# FABRICATION AND FUNCTIONALIZATION OF REACTIVE POLYMERIC COATINGS FOR BIOMEDICAL APPLICATIONS

by

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*To my family*

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# ABSTRACT


## FABRICATION AND FUNCTIONALIZATION OF REACTIVE POLYMERIC COATINGS FOR BIOMEDICAL APPLICATIONS

The work in this thesis describes the design and synthesis of novel reactive polymeric coatings that can be utilized for fabrication of functional interfaces for applications like biosensing and protective antibacterial coatings for implants. Various approaches were utilized to prepare these surface bound coatings as polymeric thin films, polymer brushes or hydrogels. In the first study, thermo-responsive polymeric films were prepared using furan containing copolymers and their reversible functionalization was demonstrated via Diels-Alder (DA)/retro Diels-Alder (rDA) strategy. The second study discloses fabrication of maleimide-containing thiol-reactive coatings on glass like surfaces and their functionalization using the nucleophilic thiol-ene reaction. In the third project, the maleimide-containing polymers were adapted for adhesion to metal surfaces via mussel inspired catechol based interaction. These surfaces were conjugated with antibacterial peptides, followed by assessment of their antimicrobial activity against bacteria. In the fourth project, maleimide containing polymer brushes were fabricated and appropriately functionalized brushes were employed for ligand-directed protein mediated immobilization of nanoparticles. In fifth study, amine-reactive polymer brushes containing succinimidyl-carbonate moieties amenable towards facile functionalization with amine-containing molecules were synthesized. In the final chapter, fabrication of multifunctional furan-protected maleimide-containing hydrogel coating on titanium surfaces that could be modified using UV-mediated thiol-ene, ieDDA, and after unmasking of the maleimide, with nucleophilic thiol-ene and DA reactions was described. In summary, in this thesis a variety of reactive polymer coated surfaces were fabricated and their efficient functionalization was demonstrated to highlight them as attractive candidates for various biomedical applications.

# ÖZET

## BİYOMEDİKAL UYGULAMALAR İÇİN REAKTİF POLİMERİK KAPLAMALARIN ÜRETİMİ VE İŞLEVSELLEŞTİRİLMESİ

Bu tezdeki çalışma, biyosensör ve implantlar için koruyucu antibakteriyel kaplamalar gibi işlevsel ara yüzeylerin üretimi için kullanılabilen yeni reaktif polimerik kaplamaların tasarımını ve sentezini açıklamaktadır. Bu yüzeye bağlı kaplamaların, polimerik ince filmler, polimer fırçalar veya hidrojeller olarak hazırlaması için çeşitli yaklaşımlar kullanılmıştır. Kaplamaların hepsinde, polimerik arayüzün özellikleri monomer kombinasyonu kullanılarak uyarlanabilmektedir. Birinci çalışmada, ısıl-duyarlı polimerik filmler, furan içeren polimerler kullanılarak hazırlanmış ve bunların geri dönüşümlü işlevselleştirilmesi, Diels-Alder (DA)/retro Diels-Alder (rDA) stratejisi vasıtasıyla gösterilmiştir. İkinci çalışmada, cam benzeri yüzeyler üzerinde tiyol-reaktif kaplamaların üretimi ve nükleofilik tiyol-en reaksiyonu ile işlevselleştirilmesi açıklanmaktadır. Üçüncü projede, maleimid içeren polimerler, midyelerden ilham alınarak katekol etkileşimi yoluyla yüzeye bağlanmak üzere uyarlanmıştır. Bu yüzeyler, antibakteriyel peptitlerin konjugasyonu için kullanılmış ve ardından bakterilere karşı antimikrobiyal aktivitelerinin değerlendirilmesi yapılmıştır. Dördüncü projede maleimid içeren polimer fırçalar maskeli maleimid monomer kullanılarak elde edilmiş, ve uygun şekilde işlevselleştirilmiş polimer fırçalar nanoparçacıkların ligand yönlendirmeli protein aracılı immobilizasyonu için kullanılmıştır. Beşinci çalışmada, amin içeren moleküller ile kolay işlevselleştirmeye elverişti süksinimidil-karbonat grupları içeren amin reaktif polimer fırçalar sentezlenmiştir. Son bölümde, UV aracılı tiyol-en ve ieDDA kullanılarak modifiye edilebilen ve maleimidin maskesinin çıkarılmasından sonra nükleofilik tiyol-ene ve DA reaksiyonları ile modifiye edilebilen çok fonksiyonlu furan korumalı maleimid içeren hidrojel kaplamanın titanyum yüzeylerde üretimi anlatılmıştır. Özetle, bu tezde çeşitli reaktif polimer kaplı yüzeyler üretilmiş ve bunların çeşitli biyomedikal uygulamalar için uygun adaylar olduklarını vurgulamak üzere etkin işlevselleştirilmeleri gösterilmiştir.

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# LIST OF ACRONYMS/ABBREVIATIONS

| | |
|---|---|
| AFM | Atomic Force Microscopy |
| AIBN | 2,2’-azobisisobutyronitrile |
| AMP | Anti-Microbial Peptide |
| ATR | Attenuated Total Reflectance |
| ATRP | Atom Transfer Radical Polymerization |
| BODIPY | 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene |
| BSA | Bovine Serum Albumin |
| CuAAC | Copper-catalyzed Azide Alkyne Cycloaddition |
| $CDCl_3$ | Deuterated Chloroform |
| $CH_2Cl_2$ | Dichloromethane |
| CTA | Chain Transfer Agent |
| DA | Diels-Alder |
| DEGMEMA | Di(ethylene glycol) methyl ether methacrylate |
| DOPA | 3,4-dihydroxy-L-phenylalanine |
| DMA | Dopamine methacrylamide |
| DMF | Dimethylformamide |
| DMPA | 4-Dimethylaminopyridine |
| DMSO | Dimethyl sulfoxide |
| EtOAc | Ethyl Acetate |

| | |
|---|---|
| EIBB | Ethyl-2- bromoisobutyrate |
| FITC | Fluorescein Isothiocynate |
| FTIR | Fourier Transform Infrared |
| FuMA | Furfuryl Methacrylate |
| FuMaMA | Furan-protected Maleimide Methacrylate |
| FTIR | Fourier Transform Infrared |
| GPC | Gel Permeation Chromatography |
| iEDDA | Inverse Electron Demand Diels–Alder |
| IR | Infrared |
| MeOH | Methanol |
| NHSMA | N-hydroxysuccinimidyl methacrylate |
| NMR | Nuclear Magnetic Resonance |
| PDI | Polydispersity Index |
| PEG | Poly(ethylene glycol) |
| PEGDMA | Poly(ethylene glycol) dimethacrylate |
| PEGMEMA | Poly(ethylene glycol) methyl ether methacrylate |
| PMDETA | N,N,N’-N’’-N’’ pentamethyldiethylenetriamine |
| PSAM | Polymeric Self-Assembled Monolayer |
| RAFT | Reversible Addition Fragmentation Chain Transfer |
| rDA | Retro Diels-Alder |
| SAM | Self-Assembled Monolayer |
| SCEMA | 2-(N-succinimidylcarboxyoxy)ethyl methacrylate |

| | |
|---|---|
| S-RAFT | Surface-Reversible Addition Fragmentation Chain Transfer |
| TEA | Triethylamine |
| TGA | Thermogravimetric Analysis |
| THF | Tetrahydrofuran |
| TMSMA | 3-(Trimethoxysilyl)propyl methacrylate |
| TRITC | Tetramethylrhodamine-isothiocyanate |
| UV | Ultraviolet |
| XPS | X-Ray Photoelectron Spectroscopy |

# 1. INTRODUCTION

## 1.1. Polymer Coatings on Solid Substrates

Functional surfaces are at the heart of various biomedical applications that range from sensing and delivery platforms to facilitating the adaptation of various implant materials. Recently, polymeric coating materials are applied to diverse fields such as corrosion protection [1], self-cleaning surfaces [2] and better mucous permeability in contact lenses [3]. Widely used applications in biomedical sciences encompass coating of orthopedic materials, cardiovascular stents, antibacterial surfaces, drug delivery devices, tissue engineering scaffolds and biosensors [4]. Apart from presenting the functional characteristics necessary for a particular application, an effective polymeric coating also requires a strong interaction between the polymeric material and the underlying substrate, as well as stability under the conditions it is expected to perform. In this thesis, polymer coatings on the solid surfaces were fabricated as thin films, brushes and 3D cross-linked hydrogel layers which are all covalently attached to the substrates (Figure 1.1). The introductory part of the thesis briefly focuses on various methods of fabricating reactive polymeric coatings and illustrates with selected examples applications of such polymer modified surfaces in areas of biosensing and antibacterial coatings, areas closely related to investigations reported in this thesis.

### 1.1.1. Polymeric Thin Films

Interactions of a surface with the materials in its surroundings are crucial in determining the performance, as well as long term stability of the material, and hence has attracted a lot of interest from the scientific community in different fields. The strength of the interaction between molecule and interface mostly depends on the chemical nature of

the materials [5]. Coatings obtained using organic molecules have been investigated for applications like modulating surface wettability and creating model surfaces e.g. exterior of a cell. One of the most thoroughly investigated molecule-substrate interaction are

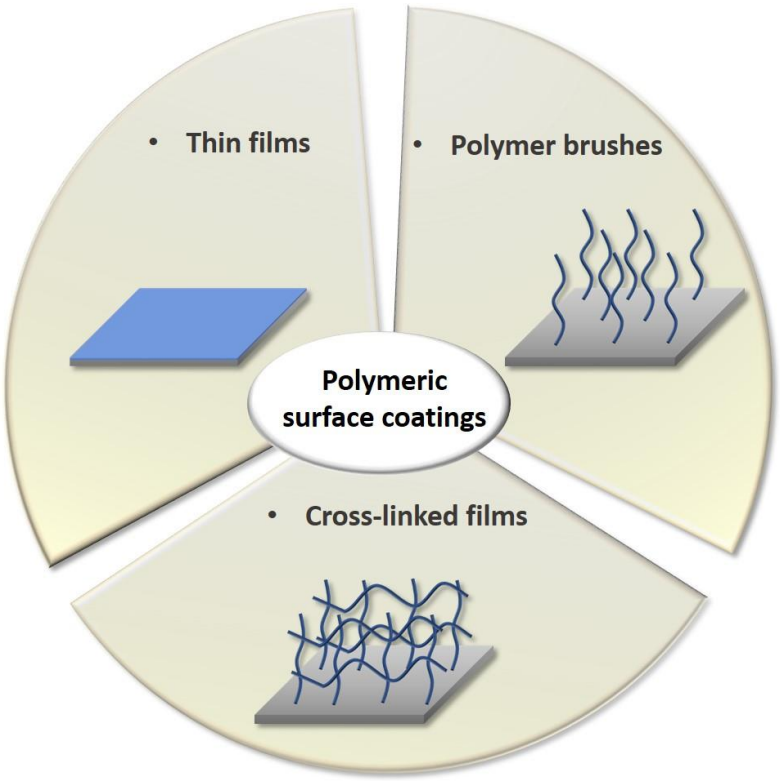


Figure 1.1. Polymeric coatings on solid substrates investigated in this dissertation.

monolayers on the surface [6,7]. They have attracted increasing attention in past decades as a versatile tool to modify the physical and chemical properties of a surface. Most common monolayers involves the one obtained by adsorption of n-alkanethiols onto gold surfaces [8,9]. For example, ethylene glycol bearing thiol derivatives ((EG)n-SH) on gold surfaces are commonly used for imparting protein resistance. However, it has been shown that such systems have limitations mostly due to oxidation of chemisorbed thiolates [10,11]. It is the existence of defects in the monolayer that reduces the stability of robustness and such coatings deteriorate over time [12,13]. So, relatively more robust alternatives such as silicon dioxide surfaces coated with trialkyl-, trichloro-, or trialkoxysilanes have been used when possible [14-16].

Similar to small molecules containing functional groups with affinity for a particular surface, macromolecular constructs like polymers containing surface-reactive end groups or side chains also tend to be adsorbed onto appropriate surfaces. Depending on the positions of anchoring units in the chain, grafting modes can be full side grafting, random side grafting, block side grafting or end grafting. When all repeating units of the polymer have reactivity towards surface, a coating with full side grafting is formed. When surface reactive units are randomly distributed along the backbone, polymer is grafted to the surface by random side grafting, and when anchoring block of a block copolymer is adsorbed parallel to the surface and the remaining block is free, block side grafting is formed (Figure 1.2) [17]. Lastly, when polymer chains that are tethered to the surface from their one end, end grafted polymeric coatings are formed. The conformation of end grafted polymers varies with the grafting density and could be in a mushroom or brush conformation depending on density of the polymer chains, as will be discussed in more detail under the next sub-heading.

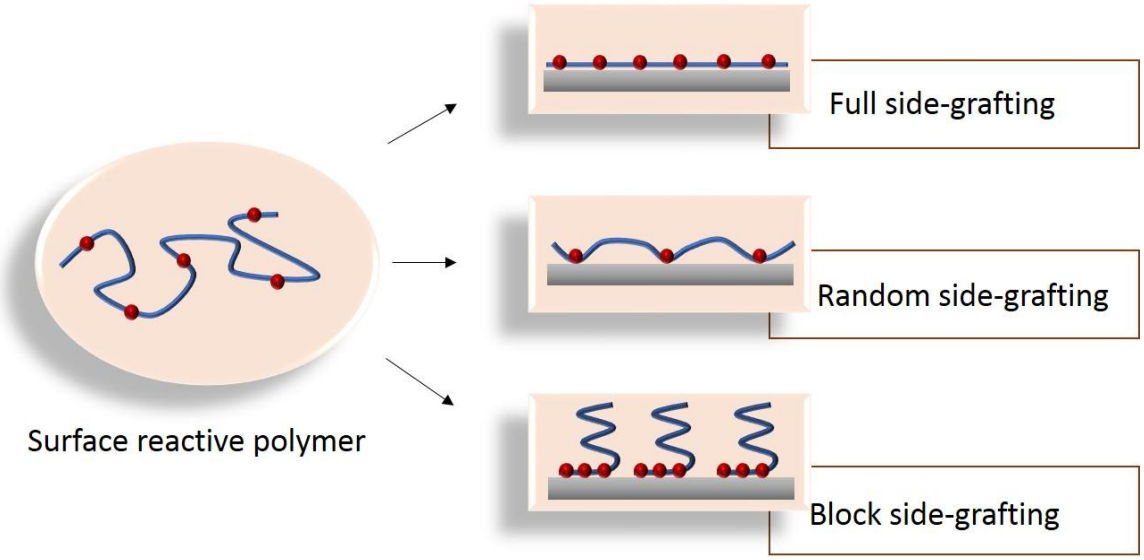


Figure 1.2. Types of polymeric thin films according to their confirmation. Adapted from [17].

Polymeric layers possess some advantages compared to small molecule monolayers e.g. higher stability, robustness and higher molecular loading. They have also some superiorities over the small molecule based monolayers due to their unique conformational

characteristics [17]. Polymeric coatings can sufficiently cover the surface even when the grafting density is low. Polymeric coatings can be imparted with higher stimuli responsiveness due to drastic change in polymeric conformation with solvent, pH or temperature.

### 1.1.2. Polymer Brushes

Polymer brushes are defined as polymer chains which are attached by their one end to a surface [18,19]. For the polymers to be in a brush conformation, polymer chains must be dense enough so that they are forced to stretch away from the surface. The thickness of the polymer brush layer is related to the degree of polymerization of the brush chains and grafting density. If the grafting density is lower than that required for brush formation, the chains collapse and such structures are called as 'mushrooms' or 'pancakes' [20,21] depending on density of the chains (Figure 1.3). The thickness of the polymer films in such cases becomes thinner than those observed for dense polymer brushes. Contrary to what is observed for side grafted polymeric chains on the surface, the effect of solvents is much more pronounced for polymer brushes [17].

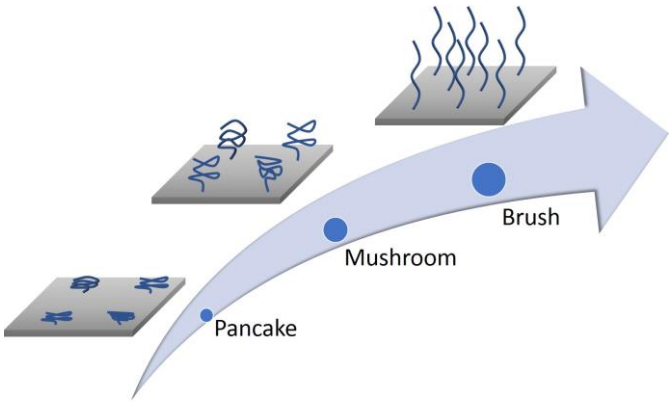


Figure 1.3. End-grafted polymers according to their grafting density. Adapted from [21].

Polymer brushes can be obtained by using one of the two approaches: a "grafting-to" and "grafting-from". In "grafting-to" method, preformed polymers which have surface

reactive end group at one of the chain-end is synthesized and attached onto the surface (Figure 1.4) [22]. The main advantage of this method is that the composition and characteristics such as molecular weight etc. of the polymers on the surface are precisely known [20]. Also, the process of modifying the surface usually involves simple operations like dip or spin coating. However, polymeric coating which was prepared using this method usually ends up with a low grafting density due to the steric hindrance toward surface attachment caused by the already attached polymer chains [21]. In the 'grafting from' approach, usually a surface anchorable initiator is attached as a monolayer to the substrate and polymers are grown from the modified surface. Importantly, if initiation efficiency is good then this technique yields polymer brushes with high grafting density since growing polymer chains do not have much room to spread due to strict steric demands of the growing chains in the vicinity. The 'grafting from' method can allow an easier and better control over brush thickness by adjusting polymerization parameters. The ultimate preference of using either a 'grafting to' or 'grafting from' method will depend on the demands of the particular application.

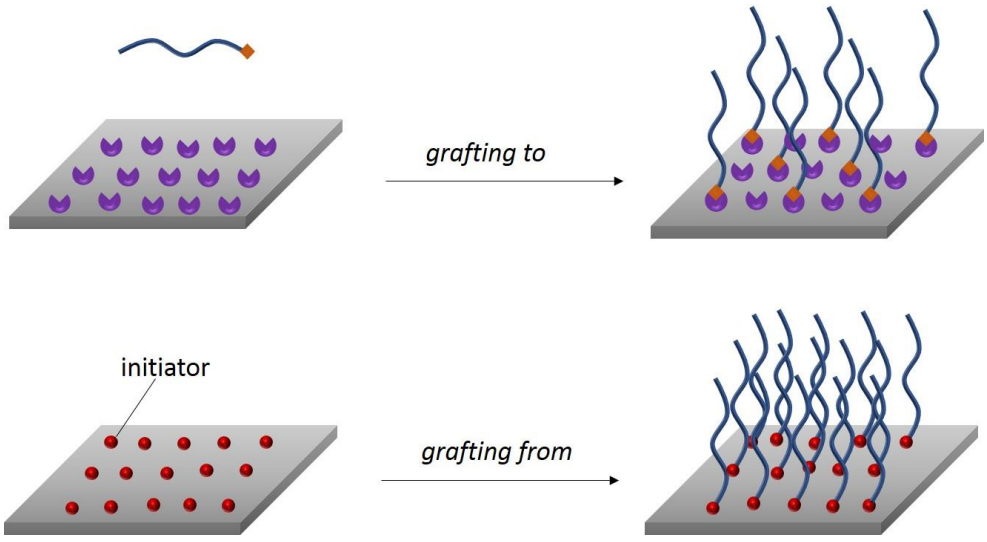


Figure 1.4. Fabrication of polymer brushes by "grafting to" or "grafting from" method. Adapted from [23].

Different polymerization methods such as cationic polymerization, anionic polymerization, ring opening polymerization and ring-opening metathesis polymerization have been employed for obtaining polymer brushes using the 'grafting from' technique. Recently, surface initiated controlled radical polymerization methods such as atom-transfer radical polymerization (ATRP) [24,25], reversible addition-fragmentation chain-transfer (RAFT) polymerization [26], nitroxide mediated polymerization (NMP) [27], and iniferter polymerization [28] have been widely investigated since proceed with good control of molecular weight with narrow polydispersity and thus allow better control over the brush thickness and composition.

1.1.2.1. SI-ATRP. ATRP has emerged as one of the most widely used controlled polymerization techniques since it was first reported in 1995 by Matyjaszewski and coworkers [29]. Later, they adapted this method for grafting of polymers from silicon substrates [30]. The polymerization proceeds through a single electron transfer from the transition metal complex (e.g. Cu(I)-bipyridine) to the halogen atom on the initiator. Thus, the catalyst complex is oxidized and radicalized initiator starts the chain growth. Subsequently, the oxidized transition metal reconverts the propagating radical chain end to the halogen-capped species. Parameters such as the type of initiator, ligand, ligand to transition metal ratio, solvent, concentration etc. affect the performance of ATRP. Adaptation of the technique as SI-ATRP was first introduced in 1997 by Huang and Wirth. In this study, they grafted poly(acrylamide) brushed from benzylchloride derivatized porous silica gel [31]. Fukuda and coworkers also reported an earliest example of polymer brushes via SI-ATRP grafting PMMA polymer brush from initiator coated silicon surfaces [32]. It has been demonstrated that SI-ATRP accelerates in the presence of polar solvents, particularly in water. Huck and coworkers accomplished the synthesis of PMMA brushes in a controlled manner within 4 h using water/methanol mixture. [33] Baker and coworkers demonstrated that it is possible to fabricate PHEMA polymer brushes using purely aqueous-based system via SI-ATRP (Figure 1.5) [34].

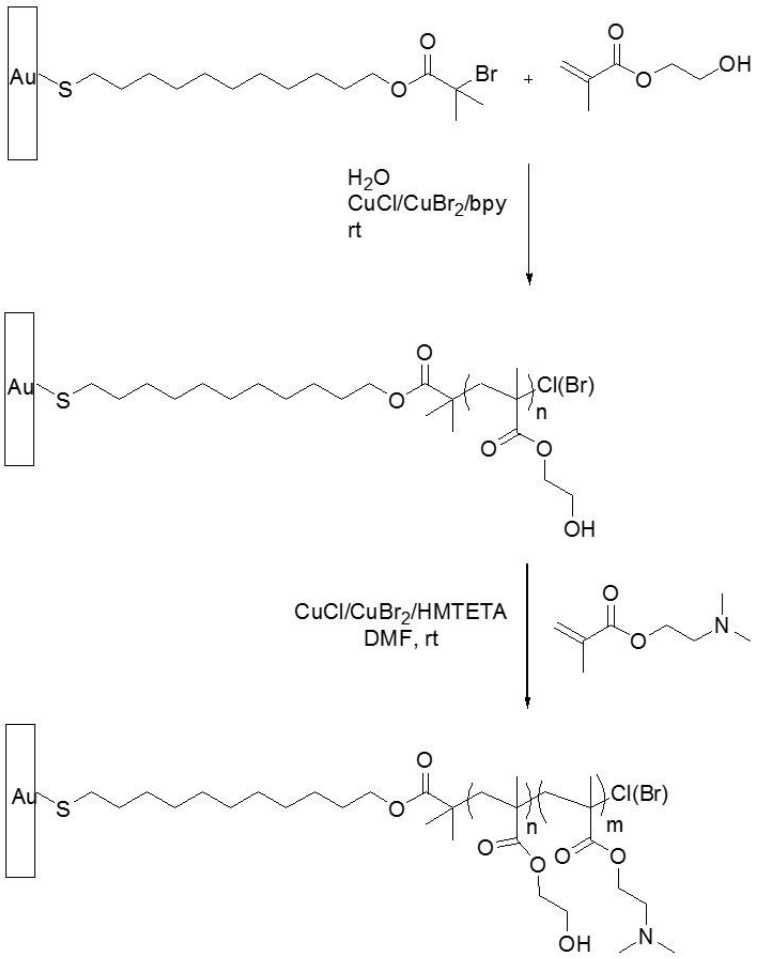


Figure 1.5. Polymerization of HEMA on gold surface via SI-ATRP. Adapted from [34].

1.1.2.2. S-RAFT. Reversible addition–fragmentation chain transfer (RAFT) polymerization involves a reversible regenerative chain transfer mechanism. It is a simple and versatile technique since traditional free radical polymerization can be easily converted into RAFT polymerization by adding a proper chain transfer agent. Additionally, this technique does not require metal catalyst and it is compatible with a wide range of monomers and reaction conditions [35,36].

Similar to other polymerization techniques, RAFT polymerization has also been adapted towards surface tethered polymerization applications. Surface RAFT (S-RAFT) polymerization can be performed either using surface-tethered free radical initiators or

surface-tethered chain transfer agent [37]. One of the earliest examples of S-RAFT was introduced by Baum and Brittain (Figure 1.6-a) [26]. They demonstrated grafting of polystyrene (PS), poly(methyl methacrylate) (PMMA) and poly(*N*,*N*-dimethylacrylamide) (PDMAM) brushes from surface-immobilized azo initiators on silicate based surfaces including silicon wafers, ATR crystals and high surface area non-porous silica gel that were modified with surface-attachable azo-type initiator. In this research, they showed that addition of free polymerization initiator simplifies polymer brush growth by increasing concentration of initiating sites with respect to monomer concentration otherwise even small amount of impurities in the reaction mixture could terminate the polymerization earlier. Additionally, after cleaving polymer brushes prepared on silica, they compared the molecular weight of polymers grown from the surface with free polymer chains grown in solution. They showed that for homopolymer brushes of PS or PMMA, molecular weights and polydispersity values of free polymer and cleaved polymer are coherent within themselves.

RAFT polymerization on surface can also be performed using surface-immobilized chain transfer agents.[26,40] Surfaces are modified with chain transfer agent in two different ways, namely, the R-group [41-44] and Z-group [39,45-48] approaches. R-group strategy includes the attachment of R group of the CTA to the surface which leaves and reinitiates during polymerization reaction. In this approach, RAFT process takes place near the free surface of brush layer (Figure 1.6-b) [38]. This method was used for grafting polymer brushes from dithiobenzoate- or trithiocarbonate-modified substrates. In the Z-group strategy, CTA is attached to surface via its stabilizing Z group (Figure 1.6-c) [39]. This makes propagating radical close to the solid surface across the barrier of polymer brush in order to undergo the RAFT reaction during polymerization. Using this strategy methacrylate, acrylate, styrene, and acrylamide-based brushes were fabricated [39,48-50].

Both R and Z strategies have some benefits and limitations [51-53]. In the R-group method, solid support is part of the leaving R group. Therefore, polymer brushes with high molecular weight and high grafted density can be obtained, however, due to possible chain coupling, polymerization may end up with chains with broad polydispersity [41,42,44]. In

the Z-group method, the polymer backbone is part of Z group, so polymerization process the reaction of linear radical chains with the reactive backbone. So, polymer brushes with narrow PDI is obtained [45,54]. However, due to steric hindrance of attached polymer chains, efficiency of the chain transfer reaction can be limited and grafting density may be lower than polymer brushes synthesized by using R approach [55-57]. Most of the reported S-RAFT studies were performed using R-supported method [41,58]. This is mostly because of the difficulty in the synthesis of surface reactive chain transfer agent for Z-supported approach and low grafting density of final end-grafted polymeric layer [59].

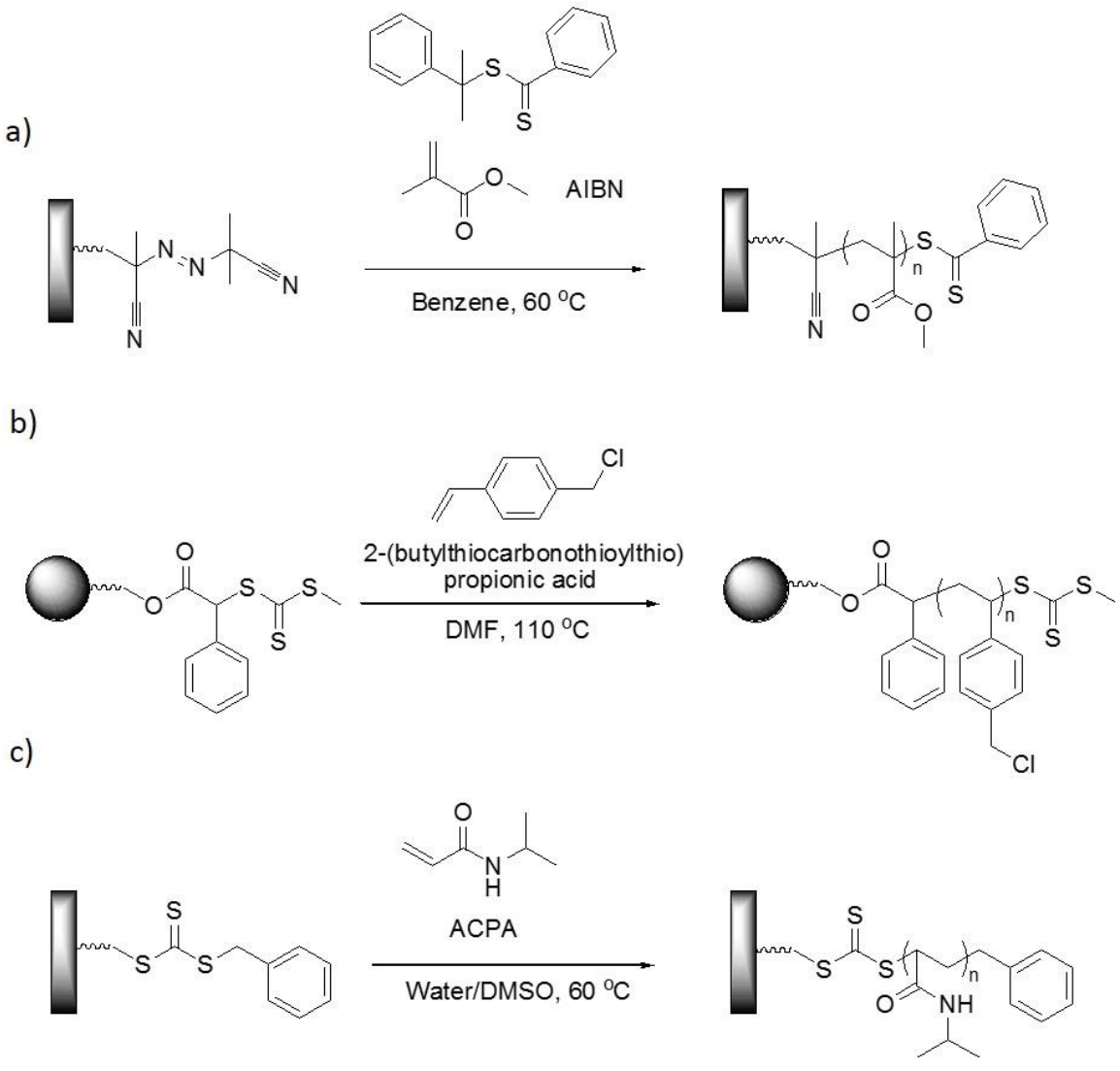


Figure 1.6. Three main methods for fabrication of polymer brushes via Surface RAFT polymerization a) using initiator modified surfaces [26] b) R-group approach [38] and c) Z-group approach [39]. Adapted from [21].

### 1.1.3. Thin Hydrogel Layer Coated Surface

Hydrogels are three-dimensional crosslinked polymeric networks which can readily absorb water several times their dry weight. They can be synthesized using natural polymers such as collagen, chitosan and hyaluronic acid or hydrophilic synthetic polymers such as poly(hydroxyethyl methacrylate), poly(ethylene glycol) and poly(vinyl alcohol). Hydrogels are vital materials for a wide range of applications such as contact lenses, wound healing dressing, biosensors and drug delivery applications [60,61].

In general there are two methods for fabrication of hydrogels which involves either physical or chemical cross-linking. Physical hydrogels are based on polymer chain entanglements or secondary forces like ionic, H-bonding or hydrophobic interactions [62,63]. However such interactions may not result in homogeneous hydrogels due to clusters of physically entangled domains. Additionally, the physical interactions may not be strong enough to offer stable gels [64]. In order to construct stable networks, chemical cross linking of polymers is achieved via covalent bond formation. They generally form stable, non-reversible and robust hydrogels.

Click chemistry based reactions are widely used for fabrication of chemically cross-linked hydrogels [65]. For example, synthesis of hydrogels with azide and alkyne modified poly(vinyl alcohol) [66] or PEG-bismaleimide and furan containing polymers [67] have been reported. Polymerization methods, in particular, photopolymerization have also been widely used for synthesis of hydrogels [67-69]. By altering the components, cross-linking methods or reaction conditions, physical properties of hydrogels such as their water uptake, cross link density, biocompatibility and biodegradability can be fine-tuned.

Hydrogels on solid substrates are also attractive materials since they give 3D porous soft materials with desired properties to the surface. For example, they provide a

higher capacity for protein immobilization [70], as well as offers a more homogenous and 'natural' environment than 2D surfaces [71].

Ruhe and co-workers reported fabrication of 3D polymeric coatings on glass surfaces by photopolymerization using a novel route [72]. In this study, they demonstrated that pendant benzophenone containing copolymers and benzophenone units on glass surface simultaneously formed hydrogel network under UV irradiation. They synthesized various hydrogels using diverse polymers including polyethyloxazoline, poly(methylmetharcrylate) and poly(hydroxyethyl methacrylate). They showed that some polymer coatings such as polyethyloxazoline showed good cell adherent property while other polymers did not attract cells. They also demonstrated such hydrogel layers can be synthesizable on porcine heart valves.

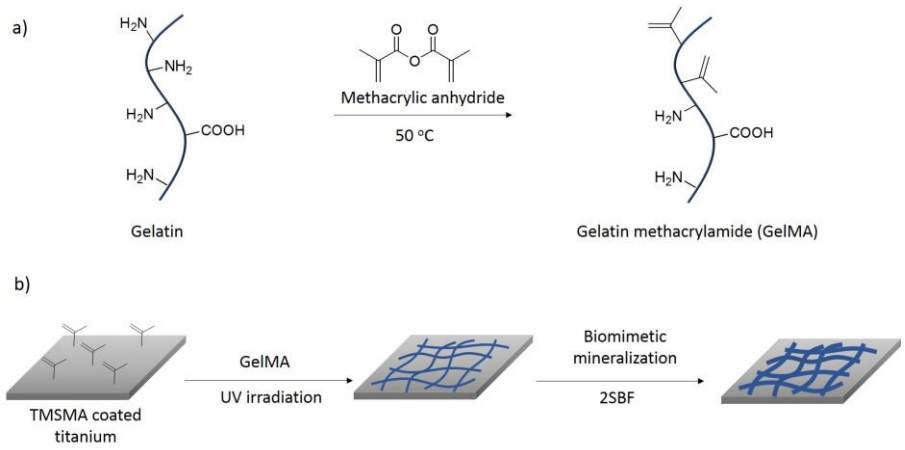


Figure 1.7. Hydrogels on titanium substrates via silanization. Adapted with permission from [73].

In another study, Chen and coworkers reported the synthesis of stable mineral–polymer composite coatings from gelatin methacrylate hydrogels for bone repair and regeneration application on titanium substrates using photochemical method (Figure 1.7). [73]. They coated titanium substrates with 3-(trimethoxysilyl) propyl methacrylate to introduce methacrylate units for strong attachment of polymeric material with the surface. Using gelatin methacrylate they obtained hydrogel layer on titanium surfaces. The

immobilized polymer layer was then mineralized to improve the osteointegration properties via immersion of the GelMA-coated Ti constructs into a solution that mimics concentrated human plasma (2X) for three days and showed that the coating on titanium is stable under aqueous conditions *in vitro* for 24 h.

