] and PS [49], respectively. Inert gases can be used to introduce radical sites on the polymer surface for subsequent graft copolymerization. For instance, Kang et al. pretreated PTFE with Ar plasma in order to graft polymerize acrylic acid [56], as did Ademovic et al. on PVDF [57] and Cheng et al. on PCL [58]. Similarly, PE, PET, and PVDF have been pretreated with Ar plasma in order to graft polymerize poly(ethylene glycol) monomethacrylate and 4-vinylpyridine [59,60]. In a process called radio frequency glow discharge (RFGD), plasma can be used to initiate surface graft polymerization of vaporized monomers [61].

Plasma can be used as a precursor to other surface modification techniques, for example, plasma activation followed by ultraviolet (UV) graft polymerization [62] or plasma activation followed by silanization [37,38]. However, with the exception of a recent development of an atmospheric plasma system [63], plasma generation requires a vacuum to empty the chamber of latent gases, which presents complications for continuous operation in a large scale industrial setting [64]. Also, results are difficult to repeat between laboratories as there are many parameters involved to optimize conditions, including time, temperature, power, gas composition/flow/pressure, orientation of reactor and distance of substrate from plasma source [43]. It should be noted that in addition to the monomers and gases intentionally introduced to the plasma chamber, latent chemicals from prior users may be present thus posing a risk of contamination. The plasma chamber should therefore be adequately cleaned, for example by oxygen plasma, before introducing polymers for surface modification.

2.1.3.2. Corona discharge. Corona discharge is a simple, low cost, continuous process in which an electrically induced stream of ionized air bombards the polymer surface. It is commercially used to improve printability and adhesion to inert polymers [44] by introducing surface oxidation products [30,65]. However, this results in a broad range of oxygenated groups and therefore may be less useful as a technique to introduce specific functionalities for bioconjugation. Because it does not operate under a vacuum, contamination may also be an issue, and variations in local temperature and humidity can affect consistency of treatment [30]. Finally, it has been reported that surface polar groups on corona treated polyolefins are particularly unstable. Materials should therefore be used shortly after treatment [45].

2.1.3.3. Flame treatment. Like corona discharge, flame treatment is a non-specific surface functionalization method that bombards the polymer surface with ionized air, generating a broad spectrum of surface oxidation products to the top several monolayers [30,44]. In this method, the reactive oxygen is generated by burning an oxygen rich gas mixture. Flame treatment has been shown to impart hydroxyl, aldehyde, and carboxylic acid functionalities to poly(ethylene) and is utilized to enhance printability, wettability, and adhesion [65]. Although fundamentally simple and inexpensive, flame treatment can reduce optical clarity to polymers, and there are many parameters (including flame temperature, contact time and composition) that must be accurately controlled to maintain consistent treatment and to avoid burning [45].

2.1.4. UV irradiation

When exposed to UV light, polymer surfaces generate reactive sites which can become functional groups upon exposure to gas or can be used to initiate UV-induced graft polymerization. This technique differs from ionized gas treatments by the ability to tailor the depth of surface reactivity by varying wavelength and thus absorption coefficient [43]. UV irradiation has been used to introduce carboxylic acid functionality to PMMA [66], as well as to activate PS surfaces for enzyme immobilization [67] and tissue engineering [68] applications. UV irradiation has also been used to initiate radical graft polymerization of bioactive compounds. For instance, *N*-vinylpyrrolidone has been photografted to the surface of PP films to generate antimicrobial materials [69]. UV treatment can, however, affect the optical properties of the polymer, and UV light can be blocked by particles, which may affect treatment consistency.

2.2. Tether molecules in bioconjugation

If initial surface functionalization does not generate enough surface reactive groups, or if the bioactive compound loses activity when linked directly to the hydrophobic polymer surface, it may be necessary to graft an intermediary between the surface and the bioactive compound. There are many factors which influence the effect of the tether molecule on biomolecule activity, as illustrated in Fig. 3. Factors such as structure–function relationships, location of functional groups available for conjugation, and the impact of binding the bioactive compound to a hydrophobic substrate must be considered. A thorough understanding of the bioactive compound of interest is therefore critical in selecting which intermediary to utilize, if any.

2.2.1. Polyfunctional reagents

Acrylic acid has been graft polymerized to polymer surfaces to generate surface carboxylic