Similarly, Tan and coworkers used hydrogel coated titanium surfaces for improving implant stability in cartilage defects aimed to provide integration between chondroitin sulphate and bone using a silane-modified titanium implant [74]. Chondroitin sulphate (CS), a polysaccharide found in cartilage and other tissues, has some important biological properties such as immunomodulatory and anti-inflammatory effects, and water and nutrient absorption. It was shown that the aldehyde groups in the hydrogel can react with the amine groups on the cartilage tissue surface [75]. This improves the bonding strength of the biomaterial with the surrounding cartilage. In this research, chondroitin sulphate (CS) was used to fabricate a tough hydrogel, bonding to surrounding tissue via a dual-bonded approach. Titanium surfaces were first modified with TMSMA, then CS-MA (chondroitin sulphate methacrylate) and CS-MA/aldehyde mixture was crosslinked on it in the presence of a photo-initiator. Hydrogels were synthesized *in situ* on pork cartilage tissue under UV irradiation. They showed that after applying physical pressure to the tissue, the hydrogel did not show any sign of breakage or detachment, which shows considerable tissue binding strength of the hydrogel. ATDC-5 cartilage cells on aldehyde containing hydrogel grew better than those grown on the control hydrogels without aldehyde, and CS-MA/aldehyde hydrogel exhibited cell viability until seven days. These results suggested that the presented approach has the potential to quickly and effectively repair cartilage defects and maintain joint function for a long time [74].

### 1.2. Reactive Polymer Coatings for Biomedical Applications

Reactive polymeric coatings on solid substrates are commonly used for fabrication of interfaces that can interact with the environment in a desired manner. A solid substrate can be coated in order to reduce unwanted interactions and/or improve desired interactions.

Especially for biomedical applications this balance is of utmost importance in terms of sensitivity. In this regard, polymeric coatings are good candidates since they can be synthesized as materials with multifunctional characteristics using combination of various monomers with different properties. Reactive polymer coatings find a range of applications in biomedical sciences such as tissue engineering, drug delivery systems, implant coatings and biosensors.[72] In this dissertation, we have focused on design of polymeric coatings that can be of potential use for biosensing and antibacterial implant coatings.

### 1.2.1. Biosensing Surfaces

Detection of nucleic acids and proteins is very important for early stage diagnostic of many health disorders. For this purpose, micro-array based biosensors have been utilized for detection of various biomarkers such as proteins, nucleic acids and carbohydrates. Microarrays can be defined as arranged probes with a known identity to catch complementary targets from the solutions. Due to ever-increasing need of robust biosensing platforms with high sensitivity, there is a need for improvement of efficient methods for immobilization of biomolecules onto glass or semiconductor or electrode surfaces for fast and effective detection of biomolecules.

Although most of the biological systems have the capacity to exhibit complex receptor recognition; they may also tend to be physically adsorbed onto solid substrates without specific receptor-recognition interactions. This causes high signal to noise (S/N) ratio or “false positives” which limits sensitivity and reliability of the sensing tool [76,77]. A good biosensor should be specific for a particular biomolecule type while inhibits other biological entities from being adsorbed non-specifically onto the substrate. In this sense, Zuilhof and coworkers called such surfaces as ‘romantic surfaces’ for the behavior of repelling all other biomolecules except the one who binds strongly as well as selectively [78], as a nice analogy to describe an ideal biosensor. Most of the biosensing surface studies utilize antifouling units such as PEG or zwitter ionic groups to enhance selectivity to the reactive surface [79,80].

In order to avoid incomplete reaction on the surface, due to their high reaction efficiency, ‘click chemistry’ based reactions have attracted increasing attention [81]. In addition, since most of biomolecules are sensitive and they usually decompose in harsh environments, efficient and specific methods for biomolecular immobilization on polymer coated surfaces with mild reaction conditions are very important for surface immobilization. Biomolecular immobilization through amine based conjugation is one of the most common conjugation methods because amine is the most abundant functional group in biomolecules. Well-known amine-reactive immobilization chemistries include N-hydroxysuccinimide (NHS) reactive esters, aldehydes and epoxides. But the presence of several amines on a single biomolecule leads to problems such as conjugation with undesired surface orientations. The most commonly used ‘click’ reaction with high specificity involves a copper(I)-catalyzed cycloaddition reaction between an azide and an alkyne group. Due to presence of the metal salt its applications are limited since residual metal impurities can interfere with subsequent application. Another set of specific reactions involve utilization of thiol-based conjugations, especially the UV-catalyzed radical thiol-ene and the nucleophilic thiol-ene reactions. Also, the Diels -Alder cycloaddition reaction offers another metal-free conjugation reaction. These reactions are desirable for biomolecular immobilization since they do not require metal catalysts and proceed under mild reaction conditions.

1.2.1.1. Amine based bioconjugation on surface. Biomolecular immobilization through amine based conjugation is very common since amine units are abundant in biomolecules and the resulting amide bond is quite stable in aqueous environment. Polymers that contains amine reactive units such as epoxy, succinimide and succinimidyl carbonate are mostly used for amine reactive surface preparation.

Schönherr and coworkers demonstrated a biomolecular microarray using side chain N-succinimide bearing copolymer on glass surface.[82] After a poly(N-succinimidyl methacrylate) homopolymer was spincoated to the oxidized silicon or glass surface, PEG-amine was attached using a patterned PDMS stamp for spatial protection of the polymeric surface from nucleophilic attack. The rest circular regions were functionalized by using

fluoresceinamine, BSA and DNA. They showed that there is no unwanted adsorption on blocked area due to antifouling property of the material while a successful immobilization took place on nonprotected region.

Likewise, Depero and coworkers reported a facile and robust amine reactive polymeric coating for DNA immobilization using a pendant DMA, NAS and TMSMA containing copolymer in aqueous environment (Figure 1.8). *N*, *N*-dimethylacrylamide was used due to its strong adhesive property on glass surface via hydrogen bonds and Van der Waals interactions and TMSMA for stabilizing the polymer on the surface through silyl etheral bonding. In this study, they demonstrated the first example of reactive polymeric coating on the glass surface by combination of physisorption and chemisorption. Usage of surface reactive groups on the polymer backbone results in highly robust polymeric coating. They demonstrated DNA immobilization on the obtained polymeric surface by using an Arrayt SpotBot spotter [83]. In the next study, they used those polymer surfaces for patterned DNA immobilization on glass surfaces. They coated 2.5 nm thick film via dip coating method and then they obtained patterned Cy3-ssDNA1 immobilization via microcontact printing method in circular, linear and square shapes. They also performed DNA hybridization study by attaching amine containing DNA strands to the surface, then immobilizing fluorescently labelled complementary target DNA matched on the surface [84].

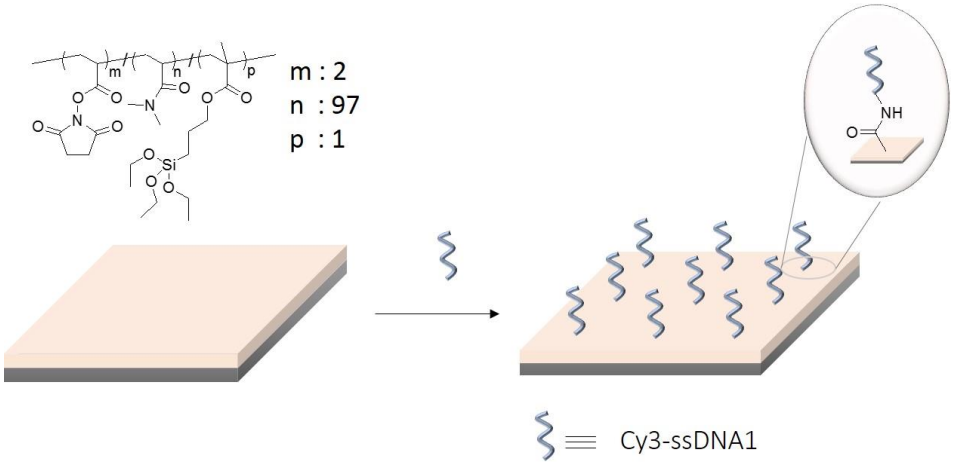


Figure 1.8. Side chain amine reactive and surface reactive polymer on glass. Adapted from [83].

Same research group incorporated a perfluoroalkylacrylate monomers during the synthesis of NHS containing surface attachable polymers to give a hydrophobic character to the coating [85]. Fluorinated polymeric coatings allow to spot smaller size of solutions avoiding spot merging and cross contamination so that fabrication of arrays with smaller dimensions and spot-to-spot distance could be achieved. They immobilized amine containing oligonucleotide and performed hybridization study. Finally, they used these DNA microarrays for the genotyping of KRAS G12 mutation.

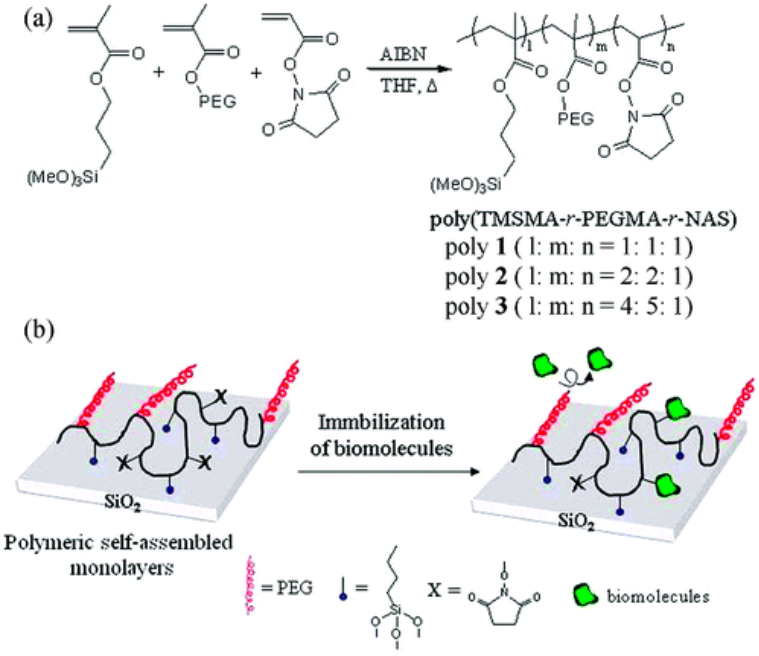


Figure 1.9. Amine reactive surface attachable antibiofouling polymer on the glass surface. Reprinted from [86].

Jon and coworkers reported a nice example of amine reactive side-grafted polymeric coating on silicon surface [86]. They synthesized a polymer similar to the one discussed above but they also incorporated PEGMEMA and TMSMA to the polymer backbone along with NHSMA. Thus obtained random copolymers have surface anchoring and bio-reactive as well as antibiofouling properties. Then they coated these polymers onto the surface via dip-coating in dichloromethane as a solvent. Since surface reactive groups are on the side chains of the copolymers, the polymeric layer on the surface is an ultra-thin

film. They performed biotin amine immobilization followed by attachment of streptavidin to these surfaces (Figure 1.9). They also showed that by changing the ratio of the monomers in the polymeric precursors, the reactive group density and amount of immobilized biomolecule on the surface can be tuned.

Maruyama and coworkers demonstrated amino group containing polymeric surfaces and functionalize them by simple dip coating method. They synthesized pendant 2-aminoethylmethacrylate (AEMA) and MMA bearing copolymer using bare AEMA and AEMA with different protecting groups which are HCl, BOC or p-toluenesulfonate. Then they coated these copolymers on PMMA plates, PET, PVC, and nylon 6 films by dip coating few seconds. They showed BOC protection effectively and reproducibly reveals amino groups on the variety of polymeric substrates. After deprotection of protecting groups they attached disulfide containing cleavable fluorescein compound via amidation and they show that it quantifies small amounts of amino groups. They also showed cleaving of fluorescein compound using DTT. Lastly, they reported a successful immobilization DNA on amine bearing surfaces [87].

Spring and coworkers fabricated amine reactive 3D small-molecule microarrays on glass surface to obtain an amine reactive slide with high loading capacity, signal sensitivity and better spot morphology when compared with some commercially available slides and 2D slide containing the same reactive group (Figure 1.10) [88]. NHSMA and PEGMEMA monomers were crosslinked on the TMSMA modified slides in the presence of PEGDA and DMPA under UV irradiation. They showed that 3D slides possessed higher loading capacity than commercial Codelink slides and 2D slides. Thereafter, different concentrations of biotin-amine were printed to the surface using commercial microarrayer. When slides were incubated with Cy3-labelled avidin, they observed that the 3D slides had higher fluorescence intensity across the concentration range of the biotin printed reactive surfaces.

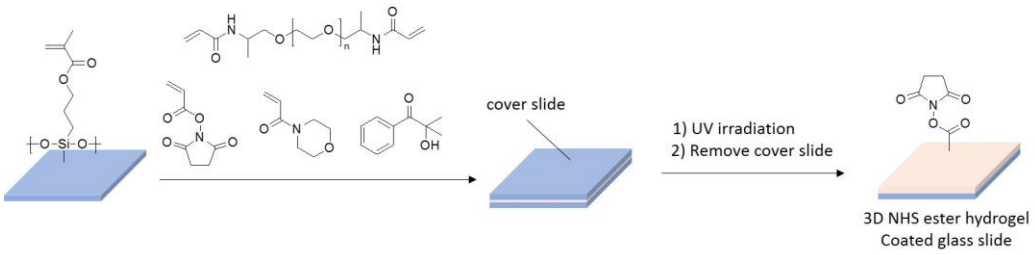


Figure 1.10. Amine reactive hydrogel on glass via photopolymerization. Adapted from [88].

1.2.1.2. Bioconjugation using Diels-Alder Chemistry. The cycloaddition between an electron rich diene and an electron deficient dienophile has been extensively used in fabrication of variety of self-healing materials [89,90], and also surface ligation on organic interfaces such as monolayers [91], nanofibers [92], and hydrogels [93]. Diels-Alder cycloaddition reaction is also an attractive candidate for biomolecular immobilization since the method does not require metal catalysts and harsh reaction conditions. Among the various advantages of the Diels-Alder reaction, its thermo-reversibility is quite relevant. The conjugation/deconjugation process can be reversible or irreversible within certain temperature ranges depending on the selected diene-dienophile combination. Another advantage of Diels-Alder reaction is the ability to proceed in aqueous media. In aqueous environment, Diels-Alder cyclo-addition reaction accelerates by a factor up to $10^4$ when compared to that in organic solvents.[94] Additionally, since diene and dienophile are not present in natural biomolecules, Diels-Alder offers a chemo-selective reaction for biomolecular immobilization.[95]

About a decade ago, Sun and coworkers described an application of Diels-Alder reaction toward biomolecular immobilization on solid substrates (Figure 1.11).[96] At first, they modified the glass surfaces with maleimide containing silane based anchoring molecule. Thereafter, they attached diene attached biotin-PEG, lactose-PEG and Protein A-PEG conjugates to modified glass surface via Diels-Alder cycloaddition reaction. These ligand conjugated surfaces were used for successful immobilization of biomolecules such as streptavidin, lectin and antibody to the complementary surfaces.

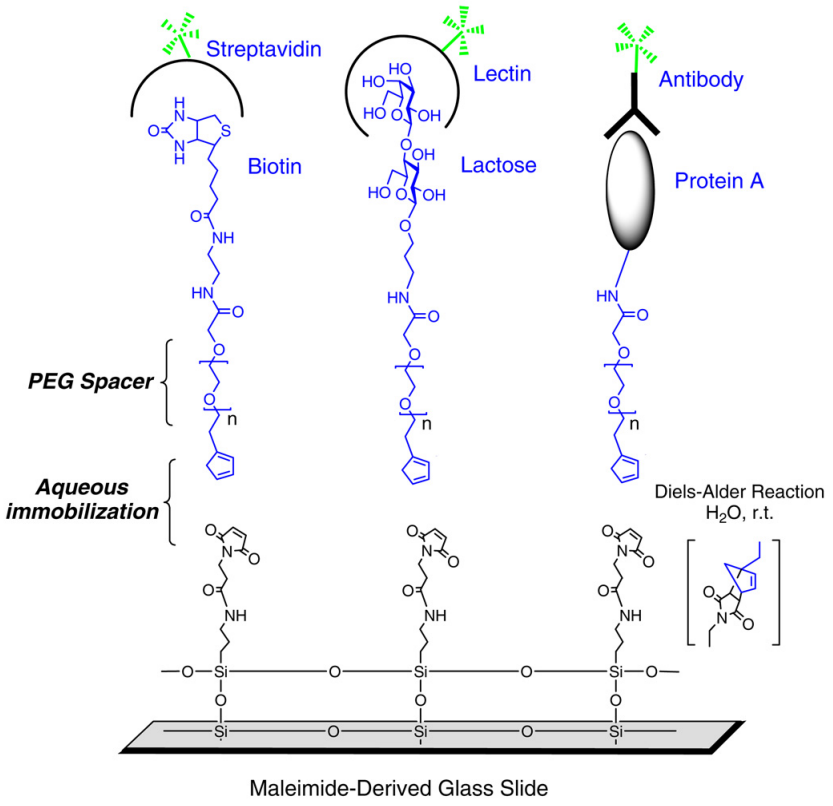


Figure 1.11. Immobilization of PEG based biomolecular ligand via Diels-Alder chemistry. Reprinted from [96].

The polymeric counterpart of a diene appended surface was recently reported by Sanyal and coworkers, where they fabricated a reactive diene appended polymer brush and utilized the Diels-Alder chemistry to functionalize them under mild and reagent free conditions (Figure 1.12).[97] Polymer brushes containing pendant furfuryl and PEG chains were grafted from silicon oxide surfaces via SI-ATRP. Facile functionalization of the furfuryl moieties in the brush with maleimide containing molecules was demonstrated. Altering the pendant reactive group density allowed control over the extent of functionalization. These reactive polymer brushes were also capable of directed immobilization of peptide coated quantum dots upon appropriate functionalization with a protein binding ligand.

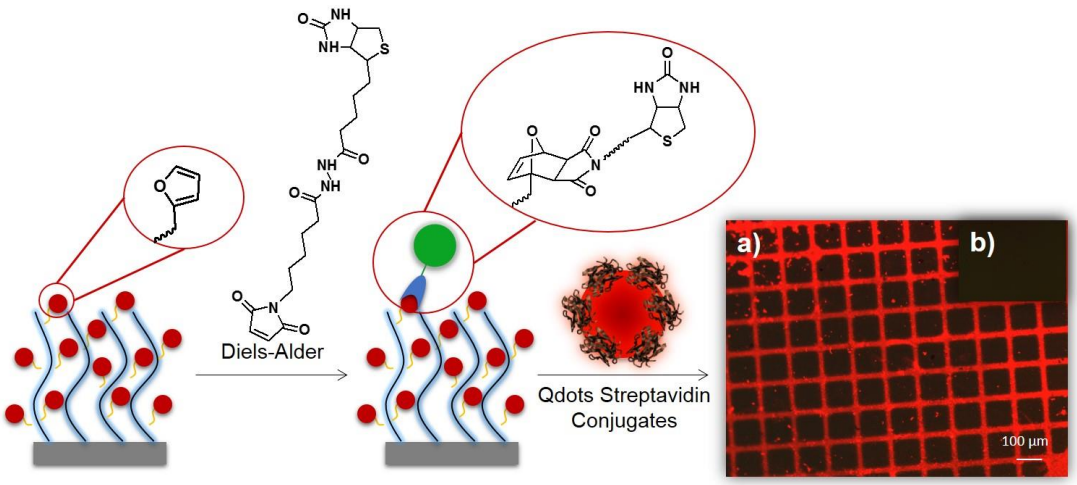


Figure 1.12. Biotin immobilization on furan bearing polymer brushes and capture of quantum dot-streptavidin conjugate. Reprinted from [97].

1.2.1.3. Thiol-based conjugation on surface. Thiol based click reactions are widely used in immobilization of biological compounds since many of the thiol-x chemistries are known to proceed with high conversion and low toxicity. The UV-light mediated radical thiol-ene/yne conjugation and the nucleophilic Michael addition are the most common thiol based conjugations. Major advantage of light based conjugation of thiols onto the alkene/ alkyne bearing surfaces that they provide spatial and temporal control of functionalization. Additionally, mild reaction conditions and tolerance for oxygen make these reactions appropriate for many applications.

In a recent example, Patton and coworkers reported the fabrication of multifunctional surfaces through a thiol-yne based post-polymerization modification (Figure 1.13) [98]. After grafting poly(propargyl methacrylate) polymer brushes from silicon surfaces, they could be modified in a one-pot setup with a mixture of two or more thiols by radical thiol-ene chemistry. This method could be used for providing a controlled level of hydrophilicity to the surface or modifying the surface with biomolecules. Additionally, they copolymerized propargyl methacrylate with 2-isocyanatoethyl methacrylate, 2-(2-bromopropanoyloxy) ethyl methacrylate or glycidyl methacrylate in order to introduce two reactive handles onto the polymer brushes. Although they

demonstrated this strategy with commercially available thiols, they concluded that this approach can be used for preparation of a multicomponent biomolecular display, similar to ones present in natural biological systems.

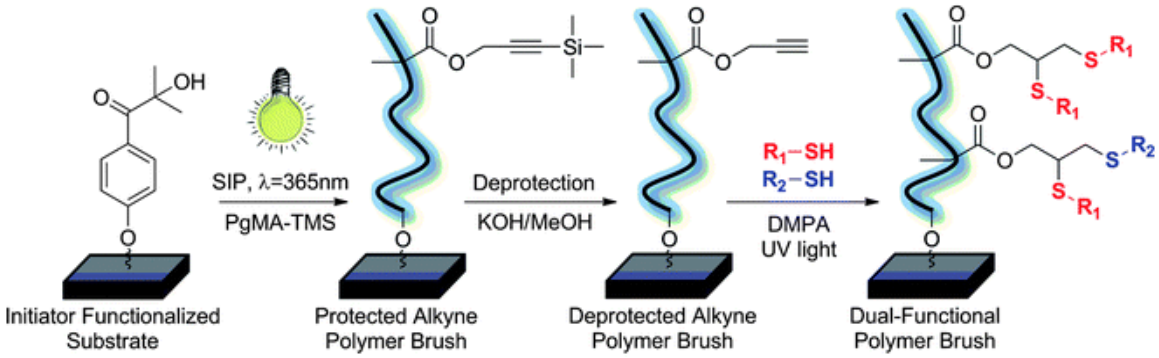


Figure 1.13. Synthesis and functionalization side chain alkyne bearing polymer brushes. Reprinted from[98].

In a related example, Huck, Gautrot and coworkers designed alkene and alkyne side chain containing polymer brush via post-polymerization modification of a poly(glycidyl methacrylate) brush(Figure 1.18) [99]. Taking advantage of site specific coupling of thiol-ene and thiol-yne reactions, they obtained patterns of various thiol containing molecules on the polymeric film using photomasks. Additionally, they attached some thiol containing molecules onto the surfaces by micro-contact printing method using patterned or non-patterned PDMS elastomers. They showed immobilization of thiol bearing molecules such as cysteamine, cysteine, reduced L-glutathione, CGGGRGDS peptide, biotin via UV mediated thiol-ene and thiol-yne chemistries.

Sanyal and co-workers demonstrated the fabrication of PEG based allyl group containing thiol reactive hydrogels using photopolymerization [100]. They obtained hydrogel micro-patterns using allyl methacrylate, PEGMEMA, and PEGDA on the TMSMA modified glass surface under UV irradiation using the micromolding in capillaries technique (Figure 1.20). Successful functionalization to these hydrogels was demonstrated by conjugation of a fluorescent dye. Furthermore, a protein binding ligand, namely, biotin was attached to the surface to demonstrate ligand directed immobilization

of streptavidin. Additionally taking advantage of spatial control of UV mediated thiol–ene chemistry, patterned immobilization of thiol-containing dye molecules through a photo-mask was successfully performed on smooth hydrogel layer. Lastly, cell attachment was also demonstrated on these allyl functional group containing hydrogels can be rendered viable substrates for cellular attachment through modification with a cell adhesive peptide via the thiol-ene reaction.

Although thiol-maleimide click reaction is widely used for immobilization of molecules and biomolecules especially in site specific manner, there could be situations that do not permit utilization of UV-irradiation. For example, when a biomolecule is not stable under UV light, or a UV initiator is not desired during conjugation, the nucleophilic thiol-ene based Michael addition provides a good alternative. Maleimide-thiol nucleophilic addition belongs to the group of 'click' reactions due to its high efficiency and lack of generation of any side products [101,102]. Thiol groups are better nucleophiles than potentially competing amine groups, hence the reaction can proceed with high selectivity in the presence of additional competing nucleophiles. In general, the nucleophilic thiol-ene reaction proceeds smoothly and with fast kinetics under mild reaction conditions.

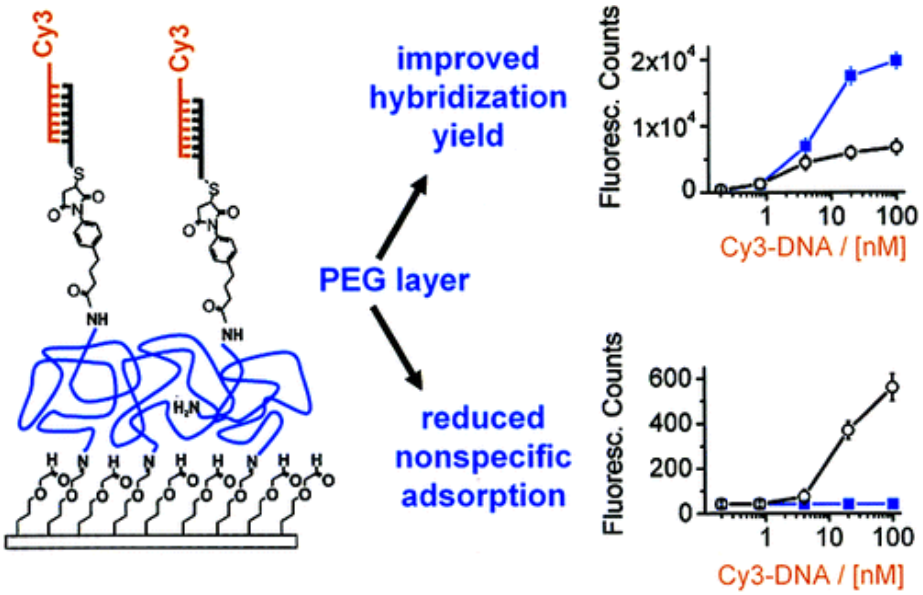


Figure 1.14. PEG based DNA oligonucleotide microarrays. Reprinted from [103].

As an early example of using thiol-maleimide conjugation based biomolecular attachment on surfaces, Howorka and coworkers fabricated PEG-based DNA oligonucleotide microarrays on glass (Figure 1.14) [103]. Surfaces were modified with 3-glycidoxypropyl trimethoxysilane and PEG-diamine was grafted on these substrates. PEG-grafted slides were then reacted with succinimidyl 4-[*p*-maleimidophenyl] butyrate to impart the glass slides thiol reactive property. They showed that PEG layer between reactive units and glass not only reduced nonspecific adsorption but also improved DNA hybridization yield when compared to non-PEGylated reactive surface.

As a thiol-based functionalizable three-dimensional polymeric material on solid substrate, Rotello, Sanyal and coworkers fabricated maleimide containing nano-imprinted polymeric substrate on silicon substrate [104] (Figure 1.15). A PEG-based, furan protected maleimide containing polymer was spin-coated on a surface and then submicron-patterns were obtained using nanoimprint lithography at 175 °C. During the imprinting process, micropattern formation is simultaneously accompanied with the unmasking of the furan protecting group to unmask the thiol-reactive maleimide units. These reactive microstructure could be functionalized with fluorescent dyes, magnetic nanoparticles, and peptides, where the later surfaces were effective for immobilization of cells in an aligned fashion.

Sanyal and coworkers later demonstrated utilization of a maleimide-containing 3D hydrogel micro-patterns on glass and silicon surfaces for biosensor applications (Figure 1.16) [105]. A furan-protected maleimide-containing monomer and PEGMEMA were photo-crosslinked in the presence of PEGDMA as cross-linker and DMPA as a photo-initiator. Micro-molding in capillaries was used to obtain hydrogel micro-patterns. After activation of the maleimide groups under vacuum at 110 oC, a protein binding ligand, namely, biotin, was attached to the maleimide groups on hydrogel patterns. Ligand directed immobilization of the protein streptavidin was demonstrated and the amount of immobilized protein could be tuned by tailoring the amount of the thiol-reactive monomer in the hydrogel.

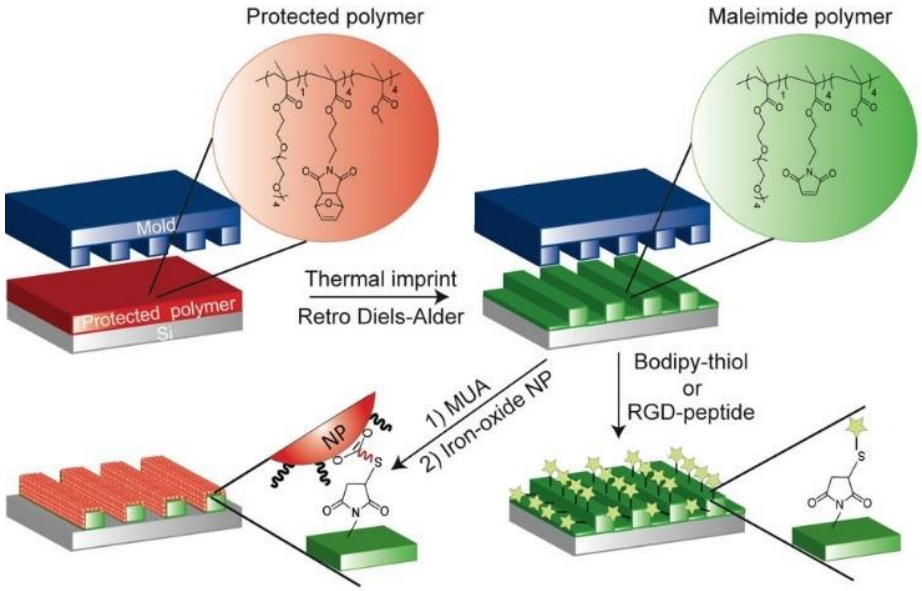


Figure 1.15. Maleimide containing thiol reactive patterned polymers on silicon substrates. Reprinted from [104].

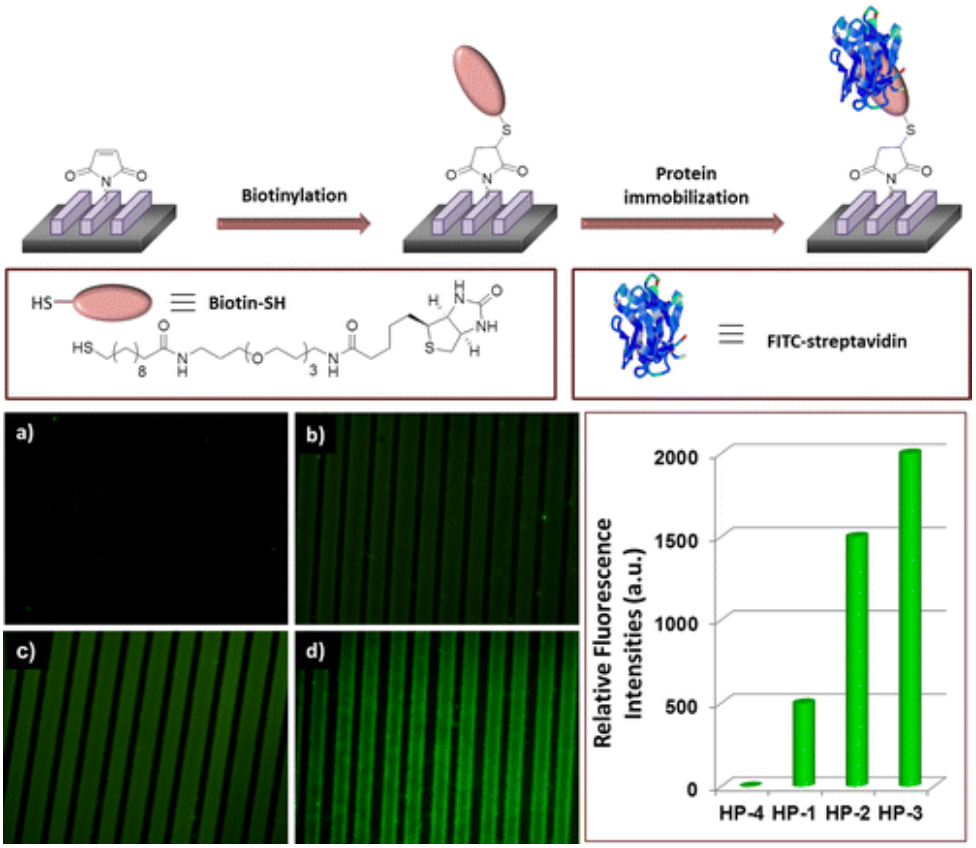


Figure 1.16. Thiol-reactive patterned hydrogels on silicon substrates. Reprinted from ref. [105].

### 1.2.2. Antimicrobial Implant Coatings

One of the applications of reactive polymeric coatings on solid substrates explored in this dissertation is fabrication of antimicrobial implant coatings. Implants are very important for treatment of many diseases but infections associated with surgical implants often cause failure of such materials. Both permanent, like orthopedic and dental implants, and temporary devices such as contact lenses and urinary tract catheters and are negatively influenced by a wide range of pathogens. Even though all devices and environments are sterilized, there is always the risk of infection during handling.

Infections mostly stem from adhesion of bacteria and proliferation on the surface of the material used for fabrication of those devices. Bacteria form colonies after they attach to the surface and then biofilms turn into a suitable media for the development of pathogenic infections (Figure 1.17). Unless bacteria strongly adhere to the substrate, biofilm formation does not occur. After attachment to the substrate, they produce a matrix of extracellular polymeric substances. This structure defends them against the host immune system and antibiotic materials. If the host cells attach irreversibly on biomaterial surface first (before adhesion of bacteria), bacteria cells cannot start biofilm formation. (Figure 1.17-2). When surface is coated with an antibacterial material such as anti-fouling polymer or bacteria repelling proteins, bacteria cannot adhere to the surface, which highly reduces the probability of biofilm formation (Figure 1.17-3). Due to altering regulation of multiple resistance genes, bacterial biofilms are between 10 and 1000-fold more resistant to most antibiotics [106]. As a result, bacterial biofilms are very difficult to eradicate. They are only eliminated by the constant removal and exchange of the implant itself, which results in discomfort to the patient and significant costs to the healthcare system.

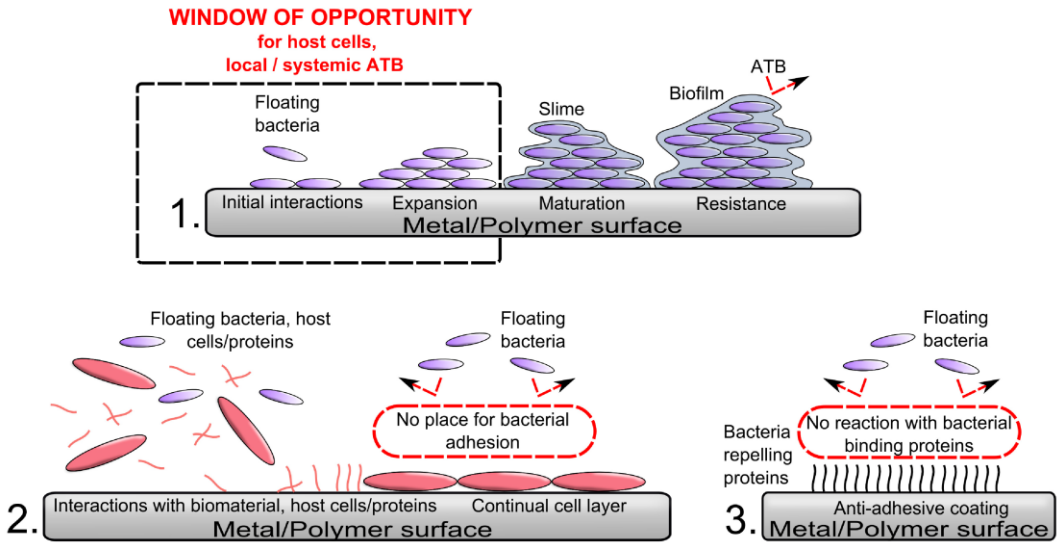


Figure 1.17. Biomaterial colonization from individual bacteria adhesion through micro-colonies towards formation biofilm (1) non-adhered bacteria do not cause biofilm formation (2,3). Reprinted from [107].

Biofilm formation on dental devices, orthopedic implants, vascular grafts and urinary catheters are observed in high rates which hampers the device performance in terms of safety and durability. So, prevention of biofilm formation is the most pragmatic method to inhibit the implant infections. For this purpose many studies have been carried out to reduce microbial adhesion onto the substrates of interest. The two main strategies that are used involves formation of either an antifouling coating or a bactericidal coating, or their combination. Coating of surface with antibiofouling material decreases or prevents cellular attachment due to unfavorable surface chemistry [108] or surface topography, while bactericidal surface disrupt the cell on contact, causing cell death [109].

1.2.2.1. Antifouling coatings as antibacterial surfaces. Antibiofouling coatings are mainly fabricated by using self-assembled monolayers (SAMs) or polymeric materials that are based on PEG or its derivatives [80]. The usage of non-adhesive materials that keeps proteins away from surface prevents initial cellular adhesion, therefore bacteria are not able to colonize there [80].

In a recent example, Rodriguez-Emmenegger demonstrated poly(MeOEGMA) and poly(HPMA) based ultra-low protein fouling polymer brushes as bacteria fouling resistance substrates on gold coated silicon surfaces and they compared biofilm formation of *P. aeruginosa* strains on SAM of polymerization initiator and polymer brushes [110]. *P. aeruginosa* is one of the most widely used bacteria in antimicrobial surface studies. Its ability to rapidly form stable biofilms makes it a very interesting model organism. This type of bacteria has many strains including the antibiotic sensitive or antibiotic multi-resistant ones. The authors examined biofilm formation on each surface, in four different media with varying nutrients. They observed that the bacterial adhesion and biofilm on SAM, while both poly(MeOEGMA) and poly(HPMA) inhibited biofilm formation even in nutrient rich media. However, when multi-resistant strain *P. aeruginosa* (PA49) was able to colonize on the prepared protein resistance. These results showed that investigation on the mechanism of bacterial adhesion should consider not only the physicochemical properties of the surface but also the biological variability of the bacteria strains.

1.2.2.2. Bactericidal surface coating. Protein resistant coatings mostly prevent bacterial adhesion to the surface and following biofilm formation, but sometimes it may not be enough to completely inhibit biofilm formation. Bactericidal coatings help to overcome the fouling-mediated risk of bacterial infection. So, in some studies implantable devices have been coated with biocidal substances. This approach is based on inactivating any cells contact with the substrate and causing death of bacteria. Bactericidal polymeric coatings can be either structural polymers grafted with antibacterial polymers, such as poly(vinyl-*N*-hexylpyridinium salts) (hexyl PVP) or polymers incorporated with antimicrobial compounds. Bactericidal surfaces have been also prepared using controlled release of biocide agents including conventional antibiotics such as tobramycin, vancomycin and gentamicin have been incorporated in controlled release devices [111]. Figure 1.18 summarizes the two different approaches for making a surface prepared to combat against bacteria using either contact-active antibiotics or antibiotic release [112].

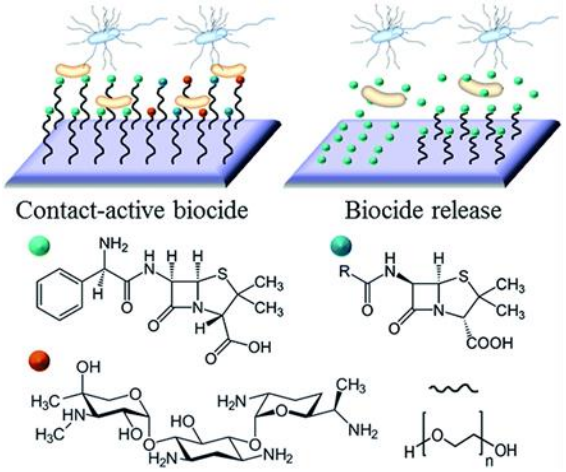


Figure 1.18. Contact-active antibiotics and antibiotic release strategies based coatings. Reprinted from [112].

Since biofilms are much more resistant to antibiotics, in order to prevent organisms in a biofilm, antibiotic dose must be 1000-times higher than to be required to fight bacteria in suspension [113]. Though controlled release of bactericidal agents provides an effective combating with infection, the release of antibiotics are mostly below the minimal inhibitory concentration (MIC) which causes bacterial resistance [114]. High doses of antibiotics may be toxic and may impair osteogenic activity [115]. Additionally, such systems are not strong enough to combat with antibiotic resistance bacteria.

1.2.2.3. Antimicrobial peptide based coatings. A promising alternative to conventional antibiotics is the short cationic antimicrobial peptides (AMPs) in bactericidal studies [116,117]. Antimicrobial peptides (AMPs), also known as host defense peptides are produced by both prokaryotes (e.g., bacteria) and eukaryotes (e.g., protozoan, fungi, plants, insects, and animals) [118-120]. In animals, AMPs are mostly found in the tissues and organs that are exposed to airborne pathogens as the first line of innate immunity. They show diverse antimicrobial activities against a broad range of targeted organisms ranging from viruses[121] to parasites.[122]

While antibiotics target specific cellular activities such as synthesis of DNA, protein, or cell wall, AMPs target the lipopolysaccharide layer of cell membrane, which is present in all microorganisms, and they disrupt this layer with a complex mechanism. Furthermore, compared to conventional antibiotics, antibacterial peptides have been shown to act at a much lower concentration. The ability to kill bacteria very rapidly and with high selectivity considerably limits the potential problems related to toxicity [119,123,124]. Also, another asset of antimicrobial peptides is their broad spectrum activity than antibiotics. More importantly, they are also quite effective against multidrug resistant and antibiotic-resistant strains [125]. Since bacterial membranes are negatively charged and AMPs are positively charged, the initial interaction between them is electrostatic. After minimum inhibitory concentration is reached, AMPs disrupt the membrane bilayer of the bacteria via various mechanisms. The disruption starts with formation of pores on the membrane, then peptides attack cytoplasm and metabolic functions of the cell and finally kill the bacteria [117].

Liskamp and coworkers reported a facile approach to immobilize AMPs onto cross-linked poly(ethylene glycol)diacrylate-based (PEGDA) hydrogels via thiol–ene photochemistry on poly(ethylene terephthalate) (PET) sheet(Figure 1.27).[126] They incorporated antimicrobial peptides in a hydrogel with a single-step immobilization-polymerization approach for the preparation of antimicrobial coatings. They used HHC10 (H-KRWWKWIRW-NH2) as antimicrobial peptide which was developed by Hancock. Antimicrobial hydrogels were synthesized by cross-linking of PEGDA and a 4-armed thiol (pentaerythritol tetrakis(3-mercaptopropionate)) in the presence of cysteine-bearing HHC10. Three different ratios of HHC10 (0.2, 1, and 10 wt %) containing hydrogels were prepared and antimicrobial activities were tested in vitro using the JIS Z 2801 assay The antibacterial activities of the hydrogel-peptide conjugates against *S. aureus, S. epidermidis,* and *E. coli* were investigated.. There were slightly bactericidal effects on 0.2% and 1 % peptide bearing hydrogels against *S. aureus, S. epidermidis,* and *E. coli* while no bacteria growth was observed on 10% peptide bearing hydrogel.

In a recent study, Kizhakkedathu and coworkers reported fabrication of antibacterial polymeric film on silicon surfaces (Figure 1.19). [127] They obtained reactive polymer brush by post-polymerization modification of side chain of a brush obtained using *N*,*N*-dimethylacrylamide (DMA) and aminopropyl methacrylamide hydrochloride (APMA) using ATRP. They converted the pendant amine groups into maleimide units by reaction with a heterobifunctional linker. To these reactive polymer brushes, they attached a series of cysteine containing antimicrobial peptides: Tet-213, 1010cys, Tet-20, Tet-21, Tet-26, HH2 and MXX226. Anti-microbial peptide (AMP) conjugated polymer brushes exhibited broad spectrum antimicrobial activity both *in vitro* and *in vivo*. They also showed that these systems resisted biofilm formation to different levels depending on the nature of immobilized peptide. The Tet-20 attached polymer brush, one of the most effective constructs *in vitro*, was also investigated *in vivo* against *S. aureus*. They demonstrated that fabricated antibacterial coating protected the rat from bacterial infection. Furthermore, the AMP conjugated polymer coatings were non-toxic to mammalian cells and they did not activate human platelets or initiate complement activation.

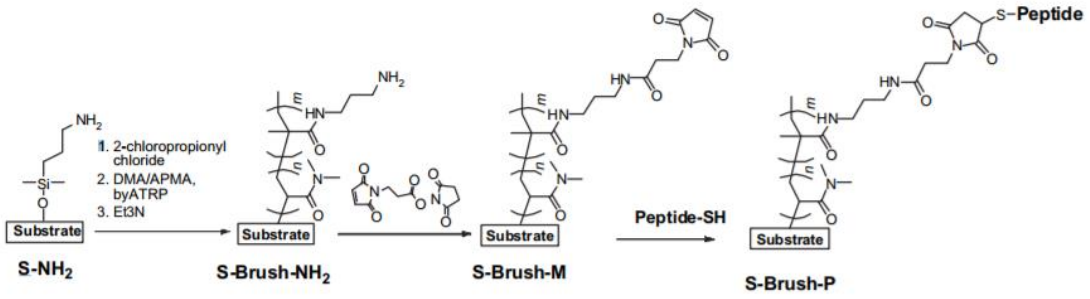


Figure 1.19. Pedant maleimide containing polymer brushes with post-polymerization modification and immobilization of antibacterial peptide. Reprinted from [127].

In another study, Kizhakkedathu and coworkers demonstrated fabrication of antibacterial polymer brush coating on polystyrene (PS) nanoparticles and titanium surfaces combining the advantages of a non-biofouling coating and antibacterial peptide (Figure 1.20) [128]. The hydrophilic monomer N-(3-Aminopropyl) methacrylamide hydrochloride (APMA) was copolymerized with three other different non-fouling monomers including (N,N-dimethylacrylamide) (DMA), 3-[(methacryloyl)amido]propyl-

N, N-dimethyl(3-sulfopropyl)ammonium hydroxide (PMPDSAH) and 2-[(methacryloyl)oxy]ethyl]-phosphorylcholine (PMPC) on PS nanoparticles and were conjugated with two cysteinylated cathelicidin-derived peptides: E6 and Tet-20. Highly efficient killing of planktonic bacteria by the antimicrobial coatings on nanoparticle surfaces was observed. Peptide E6 conjugated PDMA polymer brushes showed maximum efficiency on titanium surface, killing 50.3% of adhered bacteria while other polymer brushes with same peptide showed less antibacterial activity.

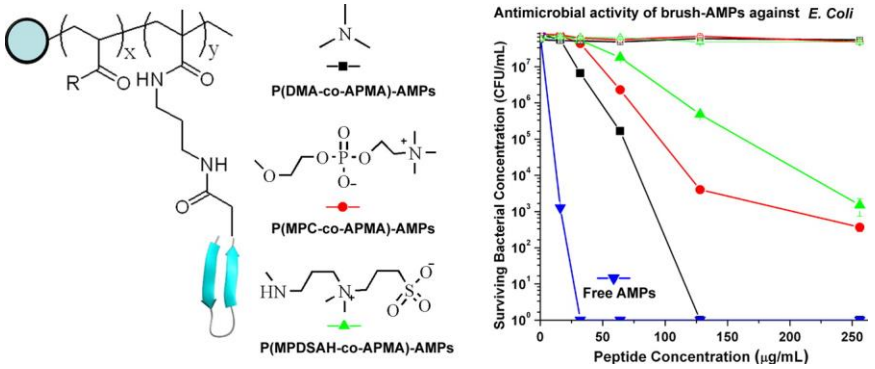


Figure 1.20. Antibacterial peptides conjugated to polymer brushes. Reprinted from [128].

In summary, as highlighted through the above examples, reactive polymeric coatings are widely used in various biomedical applications. Design of novel polymeric coatings that are robust, inherently anti-biofouling, and which can be modified effectively using under mild reaction conditions for functionalization with ligands or biomolecules of interest would help advance the field of biosensing and antimicrobial coatings.

# 2. RESEARCH OVERVIEW

This thesis encompasses six research projects under one objective which is the design and fabrication of novel reactive polymeric surface coatings for biomedical applications. This aim has been accomplished by fabrication of three different kinds of polymeric coatings on solid substrates: polymeric thin films, polymer brushes and three-dimensional hydrogels.

Approaches involve utilization of functionalizable polymers that can be anchored on glass like and metal surfaces using appropriate anchoring groups. Thin polymeric coatings bearing reactive functional groups like furan and maleimide were obtained for modification of silicon oxide surfaces using silyl ether based surface attachment. Likewise, modification of titanium surfaces with thin polymeric films was accomplished using a copolymer containing the maleimide group as a reactive handle and catechol moieties as surface anchoring groups. In another approach, maleimide- and activated carbonate group containing reactive polymer brushes were grafted from silicon oxide surfaces to obtain interfaces functionalizable with thiol and amine group containing molecules and ligands, respectively. As a final approach, thin hydrogel layers bound to titanium surfaces were obtained using a catechol based anchoring molecule to prime the surface, followed by fabrication of a hydrogel containing masked and unmasked maleimide groups as reactive groups. The hydrogel was amenable to facile modification using various click reaction based transformations such as radical thiol-ene, nucleophilic thiol-ene and normal and inverse-electron demand Diels-Alder reactions. In all of above designs, poly(ethylene glycol) based polymers were used to provide anti-biofouling matrix to these platforms.

Reactive polymeric coatings have been widely utilized for fabrication of functional surfaces that find many applications in biomedical sciences. In this thesis, we focused on platforms that can be employed for either biosensing or antibacterial surfaces.

Applicability of developed systems as biosensor for proteins was tested by using ligand directed attachment of protein. In particular, the biotin-avidin based system was used due to wide implementation in literature for validation of such systems. For fabrication of bactericidal coatings, we chose conjugation of anti-microbial peptides onto an anti-biofouling polymeric coating. The idea was to employ the anti-biofouling nature of the coating to minimize biofilm formation by preventing bacterial adhesion, along with the killing effect of the anti-microbial peptides for the adhered bacteria.

# 3. MICRO-PATTERNED FUNCTIONALIZATION OF REACTIVE POLYMERIC COATINGS VIA DIELS–ALDER REACTION

Adapted with permission from {Gevrek, T.N., R.N. Ozdeslik, G.C. Sahin, G. Yesilbag, S. Mutlu, and A. Sanyal, "Functionalization of Reactive Polymeric Coatings via Diels–Alder Reaction Using Microcontact Printing.", *Macromolecular Chemistry Physics*, Vol. 213, pp. 166−172, 2012}. Copyright {2011} John Wiley and Sons.

## 3.1. Introduction

Reactive polymeric thin films on solid surfaces continue to receive increasing attention due to their widespread applications in various areas involving biomolecular immobilization. Efficient and specific methodologies for biomolecular immobilization on polymer-modified solid surfaces are very important for biosensor technologies such as the protein and gene chips [86,129]. Areas of research related to surface functionalization have greatly benefited since the advent "click" reactions [130,131]. Usually reactions on surfaces proceed with poor efficiency due to the heterogeneity of the system. Various efficient transformations from the arsenal of "click" reactions have been utilized to date toward efficient functionalization of appropriately modified solid substrates. The Cu-catalyzed Huisgen [3 + 2] reaction is perhaps the most utilized one, but other "click" reactions such as the thiol-ene and Diels–Alder reactions are drawing attention due to their metal-free nature. Among the later reactions, the Diels–Alder reaction provides an attractive alternative due to the following attributes: (1) appropriate choice of diene and dienophile provides products in good yields in a highly predictable manner, (2) the reaction could be conducted in aqueous medium or neat without harsh chemical conditions, (3) most often no additional reagents or catalysts are required, and (4) reaction is thermoreversible [132]. As mentioned in the last point, this conjugation–deconjugation reaction harbors several reaction systems which could be irreversible or reversible over different temperature ranges based upon the molecular structure of the diene–dienophile

pair [133,134,135]. Specifically, the maleimide- furan-based systems have attracted immense attention because of the “self-healing” feature that allows the fabrication of remendable materials [136-138].

To date, most of the efforts in the area of surface functionalization using the Diels–Alder reaction have focused on the modification of self-assembled monolayers on solid surfaces such as gold or oxidized silicon. Mrksich and co-workers [139,140] demonstrated that the Diels–Alder reaction can be used to modify SAM surfaces in a spatially controlled manner. They reacted the quinone groups generated electrochemically on a self-assembled monolayer (SAM)-containing hydroquinone with cyclopentadiene-appended biotin ligands. The thus obtained biotin-appended surface was used for attachment of the enzyme streptavidin. Thereafter, the same group extended this methodology to develop carbohydrate and peptide arrays to evaluate protein binding via carbohydrate–protein interactions and protein kinase activity, respectively. More recently, Waldmann and co-workers [141] have demonstrated that diene-appended proteins can be directly immobilized onto maleimide functionalized glass slides. Interestingly, most of the research in this area has been focused upon surfaces that are decorated with a dienophile, while there are very few reports of functionalizations using diene modified surfaces. In this context, recently, surface modification of cyclopentadiene-appended cellulose was done via the hetero Diels–Alder reaction with thiocarbonyl thio-capped polymers synthesized by reversible addition–fragmentation chain transfer (RAFT) polymerization [142].

In recent years there has been an increasing interest in surface functionalization using the microcontact printing (μCP) technique [143]. This is due to the cost-effectiveness, simplicity, and effectiveness of the technique to provide patterned surfaces. In recent years, surface functionalization via μCP to obtain arrays using the Diels–Alder reaction has been explored, but to date efforts have been limited to reactions on self-assembled monolayers. Ravoo [144] has extensively explored the use of SAM surface functionalization using various “click” reactions under μCP protocols. Carbohydrate microarrays were fabricated on glass and silicon surfaces modified with a monolayer-containing maleimide group [145]. Carbohydrates modified with dienes such as

cyclopentadiene and furan were printed onto these maleimide-containing SAMs. The authors also reported a comparative study of reactivity of various dienes with surfaces bearing different dienophiles as well as their stability toward thermoreversion [146]. Studies initiated in our laboratory are aimed toward the development of thin polymeric coatings using novel reactive polymers that can be efficiently functionalized via Diels–Alder reaction.

Stimuli responsiveness of these polymeric surfaces can be a desirable attribute depending on the intended application. To date, reversibility based on pH, ionic strength, acidity, and temperature have been explored. Polymeric surfaces fabricated with thermoresponsive polymers such as poly(N-isopropylacrylamide) (PNIPAAM) have been utilized for controlled release of drugs, proteins, and cells [147,148]. Rewritable surfaces offer the ability to refunctionalize the same solid surface, which could be an electrode surface, glass surface in a sensor, and successfully reuse the same surface many times. Rewritable DNA microarrays were generated by immobilization of a thiol terminated oligonucleotide on a pulsed plasma deposited poly(allylmercaptan) film [149]. The oligonucleotides could be stripped off the surface via reduction of the disulfide linkages. The study was recently extended to fabricate rewritable glycochips or carbohydrate arrays based upon the chemically reversible disulfide or imine linkage [150]. These thiol- or amine-containing polymeric surfaces were obtained by pulsed plasma deposition of allyl mercaptan or vinylaniline. As an alternative, polymeric coatings obtained via the utilization of well-defined polymers containing reactive groups that allow such reversible immobilization and that can be simultaneously attached onto intended surfaces would provide a desirable robust platform due to possible tailorability by design of the polymer. Poly(ethylene glycol) (PEG)-based polymers has been of interest as coating materials intended for biomedical or biological applications. It is well established that PEG-based coatings renders the surfaces bioinert toward protein and cell adsorption [151]. Furthermore, recently it has been noted that these poly(ethylene glycol) monomethyl ether methacrylate (PEGMEMA)-based polymers exhibit a thermoresponsive behavior similar to PNIPAAM-based polymers [152]. In a recent study, Jaeger and co-workers [153] demonstrated that such PEG-based polymeric coatings anchored to a gold substrate can be used for nondestructive detachment of cells adhered to the surface.

In this part of the thesis, we explore the fabrication of polymeric coatings that can be easily functionalized at room temperature and can be renewed by simple heating. In particular, we report the design and synthesis of a copolymer that contains furan side chains as reactive groups for functionalization, a polyethylene glycol pendant side chains for providing bioinertness, and an alkoxy silane-based side chain for anchoring to oxidized silicon or glass surfaces. Thereafter, fabrication of reactive surfaces and their facile functionalization via μCP with maleimide-appended dye molecules is carried out. Successful immobilization of streptavidin directed by the patterned display of biotin ligands obtained via the Diels–Alder functionalization on polymer-coated surface is demonstrated. Finally, the ability to erase and rewrite on these thermoresponsive surfaces is demonstrated (Figure 3.1).

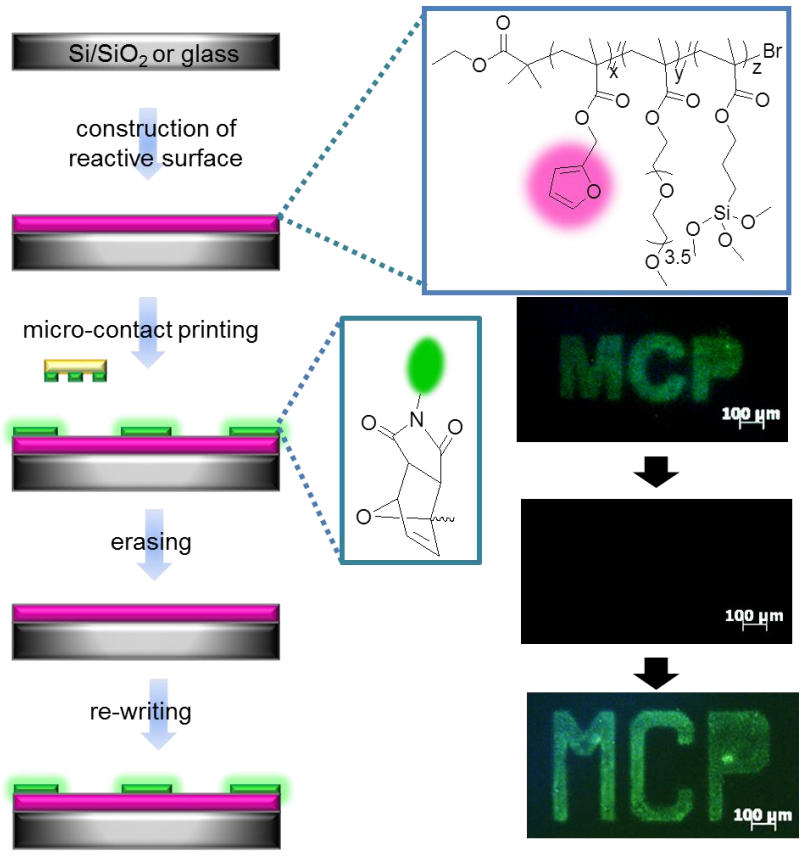


Figure 3.1. Reversible patterning on furan containing thin films by maleimide containing dye.

## 3.2. Experimental

### 3.2.1. Materials

Furfuryl methacrylate (FuMA), poly(ethylene glycol) monomethyl ether methacrylate (PEGMEMA, molecular weight 300 Da). N , N , N , N , N -pentamethyldiethylenetriamine (PMDETA), ethyl-2- bromoisobutyrate (EIBB), and 3-(trimethoxysilyl)propyl methacrylate 98% (TMSMA), N -biotinoyl- N′ -(6-maleimidohexanoyl) hydrazide (biotin-maleimide) were purchased from Aldrich. Boron-dipyrromethene (BODIPY)-maleimide was synthesized from BODIPY-bromide[154] with furan-protected maleimide, followed by cycloreversion. Glass surfaces and silicon wafers were cleaned using nonchromix solution, water, acetone, and isopropanol, respectively. Dimethyl sulfoxide (DMSO), dimethylformamide (DMF), and anisole were purchased from Merck. All organic solvents were used as received without further purifi cation. Nonchromix was purchased from Godax laboratories, Inc. Glass surfaces were purchased from Lamtek, and silicon wafers were purchased from University Wafers, USA.

### 3.2.2. Methods

For characterization of copolymer and fluorescent dye, 1H NMR spectra were recorded on a Varian 400 MHz spectrometer. The spectra were acquired using deuterated chloroform as a solvent. Trimethylsilane was used as an internal reference. The molecular weights of the copolymers were estimated by GPC analysis using a Shimadzu PSS-SDV (length/ID 8 × 300 mm, 10 mm particle size) mixed-C column calibrated with polystyrene standards (1–150 kDa) using a refractive-index detector. Tetrahydrofuran (THF) was used as eluent at a flow rate of 1 mL min $^{-1}$ at 30 ° C. The thicknesses of the monolayer films were measured with a Rudolph manual ellipsometer at a 70 ° angle of incidence. A refractive index of 1.46 was used for all films, and a three-phase model was used to calculate thicknesses. Static contact angle of a water droplet on spin-coated surfaces were

measured under open-air condition. Approximately, 50 μ L of deionized water deposited on the surface and images were taken by an integrated digital camera. The software of the camera provides contact angle measurements once the liquid is dispensed. The contact angle for each sample was independently measured at five different locations and average contact angle values were calculated. Images of dye or fluorescently labelled enzyme conjugated surfaces were recorded at room temperature on a Zeiss Observer Z1 fluorescence microscope. (ZEISS Fluorescence Microscopy, Carl Zeiss Canada Ltd., Canada). Samples were excited by 488 nm line of an Ar+ laser.

## 3.3. Results and Discussion

### 3.3.1. Synthesis of Polymers

Furan-containing reactive copolymers, poly(FuMA-TMSMA-PEGMEMA) were synthesized using Cu(I)-catalyzed atom-transfer radical polymerization (ATRP) (Figure 3.2) to obtain polymers with good control over molecular weights and polydispersities. Furfuryl methacrylate has been utilized extensively to synthesize copolymers via ATRP that are tailor made to furnish remendable materials [155-157]. In these copolymers, furfuryl methacrylate (FuMA) was chosen as reactive diene moiety, TMSMA was incorporated to ensure attachment to oxidized silicon and glass surfaces, and PEGMEMA was used in provide bioinertness, that is, reduce nonspecific adsorption of biomolecules. Changing the amount of reactive monomer in the polymer is expected to provide control over the extent of functionalization on these surfaces. Toward that end, two different random copolymers P1 and P2 which have 10 and 30% FuMA, respectively, were synthesized. The alkoxysilane-containing monomer was kept to 5%, sufficient enough to anchor the polymer onto silicon or glass efficiently. In addition, copolymer P3 devoid of any reactive furfuryl group was synthesized for control experiments.

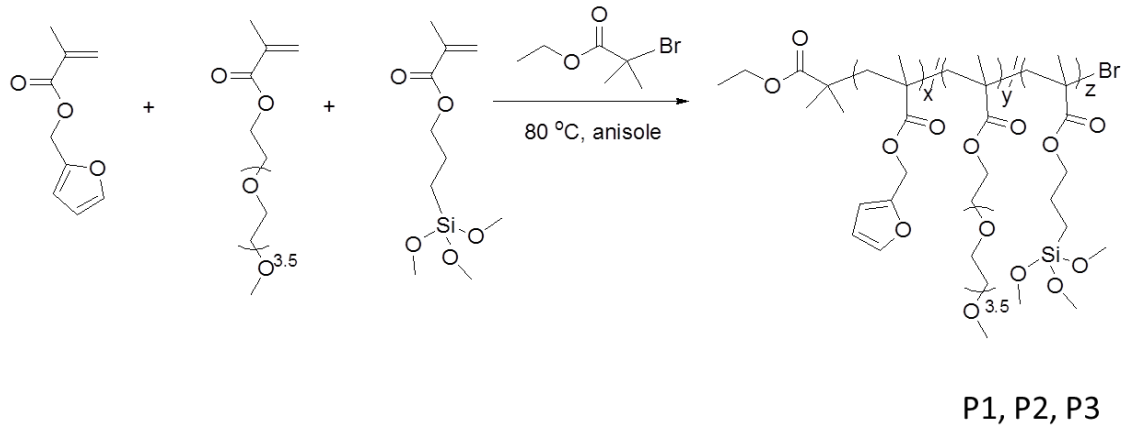


Figure 3.2. Synthesis of furan-containing reactive random copolymers.

Polymerizations were carried out using ATRP as the living radical polymerization technique to obtain the two polymers with commensurate molecular weights and only differing in the ratio of the reactive furfuryl groups to the PEG chains. Polymerizations were carried out in anisole at 80 °C under nitrogen atmosphere, using the CuBr(I)/PMDETA complex. Pure copolymers were obtained by precipitation in cold dry diethyl ether, as evident from their $^{1}$H NMR spectra (Figure 3.3). The $^{1}$H NMR spectra of the polymers clearly show the protons on the furan side chains at 7.40 and 6.31 ppm. The ratio of the furan comonomer and the PEG-based comonomer can be easily calculated by comparing the integration of proton resonances at $\delta$ = 7.42 belonging to the furan protons and the peak at $\delta$ = 3.35 belonging to the methoxy group at the terminus of PEG side chains. The actual FuMA content in each copolymer was close to the expected content based upon the feed ratio. The molecular weights of three copolymers utilized in this study were comparable (25–27 kDa) with a polydispersity index (PDI) of between 1.3 and 1.4, as measured by GPC using monodisperse polystyrene standards (Table 3.1).

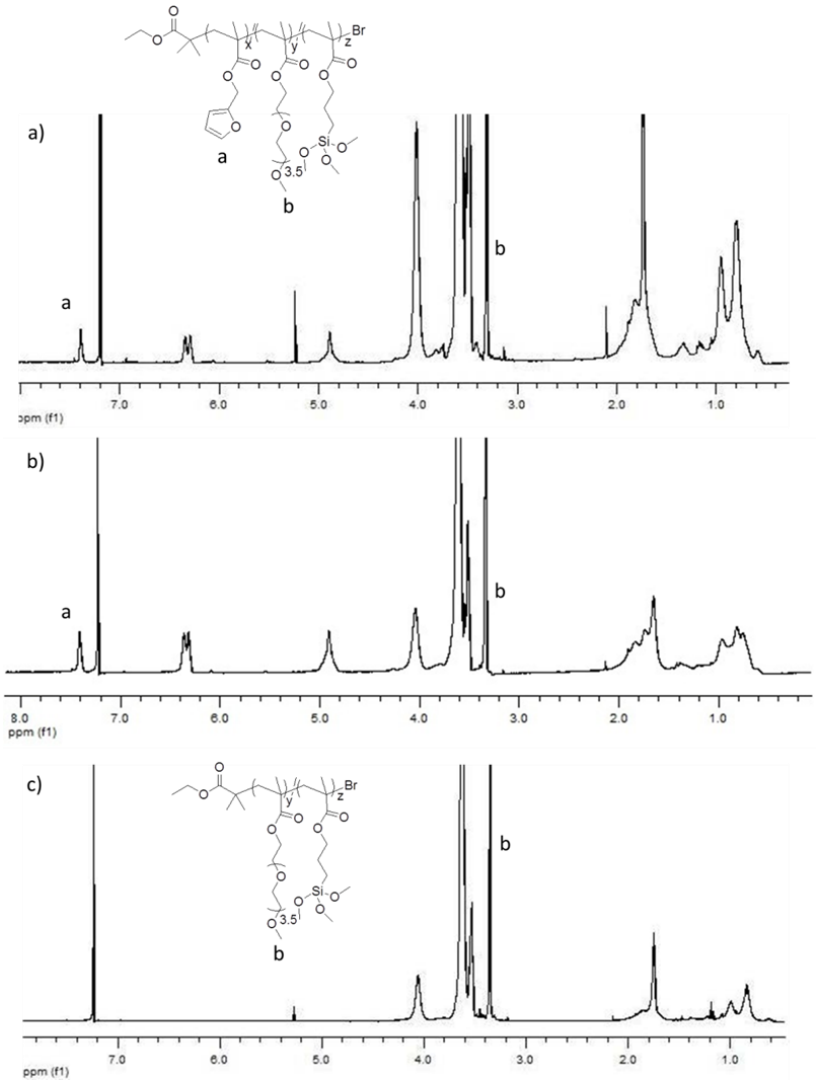


Figure 3.3. $^{1}$H NMR spectra of copolymers: (a) P1, (b) P2 and (c) P3

Table 3.1. GPC results of polymers.

| **Polymer** | **Copolymer Composition** | **$M_n$** | **PDI** |
|---|---|---|---|
| P1 | Poly(TMSMA-PEGMEMA-FuMA) | 25 K Da | 1.42 |
| P2 | Poly(TMSMA-PEGMEMA-FuMA) | 26 K Da | 1.33 |
| P3 | Poly(TMSMA-PEGMEMA) | 27 K Da | 1.40 |

### 3.3.2. Synthesis of Copolymers

PEGMEMA ( Mn =300 g mol $^{-1}$ ) was flowed through the aluminum oxide column in order to remove the inhibitor. Cu(I)Br were taken into a 10-mL round bottom fl ask with a stir bar and degassed for 20 min using $N_2$ gas stream. FuMA, PEGMEMA, anisole, and PMDETA which were also degassed separately were added in the round bottom flask. After addition of TMSMA, round bottom flask was placed in 80 °C oil bath. The initiator (EIBBr) was added and the mixture were allowed to stir at 80 °C for 30 min. Polymer in anisole poured into hexane to remove anisole and unreacted monomers. After polymer was precipitated, hexane was decanted. Polymer was dissolved in $CH_2Cl_2$ and flowed through the aluminum oxide in order to remove copper salts. 1 H NMR (400 MHz, $CDCl_3$, δ): 7.42 (s, 1H furan), 6.38 (s, 1H furan), 6.33 (br s, 1H furan), 4.92 (br s, 2H, —OC H2 —furan), 4.05 (br s, 2H, –$COOCH_2$–PEG), 3.64 (m, 12H–$OCH_2CH_2O$–), 3.35 (s, 3H,$OCH_3$), 2.00–1.60 (br m), 1.08–0.56 (br m).