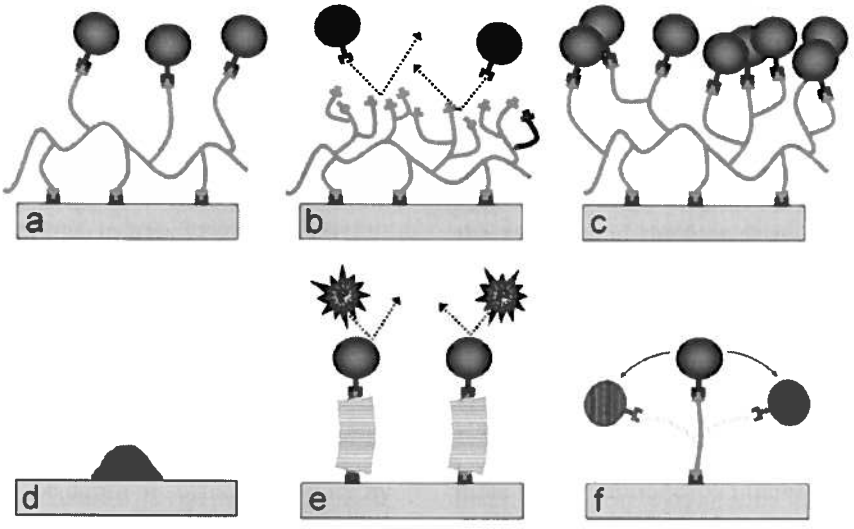

Fig. 3. Effect of tether molecule on bioactivity: (a) increased biomolecule immobilization due to branched spacer molecule, (b) reduced biomolecule immobilization due to overfunctionalization of surface, (c) reduced bioactivity due to overcrowding of bioactive compounds, (d) surface induced denaturation, (e) reduced nonspecific adsorption of proteins due to hydrophilic spacer, (f) increased mobility due to spacer molecule.

acids [1,47,52,57,58,70]. Poly(ethylenimine), poly (allylamine), and chitosan have been bound to surface aldehydes or carboxylic acids to increase functionality in the form of primary amines [6,57,59,71–73]. By using such a polyfunctional agent, one can increase the number of reactive sites available on a surface for immobilization of bioactive compounds (Fig. 3a). For example, grafting poly(ethylenimine) to a surface onto which PEG is attached results in a high PEG density resulting in reduced protein adhesion [73,74]. When bound to a chromic acid oxidized PE film, poly (ethylenimine) increased the number of available reactive functional groups from 3.3 to 15.7 nmol/ cm^2 [16]. Building a polyanionic surface with graft polymerized poly(acrylic acid) results in enhanced biocompatibility [75], added capacity to covalently immobilize proteins for medical devices [76], and improved adhesion [14]. Dendrimers offer another way to increase surface functionality [77,78]. A range of end functionalized dendrimers are available, and their step-by-step synthesis allows for control in the quantity of functional groups present on a dendrimer, depending on its generation. Various dendrimers have been employed to increase the protein and DNA immobilization density on inorganic substrates (glass slides, gold electrodes, Si wafers) in the development of high sensitivity bioanalytical assays [79–82]. Reports are limited for use of dendrimers in polymer surface modification, however, they would be suitable for use in increasing polymer surface functionality.

The major risk in utilizing a highly branched or dendritic tether is overcrowding, which may have dual impact. After a certain point, continuing to increase the number of reactive functional groups in a given area may lead to overcrowding of the functional groups which may reduce the immobilization of bioactive compounds (Fig. 3b). In addition, if bioactive compounds are overcrowded on a surface, they may be sterically hindered, thus reducing bioactivity (Fig. 3c).

2.2.2. Poly(ethylene glycol) (PEG)

Many bioactive compounds, such as enzymes, lose activity when linked directly to a solid hydrophobic substrate (Fig. 3d). Using a hydrophilic spacer molecule such as PEG may shield the compound from denaturation and improve bioactivity (Fig. 3e) [83–85]. For example, when PEG was used to tether immunogens to a solid surface, not only was non-specific protein adsorption reduced, but the enzyme-linked anti-immunogen was more stable against denaturation. Both of these factors are critical in optimizing the specificity and sensitivity of a bioanalytical assay [84,86]. In another study, the anticoagulation properties of immobilized heparin were improved by utilizing a hydrophilic PEG spacer, when compared to heparin immobilized directly to the polymer surface [87].

Linking a bioactive compound directly to a solid substrate also introduces steric constraints, which can then reduce or eliminate bioactivity. Using PEG as a spacer molecule to tether the bioactive compound to the surface has been shown to improve peptide bioactivity [85,88], DNA hybridization in microarrays [89], antigen detection in biosensors [86], and heparin bioactivity in hemocompatible devices [47,71,90,91]. When immobilized on surfaces, PEG has a unique ability to resist protein adhesion, as discussed further in Section 5.1. This property can help improve the biospecificity of PEG-tethered bioactive compounds (Fig. 3e). A variety of end-functionalized PEGs are commercially available, however these reagents can be high in cost and limited in availability. Inconsistencies in degree of functionalization and purity are issues with "home-made" functionalized PEGs.

3. Immobilization of bioactive compounds

The specific functionality imparted to the inert surface must be compatible with the reactive sites on the compound to be covalently attached to that surface; common functional groups in bioconjugation chemistry include thiols, aldehydes, carboxylic acids, hydroxyls, and primary amines. The development of numerous cross-linking agents (Tables 3a–e) has expanded the array of usable conjugation chemistries [36,75,92–94]. A detailed description of these chemistries can be found in *Bioconjugate Techniques*, by Hermanson [92]. Like bioactive compounds, cross-linkers differ in their optimum pH values, temperatures, and solubilities and must be selected based on the conditions needed for polymer : biomolecule conjugation. Cross-linkers can link the bioactive compound directly to the functionalized substrate (zero-length cross-linkers), or they can introduce a spacer of several angstroms.

It may be of interest to block certain functional groups in order to ensure proper coupling between targeted functional groups. In addition, functional groups are often charged and thus may lead to non-specific binding or contribute to a shift in bioactive