### 3.3.3. Fabrication of Thin Film on Surface

The reactive polymeric thin films were prepared by spin coating on $Si/SiO_2$ wafers or glass slides. Then, these coated surfaces were cured at vacuum oven at 60 °C for 1 h to ensure silyletheral bonding via dehydration between the siloxane groups of the copolymer and the hydroxyl groups of the substrate. After cooling to the room temperature, surfaces were rinsed with $CH_2Cl_2$ to wash off unattached polymer from solid surfaces. Ellipsometric measurements revealed thus obtained reactive thin films on silicon surface had an average thickness of 2–3 nm. Contact angle goniometry was used to probe the relative hydrophilicity of the reactive polymeric surfaces obtained. Measurements were conducted on static water droplet at ambient temperature. Contact angles were found to be 57.2, 74.1, and 52.3° for surfaces coated with the copolymers P1, P2, and P3, respectively. As expected the contact angle increased with increasing amount of the relatively hydrophobic furfuryl methacrylate-based side chains.

### 3.3.4. Functionalization of Surfaces via Micro Contact Printing Method

Initial functionalizations of these polymers modified surfaces were examined via micro patterning of the fluorescent dye molecule (BODIPY-maleimide) via the μCP technique using a PDMS stamp (Figure 3.8). Clean glass surfaces coated with the reactive polymers were brought in conformal contact with a PDMS stamp inked with the maleimide-containing dye. Optimum contact time was explored by printing for 5, 15, 30, 60, and 120 min onto the glass surfaces coated with the copolymer P1 containing 10% furan moieties. Thereafter, the surfaces were rinsed with and sonicated in $CH_2Cl_2$ for 3 min to remove any unbound dye, and dried under nitrogen stream. The surfaces were visualized under a fluorescence microscope to reveal that the functionalization reaches a constant value after 30 min of printing time. Similar studies were carried out for the polymeric surface P2 to realize that 30 min was sufficient to achieve saturation of functionalization. Control experiments were carried out in a similar fashion on a surface coated with the copolymer P3, to reveal no residual fluorescent patterns after the rinsing step. Also, μCP with BODIPY-dye devoid of the maleimide group on P1-coated surfaces did not lead to any patterns. Hence, it can be inferred that the attachment of the fluorescent dyes on the polymeric surface is due to the Diels–Alder cycloaddition reaction. More importantly, the

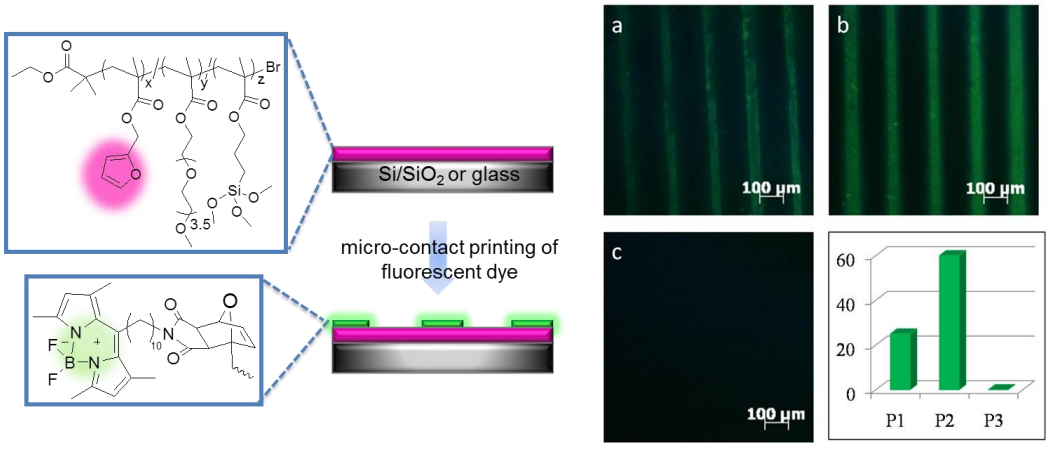


Figure 3.4. Surface functionalization via DA reaction. Fluorescence microscopy images of BODIPY-maleimide patterns on different polymer surfaces: (a) P1, (b) P2, and (c) P3 surfaces and their normalized relative fluorescence intensities.

fluorescence intensity of the patterns on surface P2 was higher than on surface P1 (Figure 3.4). This is expected because the copolymer P2 contains more of the reactive furan groups compared to the copolymer P1, hence leading to higher level of functionalization.

### 3.3.5. Biomolecular Immobilization

To examine the feasibility of these reactive polymeric surfaces for the purpose of bio-immobilizations, FITC-labeled streptavidin was chosen as a model enzyme (Figure 4). In the first step, biotin ligands were immobilized onto the surface by contact-printing a maleimide-appended biotin for 60 min onto the copolymer P2-coated surfaces. After rinsing off any unbound biotin, the surface was exposed to an FITC-labeled streptavidin solution for 30 min. Thereafter, the surface was washed with ample water to eliminate any unbound streptavidin adhering via nonspecific physisorption. Nice arrays of streptavidin patterns were generated on copolymer P2-coated surfaces as revealed by the fluorescence images (Figure 3.7).

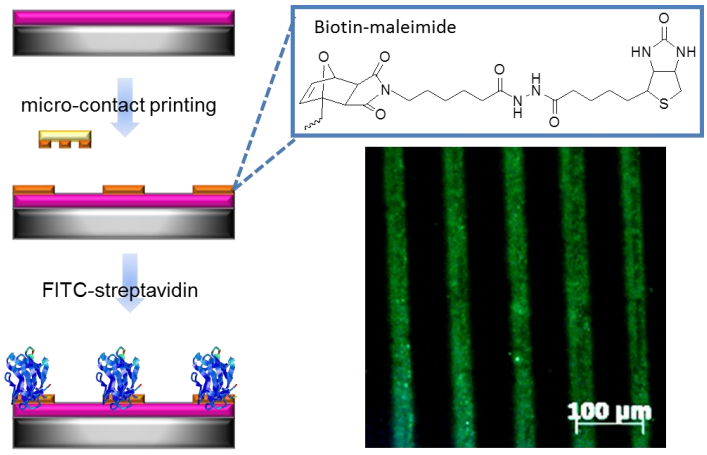


Figure 3.5. Immobilization of streptavidin directed by printed biotin patterns on surfaces coated with copolymer.

The choice of the furan-maleimide dyad as a diene–dienophile pair offers a handle to thermo-reversibility at elevated temperatures. Thus these thin polymeric platforms

should be amenable to write–erase–rewrite process. In order to test that, the thermo-reversibility of the patterns obtained via the Diels–Alder reaction on surfaces was investigated. Various printed patterns were erased by heating the surfaces to 125 °C for 2 h in DMF. Patterns were reprinted via the μCP technique. This protocol was repeated for many surfaces for five times. The surfaces subjected to print, erased, and reprint protocols were examined with fluorescence microscope. As expected, complete loss of patterns were observed after heating cycles. Furthermore, the reprinted patterns exhibited comparable fluorescence intensities, thus ensuring minimal loss of efficiency of conjugation during the process. As a particular example, a PDMS stamp was fabricated bearing the letters MCP in two different sizes and fonts. Initially, the letters with round corners were printed onto the thin film obtained from copolymer P1. Thereafter, the contact printed surfaces were heated to 125 °C for 3 h. As expected, the patterns were completely erased. Recontact printing was performed on the same surface using a different PDMS stamp that possessed the reliefs “MCP” with a different font to successfully re-functionalize the surface (Figure 3.8). This cycle was repeated four times to demonstrate the robustness of this platform.

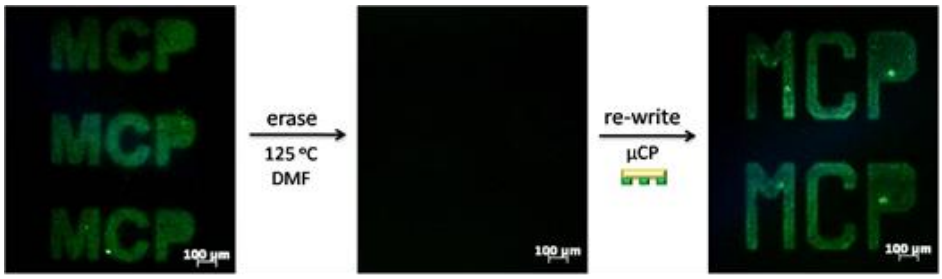


Figure 3.6. Reversible reactive micropatterning on polymer surface.

## 3.4. Conclusions

In this part, we have reported rewritable surfaces utilizing Diels–Alder/retro Diels–Alder strategy. We synthesized surface attachable reactive polymers which has furan functionality and coated them on the glass surfaces. Using maleimide-containing dye molecules we performed μCP to demonstrate small molecule micro-patterns on the surface can be done. We also generated biomolecular immobilization on the biotin-maleimide

patterned surface. Furthermore, these polymeric platforms were thermoresponsive and thus present write–erase–rewrite ability. Choice of diene–dienophile combinations that will allow the retro Diels–Alder reaction to proceed under lower temperatures will further expand the scope of this approach of surface functionalization.

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# 4. THIOL-REACTIVE POLYMERIC FILMS FOR BIOSENSOR APPLICATIONS



## 4.1. Introduction

The worldwide acceptance of "prevention is better than cure" mantra has fueled widespread interest in fabrication of highly sensitive and effective diagnostic platforms. Many health disorders can be rapidly analyzed at an early stage by efficient detection of proteins and nucleic acids. Various microarray-based biosensor platforms utilize a variety of biomarkers such as antibodies, carbohydrates and nucleic acids for detection [158-165]. This necessitates the development of efficient methods for biomolecular immobilization onto glass-like or electrode surfaces. Because many biomolecules undergo denaturation under challenging conditions, it is desirable that immobilizations are effective under mild and preferably reagent-free conditions. In this regard, the maleimide functional group has been used in immobilization studies due to its high reactivity toward thiol groups under benign conditions [104,166-171]. For example, the maleimide-modified self-assembled monolayer (SAM) on gold surfaces has been employed for immobilization of several ligands, biomolecules and oligonucleotides [172-175]. However, SAM-based coatings are often unstable over long periods of time due to chemical oxidation of the thiol functional groups,[10] as well as their facile desorption from the surface [13,176]. In recent years, maleimide terminated monolayers have also been fabricated on glass and $SiO_2$ surfaces, followed by molecular immobilization using techniques like microcontact printing [146,177,178]. Although the chemistry of the reactive handle for bioimmobilization is crucial for effective conjugation, the adjoining surface environment is also important to

minimize denaturation and nonspecific absorption. As an attractive alternative to SAMs, polymeric coatings on glass-like surfaces have been investigated as an engineered adlayer between the surface and the ligand or biomolecule utilized for sensing [17,179,180].

To achieve detection with high sensitivity and selectivity, it is imperative that these platforms exhibit minimal nonspecific adsorption of analytes. It is well established that antibiofouling characteristics can be imparted to surfaces by utilization of poly(ethylene glycol) (PEG)-based materials due to their inherent bioinertness [181-183]. To date, a variety of different reactive functional groups such as activated esters and epoxides have been incorporated into PEG-based copolymers to enable conjugation of various ligands and biomolecules. For example, polymeric thin films containing *N*-hydroxysuccinimide (NHS) activated carboxylic-acid esters were utilized by Jon and co-workers to immobilize proteins [86]. In this study, the polymers containing NHS-activated esters and trimethoxysilyl group-based side chains were anchored onto silicon and glass surfaces. A similar strategy was utilized by Choi, Oh and co-workers to obtain NHS-ester containing PEG-based polymeric films to coat titanium surfaces using dopamine-based anchoring units [184]. In another study, epoxy bearing amine reactive polymeric coatings with antibiofouling properties were fabricated and amine bearing biomolecules were attached via epoxy chemistry [185]. Though most of the conjugation studies have focused on utilization of amine-based chemistry, implementation of alternative chemistries have been scarce. In recent years, several studies have shown that various “click” type conjugations such as the copper mediated and strain promoted alkyne–azide and the Diels–Alder cycloadditions can be used for this purpose [186-189]. Utilization of the highly efficient nucleophilic thiol–maleimide conjugation has been rarely exploited on polymeric thin films on surfaces. One recent example includes modification of maleimide containing polymeric brushes that were grown from silicon oxide surfaces using surface initiated polymerization, a process that involves direct growth of polymers from surfaces, which can be difficult to adapt as a coating strategy for large surfaces [170]. A simpler fabrication approach toward thiol-reactive polymeric coatings where appropriately functionalized polymers can be simply anchored to surfaces will provide a modular approach for obtaining such interfaces.

Herein, we report the fabrication of maleimide-containing thiol-reactive polymeric thin films and demonstrate their functionalization with thiol-containing molecules and ligands for biomolecular immobilization/sensing (Figure 4.1). For this purpose, PEG-based copolymers containing reactive functional groups were synthesized and coated on $SiO_2$ or glass surfaces. The maleimide group in the copolymer was masked with a furan moiety using the Diels–Alder reaction to protect it during the polymerization step. A trialkoxysilyl group containing comonomer was used to provide surface anchoring to glass like substrates. Surfaces coated with these copolymers were rendered thiol-reactive upon heating, through unmasking of the maleimide group via the retro Diels–Alder reaction (rDA). Thus, obtained surfaces could be easily functionalized with thiol-containing fluorescent dye and ligands. Functionalization of surfaces could be achieved in a spatially controlled manner using microcontact printing. Micropatterns of a ligand, namely biotin, were used to direct the immobilization of the protein Streptavidin. Furthermore, it was established that the degree of immobilization onto such polymeric surfaces can be tailored by adjusting the amount of reactive functional groups in the parent copolymers.

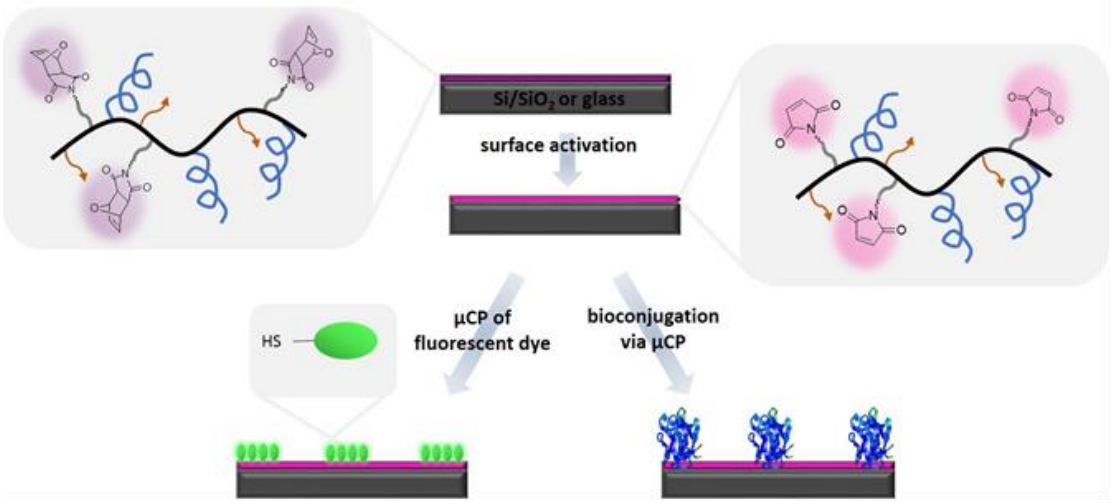



Figure 4.1. Scheme of fabrication and functionalization of reactive polymeric surface by microcontact printing.

## 4.2. Experimental

### 4.2.1. Materials

Poly(ethylene glycol) methyl ether methacrylate (PEGMEMA, Mn = 300 g/mol), ethyl α-bromoisobutyrate (EIBBr), bis(2-dimethylaminoethyl)methylamine 99% (PMDETA), 3-(trimethoxysilyl)propyl methacrylate 98% (TMSMA) and tetraethylrhodamine isothiocyanate (TRITC) conjugated extravidin were obtained from Sigma-Aldrich. Biotinylated (triethylene glycol) undecanethiol (Biotin-SH) was obtained from Nanoscience Instruments (Phoenix, AZ). Fluorescein (FITC) conjugated streptavidin was obtained from Pierce. Furan protected maleimide methacrylate monomer (FuMaMA)[189] and thiol containing dye 4,4-difluoro-1,3,5,7-tetramethyl-8-[(10-mercapto)]-4-bora-3a,4a-diaza-s-indacene (BODIPY-SH) [154]were synthesized according to literature procedure. Nochromix was obtained from Godax Laboratories, Inc. All organic solvents were used as received without further purification. PDMS stamps were prepared using standard photolithography using previously reported procedures.[190]

### 4.2.2. Methods

For copolymer composition characterizations, 1H NMR spectroscopy (Varian 400 MHz) was used. The molecular weights were estimated using size exclusion chromatography (SEC) on a Viscotek instrument equipped with a refractive index detector using polystyrene standards for calibration and THF as solvent. Static water contact angle values were measured in air via the sessile-drop method using a goniometer (CAM 101 KSV instruments). Approximately 5 μL of deionized water was deposited on the surface, and images were taken by an integrated digital camera. The software of the camera provides contact angle measurements once the liquid is dispensed. The contact angle value for each sample was independently measured at five different locations and average contact angle values were measured. The thicknesses measurement of the polymer coated

surfaces films were performed with a Rudolph manual ellipsometer at a 70° angle of incidence. A refractive index of 1.46 was used, and a three-phase model was used to calculate thicknesses. Attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy was performed on a Thermo Scientific Nicolet 380 FT-IR spectrophotometer equipped with Harrick Scientific GATR accessory and a Ge crystal. A clean silicon wafer was used as a background during measurements on polymer coated substrates. XPS spectra were recorded on the Kratos Analytical Axis Ultra X-ray photoelectron spectrometer (XPS/ESCA) with an Al Kα-monochromatized source of 1486.71 eV. All measurements were carried out on silicon substrates. Fluorescence microscopy images of dry samples on glass surfaces and were recorded at room temperature on a Zeiss Observer.Z1 fluorescent microscope (ZEISS Fluorescence Microscopy, Carl Zeiss Canada Ltd., Canada). BODIPY-SH and FITC conjugated streptavidin were excited by 488 nm line of an Ar+ laser, TRIT-C conjugated extravidin was excited by a 546 nm HeNe laser.

### 4.2.3. Synthesis of Latent Reactive Polymers

Prior to polymerization, PEGMEMA ($M_n$=300 g/ mol) monomer was filtered through a short aluminum oxide column to remove the inhibitor. Furan-protected maleimide-containing monomer (FuMaMA) (0.7 mmol, 203.8 mg), Cu(I)Br (0.035 mmol, 5 mg) and anisole (1.5 mL) were taken in a round bottom flask with a stir bar and purged with a stream of $N_2$ gas. Degassed TMSMA (0.175, 0.42 µL), PEGMEMA (2.625 mmol, 0.75 mL) and PMDETA (0.07 mmol, 12.1 µL) was added into the mixture. The round bottom flask was immersed into an oil bath at 80 $^oC$. The initiator (EIBBr) (0.035 mmol, 5.2 µL) was added to the flask, and the mixture was allowed to stir at 80 $^oC$ for 15 minutes. After immediate cooling to room temperature, the polymer was precipitated in cold diethyl ether to remove anisole and unreacted monomers. The polymer was then dissolved in $CH_2Cl_2$ and quickly filtered through a short aluminum oxide plug to remove Cu-based impurities. Using this procedure, three different poly(TMSMA-PEGMEMA-FuMaMA) bearing different initial molar ratio of the FuMaMA monomer (P1=5:75:20, P2=5:55:40, P3=5:35:60) were prepared. Monomer incorporations in the copolymers were calculated using $^1H$ NMR spectroscopy (400 MHz, $CDCl_3$). The peaks at δ = 3.35 (O−C$H_3$ in

PEGMEMA, 3H, s), the peak at δ = 0.61 (C*H*$_2$-Si(OCH$_3$)$_3$ in TMSMA, 2H, br s) and the peak at δ = 6.51 (C*H*=C*H* in FuMaMA, 2H, s) were used for calculation and compositions were found as 8.1:74.0:16.9, 4.5:57.7:37.8 and 4.4:41.1:54.5 for P1, P2 and P3, respectively. For $^1$H NMR spectra of polymers see supporting information (Figure S1). Molecular weight and molecular weight distributions obtained using size exclusion chromatography were obtained as P**1** ($M_n$: 15.9 kDa with $M_w/M_n$: 1.42), P**2** ($M_n$: 14 kDa with $M_w/M_n$: 1.66) and P**3** ($M_n$:12.6 kDa with $M_w/M_n$: 1.55).

### 4.2.4. Coating of Glass and Silicon Surfaces

Prior the use, Si/SiO$_2$ wafer or glass surfaces (2x2.5 cm$^2$) were cleaned using nocromix solution in H$_2$SO$_4$. Glass or silicon surfaces were spin coated with polymer (20 µL of 10 mg/mL polymer solution in DMSO). The substrates were spin-coated at 500 rpm for 10 seconds followed by 4000 rpm for 30 seconds. The resulting films were baked at 40 ºC under vacuum for 1 hour in order to promote adhesion between the polymer and underlying surface. Thereafter, the surfaces were heated to 110 ºC for 15 minutes to unmask the maleimide groups to their thiol-reactive form.

### 4.2.5. Micro-Contact Printing of Thiol-Containing Dye and Ligand

Solution of BODIPY-SH in THF (1 mg/mL) was used to wet a 1x1 cm$^2$ PDMS stamp. The stamp was left to dry for 10 min, followed by further drying under a gentle stream of N$_2$. The stamp was placed onto the polymer coated surface for 1.5 h. After printing, surfaces were washed with copious amounts of THF to remove non-conjugated materials. For micro-contact printing of Biotin-SH, solution of the ligand in 50:50 methanol/THF mixture (1mg/ mL) was used and the stamp was kept in contact with the surface for 4h. After printing, surfaces were washed with copious amounts of methanol to remove unbound ligand.

### 4.2.6. Ligand Mediated Bio-Immobilization of Protein

Biotin immobilized surfaces were immersed in PEG-SH solution in THF (2 mg/mL) for 12 hours. After washing with copious amount of THF, aqueous solution of dye conjugated streptavidin or extravidin (20-30 µL, 0.1 mg/ mL) was dropped on the biotin printed polymeric surface and covered with a microscope glass to make the protein solution spread homogenously all over the surface. After waiting for 15 minutes in the dark, the surface was rinsed with water several times to remove unbound protein.

## 4.3. Results and Discussion

### 4.3.1. Synthesis of Copolymers

The latent reactive copolymer poly(TMSMA-r-PEGMEMA-r-FuMaMA) was synthesized from three kinds of monomers by copper-I catalyzed Atom Transfer Radical Polymerization (Figure 4.2). In this polymer, 3-(trimethoxysilyl)propyl methacrylate (TMSMA) is used as surface reactive part, furan-protected maleimide methacrylate (FuMaMA) as latent reactive part and poly(ethylene glycol)methyl ether methacrylate (PEGMEMA) is used in order to reduce unspecific adsorption and give surface a protein resistant property. Changing ratio of each monomer in the polymer synthesis, three different poly(TMSMA-PEGMEMA-FuMaMA) bearing different initial molar ratio of the FuMaMA monomer (P1=5:75:20, P2=5:55:40, P3=5:35:60) were prepared (Figure 4.2). Monomer incorporations in the copolymers were calculated using $^{1}$H NMR spectroscopy (400 MHz, $CDCl_3$). The peaks at δ = 3.35 (O−C$H_3$ in PEGMEMA, 3H, s), the peak at δ = 0.61 (C$H_2$-Si$(OCH_3)_3$ in TMSMA, 2H, br s) and the peak at δ = 6.51 (C*H*=C*H* in FuMaMA, 2H, s) were used for calculation and compositions were found as 8.1:74.0:16.9, 4.5:57.7:37.8 and 4.4:41.1:54.5 for P1, P2 and P3, respectively. For $^{1}$H NMR spectra of polymers see supporting information (Figure 4.3). Molecular weight and molecular weight distributions obtained using size exclusion chromatography were obtained as P**1** ($M_n$: 15.9

kDa with $M_w/M_n$: 1.42), P**2** ($M_n$: 14 kDa with $M_w/M_n$: 1.66) and P**3** ($M_n$: 12.6 kDa with $M_w/M_n$: 1.55).

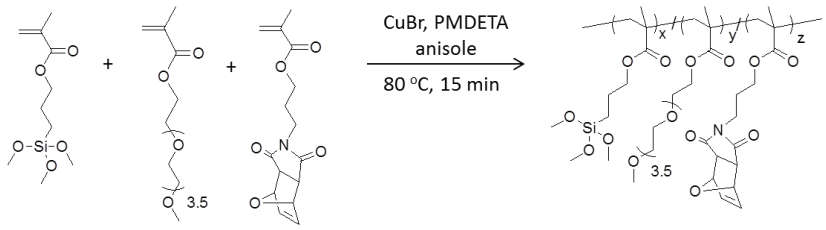



Figure 4.2. Synthesis of poly (TMSMA-r-PEGMEMA-r-FuMaMA) via ATRP.

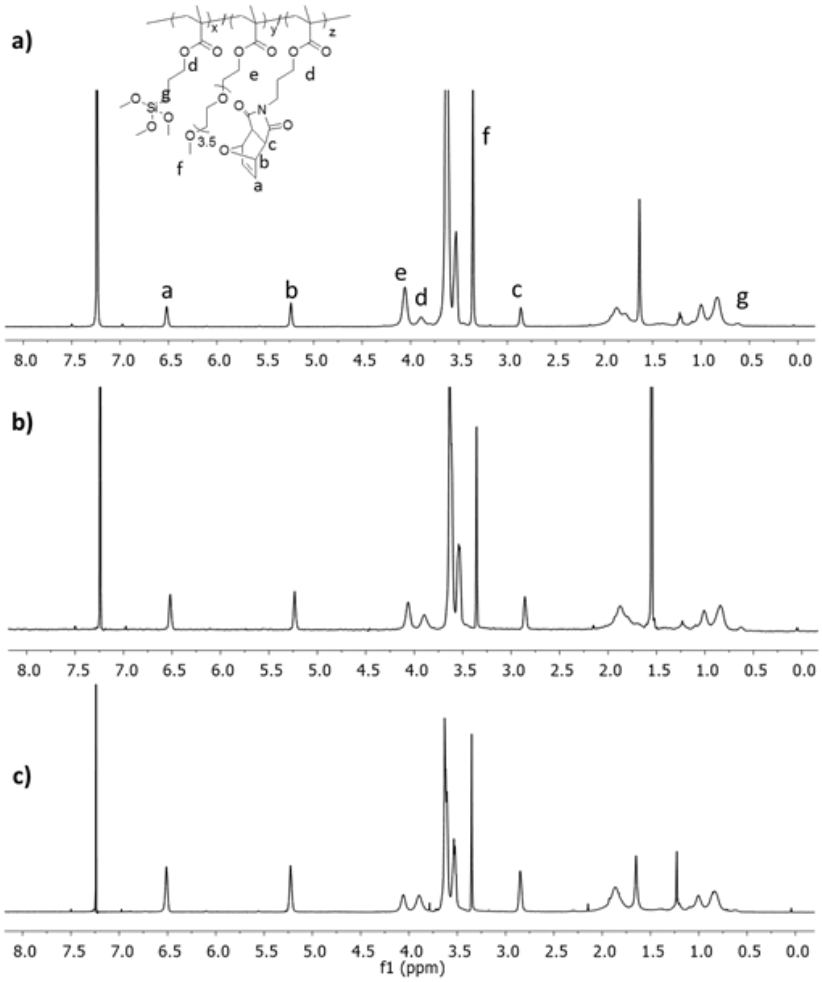



Figure 4.3. 1HNMR spectra of (a) P1, (b) P2 and (c) P3.

### 4.3.2. Fabrication of Thiol-Reactive Polymeric Coatings

The polymer coated surfaces were prepared by spin coating the masked maleimide group containing copolymers onto $Si/SiO_2$ wafer or glass slide, followed by curing under vacuum at 40 °C for 60 min to ensure silyl ether bonding between the alkoxysilane groups of the copolymer and the hydroxyl groups on the substrate. Thereafter, the surfaces were heated to 110 °C for 15 min for deprotection of the maleimide groups on the copolymer. After cooling to room temperature, surfaces were rinsed with copious amounts of $CH_2Cl_2$ to remove any unbound polymer from the surfaces. After drying in a stream of nitrogen, thickness of the thin films of the copolymers was determined to be between 6 and 8 nm using ellipsometry. The wettability of the polymeric surface was determined using static water contact angle measurement at ambient temperature. Contact angle decreased from 81° to 72° with the increasing ratio of hydrophilic PEG monomer in the copolymer (Table 4.1). Although lower contact angle values due to hydrophilic PEG side chains were expected, the hydrophobic maleimide groups decrease the hydrophilicity of the surfaces.

Surfaces coated with copolymers containing the masked maleimide groups were analyzed using XPS to determine their chemical compositions. The existence of protected maleimide units on the surfaces was confirmed from the presence of the N 1s signal at 400.5 eV in the survey scan. With increasing amount of maleimide in the parent copolymers, an incremental increase of intensity of N 1s was observed (Figure 4.4) Because coatings are less than 10 nm, Si 2s and 2p peaks from the underlying substrates were also observed as expected. Static water contact angle values of polymer coated surfaces were obtained as 70, 76 and 79 for P1, P2 and P3, respectively. With decreasing ratio of PEG groups, an increase in contact angle was witnessed. After deprotection of the maleimide groups, no significant changes in contact angle values were observed, and were determined as 72, 77 and 81 for P1, P2 and P3 coated surfaces, respectively.

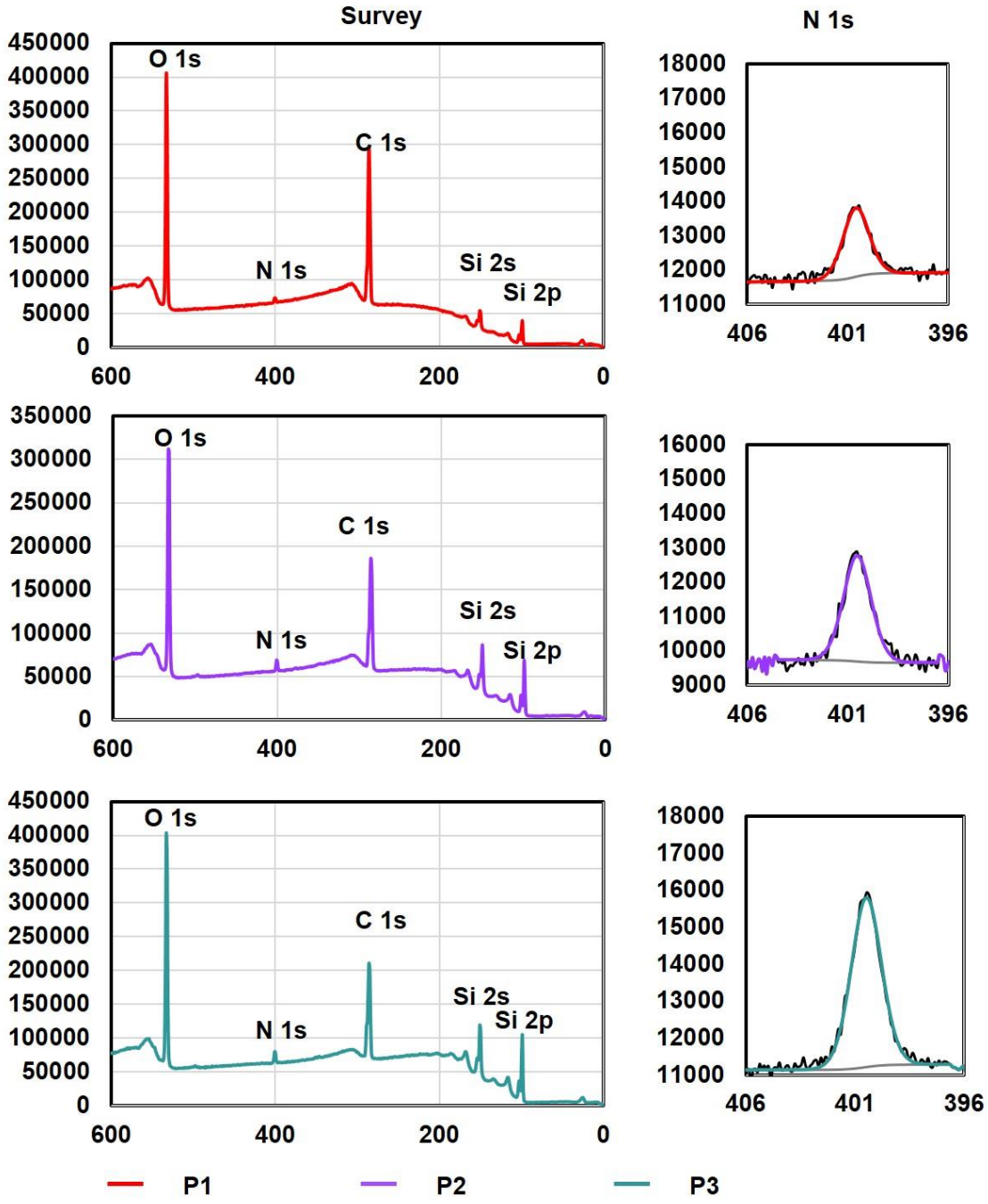



Figure 4.4. Fabricating polymeric layer on the substrates (a) and N1s peaks from XPS multiplex scans, of copolymer P1, P2 and P3 coated surfaces on $Si/SiO_2$.

Table 4.1. Thickness (after retro) , mass concentration of N (after retro Diels-Alder) and contact angle of P1, P2 and P3 layers on $Si/SiO_2$ wafers measured by ellipsometry, XPS and goniometry.

| Polymer coated surfaces | Thickness (Å) | N/C (Theoretical)[a] | N/C (XPS) | N/C (rDA) (Theoretical)[a] | N/C (rDA) (XPS) | Contact Angle (°) | Contact Angle (°) (rDA) |
|---|---|---|---|---|---|---|---|
| **P1** | 71.21 | 0.013 | 0.013 | 0.013 | 0.014 | 70 | 72 |
| **P2** | 76.62 | 0.029 | 0.032 | 0.030 | 0.031 | 76 | 77 |
| **P3** | 63.13 | 0.038 | 0.043 | 0.045 | 0.049 | 79 | 81 |

Successful activation of the maleimide functional groups on the surface was confirmed using ATR FTIR. Thicker polymeric coating was prepared on the surfaces by using a concentrated polymer solution (10×). The FTIR spectra showed C═O stretching band belonging to ester groups at ∼1727 cm–1 for all surfaces. In addition, the spectra revealed the presence of the out-of-phase C═O stretching vibration at 1702.5, 1700.8 and 1698.8 cm–1 corresponding to cyclic imides due to furan protected maleimide units of copolymer P1, P2 and P3, respectively (Figure 4.5). In addition, weak band at ∼1773 assigned to the in-phase C═O stretching vibration of maleimide units [97,191] was also observed (Figure 4a). The rDA reaction to unmask the furan-protected maleimide units was confirmed from the shift of C═O stretching vibration band to ∼6 cm–1 higher wavenumber for each polymer film.[192,193] Also, significant decrease of the in-phase vibration (∼1773 cm–1) supports the decomposition of furan maleimide adduct (Figure 4.5-b). Since efficient activation through the rDA reaction on thicker films was clear from the ATR FTIR analysis, it can be assumed that activation of thinner polymeric films used for surface functionalization was also achieved since the same conditions was used in both cases. No degradation of the film during thermal treatment under vacuum was evident from the XPS analysis from the preservation of expected nitrogen atom content (Figure 4.6).

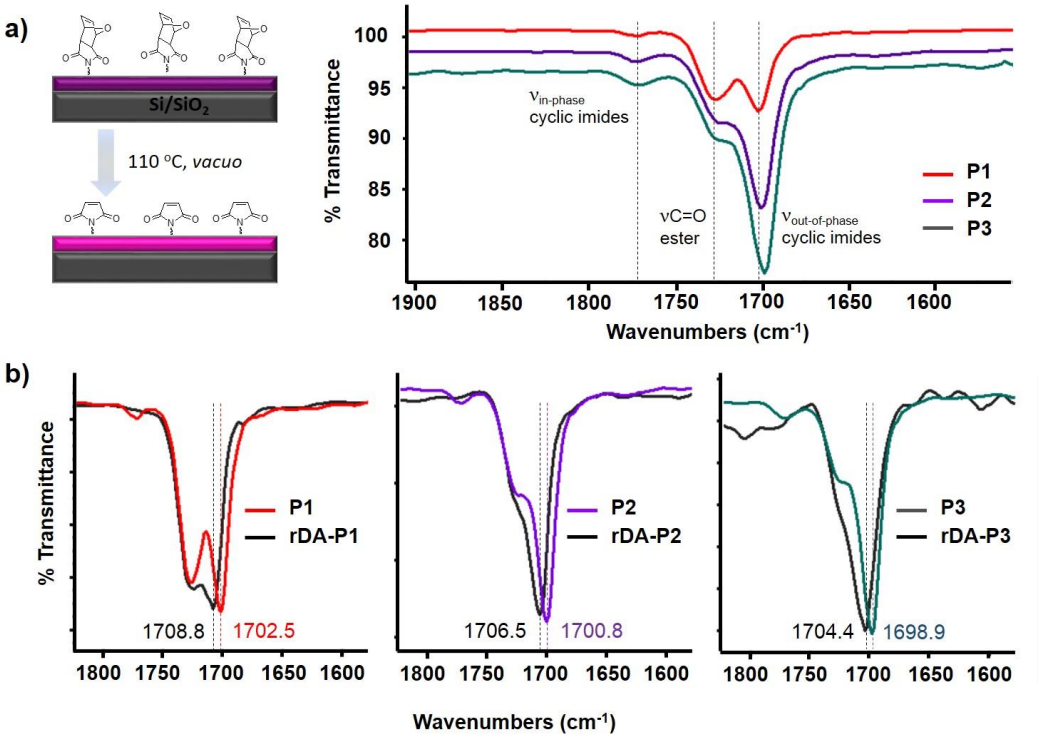



Figure 4.5. a) FTIR spectra in the 1905–1555 $cm^{-1}$ spectral range for copolymers P1, P2 and P3 on $Si/SiO_2$ surface. b) FTIR spectra in the 1820–1580 $cm^{-1}$ spectral range for copolymers (P1, P2 and P3) and activated copolymers rDA-P1, rDA-P2 and rDA-P3.

### 4.3.3. Surface Functionalization with Fluorescent Dye

To evaluate the feasibility of these polymeric surfaces as reactive platforms, immobilization of a thiol-containing fluorescent dye (BODIPY-SH) using a PDMS elastomeric stamp via microcontact printing was explored. The thiol-containing dye was contact printed for 90 min onto the polymeric surfaces rDA P1–P3 using an inked PDMS stamps. Thereafter, the surfaces were rinsed using copious amounts of THF and dried with a gentle stream of nitrogen. These dye-appended surfaces were visualized using a fluorescence microscope. It was observed that the utilization of well-defined copolymers for surface modification allows tunability of the reactive functional group on the surface. As expected, as concentration of maleimide group increases on the surface, a corresponding increase in the fluorescence intensity was observed (Figure 4.7). Control experiment performed using nonactivated surface, i.e., surfaces before rDA reaction did

not give significant fluorescence (Figure 4.8), which suggests that the reaction proceeds through thiol–maleimide conjugation.

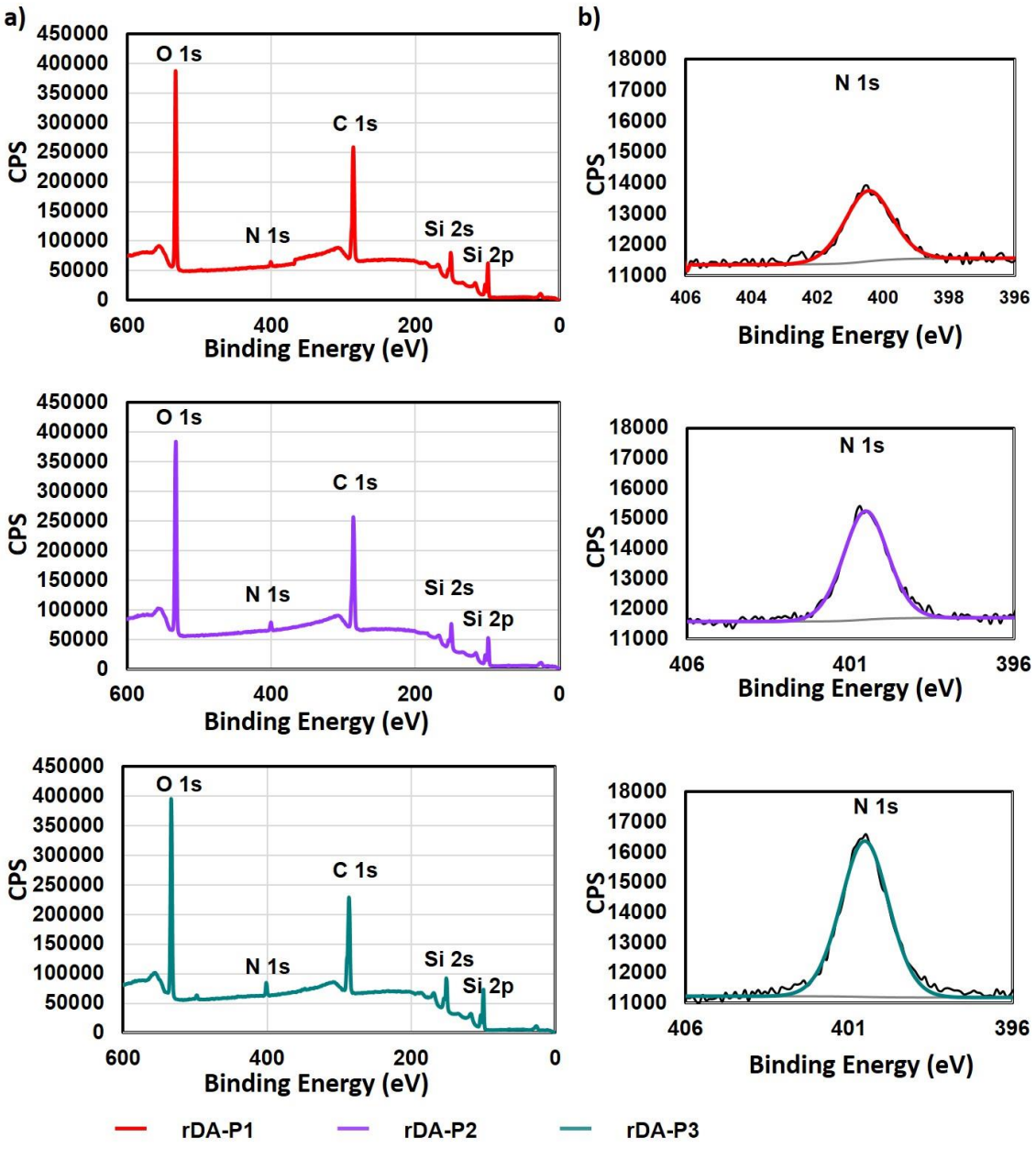



Figure 4.6. a) XPS survey spectra of rDA-P1, rDA-P2 and rDA-P3 surfaces, b) high-resolution XPS spectra for N 1s.

### 4.3.4. Surface Functionalization with Biotin-thiol and Immobilization of Avidin

After successful attachment of the fluorescent dye, the viability of ligand directed biomolecular immobilization/detection was explored. First, Biotin-SH was contact printed on a surface coated with copolymer P2. Thereafter, the biotinylated surface was incubated with fluorescently labeled avidin solution for 15 min, followed by rinsing with water to remove unbound protein. To our surprise, appreciable absorption had occurred on the nonbiotinylated regions (Figure 4.9). It is anticipated that numerous nucleophilic functional groups such as amines on the protein surface, react with electron-deficient maleimide units that are left over in the nonbiotinylated regions of the surface. To prevent the unwanted binding of avidin, we backfilled the nonbiotinylated area using a PEG5000-SH after printing of Biotin-SH ligand. Thus, modified surfaces were immersed in fluorescently labeled extravidin for 15 min and washed with water several times. Fluorescence microscopy analysis of surfaces revealed micropatterns of immobilized protein with much higher contrast (Figure 4.9-b).

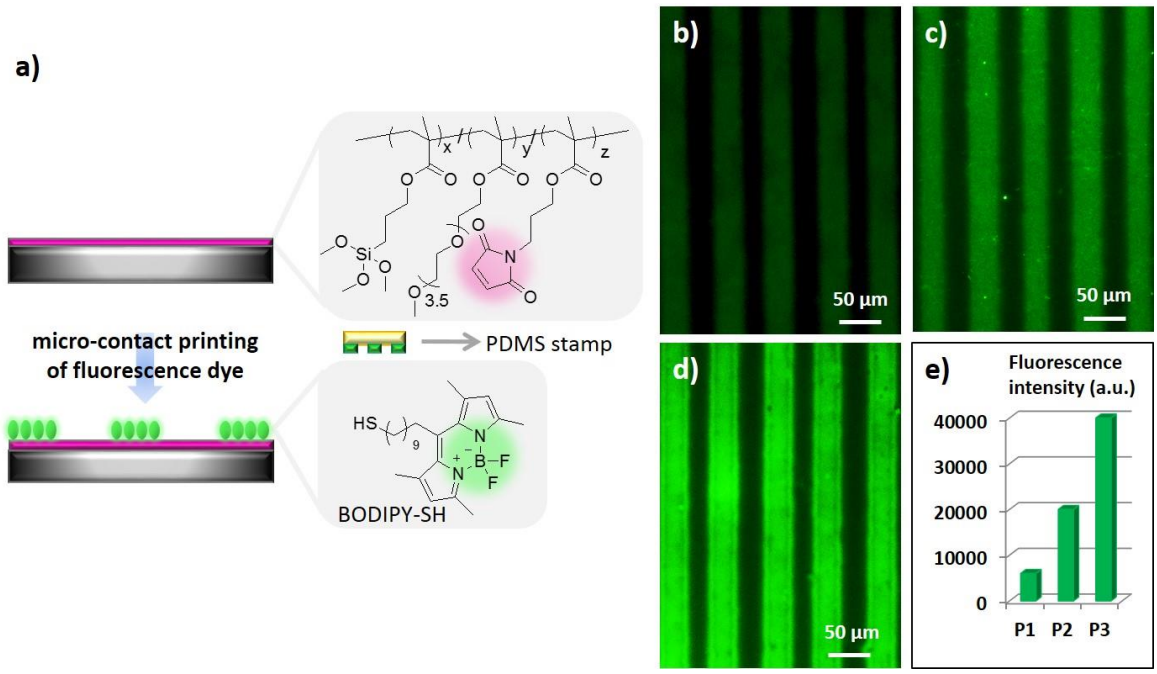



Figure 4.7. (a) Schematic of the microcontact printing of BODIPY-SH fluorescent dye, fluorescence microscope images of BODIPY-SH patterns on (b) rDA-P1, (c) rDA-P2, (d) rDA-P3 coated glass surfaces and (e) normalized fluorescence intensities.

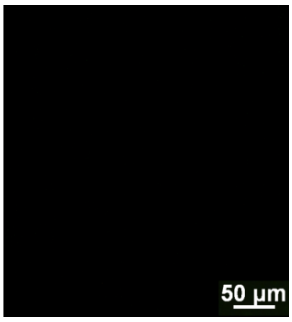



Figure 4.8. Fluorescence microscopy image of microcontact printing of BODIPY-SH on non-reactivated P1 surface.

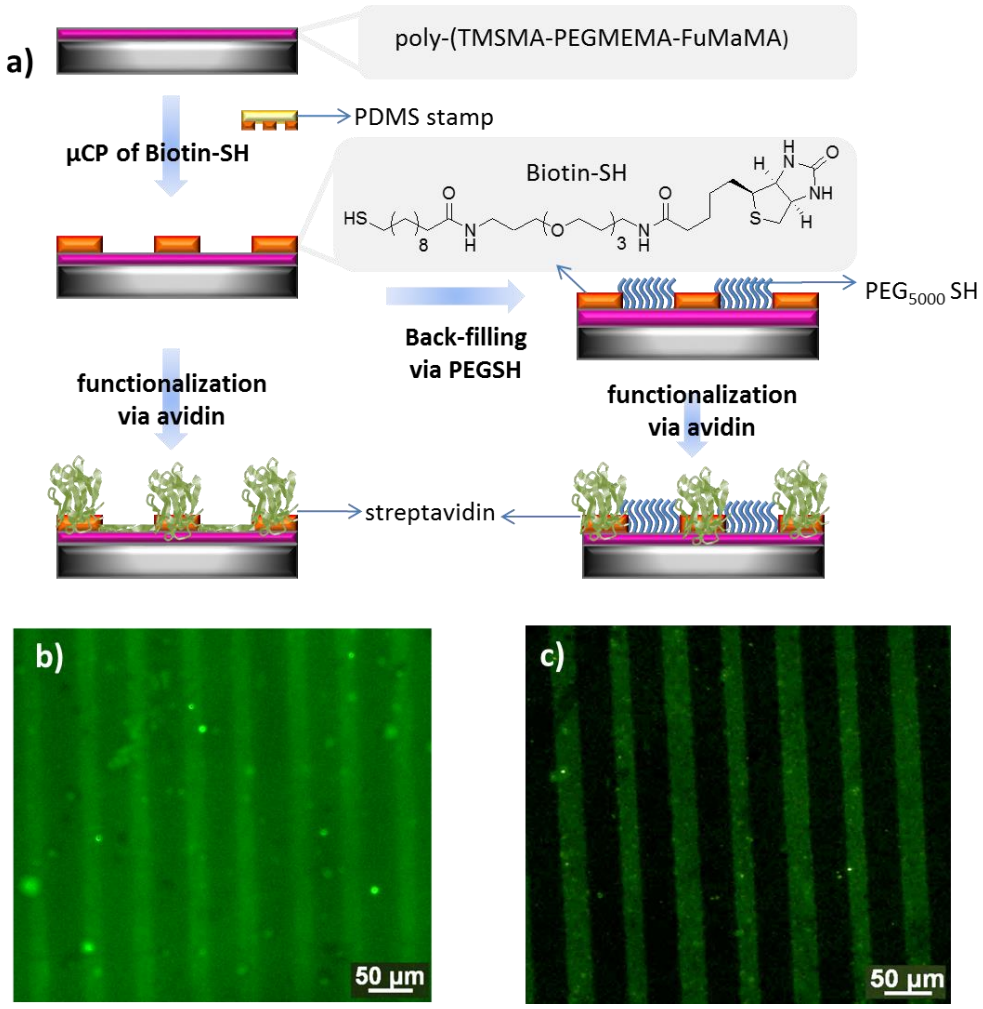



Figure 4.9. (a) Schematic illustration of immobilization of fluorescently labeled avidin on biotinylated micropatterns on polymeric film, and fluorescence microscopy images of rDA-P2 coated surface (b) before and (c) after backfilling with PEG5000-SH.

As an extension, to probe the tunability of protein immobilization on surfaces containing biotinylated domains with varying ligand density, surfaces were treated with TRITC-labeled extravidin. A clear increase in fluorescence intensity was observed upon survey of the surfaces coated with copolymers P1 to P3 (Figure 7). It is gratifying to note that the extent of biomolecular immobilization was in agreement with the maleimide functional group composition of the polymer coated surfaces.

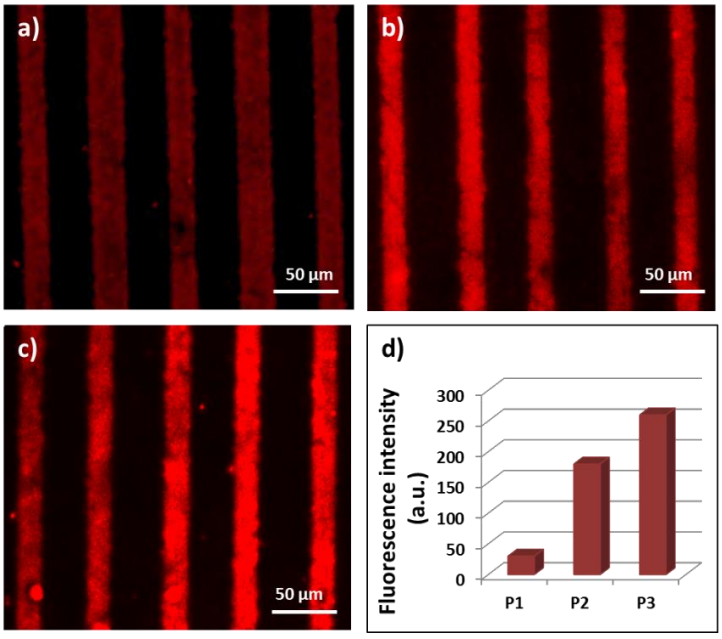



Figure 4.10. Fluorescence microscope images of extravidin patterns on (a) rDA-P1, (b) rDA-P2 and (c) rDA-P3 surfaces and (d) normalized relative fluorescence intensities.

## 4.4. Conclusions

Facile fabrication and functionalization of thiol reactive polymeric thin films on $Si/SiO_2$ and glass surfaces is demonstrated. Copolymers containing surface anchoring, bioinert and latent thiol-reactive units were coated onto silicon and glass surface. Activation of these polymeric coatings via thermal treatment provides surfaces with high reactivity toward thiols. Functionalization could be undertaken with spatial control using via microcontact printing of thiol containing dye and protein binding ligand. Notably, the functional group density on these polymeric surfaces could be effectively tailored by

tuning the feed ratio of monomers in the parent polymers. It can be envisioned that the simplicity of fabrication and effective functionalization of these thiol reactive soft polymeric coatings will find them attractive for various applications that employ biomolecular immobilization.

Acknowledgment: T.N.G. thanks the Bogazici University Foundation (BUVAK) and TUBITAK BIDEB (2211-D) for fellowship. This work was supported by The Scientific and Technological Research Council of Turkey (TUBITAK Project No. 110T068)

# 5. FABRICATION OF ANTIMICROBIAL TITANIUM SURFACES USING THIOL REACTIVE POLYMERIC COATINGS

## 5.1. Introduction

Bacterial adhesion onto surfaces leads to biofilm formation which possesses a strong resistance towards the host immune system [194]. Such bacterial colonization on implanted devices causes infections that are difficult to treat [195]. Treatment of infections associated with surgical implant is an immense burden for both patient and the medical staff since such failures may require implant replacement and in some cases lead to even more catastrophic situations such as amputation and mortality [196,197]. Hence, the control of biofilm formation is of utmost importance for ensuring safety of implantable medical devices. For this purpose, antimicrobial surface coatings have been developed to address this problem. In general, surfaces can be imparted with antimicrobial attributes by using the following strategies either alone or in combination: creating anti-adhesive (or anti-biofouling) and bactericidal surface [198]. The first approach includes coating the substrate with polymeric materials that deter the adhesion of microbes onto its surface, while the latter aims to kill the bacteria using either contact-active or release-based strategies.

Titanium is extensively used for dental and orthopedic implants as a raw material because of its good mechanical properties, high compatibility, corrosion resistance and low specific weight [199]. However, since bare titanium adsorbs serum proteins upon contact with body fluids, it has weak integration with surrounding bone environment which may cause inflammation. Therefore, for biomedical applications, particularly for *in vivo* applications titanium needs to be appropriately modified for long term performance.

Polymeric materials with antibiofouling properties are used as surface coatings to enhance *in vivo* compatibility and provide a layer for inhibition of bacterial attachment [200]. PEG is one of the most widely employed polymers to impart antifouling characteristics. It resists biomolecule attachment by creating a barrier of structured water associated with PEG and through chain compression which generates entropic barrier.[201-203] In order to modify surfaces with PEG, self-assembly, physorption and silanization are widely used methods. The robust attachment of the polymeric coating on the surface is crucial to warrant reliable performance of these materials under demanding conditions. Recently, surface chemistry has inspired by biological organisms which provides robust, nontoxic and permanent adhesive properties to the surfaces [204]. Especially, mimicking of mussel adhesive proteins by dopamine and its derivatives has attracted significant interest [205]. Mussels adhere to practically all types of inorganic and organic surfaces (Figure 8.1) [206], even adhesion-resistant substrates such as poly(tetrafluoroethylene) (PTFE) [205]. Mussels' adhesive property stems from the amino acid composition of proteins found near the plaque-substrate interface, which are rich in 3,4-dihydroxy-L-phenylalanine (DOPA) and lysine amino acids [207]. In addition to participating in reactions leading to bulk solidification of the adhesive [208,209], DOPA forms strong covalent and noncovalent interactions with substrates [210]. Not only DOPA but subunits of this adhesive protein, catechol, has been showed to bind strongly to metal or metal oxide surfaces [211-215].

Messersmith and coworkers showed that immobilization of PEG based molecules on the $TiO_2$ surfaces when using at least two catechol units [213,216]. The stability of the surface further increased when three repeating DOPA units were used as a multiple adhesion foot and such adlayers were capable of imparting non-fouling character to the surfaces. Similarly, Textor and coworkers obtained a non-fouling coating on the titanium surface. They modified titanium surface by dendrons containing catechol groups in their core containing at least three catechol units and PEG arms on the periphery. They also prepared linear PEG surface to compare some of their mechanical properties. Dendritic adlayers were found to have lower hydration and much lover dissipation than linear PEG surface. Despite their different mechanical properties, both dendritic adlayer and linear PEG coating exhibited excellent nonbiofouling property to titanium substrates [217].

Rodriguez-Emmenegger and coworkers demonstrated that ultra-low protein fouling polymer brushes act as bacteria fouling resistance substrates on gold coated silicon surfaces and they compared biofilm formation of *P. aeruginosa* strains on self-assembled monolayer (SAM) of polymerization initiator and polymer brushes [110]. *P. aeruginosa* bacteria that cause nosocomial infections, is a very good model organism since it can rapidly form stable biofilm and has many strains including antibiotic sensitive or antibiotic multi-resistant. In this study they compared biofilm formation of environmental, antibiotic-sensitive and antibiotic multi-resistant strain of *P. aeruginosa* on poly(MeOEGMA) and poly(HPMA) containing surface, in four different media with varying nutrient content. They observed bacterial adhesion and biofilm formation on SAM, while both poly(MeOEGMA) and poly(HPMA) inhibited biofilm formation even in nutrient rich media. Bacterial staining with a fluorescent marker showed that the fluorescence intensity on both brushes after 7 days contact with environmental strain suspended in low nutrient media (BM2 and M9) was below the controls, which suggests that biofilm did not form. However, multi-resistant strain *P. aeruginosa* (PA49) was able to colonize on the prepared protein resistance surfaces. These results showed that the investigation on the mechanism of bacterial adhesion should consider not only the physicochemical properties of the surface but also the biological variability of the bacteria strains.

Polymers conjugated with cationic biocides, antibiotics, or antimicrobial peptides can kill bacteria on contact [218]. Contrary to other bactericides, antimicrobial peptides are effective to drug resistance strains as well as antibiotics-sensitive bacteria [219-221]. Antimicrobial peptides target bacterial cell membranes and disrupt the lipid bilayer structure [222]. One third of the total proteins of the cells are connected to the membrane. These cells are responsible for critical vital activities such as adenosine triphosphate (ATP) generation and transport of nutrients. Since AMPs can change the function of these proteins, they kill the bacteria even if complete disruption of the cells does not occur [223]. Therefore, it is envisaged that such antibacterial peptide modified surfaces would result in better bactericidal activity than antibiotics containing surfaces.

In a recent study, Kizhakkedathu and coworkers reported fabrication of antibacterial polymeric film on silicon surfaces [224]. They obtained reactive polymer brush by post-modification of side chain *N*,*N*-dimethylacrylamide (DMA) and aminopropyl methacrylamide hydrochloride (APMA) using ATRP. Then they converted pendant amine groups into maleimide units via post-polymerization modification. To these reactive polymer brushes, they attached a series of cysteine containing antimicrobial peptides: Tet-213, 1010cys, Tet-20, Tet-21, Tet-26, HH2 and MXX226. Anti-microbial peptide (AMP) conjugated polymer brushes exhibited broad spectrum antimicrobial activity *in vitro* and *in vivo*. They also showed that these systems resisted biofilm formation to different levels depending on the nature of immobilized peptide. The Tet-20 attached polymer brush, one of the most effective constructs *in vitro*, was also tried *in vivo* against *S. aureus.* They demonstrated that fabricated antibacterial coating protected the rat from bacterial infection. Furthermore, the AMP conjugated polymer coatings were non-toxic to mammalian cells, did not activate human platelets or initiate complement activation.

In another study, Kizhakkedathu and coworkers demonstrated fabrication of antibacterial polymer brush coating on polystyrene (PS) nanoparticles and titanium surfaces combining the advantages of nonbiofouling coating and antibacterial peptide [128]. The hydrophilic monomer *N*-(3-Aminopropyl) methacrylamide hydrochloride (APMA) was copolymerized with three other different nonfouling monomers including (N,N-dimethylacrylamide) (DMA), 3-[(methacryloyl)amido]propyl-*N*, *N*-dimethyl(3-sulfopropyl)ammonium hydroxide (PMPDSAH) and 2-[(methacryloyl)oxy]ethyl]-phosphorylcholine (PMPC) on poly styrene nanoparticles and were conjugated with two cysteinylated cathelicidin-derived peptides: E6 and Tet-20. Highly efficient killing of planktonic bacteria by the antimicrobial coatings on nanoparticle surfaces was observed. Peptide E6 conjugated PDMA polymer brushes showed maximum efficiency on titanium surface, killing 50.3% of adhered bacteria while other polymer brushes with same peptide showed less antibacterial activity. Investigations revealed that the flexibility of the secondary structure of the tethered peptides are greatly affected by polymer structure which impacts the total antibacterial effect.

In this study, we aimed to prepare polymeric coatings anchored to titanium surface which possess both anti-adhesive and bactericidal properties. PEG based copolymers were designed for this purpose due to their well-established anti-adhesive property. In order to provide a modular approach for incorporation of different anti-bacterial agents onto the polymer coating, a thiol-reactive handle was used. The high efficiency of the thiol-maleimide addition reaction prompted us to use maleimide group containing polymers. In order to obtain a coating precursor with long term stability, furan-protected maleimide group containing polymers were designed. These masked maleimide group containing polymers was used to fabricate the coating, followed by a thermal treatment of coated surface to unmask the maleimide groups to their thiol-reactive forms. Dopamine unit containing side chains were incorporated as anchoring groups into the parent copolymer to provide a robust surface attachment. For this purpose, a series of copolymer containing pendant PEG chains, furan-protected maleimide groups and dopamine groups were synthesized to modify titanium substrates simulating implant surfaces (Figure 5.1). Titanium surfaces coated with these copolymers were rendered thiol-reactive upon heating through the removal of the furan moiety via the retro Diels-Alder reaction. Lastly, thiol-containing antibacterial peptides were conjugated onto these polymer coated surfaces and adhesion and proliferation of gram-negative and gram-positive bacteria were investigated.

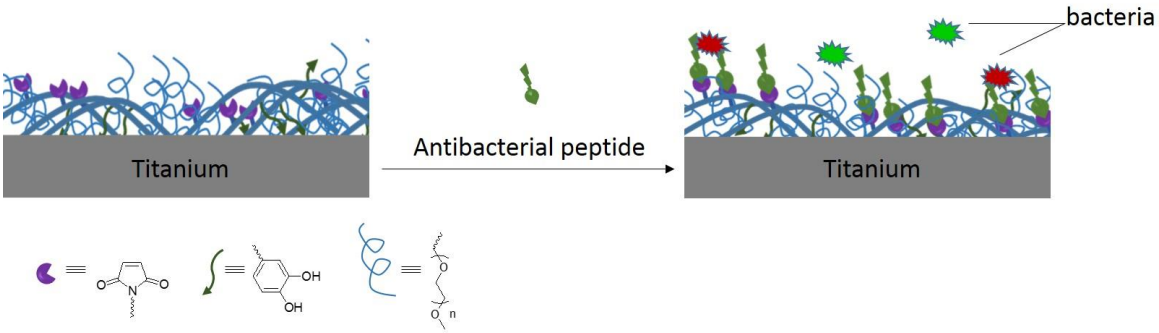



Figure 5.1. Fabrication of antibacterial surface coatings on titanium.

## 5.2. Experimental

### 5.2.1. Materials

PEGMEMA ($M_n$ 500 $gmol^{-1}$) was obtained from Sigma-Aldrich. Dopamine methacrylamide (DMA)[225] and furan protected maleimide monomer (FuMaMA) [189] were synthesized according to literature procedures. Cysteine-containing E6 peptide (RRWRIVVIRVRRC) was synthesized by CanPeptide Corp. (>95% purity by high performance liquid chromatography; Montreal, Quebec, Canada) and used as supplied. Solvents are purchased from Merck and used as received.

### 5.2.2. Methods

HNMR spectra were recorded on Bruker Avance Ultrashield 400 (400 MHz). Molecular weights of the synthesized polymers were estimated by size exclusion chromatography (SEC) using a PSS-SDV (length/ID 8 × 300 mm, 10 mm particle size) linear Mixed C column calibrated with poly(methyl methacrylate) (PMMA) standards using a refractive-index detector with a mobile phase solution of 0.05 M lithium bromide in DMAc as eluent at a flow rate of 1 mL/min at 30 °C. Attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy was collected on a Thermo Scientific Nicolet 380 FT-IR spectrophotometer. For surface analysis, ATR-FTIR was equipped with Harrick Scientific GATR accessory and a Ge crystal. XPS spectra were recorded on the Kratos Analytical Axis Ultra X-ray photoelectron spectrometer (XPS/ESCA) with an Al Kα-monochromatized source of 1486.71 eV. Thickness measurement was performed by collecting the variable-angle spectroscopic ellipsometry (VASE) spectra using an M- 2000 V spectroscopic ellipsometer from J. A. Woollam Co. Inc., Lincoln, NE at 50°, 60°, and 70° at wavelengths from 480 to 700 nm with an M-2000 50Wquartz tungsten halogen light source. For water contact angle analysis an image of the 3.5 uL water droplet was captured with Retiga 1300, Q-imaging Co digital camera and its contact angle with surface was analyzed using Northern Eclipse software. For each sample three different regions were tested.

### 5.2.3. Synthesis of Copolymer P-0

Dopamine methacrylamide (1.14 mmol 252.24 mg), PEGMEMA (4.56 mmol, 2280 mg), AIBN (0.0076 mmol, 1.25 mg) and 4-cyano-4-(phenyl carbonothioylthio)pentanoic acid (0.038 mmol, 10.62 mg) were dissolved in 2.8 mL of DMF in a round bottom flask containing a stir bar and the mixture was purged with $N_2$ gas for 20 minutes. The round bottom flask was immersed into an oil bath at 75 $^oC$ and reaction was stirred for 18 hours. Thereafter, the polymer was precipitated in cold diethyl ether in order to remove unreacted monomers and reagents. $^1H$ NMR (300 MHz, $CDCl_3$ δ (ppm)): 6.80-6.59 (m, 3H, Ph), 4.1 (s, 2H, OCH2 ester protons of PEGMEMA), 3.9 (br s, 2H, $OCH_2$ ester protons of DMA), 3.81–3.54 (m, (4n+2) H, $OCH_2$ of PEGMEMA), 3.40 (s, 3H, $OCH_3$ of PEGMEMA), (3.1-2.4, 2.2-1.5 and 1.12–0.58 (m, $CH_2$ and $CH_3$ along polymer backbone).

### 5.2.4. Synthesis of Copolymer P-10

Dopamine methacrylamide (1.14 mmol 252.24 mg), PEGMEMA (3.99 mmol, 1995 mg), FuMaMA (0.57 mmol, 165.96 mg), AIBN (0.0076 mmol, 1.25 mg) and 4-cyano-4-(phenyl carbonothioylthio)pentanoic acid (0.038 mmol, 10.62 mg) were dissolved in 2.8 mL of DMF in a round bottom flask containing a stir bar and the mixture was purged with $N_2$ gas for 20 minutes. The round bottom flask was immersed into an oil bath at 75 $^oC$ and stirred for 18 hours. Thereafter, the polymer was precipitated in cold diethyl ether in order to remove unreacted monomers and reagents. $^1H$ NMR (300 MHz, $CDCl_3$ δ (ppm)): 6.85-6.65 (m, 2H, Ph), 6.51 (s, 2H, C*H*═C*H* and m, 1H, Ph) 5.21 (s, 2H, C*H* bridgehead protons), 4.05 (s, 2H, OCH2 ester protons of PEGMEMA), 3.9 (br s, 2H, $OCH_2$ ester protons of DMA and FuMaMA), 3.79–3.50 (m, (4n+2) H, $OCH_2$ of PEGMEMA), 3.35 (s, 3H, $OCH_3$ of PEGMEMA), 2.87 (s, 2H, C*H*–C*H*, bridge protons), (2.75-2.12, 2.1-1.5 and 1.12–0.58 (m, $CH_2$ and $CH_3$ along polymer backbone).

### 5.2.5. Synthesis of Copolymer P-30

Dopamine methacrylamide (1.14 mmol 252.24 mg), PEGMEMA (2.85 mmol, 1425 mg), FuMaMA (1.71 mmol, 497.83mg), AIBN (0.0076 mmol, 1.25 mg) and 4-cyano-4-(phenyl carbonothioylthio)pentanoic acid (0.038 mmol, 10.62 mg) were dissolved in 2.8 mL of DMF in a round bottom flask containing a stir bar and the mixture was purged with $N_2$ gas for 20 minutes. The round bottom flask was immersed into an oil bath at 75 $^oC$ and stirred for 18 hours. The polymer was then precipitated in cold diethyl ether to remove unreacted monomers and reagents. $^1H$ NMR (300 MHz, $CDCl_3$ δ (ppm)): 6.85-6.65 (m, 2H, Ph), 6.51 (s, 2H, C*H*═C*H* and m, 1H, Ph) 5.21 (s, 2H, C*H* bridgehead protons), 4.05 (s, 2H, $OCH_2$ ester protons of PEGMEMA), 3.9 (br s, 2H, $OCH_2$ ester protons of DMA and FuMaMA), 3.81–3.45 (m, (4n+2) H, $OCH_2$ of PEGMEMA), 3.35 (s, 3H, $OCH_3$ of PEGMEMA), 2.85 (s, 2H, C*H*–C*H*, bridge protons), (2.93-2.77 and 2.20-1.55 (m, $CH_2$ and $CH_3$ along polymer backbone).

### 5.2.6. Polymeric Coating on Titanium Surfaces

Polymer solution (25 µL, 400 mg/mL in methanol) was spread over each surface (1x1 $cm^2$ titanium). They were left 1 hour at room temperature to let the solvent evaporate. Surfaces were placed into the vacuum oven at 110 $^oC$ for 30 minutes and they were removed after 1.5 hours when the oven's temperature dropped below 60 $^oC$. Lastly, surfaces were washed and sonicated in methanol for 30 minutes to remove non-adhered polymer from surface and then surfaces were dried under stream of nitrogen. Curing the polymer coated substrates at high temperature resulted in strongly attached polymer coating on the surface as well as activation of the maleimide groups via retro Diels-Alder cycloreversion reaction.

### 5.2.7. Bacterial Viability Estimation via Live/Dead Assay

Bacteria were grown in Lysogeny broth (LB; 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl/L) from freezer stocks at 37 °C O/N, and used at approximately 1 × 105 CFU/mL (CFU = colony-forming unit), as determined by OD600 readings using the approximate equation of 0.1OD600 = 108 CFU/mL in 96-well plates. Live/Dead BacLight bacterial viability kit (L-7012; Molecular Probes, Eugene, OR) was used to determine the bacterial cell viability on polymer coated surfaces. Polymer coated titanium substrates and bare titanium substrates were each placed in a 24-well plate. The samples were then sterilized with 1 mL of 70% ethanol by incubating it for 2 minutes and the process was repeated three times. The samples were washed with sterilized water three times. *S. aureus* and *P. aeruginosa* strain was grown in LB broth (4 mL, pH 7.2) at 37 °C overnight to a concentration of around 108 CFU/mL. The bacteria (12 μL) were then diluted by a LB medium (12 mL). A diluted bacteria suspension (1 mL) was then introduced to each well, and the substrates were placed on a shaker at a speed of 120 rpm to provide a homogeneous liquid environment for the interaction and incubated at 37 °C for 4 h. The substrates were then washed with 1 mL of PBS buffer consecutively. A solution of the SYTO 9 (2.4 μL) and propidium iodide (PI; 12 μL) dyes in PBS buffer (12 mL) was prepared. After incubation of the substrate with a dye solution at room temperature in a dark environment for 15 min, the substrates were washed with sterilized water and dried. The samples were then examined using a fluorescence microscope equipped with a fluorescence illumination system (AttoArc 2 HBO) and appropriate filter sets. Images were taken a 20× objective lens. The pictures were taken using fluorescein isothiocyanate (filter 1) and rhodamine (filter 2) filters to visualize the total bacterial and dead bacteria respectively.

## 5.3. Results and Discussion

### 5.3.1. Synthesis of Copolymers

The latent reactive copolymers poly(DOPA-r-PEGMEMA-r-FuMaMA) were synthesized from three different monomers via reversible addition fragmentation chain transfer (RAFT) copolymerization (Figure 5.2). In these polymers, dopamine methacrylamide (DMA) was used as surface reactive part, furan-protected maleimide methacrylate (FuMaMA) as latent reactive part and poly(ethylene glycol)methyl ether methacrylate (PEGMEMA) to provide surfaces with resistance to nonspecific adsorption.

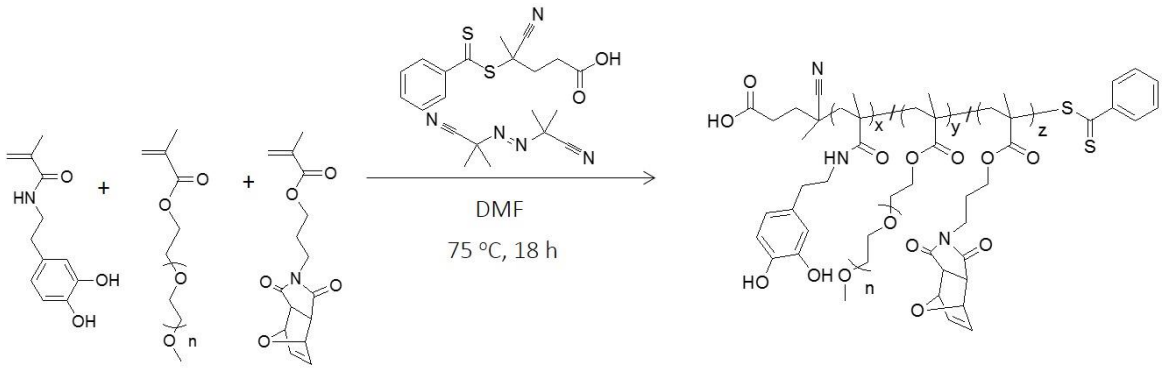



Figure 5.2. Synthesis of copolymer poly(DOPA-r-PEGMEMA-r-FuMaMA) via RAFT polymerization.

Monomer composition in copolymers was determined using $^{1}$H NMR spectroscopy (Figure 5.3). The proton resonance at 3.35 ppm (s, 3H, PEGMEMA), 6.78 ppm and 6.71 ppm (d, 1H, Aryl–Hh and s, 1H, Aryl–Hf in catechol) and 5.22 ppm (s, 2H, CH bridgehead protons in furan-maleimide cycloadduct) were used for calculation. Actual monomers ratio obtained in the copolymers were calculated according to integration values in their 1H NMR spectra (Table 5.1). The molecular weights of the copolymers are between 45-58 kDa with a polydispersity index of nearly 1.3, as measured using size exclusion chromatography (SEC) (Table 5.1).

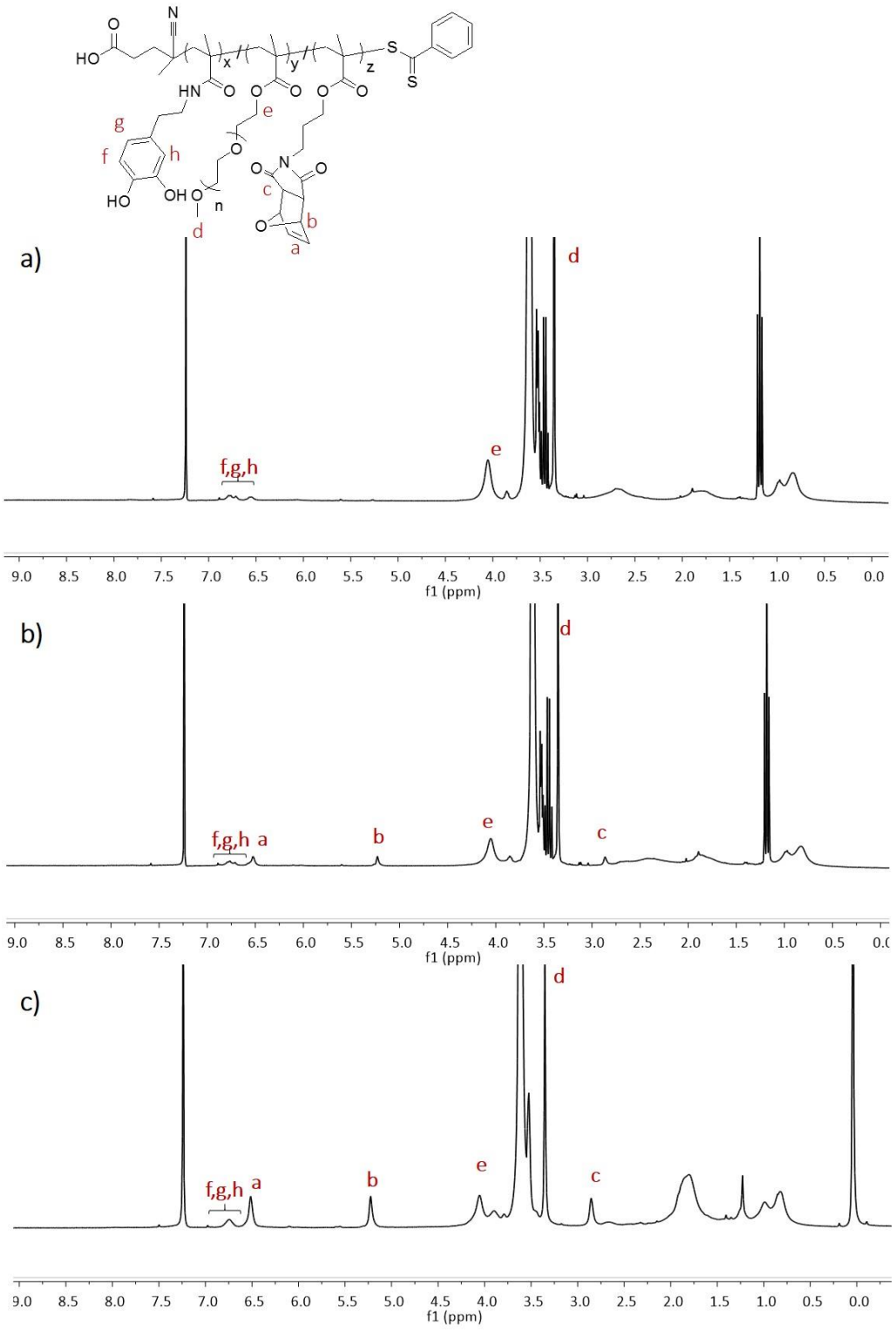



Figure 5.3. 1H NMR spectra of the polymers a) P-0, b) P-10 and c) P-30 (Peaks at 1.18 and 3.46 ppm belong to ether)

Table 5.1. Monomer compositions of synthesized polymers and molecular weight analysis using size exclusion chromatography.

| **Copolymer[a]** | **Theoretical ratio[b] DMA:PEGMEMA: FuMaMA** | **Obtained ratio [c] DMA:PEGMEMA: FuMaMA** | **$M_n$[d]** | **PDI** |
|---|---|---|---|---|
| P-0 | 20:80:0 | 13.85: 86.15:0 | 48 kDa | 1.20 |
| P-10 | 20:70:10 | 15.54: 73.12:11.34 | 48 kDa | 1.36 |
| P-30 | 20:50:30 | 17.24: 57.24: 25.52 | 45 kDa | 1.26 |

[a]$[I]_o/[CTA]_o$: 1/5; $[M]_o$: 2M; Initiator: AIBN. CTA: 4-cyano-4-(phenyl carbonothioylthio)pentanoic acid. Reaction time: 18 h; 75°C; solvent: DMF.
[b]Based on feed ratio DMA:PEGMEMA: FuMaMA
[c]Determined by 1H NMR.
[d]Estimated by SEC eluted with DMAc, using PMMA standards.

Incorporation of the FuMaMA monomer in copolymers was also examined using ATR-FTIR. For P-0, the FTIR spectra displayed C=O stretching band belonging to ester groups of at 1726.8 $cm^{-1}$. For copolymer P-10, similar stretching band was observed at 1726.34, with additional out-of-phase C=O stretching vibration at 1703.68 and in-phase C=O stretching vibration at 1770.59 $cm^{-1}$ corresponding to furan protected maleimide units of copolymer. Similarly, spectrum belonging to copolymer P-30 displayed a C=O stretching band from polymer backbone at 1724.54, and out-of-phase C=O stretching vibration at 1699.17 and in-phase C=O stretching vibration at 1771.54 $cm^{-1}$. As expected, due to higher amount of the masked maleimide monomer, the latter two vibration bands were more intense than those observed for copolymer P-10 (Figure 5.4).

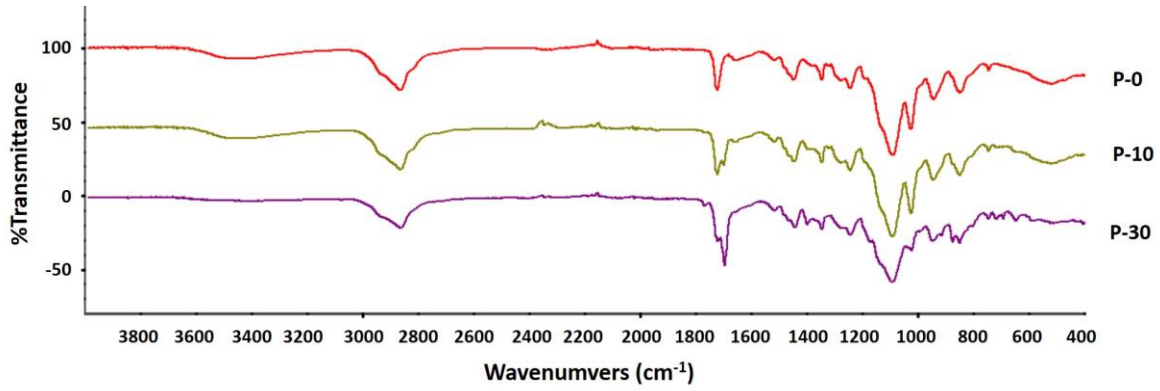



Figure 5.4. ATR-FTIR spectra of the copolymers a) P-0, b) P-10 and c) P-30.

Changes in layer thickness and water contact angle data are shown in Table 5.2. Since obtaining reliable thickness data from ellipsometry was challenging due to instability of oxide layer during the coating process, we coated polymers on both $Si/SiO_2$ and titanium substrates and collected thickness data from $Si/SiO_2$ surfaces. Contact angle values of polymer coated titanium and silicon surfaces were similar. Therefore, we assumed that thicknesses of polymeric coatings on titanium surface was around 4-5 nm as those on silicon surfaces. Water contact angle increased from 37° to 43° with the incorporation and increasing of maleimide units on the surfaces due to the decrease of the ratio of hydrophilic PEG ratio (Table 5.2).

### 5.3.2. Polymer Coating on Titanium Surfaces

First, 25 µL of polymer solution (400 mg/mL in methanol) was spread over the surfaces. They were left for 1 hour at room temperature to allow evaporation of the solvent. Then the surfaces were placed into a vacuum oven at 110 $^{o}$C for 30 minutes and they were removed after 1.5 hours when the oven's temperature dropped below 60 $^{o}$C. Surfaces were washed and sonicated in methanol for 30 minutes and then dried under a gentle stream of nitrogen. Curing the polymer coated substrates at high temperature resulted in fabrication of robust polymer film on the surface as well as activation of the maleimide groups via the retro Diels-Alder cyclo-reversion reaction (Figure 5.5-a). Hereafter, copolymer P-0, P-10, and P-30 coated titanium surfaces are referred as S-0, S-10, and S-30, respectively.

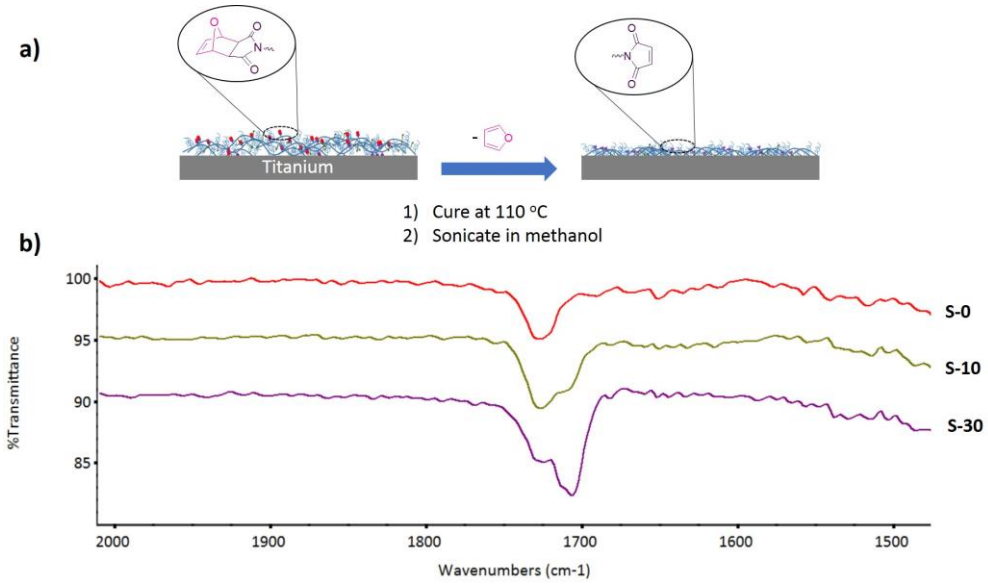
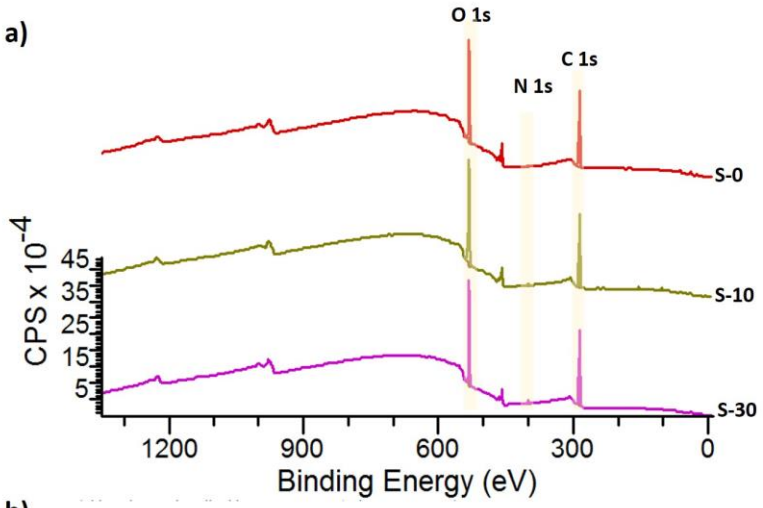



Figure 5.5. a) Coating of polymers onto titanium. b) ATR-FTIR spectra of the polymers coated surfaces a) S-0, b) S-10 and c) S-30.

Deprotection of the maleimide functional group during coating process was confirmed via ATR-FTIR analysis of freshly coated and baked surfaces. Analysis was done on surfaces before washing off excess polymers so that there is appreciable amount of polymers for obtaining proper spectra. After the thermal treatment, the carbonyl stretching bands from the ester units of all side chains were observed at 1727.72, 1727.08 and 1725.44 $cm^{-1}$ for copolymers on titanium surfaces S-0, S-10 and S-30 respectively. Shift of C═O stretching vibration band to 1711.96 from 1703.68 and 1707.14 from 1699.17 for maleimide containing samples S-10 and S-30 confirms that maleimide groups are unmasked after coating process (Figure 5.5-b).

XPS analysis of S-0 surface which is devoid of any maleimide units showed 0.81% nitrogen atom content, which originates from the nitrogen atom in the dopamine units. Incorporation of masked maleimide units on the polymeric surface resulted in an increase of the nitrogen atom content. As expected when higher amount of the FuMaMA monomer was used in the synthesis of copolymers, an increase in N atom content on the polymeric coated surface was observed (Figure 5.6.).




b)

| Polymer samples on Ti | Atomic % concentration | | |
|---|---|---|---|
| | C 1s | O 1s | N 1s |
| S-0 | 65.79 | 33.41 | 0.81 |
| S-10 | 65.25 | 33.83 | 0.92 |
| S-30 | 66.24 | 31.90 | 1.86 |

Figure 5.6. XPS survey spectra and relative concentrations of C, O and N of polymeric coatings.

### 5.3.3. Peptide Immobilization on Polymeric Coated Titanium Surfaces

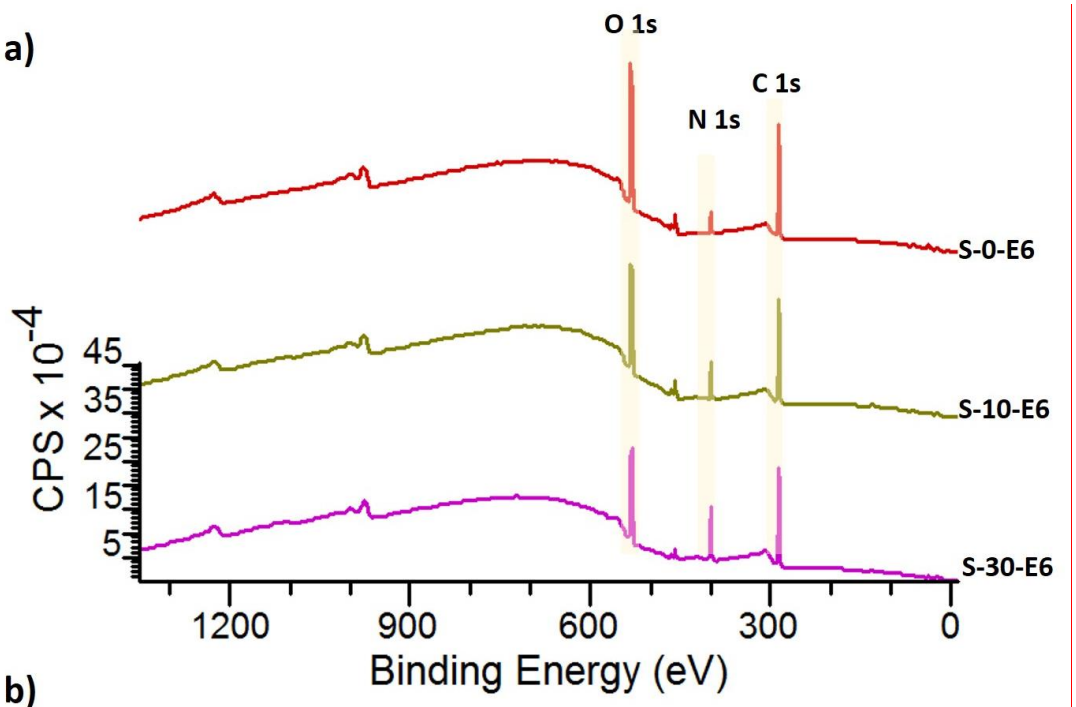
Antibacterial peptides were attached to the surfaces using thiol-maleimide conjugation chemistry. Maleimide functional group containing polymers were incubated in a solution containing the cysteine containing peptide E6 (RRWRIVVIRVRRC) (0.2 mg/mL) for 18 h. Surfaces were thoroughly washed with PBS and water, and sonicated in water for 30 seconds to remove any non-conjugated peptide, before drying them under stream of $N_2$.

Immobilization of thiol bearing antimicrobial peptides to the surface was confirmed with change of water contact angle and thickness. After peptide attachment, thickness of the layer on $SiO_2$ increased. Peptide conjugation caused an increase in water contact angle due to decrease of the hydrophilic PEG units exposed on the polymeric coating.

Table 5.2. Surface characterization of polymeric surfaces and E6 conjugated surfaces.

| Surface | Thickness of polymers[a] (nm) | CA ° (on $Si/SiO_2$) | CA ° (on titanium) | Thickness (polymer- E6)[a] (nm) | CA ° (polymer- E6) |
|---|---|---|---|---|---|
| S-0 | 4.2 ± 0.01 | 36 ± 0.5 | 38 ± 0.1 | 15 ± 0.2 | 44 ± 0.7 |
| S-10 | 5.2 ± 0.08 | 39 ± 0.8 | 40 ± 0.10 | 17 ± 0.2 | 55 ± 1.3 |
| S-30 | 4.5 ± 0.10 | 42 ± 1.2 | 44 ± 0.48 | 19± 0.2 | 63.87 ± 0.1 |

[a]Determined by ellipsometer using polymeric coating on $Si/SiO_2$




b)

| Polymer samples on Ti | Atomic % concentration | | |
|---|---|---|---|
| | C 1s | O 1s | N 1s |
| S-0-E6 | 65.74 | 29.04 | 5.21 |
| S-10-E6 | 64.77 | 26.08 | 9.15 |
| S-30-E6 | 64.21 | 22.81 | 12.98 |

Figure 5.7. XPS survey spectra and relative concentrations of C, O and N of peptide conjugated polymeric coatings.

Additionally, peptide attachment caused an increase in nitrogen content (Figure 5.7) for all surfaces including S-0 which does not possess maleimide. Presence of nitrogen on peptide exposed S-0 surface suggests that conjugation of thiol containing peptides with oxidized catechol moiety takes place to a certain degree. Increasing the maleimide content on the polymeric surface resulted in a higher increase in nitrogen content observed in the XPS spectra.

### 5.3.4. Antibacterial Activity of Peptide Conjugated Polymer Coatings on Titanium Surfaces

The antimicrobial activity of immobilized E6 peptide on surfaces against Gram-positive (*S. aureus*) and Gram-negative (*P. aeruginosa*) bacteria was evaluated by live/dead assay. Firstly, total adhered bacteria and dead bacteria ratios were compared on titanium surfaces using *S. aureus*. A Live/Dead BacLight bacterial assay was used to observe the degree of bacterial adhesion to the surfaces and the viability of adhered bacteria. In this assay, SYTO9 with green fluorescence, is able to enter both live and dead cells was used for counting total adhered bacteria to the surfaces, while red fluorescent PI was used for visualizing dead bacteria since it enters those with a disrupted cytoplasmic membranes. For each surfaces average of three images were taken with a 20x objective lens were used for comparison. On clean titanium surface average 13% of the adhered bacteria were dead (Figure 5.8-a). The copolymer coated surfaces (S-0, S-10 and S-30) attracted less bacteria than bare titanium. We counted on average 10-12 bacteria from the images, where 3-7 of them were dead (Figure 5.8-b, c and d). It was observed that E6 conjugated surfaces attracted much more bacteria than the control samples, however, 78 % of the adhered bacteria were dead (Figure 5.8-f). The surface S-30-E6 attracted more bacteria than S-10-E6, and 83% of them were dead. These results show that peptide conjugated surfaces worked very effectively as antibacterial coating (Figure 5.8-g). Interestingly, even though there is no maleimide group on S-0 surface, it appears that conjugation of peptides to possibly-oxidized catechol units on the polymer takes place and this results in adhesion of bacteria and is able to kill 71% of the adhered bacteria (Figure 5.8-e).

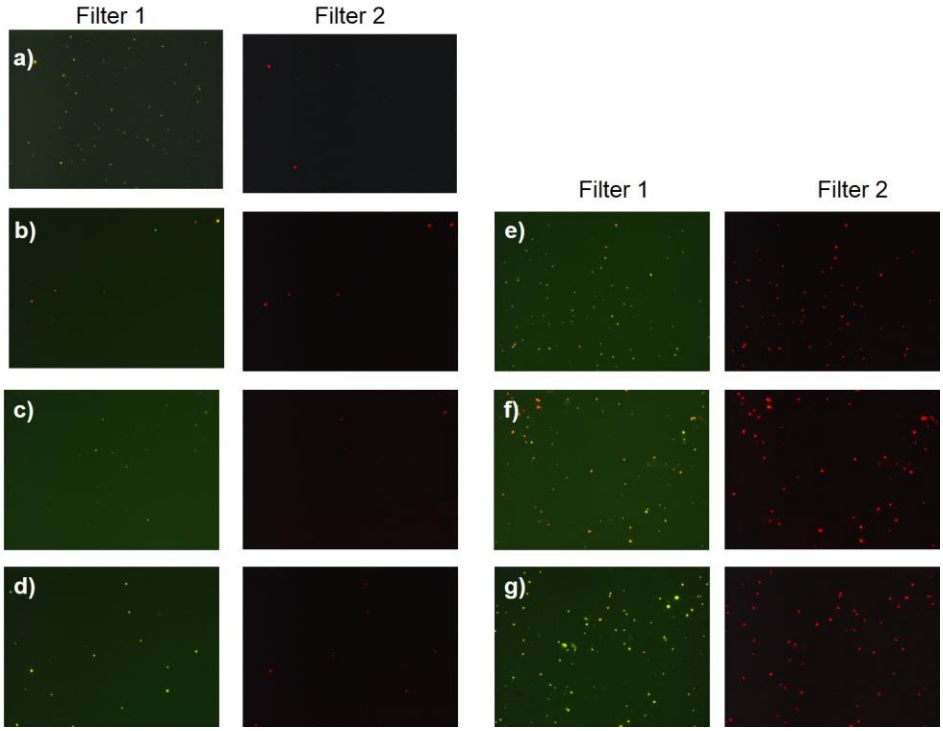



Figure 5.8. Fluorescence microscopy images of (a) bare titanium (b) S-0 (c) S-10 (d) S-30 (e) S-0-E6 (f) S0-10-E6 (g) S-30-E6 films on titanium surfaces by live/dead bacteria staining after a 4 h of incubation with *S. aureus.*

For easier comparison, the numbers of live and dead *S. aureus* on E6 immobilized surface are presented in Figure 5.9. According to this graph, while the polymer coated surfaces displayed quite anti-biofouling characteristics, the peptide conjugated surfaces attracted more bacteria than bare polymer surfaces. An increasing amount of maleimide units on the surface number corresponds to higher adhesion of bacteria and higher killing efficiency.

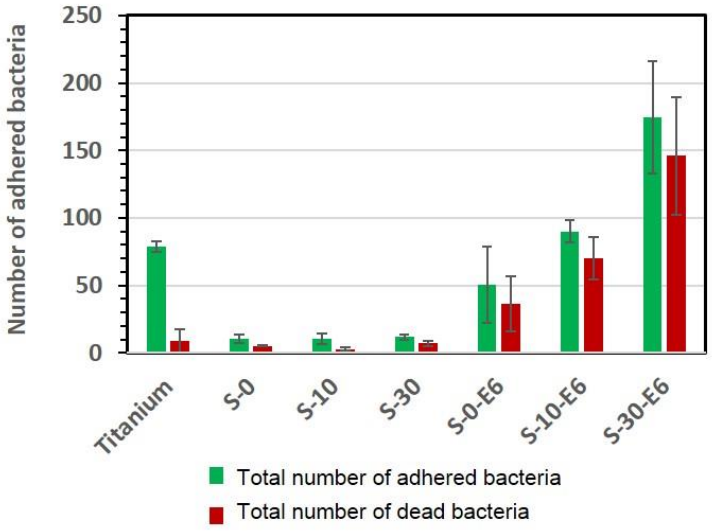



Figure 5.9. Number of total and dead *S. aureus* on bare titanium and polymer coated E6 immobilized surfaces. Values are average of three images taken with a 20x objective.

Antibacterial effect of fabricated surfaces was also studied using gram negative bacteria: *P. aeruginosa.* It was observed that both control and peptide E6 conjugated surfaces attracted more *P. aeruginosa* than *S. aureus.* Only 13% of adhered bacteria were dead onto the bare titanium surface (Figure 5.10-a). After polymer coating, the titanium surfaces did not attract as many bacteria as bare titanium due to the antibiofouling PEG units on the polymers (Figure 5.10-b, c and d). Peptide E6 conjugated S-10 and S-30 killed 77% and 88% of adhered bacteria respectively, while 70% of bacteria were dead on S-0-E6 surface (Figure 5.10-e, f and g). Representative micrographs with total and dead number of *P. aeruginosa* on various surfaces can be seen in Figure 5.11. These surfaces were found be quite effective in killing the adhered bacteria efficiently. Further investigations on these surfaces to evaluate biofilm formation should be carried out to evaluate their long term efficacy as anti-bactericidal coatings.

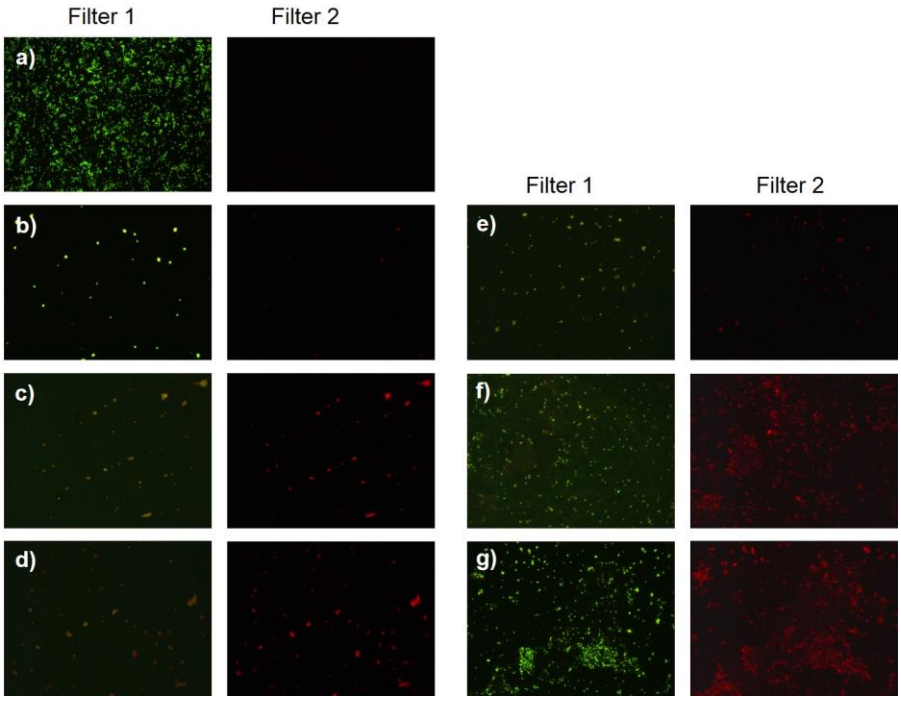



Figure 5.10. Fluorescence microscopy images of (a) bare titanium (b) S-0 (c) S-10 (d) S-30 (e) S-0-E6 (f) S-10-E6 (g) S-30-E6 on titanium surfaces by live/dead bacteria staining after incubation with *P. aeruginosa.*

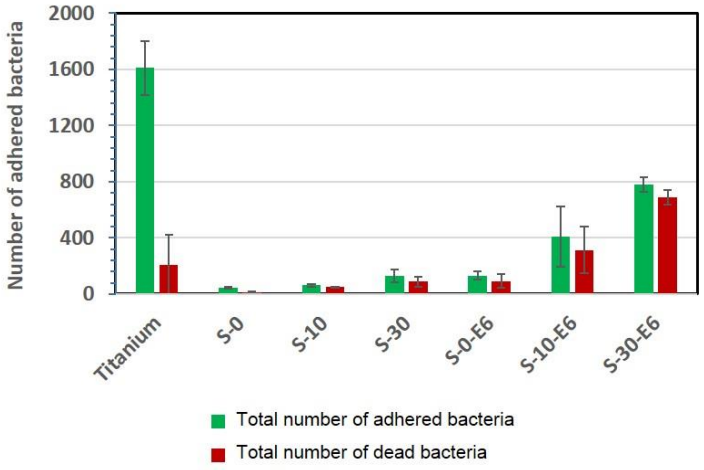



Figure 5.11. Number of total and dead *P. aeruginosa* on bare titanium and polymer coated and E6 immobilized surfaces. Values are average of three images taken with a 20x objective lens.

## 5.4. Conclusions

In conclusion, a maleimide bearing polymeric coating on titanium surfaces was fabricated using copolymers containing a masked maleimide group and surface anchoring units. Polymeric precursors were coated on titanium surfaces and after the fin formation, the maleimide groups were activated to their thiol-reactive forms. Thereafter, antibacterial peptides were conjugated to these polymer films using the thiol-maleimide chemistry. It was observed that all surfaces showed antibacterial properties in varying degree with antifouling characteristics. Coatings fabricated with copolymers containing increased amount of thiol-reactive maleimide group demonstrated increased antibacterial property since higher amounts of peptides were conjugated. In addition, it was observed that peptide E6 containing surfaces attracted more *P. aeruginosa* than *S. aureus*. The surfaces showed good bactericidal properties since on an average 83% and 88% of adhered *S. aureus* and *P. aeruginosa* were killed on the peptide containing surface S-30-E6.

# 6. FABRICATION AND FUNCTIONALIZATION OF THIOL REACTIVE POLYMER BRUSHES

Adapted with permission from {Gevrek, T.N., T. Bilgic, H.-A. Klok, and A. Sanyal, “Maleimide-Functionalized Thiol Reactive Copolymer Brushes: Fabrication and Post- Polymerization Modification”, *Macromolecules*, Vol. 47, pp. 7842-7851, 2014}. 

## 6.1. Introduction

Recent years have witnessed significant advances in the field of thin polymeric coatings due to the crucial role they play in determining the interaction of the underlying materials with their surrounding environment. Over the years, thin polymeric coatings have evolved from playing a role as a merely a protective barrier for bulk materials to designer interfaces that allow to endow the underlying material with desirable functional attributes. Among the various strategies available for the fabrication of polymeric thin films, the utilization of polymer brushes, i.e., polymeric coatings where the polymer chains are tethered to the substrate by one of the chain-ends is rapidly increasing [19]. Polymer brushes have found application in various areas such as biomolecule immobilization, controlled cell adhesion and growth, and non-biofouling and antibacterial surfaces as well as interfaces for detection and sensing [226,227].

Polymer brushes can be prepared by using either the “grafting-to” or the “grafting-from” approach, where the latter provides dense coatings with a high grafting density. To date, a variety of surface-initiated radical polymerization techniques has been used for the preparation of polymer brushes. In particular, controlled radical polymerization techniques such as nitroxide-mediated polymerization (NMP) [228-232] and atom transfer radical polymerization (ATRP),[233-238] as well as reversible addition–fragmentation chain

transfer (RAFT) polymerization,[26,39,239-241] are very attractive methods for the preparation of polymer brushes due to their ability to control the film thickness as well as the chemical composition and architecture of the surface tethered polymers.

Functional polymer brushes often can be prepared via direct surface-initiated polymerization of the appropriate functional monomer. When the desired monomers are only available in small quantities, such as in the case of peptide- or other biofunctional monomers, the direct synthesis of functional polymer brushes can become challenging. Furthermore, while (controlled) radical polymerization reactions generally are tolerant to quite a diverse range of functional groups, there are still monomers with functional groups that can interfere with these polymerization processes. An attractive alternative approach that circumvents the aforementioned concerns is to fabricate polymer brushes with reactive side chain functional groups that can be further modified with the molecule of interest in a subsequent post-polymerization modification step.

Post-polymerization modification of brushes incorporating reactive functional groups in their side chain provides an efficient approach to design functional polymeric brushes. To date, efficient synthesis as well as post-polymerization functionalization of a wide variety of polymer brushes containing various reactive functional groups such as activated esters [242-244], epoxides [245-248], isocyanates [249], azides [250], alkenes [251], and alkynes [98,187,252] has been demonstrated. Activated esters, epoxides and isocyanate-containing polymer brushes are often post-modified with functional amines. Thiol-based conjugation reactions, in contrast, have been much less explored for the post-polymerization modification of polymer brushes. Thiol-mediated post-polymerization modification reactions, however, are very interesting since they allow site-specific conjugation of peptides and proteins, among others. In one example, Patton and co-workers synthesized alkyne bearing polymer brushes that were shown to undergo efficient derivatization using thiol containing molecules using the radical-initiated thiol–ene “click” reaction [253].

Thiol-based conjugation reactions are often performed using thiol–ene and thiol–yne "click" reactions. Thiol-maleimide conjugation chemistry, however, represents an interesting alternative that has been demonstrated to proceed in an efficient manner under reagent free, mild conditions [104,166-168,254-257]. Since the maleimide moiety cannot be incorporated via a direct (controlled) radical polymerization reaction, the use of thiol-maleimide conjugation reactions often involves the post-polymerization modification of another reactive precursor polymer with a heterobifunctional, maleimide containing linker, such as, e.g., succinimidyl trans-4-(maleimidylmethyl)cyclohexane-1-carboxylate [258]. Alternatively, polymers containing maleimide chain end or side chain functional groups can also be obtained by using a furan-protected maleimide based monomer or initiator [189,259]. In this strategy, the maleimide group is masked via a Diels–Alder reaction with a furan moiety prior to the polymerization. The maleimide group can be unmasked by removal of the furan group via the thermally promoted retro Diels–Alder reaction. Whereas this approach has been extensively used for the synthesis of bioconjugates and for the solution postpolymerization modification of synthetic (co)polymers [104,166,168], its use for the postpolymerization modification of surface-grown polymer brushes is unprecedented.

While maleimide containing polymer brushes have been prepared by post-polymerization modification of *N*-(3-aminopropyl)methacrylamide) containing brushes with 3-maleimidopropionic acid *N*-hydroxysuccinimide ester [224], the direct surface-initiated copolymerization of maleimide functionalized monomers is unprecedented. This would, however, be a very powerful additional tool that could further enhance control over the composition of reactive species in the polymer brush and would also offer additional possibilities to engineer the brush architecture. Herein, we report the first example of the direct fabrication of maleimide containing polymer brushes and demonstrate their efficient post-polymerization modification with thiol containing molecules. The strategy presented in this manuscript is outlined in Figure 6.1. In a first step, a furan-protected maleimide based methacrylate monomer is copolymerized using surface-initiated atom transfer radical polymerization (SI-ATRP). A poly(ethylene glycol) based hydrophilic monomer was employed as a comonomer to impart hydrophilic properties to the polymer brushes and render them bioinert, i.e., minimize nonspecific absorption of biomolecules. The

maleimide groups in the resulting polymer brushes were subsequently efficiently unmasked to their reactive form to furnish thiol-reactive polymer brushes that are ready for conjugation with thiol-bearing molecules under reagent free conditions. It is shown that tuning the feed ratio of the monomers during surface-initiated polymerization yields polymer brushes with varying amount of the reactive functional group. To explore the feasibility of the maleimide brushes as a platform for the fabrication of functional polymer coatings, post-polymerization modification with a diverse variety of thiols including cysteamine hydrochloride, cysteine hydrochloride, and glutathione as well as a thiol-containing fluorescent dye (BODIPY-SH) and a thiol-modified biotin derivative was investigated. The latter surfaces were subsequently used for the immobilization of streptavidin-coated quantum dot nanoparticles.

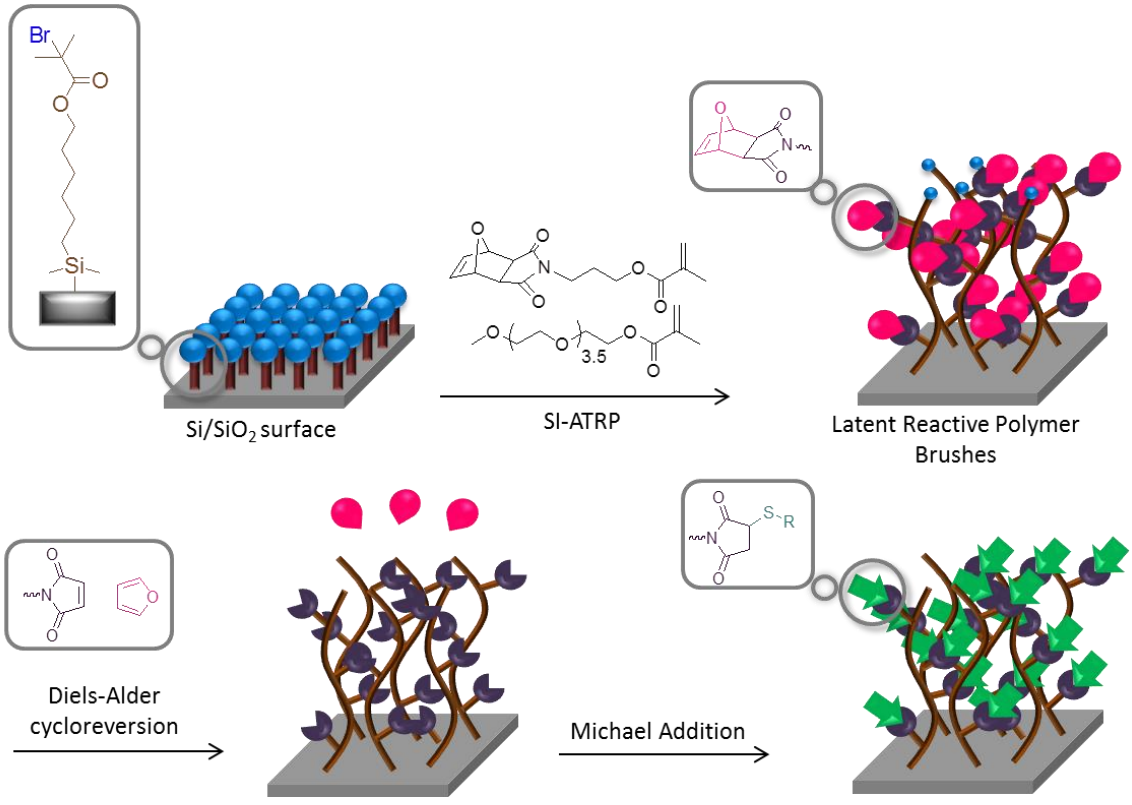



Figure 6.1. Preparation and post-polymerization modification of the maleimide-containing copolymer brushes.

## 6.2. Experimental

### 6.2.1. Materials

All chemicals were used as received unless specified. Poly(ethylene glycol) methyl ether methacrylate (PEGMEMA; Mn = 300 g/mol) and 2,2′-bipyridine were obtained from Sigma-Aldrich. CuCl (99%) was purchased from Acros Organics. The inhibitor in the monomer was removed by passing the monomer through a column of aluminum oxide. Milli-Q water (18.2 MΩ cm) was obtained from a Millipore Direct-Q 5 ultrapure water system. Organic solvents were purchased from Merck and used without purification. Cysteamine hydrochloride, l-cysteine hydrochloride and l-glutathione were purchased from Sigma-Aldrich. Biotinylated hexa(ethylene glycol)undecanethiol ($HS(CH_2)_{11}(OCH_2CH_2)_6NH$Biotin, Biotin-SH) was purchased from Nanoscience Instrument (Phoenix, AZ). Qdot 605 streptavidin conjugate was obtained from Invitrogen molecular probes. Furan-protected maleimide monomer (FuMaMA),[189] (6-(2-bromo-2-methyl)propionyloxy)hexyldimethylchlorosilane, [260] and the thiol-containing fluorescence dye 4,4-difluoro-1,3,5,7-tetramethyl-8-[(10-mercapto)]-4-bora-3a,4a-diaza-s-indacene (BODIPY-SH)[154] were synthesized as previously described.

### 6.2.2. Methods

X-ray photoelectron spectroscopy (XPS) was carried out using an Axis Ultra instrument from Kratos Analytical or a K-Alpha instrument from Thermo Scientific. The X-ray source employed was a monochromatic Al Kα (1486.6 eV) source operating at 100 W and $10^{-9}$ mbar for Kratos Analytical and at 72 W and $10^{-9}$ mbar for K-Alpha instrument from Thermo Scientific. All XPS spectra were calibrated on the aliphatic carbon signal at 285.0 eV. Relative sensitivity factors (RSF) of 0.278 (C1s), 0.78 (O1s), 0.477 (N1s), 0.668 (S2p) were used to correct peak area ratios. Water contact angles were determined using a DataPhysics OCA 35 contact angle measurement instrument. Attenuated total reflectance

Fourier transform infrared spectroscopy (ATR-FTIR) was performed on a nitrogen purged Nicolet 6700 FT-IR spectrometer equipped with a SmartiTR™ (Thermo Fisher Scientific Inc., Waltham, MA, USA) accessory and a diamond crystal. Atomic force microscopy (AFM) was performed in tapping mode on a Veeco Multimode Nanoscope IIIa SPM controller (Digital Instruments, Santa Barbara, CA) using NSC14/no Al MikroMasch (Tallinn, Estonia) cantilevers. In addition, an Ambios-Quesant Q-Scope Universal SPM (Scanning Probe Microscope) was also used for several analyses. Fluorescence microscopy was performed using LD-A-Plan 10x/0.30 objective in Zeiss Axio Observer inverted microscope (ZEISS Fluorescence Microscopy, Carl Zeiss Canada Ltd, Canada). Filter set 38 (Excitation BP 470/40, Emission BP 525/50) was used for imaging of BODIPY-SH functionalized polymer brushes and filter set 43 (Excitation BP 545/25, Emission BP 605/70) for imaging of Qdot ® 605 immobilized polymers brushes. Fluorescence images were processed using Zeiss AxioVision software.

### 6.2.3. Pre-Treatment of Silicon Surfaces, Activation and Grafting of Initiator

Initiator-modified substrates were prepared following a previously reported procedure [261]. Briefly, silicon wafer pieces (0.8 cm × 1.0 cm) were sonicated for 5 min in acetone, 5 min in ethanol, and 5 min in deionized water and dried under a stream of air. Subsequently, the silicon wafers were exposed to a microwave-induced oxygen plasma system (200 W, Diener Electronic GmbH, Germany) for 15 min. Next, the silicon wafers were immersed into a 2 mM solution of the SI-ATRP initiator ((6-(2-bromo-2-methyl)propionyloxy)hexyldimethylchlorosilane) in dry toluene for 16 h at room temperature under an inert atmosphere. After that, the slides were rinsed extensively with dichloromethane (DCM) and methanol. Finally, the initiator-functionalized slides were dried under a flow of nitrogen. Micropatterned initiator-coated substrates were prepared using a protocol previously reported in the literature [262].

### 6.2.4. Surface-Initiated Atom Transfer Radical Copolymerization

PEGMEMA (27.81 mmol, 8 mL), FuMaMA (3.09 mmol, 900 mg) and bipyridine (3.22 mmol, 503 mg) were dissolved in 8.8 mL of a water-methanol mixture (3 : 2 v/v). The solution was degassed by three freeze-pump-thaw cycles. After addition of CuCl (1.14 mmol, 113 mg) degassing was continued for 15 minutes using a stream of $N_2$. Aliquots of this solution (2 mL) were transferred to nitrogen purged reaction vessels containing the initiator-modified silicon surfaces via a nitrogen-flushed syringe. The polymerization was allowed to proceed for a defined period of time at 60 $^{o}$C. Subsequently, the monomer/catalyst solution was removed, and the polymer brush coated slides were rinsed with copious amounts of methanol and water, and finally dried using a gentle stream of nitrogen.

### 6.2.5. Unmasking of Maleimide Functionalized Copolymer Brushes

Furan protected maleimide containing copolymer brushes grafted from silicon wafers were unmasked by heating at 120 $^{o}$C for 90 minutes under vacuum to allow elimination of furan via the retro Diels-Alder reaction.

### 6.2.6. Post-Polymerization Modification with Cysteamine.HCl, Cysteine.HCl and *L*-Glutathione

Maleimide containing copolymer brush coated surfaces (d = 38 ± 5 nm) were dipped in 5 mM aqueous solutions of cysteamine.HCl, cysteine.HCl or glutathione for 16h at ambient temperature. After the reaction, the slides were washed with water and dried under a stream of nitrogen.

### 6.2.7. Post-Polymerization Modification with BODIPY-SH

Maleimide-containing polymer brush coated surfaces (d = 40 nm) were dipped into 1 mg/mL (2.3 mM) BODIPY-SH solution in THF and left overnight. Afterwards, surfaces were washed using THF several times and dried under nitrogen flow. As a control experiment, a polymer brush coated surface was treated with a solution containing BODIPY-Br (a dye molecule devoid of reactive thiol group), incubated and rinsed with THF. All surfaces were analyzed using fluorescence microscopy for assessing dye attachment.

### 6.2.8. Post-Polymerization Modification with Streptavidin-Coated Quantum Dot Nanoparticles

A maleimide containing polymer brush coated silicon substrate (1 cm x 0.8 cm) was treated with 5 µL of a 37.5 mM solution of biotinylated tri(ethylene glycol) undecene thiol (biotin-SH) in methanol for 12 hours. After that, the surface was rinsed with copious amounts of methanol to remove any residual ligands and a solution of Qdot (10 µL, 1 µM) dissolved in 10 µL water was placed onto the biotinylated surface and incubated for 30 minutes. Thereafter, the surface was gently rinsed with water several times to remove any physiosorbed nanoparticles.

## 6.3. Results and Discussion

### 6.3.1. Preparation of Maleimide Containing Copolymer Brushes

The synthetic strategy for the preparation of the maleimide containing copolymer brushes is outlined in Figure 6.2. To enable the installation of thiol-reactive maleimide

functional groups as side chains on polymer brushes, a furan-protected maleimide containing methacrylate monomer (FuMaMA) was utilized. Poly(ethylene glycol) methylether methacrylate (PEGMEMA) was used as a comonomer to impart hydrophilic and non-biofouling properties to the brush matrix. Due to hydrophilic nature of the PEGMEMA monomer, a 3 : 2 water-methanol mixture was used as solvent system. All polymerizations were conducted using a CuCl/bipyridine catalyst system. To allow the determination of film thicknesses via AFM as well as to enable visualization of the post-polymerization modification with fluorescent dyes or quantum dots (*vide infra*), micropatterned copolymer brushes were prepared from photolithographically structured substrates, which were obained according to a previously reported protocol.[262,263]

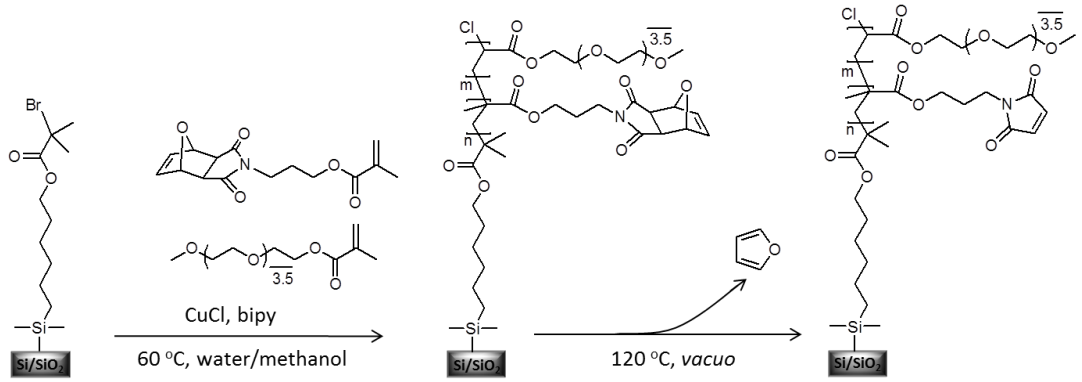



Figure 6.2. Synthesis of maleimide functionalized polymer brushes via SI-ATRP.

A series of P[PEGMEMA$_y$-*co*-FuMaMA$_x$] copolymer brushes (the subscripts y and x refer to the composition of the monomer feed) containing various amounts of the furan-protected maleimide containing monomer were prepared by SI-ATRP (Table 6.1, P1 – P7). Polymer brush thicknesses were determined using AFM by evaluation of step heights of cross-sectional profiles of micropatterned samples. For all the investigated monomer compositions, brush thicknesses were found to increase as a function of polymerization time, as it is expected for a surface-initiated controlled polymerization reaction. (Figure 6.3)

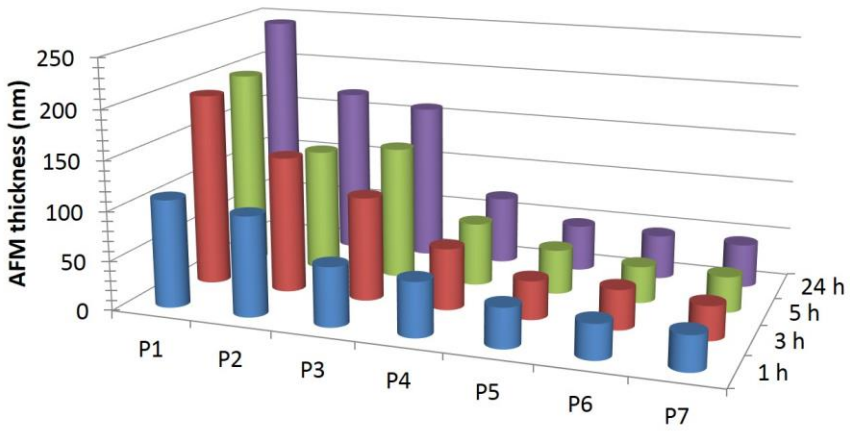



Figure 6.3. AFM thichness of PEGMEMAx-co-FuMaMAy polymer brushes P1 to P6.

The brush thicknesses, however, at any given polymerization time were found to be strongly dependent on the monomer feed composition and decreased from 243 nm after 24 hours for brushes generated from only PEGMEMA to 44 nm for copolymer brushes obtained from a 50/50 mixture of PEGMEMA and FuMaMA.

The composition of the P[PEGMEMA$_y$-*co*-FuMaMA$_x$] copolymer brushes was investigated using XPS. As a typical example, Figure 6.4. presents XPS survey spectra and O1s high resolution scans of a PPEGMEMA homopolymer brush (P1) as well as of a P[PEGMEMA$_{50}$-co-FuMaMA$_{50}$] copolymer brush (P7). The incorporation of the maleimide containing comonomer is evident from the N1s signal at 400.9 eV in the survey scan of the copolymer brush. Further evidence for the successful incorporation of the FuMaMA comonomer comes from the O1s high resolution XPS spectra. The $O_{1s}$ high resolution spectrum of the PPEGMA brush P1 can be fitted with three Gaussian/Lorentzian curves with the expected peak area ratios, and which correspond to the C-O-C (532.9 eV), O-C=O(533.9 eV) and O=C-O (532.0 eV) oxygen atoms. In contrast, fitting the $O_{1s}$ high resolution signal of the P[PEGMEMA$_{50}$-co-FuMaMA$_{50}$] copolymer brush requires one additional component due to the C-O-C (533.1 eV) oxygen atom in the bicylic moiety. The incorporation of the FuMaMA monomer is also evident from the O1s high resolution signal from the increase in relative intensity of the O1s

Table 6.1. Characteristics of the furan-protected maleimide monomer containing polymer brushes.

| **Sample** | **Monomer Feed Composition (mol %)** | | **Surface Composition (mol%)[a]** | | **Water CA(º)** | **Thickness (nm)[b]** |
|---|---|---|---|---|---|---|
| | PEGMEMA | FuMaMA | PEGMEMA | FuMaMA | | |
| **P1** | 100 | 0 | 100 | 0 | 50 | 243 |
| **P2** | 95 | 5 | 98.2 | 1.8 | 55 | 171 |
| **P3** | 90 | 10 | 95.9 | 4.1 | 56 | 170 |
| **P4** | 80 | 20 | 87.5 | 12.5 | 60 | 67 |
| **P5** | 75 | 25 | 80.9 | 19.1 | 63 | 47 |
| **P6** | 60 | 40 | 66.7 | 33.3 | 74 | 45 |
| **P7** | 50 | 50 | 59.6 | 40.4 | 75 | 44 |

[a] Values determined based on XPS [N1s]/[C1s] ratio. [b] After a polymerization time of 24 h. Thicknesses were determined by AFM measurements on micro-patterned samples.

contribution due to the carbonyl oxygen groups (O=C-O / O=C-N-C=O) at 532.1 eV. Table 6.1 indicates the surface chemical composition of the P[PEGMEMA$_y$-*co*-FuMaMA$_x$] brushes expressed as mol% PEGMEMA and FuMaMA as determined from the XPS [N1s]/[C1s] ratios. The results listed in Table 6.1 indicate a fairly good agreement between the composition of the monomer feed and the composition of the copolymer brushes. The copolymer brushes, especially with increasing FuMaMA content in the monomer feed, are slightly enriched with respect to the PEGMEMA monomer.

Additional evidence for the successful incorporation of the FuMaMA comonomer and the possibility to tune the brush composition by varying the monomer feed was

obtained from FTIR spectroscopy as illustrated in Figure 6.5. The FTIR spectra of all the polymer brushes reveal the ester carbonyl (C=O) stretch at ~ 1727 $cm^{-1}$. The FuMaMA

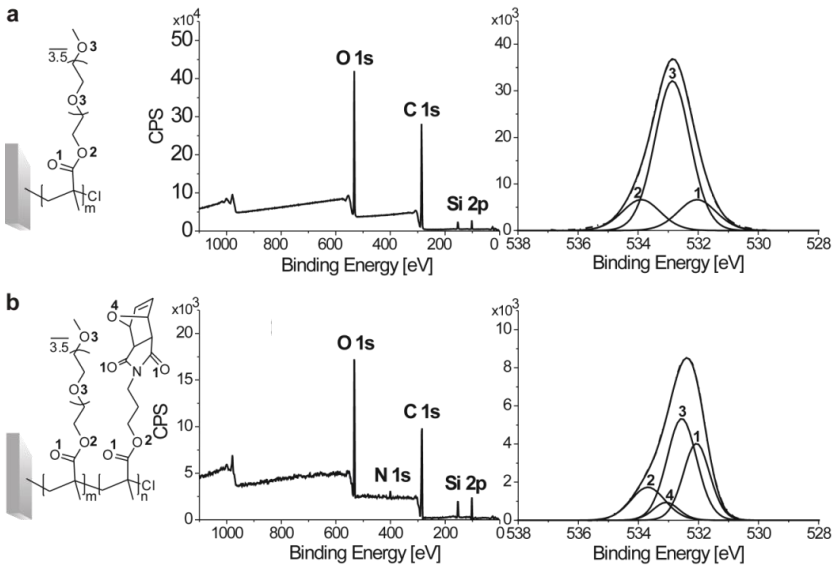



Figure 6.4. XPS survey spectra and high resolution $O_{1s}$ elemental scans of: (a) a PPEGMEMA homopolymer brush (d = 102 nm) and (b) a P[PEGMEMA$_{50}$-*co*-FuMaMA$_{50}$] copolymer brush (d = 36 nm).

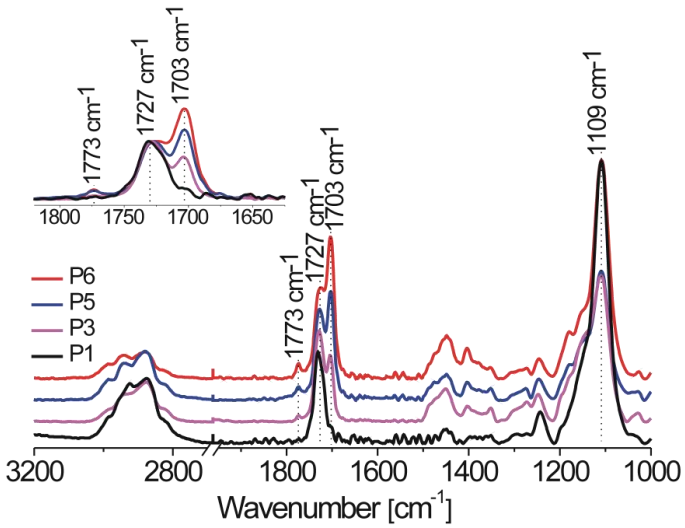



Figure 6.5. ATR-FTIR spectra of polymer brushes P1 (d = 57 nm), P3 (d = 39 nm), P5 (d = 38 nm) and P6 (d = 41 nm). All spectra were normalized with respect to the carbonyl at 1727 $cm^{-1}$. Insert represents details of the 1820 - 1625 $cm^{-1}$ region.

containing brushes in addition show a band at ~ 1703 cm-1, which is due to the maleimide carbonyl groups and the intensity of which increases with increasing FuMaMA content in the monomer feed. Water contact angle analysis of the different copolymer brushes is consistent with the results of XPS and FTIR and indicates a gradual increase in water contact angle with increasing FuMaMA content.

In a final step, the maleimide groups were unmasked by heating the polymer brushes at 120 °C under vacuum for 90 minutes. To investigate the unmasking reaction, a series of P[PEGMEMA$_y$-*co*-FuMaMA$_x$] copolymer brushes was produced, the film thicknesses and compositions of which are summarized in Table 6.2. Polymer brushes with film thickness between 30-40 nm were obtained by varying polymerization times, and brushes with comparable thickness were utilized for the maleimide activation and subsequent functionalization steps. The progress of the retro Diels-Alder reaction was monitored with XPS spectroscopy. As a typical example, Figure 6.6 compares the survey scans as well as O1s and N1 high resolution scans of copolymer brush **P6** before and after the retro Diels-Alder reaction.

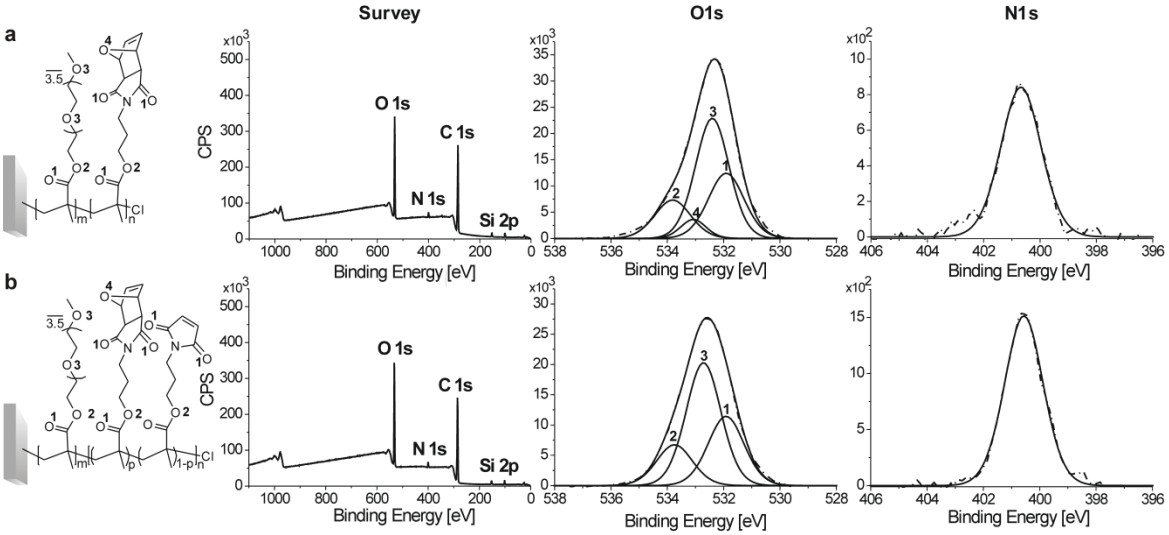



Figure 6.6. XPS survey scans as well as $O_{1s}$ and $N_{1s}$ high resolution spectra of copolymer brush P6 (a) before and (b) after retro Diels-Alder reaction.

From the N and C atomic percentages obtained from the XPS analysis, the surface compositions as well as extent of deprotection reaction were calculated (Table 6.2). The results in Table 6.2 show that heating at 120 °C for 90 min was sufficient for near to quantitative unmasking. The very high conversions of the deprotection reaction are also reflected in the O1s high resolution XPS scans of the unmasked brushes. Fitting the O1s signal of the P[PEGMEMA$_{60}$-co-FuMaMA$_{40}$] brush required four Gaussian/Lorentzian curves, corresponding to the C-O-C (532.4 eV), O-C=O (533.8 eV), carbonyl groups (O=C-O / O=C-N-C=O) (531.9 eV) and *exo*-oxa C-O-C (533.1 eV) oxygen atoms. The O1S signal of the unmasked analogue, in contrast, could be deconvoluted with three residuals representing the oxygen atoms from the C-O-C (532.7 eV), O-C=O (533.7 eV) and carbonyl groups (O=C-O / O=C-N-C=O) (531.9 eV) (Figure 4) and did not require a component at 533.1 eV that would represent the *exo*-oxa oxygen atoms of residual masked maleimide groups.

Table 6.2. Composition of P(PEGMEMA$_y$-co-FuMaMA$_x$) copolymer brushes and the extent of the deprotection of maleimide groups upon retro Diels-Alder reaction.

| **Sample** | **Monomer Feed Composition (mol%)** | | **Surface Composition (mol%)[a]** | | **Thickness (nm)[b,c]** | **Deprotection (%)[a]** |
|---|---|---|---|---|---|---|
| | PEGMEMA | FuMaMA | PEGMEMA | FuMaMA | | |
| **P3** | 90 | 10 | 95.3 | 4.7 | 39 | 94 |
| **P5** | 75 | 25 | 77.7 | 22.3 | 36 | 90 |
| **P6** | 60 | 40 | 65.1 | 34.9 | 41 | 99 |

[a] Values determined based on XPS [N1s]/[C1s] ratio. [b] Thicknesses were determined before the unmasking of the maleimide groups via retro Diels-Alder reaction. [c] Polymerization times for utilized samples: P3(1h), P5 and P6 (24 h).

### 6.3.2. Post-Polymerization Modification of Polymer Brushes

The maleimide containing (co)polymer brushes are a potentially interesting platform for mild, reagent-less post-polymerization modification reactions with thiol-functional molecules (Figure 6.7). In a first series of experiments, the reactivity of the maleimide containing copolymer brushes towards 3 different small molecule thiols, viz. cysteamine, cysteine and glutathione was investigated. These experiments were carried out by treating P(PEGMEMA$_y$-co-MaMA$_x$) copolymer brushes (with y = 90, 75 and 60, i.e. P3, P5 and P6) with a 5 mM aqueous solution of the appropriate thiol at room temperature for 16 h. After washing the derivatized surfaces with copious amounts of water, the samples were dried and analysed using XPS.

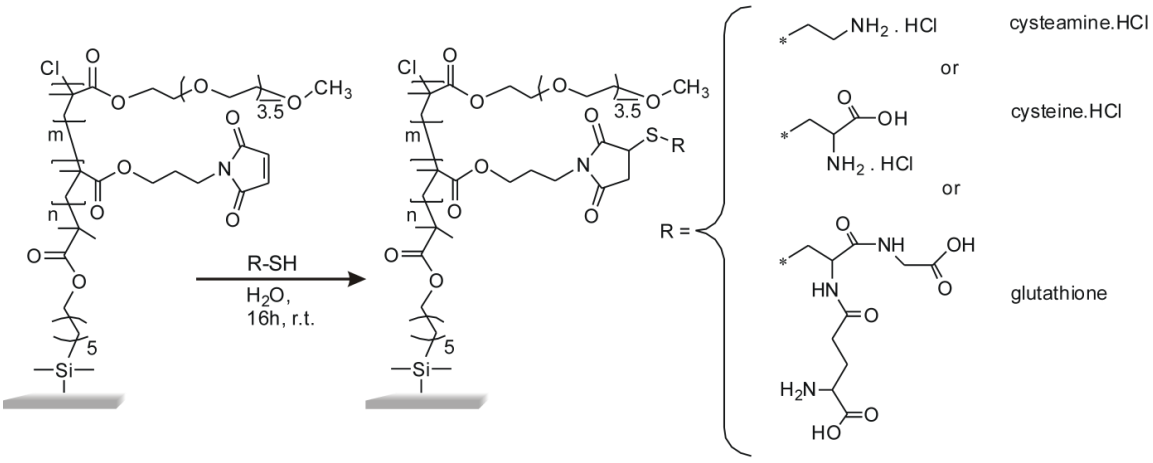



Figure 6.7. Post-polymerization modification of P(PEGMEMA$_y$-co-MaMA$_x$) copolymer brushes with various small molecule thiols.

Figure 6.8 shows survey scans as well as N1s and S2p high resolution XPS spectra of P(PEGMEMA$_y$-co-MaMA$_x$) copolymer brushes P3, P5 and P6 before as well as after post-polymerization modification with cysteamine, cysteine and glutathione. The success of the post-polymerization modification reaction is evident from the increase in intensity of the N1s and S2p signals. The conversions of the maleimide group for each of the post-polymerization modification reactions were estimated from the N and S atomic percentages and are listed in Table 6.3. The post-polymerization modification reactions in

most cases proceeded with reasonable to good conversion. While some variations between the different thiols can be observed, the conversions seemed to most strongly depend on the maleimide content of the polymer brush. When the maleimide content, for example, is decreased from 40 to 25 to 10 %, the maleimide conversion dropped from 63 - 87 % to 23 - 48 %. The effect of the size of the thiol on the post-polymerization modification reaction is particularly evident for the brush sample with the lowest maleimide content, where only 23 % conversion is observed for reactions carried out with the largest thiol (glutathione), as compared to 35 – 48 % for the smaller, less sterically demanding thiols cysteamine and cysteine.

Table 6 3. Maleimide conversion of P(PEGMEMA$_y$-co-MaMA$_x$) copolymer brushes of various composition upon reaction with three, small thiol-containing molecules.

| **Thiol** | **Sample** | **Monomer Feed Composition (mol%)** | | **Thickness (nm)** [a] | **Conversion (%)** [b] |
|---|---|---|---|---|---|
| | | PEGMEMA | FuMaMA | | |
| Cysteamine.HCl | **P3** | 90 | 10 | 39 | 48 |
| | **P5** | 75 | 25 | 43 | 64 |
| | **P6** | 60 | 40 | 41 | 63 |
| Cysteine.HCl | **P3** | 90 | 10 | 39 | 35 |
| | **P5** | 75 | 25 | 38 | 70 |
| | **P6** | 60 | 40 | 35 | 73 |
| Glutathione | **P3** | 90 | 10 | 39 | 23 |
| | **P5** | 75 | 25 | 32 | 87 |
| | **P6** | 60 | 40 | 34 | 63 |

[a] Thicknesses were determined before the unmasking of the maleimide groups via retro Diels-Alder reaction. [b] Values determined based on XPS [S2p]/[N1s] ratio.

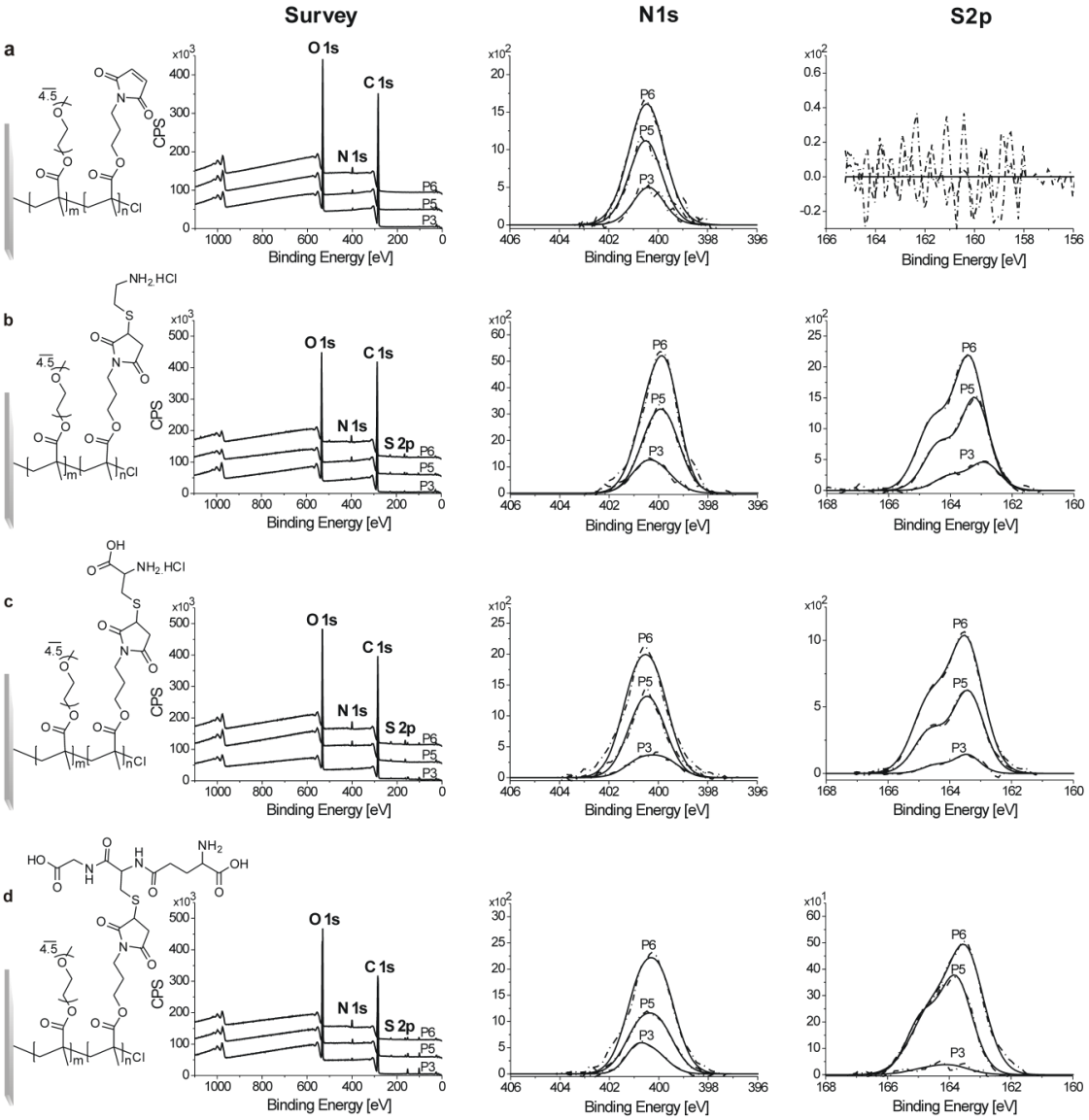



Figure 6.8. XPS survey scans as well as $N_{1s}$ and $S_{2p}$ high resolution spectra of maleimide bearing copolymer brushes P3, P5 and P6 before (a) and after post-polymerization modification with cysteamine.HCl (b), cysteine.HCl (c) and glutathione (d).

### 6.3.3. Functionalization with Fluorescent Dye Molecules and Biomolecules

As a further proof-of-concept experiment, the maleimide containing polymer brushes were used as a platform for the conjugation of a thiol modified fluorescent dye, BODIPY-SH (Figure 6.9). The use of a fluorophore is attractive as it allows, in addition to

XPS, to monitor the post-polymerization modification reaction by fluorescence microscopy. To this end, a 40 nm thick micropatterned P(PEGMEMA$_{90}$-co-MaMA$_{10}$) brush was incubated in a 2.3 mM BODIPY-SH solution in THF for 24 hours. As control experiments, the maleimide functionalized polymer brush was also treated with under the same conditions BODIPY-Br, an analogue that does not contain the SH group, and a protected maleimide containing polymer brush (P(PEGMEMA$_{75}$-co-FuMaMA$_{25}$; P5) was incubated in BODIPY-SH solution in THF. The fluorescence microscopy image in Figure 6b demonstrates the successful attachment of BODIPY-SH. Whereas the brush-covered areas of the micropatterned substrate show the typical green BODIPY fluorescence, the parts of the substrate that are not covered with the maleimide-functionalized brush do not reveal any fluorescence.

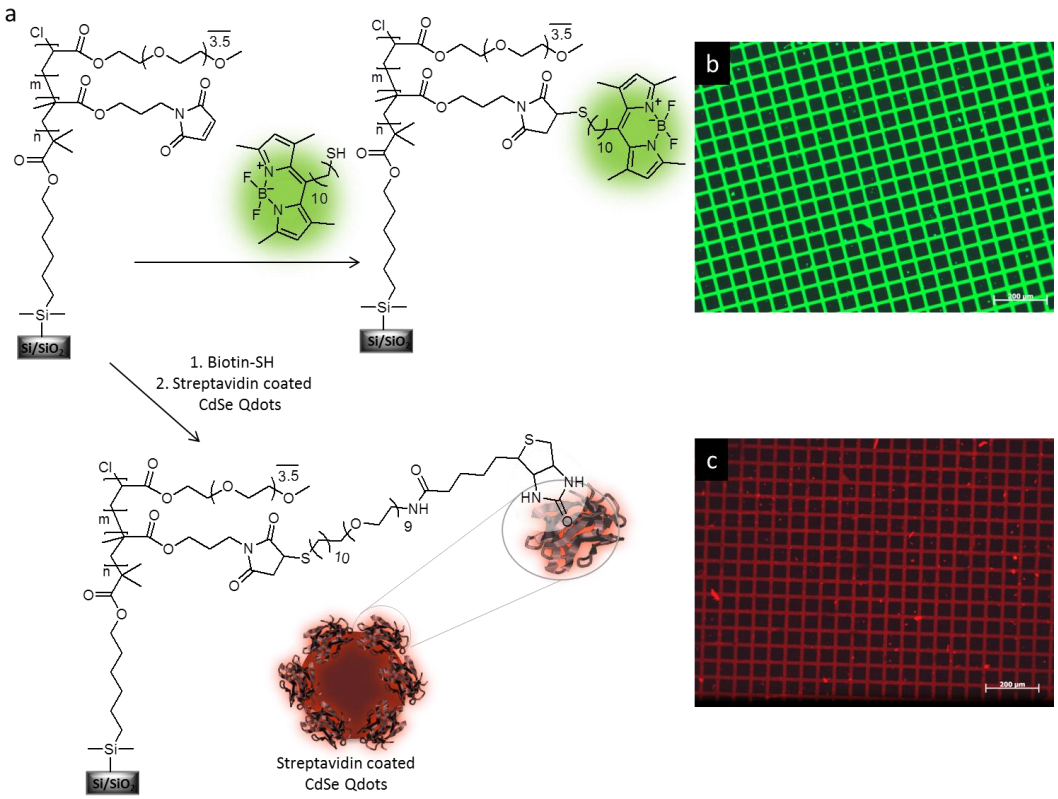



Figure 6. 9. Scheme of post-polymerization modification of copolymer brushes with BODIPY-SH and biotin-SH/streptavidin-coated Qdots (a) and fluorescent microscope image of BODIPY-SH (b) and biotin-SH/streptavidin coated Qdots. Scale bars are 200 µm.

Further evidence for the thiol-specific attachment of the BODIPY-SH probe was obtained from the fluorescence microscopy analysis of the P(PEGMEMA$_y$-co-MaMA$_x$) and P(PEGMEMA$_y$-co-FuMaMA$_x$) substrates that were treated with BODIPY-Br (Figure 6.10) and BODIPY-SH (Figure 6.11), respectively and which do not reveal any BODIPY-related fluorescence.

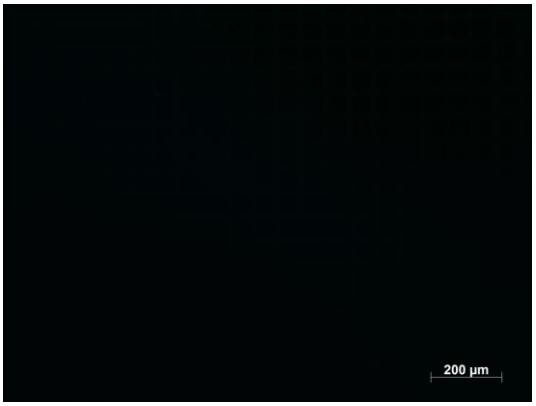



Figure 6.10. Fluorescence microscopy image of BODIPY-Br treated reactivated polymer brush P3.


Figure 6.11. Fluorescence microscopy image of BODIPY-SH treated non reactivated polymer brush P5.

In a final proof-of-concept experiment, the maleimide containing polymer brushes were explored for the biotin-mediated immobilization of streptavidin-coated CdSe quantum dots (Figure 6.9). This experiment first involves modification of a 45 nm thick

micro-patterned P(PEGMEMA$_{90}$-co-MaMA$_{10}$) brush with biotin-SH followed by treatment with a solution containing streptavidin-coated CdSe quantum dots. The fluorescent image in Figure 5c evidences the successful immobilization of the streptavidin-coated CdSe nanoparticles on the biotinylated polymer brushes. The areas of the substrate that are not coated with the maleimide containing brush, and thus not biotinylated, do not reveal any fluorescence upon exposure to the streptavidin-coated quantum dots. The latter is particularly interesting, since it underlines that these brushes combine the properties imparted by the two monomers, i.e. the non-biofouling properties due to the PEGMEMA monomer and the chemoselective reactivity of the maleimide-containing FuMaMA. The combination of these two properties makes these P(PEGMEMA$_y$-co-FuMaMA$_x$) copolymer brushes a potentially very attractive platform for the immobilization of a range of biomolecules.

## 6.4. Conclusions

Polymer brushes containing thiol-reactive maleimide side chains were fabricated using a Diels-Alder/retro Diels-Alder reaction based strategy. Brushes will varying thickness and chemical compositions were synthesized using the surface-initiated ATRP. Efficient post-functionalization of polymeric brsuhes with varying amount of maleimide content enabled functionalization with thiol-containing molecules. Surfaces containing micro-patterned polymer brushes were shown to undergo facile functionalization with a thiol-containing dye molecule BODIPY, as well as thiol containing biotin, a well known ligand for the protein streptavidin. The biotin-streptavidin conjugation was utilized towards immobilization of streptavidin coated quantum dots. It is envisioned that due to the simplicity in fabrication and efficiency in their functionalization under mild conditions, this novel class of polymeric brushes will find applications in various areas in materials and biomedical sciences.

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*The study described in this chapter were performed in collaboration with Prof. Harm-Anton Klok and his PhD student Tugba Bilgic. My contribution in the project is synthesis of FuMaMA and BODIPY-SH, fabrication of maleimide-containing copolymer brushes, contact angle, post-polymerization modification of polymer brushes, fluorescence imaging. During the project, Tugba Bilgic supplied initator coated surface and also performed XPS, AFM and ATR-FTIR analyses of the polymer brushes.

## 6.5. Extending The Approach: Grafting Side Chain Maleimide Containing Polymer Brush From Plastic Substrates

Afterwards, maleimide containing polymer brushes by protection-deprotection strategy was extended by Padeste and coworkers as a collaborative endeavour [192] (Figure 6.12). Thiol reactive polymer brushes were grafted from a polymeric substrate, namely, poly(ethylene-alt-tetrafluoroethylene) (ETFE). After creating radical patterns on ETFE substrates under EUV radiation using interference lithography, grafting of patterned pendant furan protected maleimide bearing copolymers from fluoropolymer foils with, MMA, ethylene glycol methyl ether methacrylate (EGMA), or PEGMEMA were undertaken. Nano-patterned line structures, dot structures and hexagonal structures of copolymers were designed using interfering EUV-beams. After polymerization, surfaces were heated under vacuum to activate the maleimide groups via rDA reaction. Fabricated thiol-reactive films on PTFE was conjugated with a thiol-containing photo-responsive spiropyran (SP) to obtain a light responsive surface. Switcing of transparent polymer brushes into purple was demonstrated via UV irradiation.

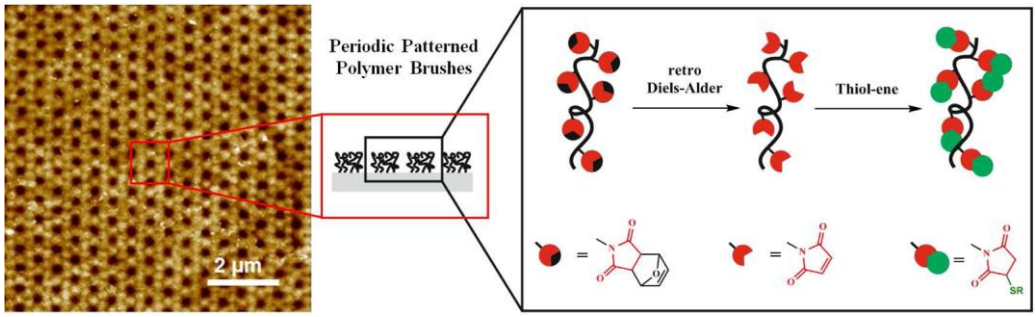



Figure 6.12. Side chain thiol reactive patterned polymer brushes on fluoropolymer film. Adapted with permission from [192].

In a following study, using similar approach they reported stimuli-responsive orthogonally functionalizable polymer brushes using glycidyl methacrylate and a furan-protected maleimide-containing monomer [264]. Polymer brushes were grafted from radical initiator containing fluoropolymer foils. After modification of glycidyl units with enzymatically active microperoxidase-11 and maleimide units with light-responsive spiropyran group, obtained polymer brushes were able to catalyze the oxidation of 3,3′5,5′-tetramethylbenzidine. Exposure to either UV or visible-light allowed switch in enzymatic turnover which provided transition between light-induced spiropyran and merocyanine (Figure 6.13). The modified samples were also integrated into an optofluidic device that allowed the dynamic enzymatic activity of the biofunctionalized microchannel. In order to show enzymatic activity in a modified microchannel of the device under continuous flow, three cycles of alternating exposure of visible (orange) or UV-light to functionalized polymer brush were performed. The outcomes were similar with results under static conditions. These results confirmed the potential application of this platform in smart lab-on-a-chip systems. It is anticipated that such responsive bioconjugated polymer coatings can find applications in smart diagnostic systems in which metabolic events can be investigated in areas of interest.

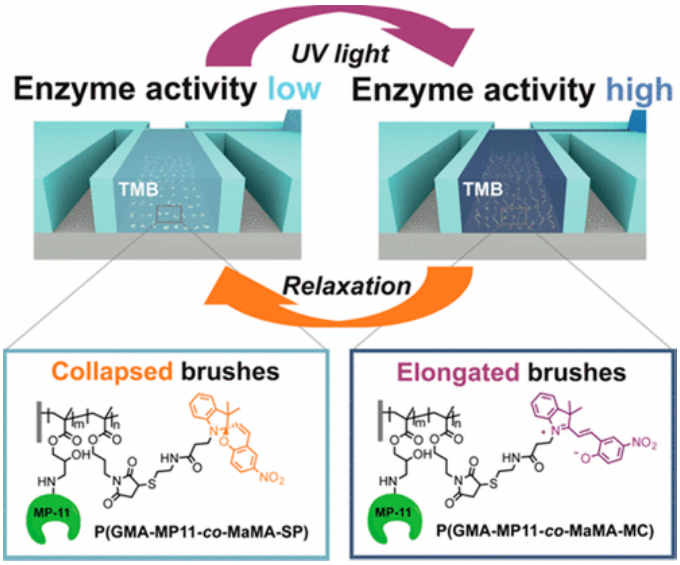



Figure 6.13. Orthogonally functionalized polymer brushes with light-switchable enzymatic activity. Adapted with permission from [264] .

# 7. SUCCINIMIDYL-CARBONATE CONTAINING AMINE-REACTIVE POLYMER BRUSHES

## 7.1. Introduction

Polymer brushes are dense polymer chains that are covalently bound to the surfaces with their one end [265,266]. Since they are strongly attached to the surfaces they are in general more robust then polymer films that are prepared by physisorption techniques such as spray, dip and spin coating methods. Polymer brushes can be obtained by two methods which are ‘grafting to’ and ‘grafting from’. In ‘grafting to’ methods, pre-prepared polymers containing surface reactive groups are coated onto the surface [267]. In the latter technique polymerization reaction starts from the polymerization initiator coated surface [265,26]. End-grafted polymers fabricated by grafting from method has higher grafting density than grafting to method [21]. Recently, polymer brushes have been fabricated by using contemporary polymerization methods like nitroxide-mediated polymerization [268], living anionic or cationic polymerization [269], ATRP [21] and RAFT polymerization [270].

In recent years RAFT has emerged as an attractive polymerization technique due to its simplicity and versatility. Traditional free radical polymerization can be easily converted into RAFT polymerization by addition of an appropriate chain transfer agent [271]. Moreover, this polymerization method does not require metal catalyst and it is compatible with a wide range of monomers and reaction conditions. RAFT polymerization on solid substrates can be prepared by modification of surface by radical initiator or chain transfer agent. Chain transfer agents are immobilized to the surface with Z group or R group strategies. In the Z-group approach, CTA is bound to surface via the stabilizing Z group [39]. In this approach, where the polymer backbone is part of Z group, makes propagating radical close to the solid surface and brushes with narrow PDI is obtained.

However, due to steric hindrance of polymer chains, efficiency of the chain transfer reaction can be limited and grafting density may be low. In the R group approach, R group of the CTA attached to the surface and polymerization takes place near the free ends of polymer brushes so solid support is part of the leaving R group [38]. Therefore, polymer brushes with high molecular weight and higher grafting density can be obtained, however, due to possible chain coupling, polymerization reactions result in chains with broad poly dispersity.

As explained in previous chapters, reactive polymeric surfaces are widely used in the biomedical field, especially in biosensing applications. Immobilization of biomolecules through their amine groups is one of the most commonly used approaches in bioconjugation. Surfaces that contain succinimide and epoxy units are frequently used for immobilization of amine bearing molecules., As an alternative amine reactive group, apart from the succinimide based activated ester group, quite rarely though, succinimidyl carbonates have also been investigated for surface modification studies. For example, Horiike, Yamaguchi and coworkers reported terminal succinimidyl carbonate containing silane based self-assembled monolayers on glass and silicon surfaces for protein immobilization [272]. Although synthetic straightforwardness and molecular order makes SAMS be attractive, polymer brushes have superiority over them in aspects such as the stability. Vaia and coworkers synthesized side chain succinimidyl carbonate polymer brushes via post-polymerization modification of poly(2-hydroxyethyl methacrylate) brushes on silicon substrates [242]. They demonstrated post-modification of pendant succinimidyl carbonate units via amine containing molecules bearing C16, PEG20, PEG50 and $C_8F_{15}$ groups. Using these modified surfaces, they demonstrated the immobilization of citrate coated Au NPs on surfaces. These nanoparticles were attached to PEG containing polymer brushes while $C_8F_{15}$ attached surfaces repelled them. In another example, Homola and coworkers fabricated polymer brushes via SI-ATRP, one made from hydroxylated (HEMA or pHOEGMA) and the other from zwitter-ionic poly(carboxybetaine acrylamide) (CBAA) monomers [273]. They modified CBAA containing polymer brushes with NHS and thus activated hydroxylated polymer brushes with disuccinimidyl carbonate. After immobilization with amine containing proteins, they deactivated free reactive units back to carboxyl and hydroxyl units to increase antibiofouling property. They showed that polymer

brushes have higher surface reactive property than self-assembled monolayer. pCBAA polymer brushes showed antibiofouling property before and after post-modification with NHS and also after post functionalization with proteins.

As summarized above side chain succinimidyl carbonate bearing polymer brushes have been fabricated by post-polymerization modification of side chain OH bearing polymers with *N*,*N*′-disuccinimidyl carbonate, however, the direct surface-initiated copolymerization of succinimidyl carbonate functionalized monomers have not been reported. Recently, Sanyal and coworkers introduced synthesis of succinimidyl carbonate monomer (SCEMA) by the reaction of HEMA with *N*,*N*′-disuccinimidyl carbonate (DSC) in the presence of triethylamine at room temperature [274]. The novel monomer was successfully polymerized with MMA and PEGMEMA using conventional free radical polymerization. To show that this monomer can be incorporated and orthogonally functionalized in the presence of other reactive monomers, they copolymerized SCEMA with an azide-containing monomer AHMA and *N*-hydroxysuccinimide-containing methacrylate monomer (NHSMA). They showed that side chain succinimidyl carbonate groups were easily functionalized with amine containing model compounds to yield various carbamates. In another study, Sanyal and coworkers reported synthesis of PEG based amine reactive 3D hydrophilic crosslinked materials via photopolymerization using previously reported monomer SCEMA [275].

In this part of the thesis, design of a pendant succinimidyl carbonate bearing reactive polymer brushes that are functionalizable with amine containing molecules are presented (Figure 7.1). A succinimidyl carbonate containing monomer was copolymerized with hydrophilic DEGMEMA to minimize non-specific adsorption of biomolecules. Though pendant succinimidyl carbonate bearing polymers were reported by post-polymerization modification, there is no example of surface-initiated polymerization of succinimidyl carbonate containing monomers. Direct polymerization of such a reactive monomer would be simpler and more controlled compared to post-polymerization modification approach. In this study, the density of reactive units on the grafted polymer brushes was shown to be well-tuned by varying the monomer ratios in the feed. First,

successful functionalization of polymer brushes was demonstrated using 4-(trifluoromethyl)benzylamine as a model compound. After conjugation with the ligand biotin-$NH_2$, specific immobilization of the target protein streptavidin was demonstrated. Additionally, taking advantage of R group approach of S-RAFT, terminal dithiocarbonyl units were functionalized after reduction to thiol units, thus opening up a route to orthogonal functionalization of chain end and side chain functional groups.

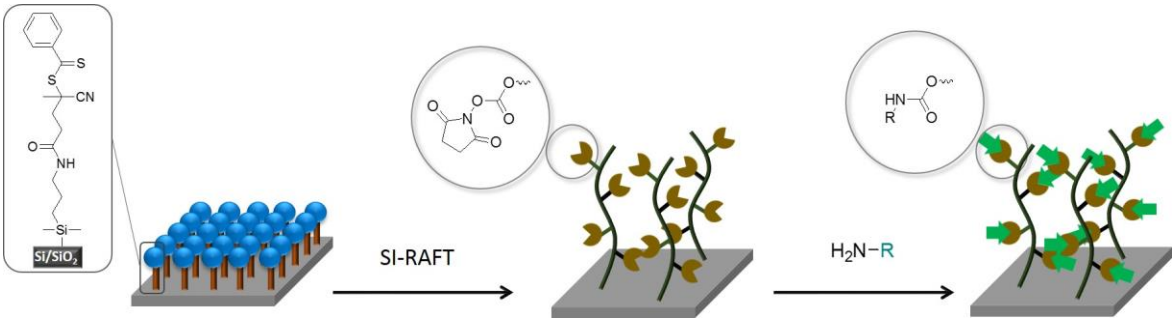



Figure 7.1. Fabrication and functionalization of amine reactive polymer brushes.

## 7.2. Experimental

### 7.2.1. Materials

Di(ethylene glycol) methyl ether methacrylate (DEGMEMA) was obtained from Sigma-Aldrich. The inhibitor in the monomer was removed by passing the monomer through a column of basic aluminum oxide. Amine-$PEO_3$-Biotin (Biotin-$NH_2$) was obtained from EZ-Link. Pierce Streptavidin, (FITC) was purchased from Thermo Scientific. Qdot 605 streptavidin conjugate was obtained from Invitrogen molecular probes. Tetramethylrhodamine-5-maleimide was obtained from Sigma. Surface-reactive chain transfer agent [276] and SCEMA [274] were synthesized following previously described procedures.

### 7.2.2. Methods

Prior to initiator immobilization, substrates were cleaned using a Novascan PSD Series UV/Digital Ozone System for 30 minutes. Attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy was determined on a Thermo Scientific Nicolet 380 FT-IR spectrophotometer equipped with Harrick Scientific GATR accessory and a Ge crystal. A clean silicon wafer was used as a background during measurements of polymer brush coated substrates. Static water contact angles were performed using a KSV's CAM 101. Atomic Force Microscopy was obtained from Nanosurf. X-ray photoelectron spectroscopy (XPS) was performed on the non-patterned regions of a polymer brush by means of a K-Alpha instrument from Thermo Scientific. The X-ray source employed was a monochromatic Al Kα (1486.6 eV) source operated at 100 W and 1 × 10-9 mbar. Fluorescence microscopy was realized using LD-A-Plan 10x/0.30 objective in Zeiss Axio Observer inverted microscope (ZEISS Fluorescence Microscopy, Carl Zeiss Canada Ltd, Canada). Filter set 38 (Excitation BP 470/40, Emission BP 525/50) was used for imaging of Streptavidin functionalized polymer brushes and filter set 43 (Excitation BP 545/25, Emission BP 605/70) for imaging of Qdot ® 605 immobilized polymers brushes. Patterned polymer brushes were prepared by UV irradiation of RAFT-CTAmodified substrates using literature protocols.

### 7.2.3. Surface-initiated Reversible Addition Fragmentation Chain Transfer Polymerization (S-RAFT)

7.2.3.1. Fabrication of polymer brush P1 (100/0) (DEGMEMA homopolymer brush). DEGMEMA (3.65 mmol, 0.64 mL), and AIBN (0.0073 mmol, 1.2 mg), were dissolved in anhydrous DMF (2.0 mL). The solution was degassed by introducing bubbled nitrogen gas for 30 minutes. The S-RAFT-CTA modified $Si/SiO_2$ wafer was placed in a vial and purged with $N_2$. Monomer containing solution was then transferred to this vial via a nitrogen-flushed syringe and the vial was placed in an oil bath at 75 ºC for 5 hours. After this residual monomer containing solution was removed and polymer brush coated surfaces

were thoroughly washed with DMF and THF, with brief sonication and dried using a gentle stream of nitrogen.

7.2.3.2. Fabrication of polymer brush P2 (80/20). DEGMEMA (2.91 mmol, 511 µL), SCEMA (0.73 mmol, 197.45 mg) and AIBN (0.0073 mmol, 1.2 mg), were dissolved in 2 mL of anhydrous DMF. The solution was degassed by introducing bubbled nitrogen gas for 30 minutes. The S-RAFT-CTA modified $Si/SiO_2$ wafer was placed in a vial and purged with $N_2$. Monomer containing solution was then transferred to this vial via a nitrogen-flushed syringe and the vial was placed in an oil bath at 75 ºC for 5 hours. After this, residual monomer containing solution was removed and polymer brush coated surfaces were washed with DMF and THF, with brief sonication and dried using a gentle stream of nitrogen.

7.2.3.3. Fabrication of polymer brush P3 (60/40). DEGMEMA (1.095 mmol, 210 µL), SCEMA (0.73 mmol, 197.45 mg) and AIBN (0.00365 mmol, 0.6 mg), were dissolved in 1 mL of anhydrous DMF. The solution was degassed by introducing bubbled nitrogen gas for 30 minutes. The RAFT-CTA modified $Si/SiO_2$ wafer was placed in a vial and purged with $N_2$. Monomer containing solution was then transferred to this vial via a nitrogen-flushed syringe and the vial was placed in an oil bath at 75 ºC for 5 hours. After this, residual monomer containing solution was removed and polymer brush coated surfaces were washed with DMF and THF, with brief sonication and dried using a gentle stream of nitrogen.

7.2.3.4. Fabrication of polymer brush P4 (40/60). DEGMEMA (0.486 mmol, 91.6 µL), SCEMA (0.73 mmol, 197.45 mg) and AIBN (0.00243 mmol, 0.4 mg), were dissolved in 1 mL of anhydrous DMF. The solution was degassed by introducing bubbled nitrogen gas for 30 minutes. The S-RAFT-CTA modified $Si/SiO_2$ wafer was placed in a vial and purged under $N_2$. Monomer containing solution was then transferred to this vial via a nitrogen-flushed syringe and the vial was placed in an oil bath at 75 ºC for 5 hours. After this, residual monomer containing solution was removed and polymer brush coated surfaces

were washed with DMF and THF, with brief sonication and dried using a gentle stream of nitrogen.

7.2.3.5. Fabrication of polymer brush P5 (0/100) (SCEMA homopolymer brush). SCEMA (0.73 mmol, 197.45 mg) and AIBN (0.00146 mmol, 0.239 mg), were dissolved in 0.4 mL of anhydrous DMF. The solution was degassed by introducing bubbled nitrogen gas for 30 minutes. The S-RAFT-CTA modified $Si/SiO_2$ wafer was placed in a vial and purged under $N_2$. The monomer containing solution was then transferred to this vial via a nitrogen-flushed syringe and the vial was placed in an oil bath at 75 ºC for 5 hours. After this, residual monomer containing solution was removed and polymer brush coated surfaces were washed with DMF and THF, with brief sonication and dried using a gentle stream of nitrogen.

### 7.2.4. Functionalization of Polymer Brush with 4-(Trifluoromethyl)benzylamine

4-(Trifluoromethyl)benzylamine (0.01 mmol, 1.75 mg) and TEA (0.01 mmol 1.01 mg) were dissolved in anhydrous DMF and polymer brush coated surfaces were placed in an orbital shaker at room temperature for 18 hours. Then surfaces were washed with DMF and THF to remove any unreacted materials.

### 7.2.5. Functionalization of Polymer Brush with Biotin-PEG-Amine

Polymer brush coated surfaces were incubated in Biotin-PEG-amine (0.048 mmol, 2 mg) solution in anhydrous DMF in the presence of TEA (0.048 mmol 0.48 mg) in an orbital shaker at room temperature for 18 h. Surfaces were then washed with DMF and THF to remove any unreacted materials.

### 7.2.6. Immobilization of Extravidin and Streptavidin Coated Qdots

0.2 M Qdot 605 streptavidin solution was prepared by diluting 1 M commercial solution with 1x PBS. 20 µL of solution was dropped onto the polymer brush coated surface and left for 30 minutes in a dark place. Substrate was washed with copious amount of 1x PBS and water.

### 7.2.7. Orthogonal Functionalization with Maleimide Containing Dye Molecules

At first, P2 coated surface was reacted with propanol amine by incubating in solution of propanol amine (0.0576 mmol, 4.4 µL) in DMF (1 mL) in the presence of TEA (0.0576 mmol, 8.4 µL). 10 µL of Tetramethylrhodamine-5-maleimide solution (1 mg/mL in MeOH) was dropped onto a clean PDMS stamp and left to dry for 10 minutes in dark. After gently drying the surface of the stamp in a stream of nitrogen, it was pressed onto the polymer brush coated surface and kept in contact for 1 day. As a control, same experiment was conducted using non-modified P2 coated surface. Afterwards, unreacted dyes were washed away form surface by rinsing with MeOH and drying under a stream of $N_2$.

## 7.3. Results and Discussion

### 7.3.1. Preparation of Carbonate Containing Copolymer Brushes

Synthesis of amine reactive polymer brushes was shown in Figure 7.2. In order to synthesize side chain carbonate bearing polymer, di(ethylene glycol)methylether methacrylate (DEGMEMA) and 2-(*N*-succinimidylcarboxyoxy)ethyl methacrylate (SCEMA) were used. SCEMA was used as amine reactive monomer while DEGMEMA was used as a low biofouling comonomer to minimize nonspecific adsorption. Polymer

brushes were synthesized using surface initiated RAFT polymerization at 75 $^{o}$C in DMF in the presence of AIBN (Figure 7.2).

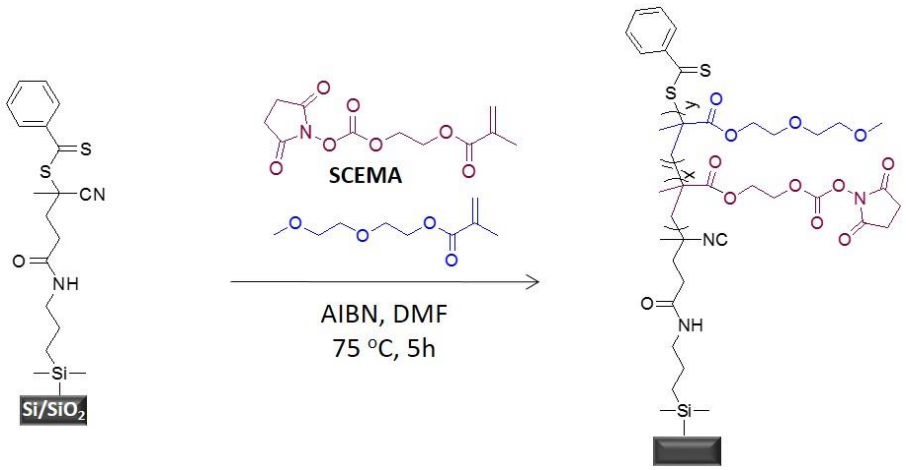



Figure 7.2. Synthesis of SCEMA functionalized polymer brushes via S-RAFT and its functionalization with amine containing molecules.

Before grafting polymer from the surface RAFT chain transfer agent modified silicon substrates, they were patterned using UV irradiation. Due to photolytic cleavage of C-S bond of the dithioester unis of the CTA, polymers cannot be grafted at regions that are exposed to high intensity UV light. A series of P[DEGMEMA$_y$-*co*-SCEMA$_x$] copolymer brushes (the subscripts y and x refer to the composition of the monomer feed) containing various amounts of SCEMA were prepared by surface initiated RAFT polymerization (Figure 7.3, P1 – P5). Polymer brush thicknesses were determined using AFM as in between 35-46 nm by evaluation of step heights of cross-sectional profiles of micropatterned samples. Incorporation of SCEMA monomer was determined using ATR-FTIR spectroscopy. ATR-FTIR spectra of P1 which is a homopolymer brush of DEGMEMA showed a carbonyl (C=O) stretch at 1728 cm$^{-1}$. Newly formed carbonyl (C=O) stretches around 1790 and 1814 cm$^{-1}$ belonging to the succinimidyl carbonate groups were observed in the ATR-FTIR spectra of copolymers P2-P5 (Figure 7.3).

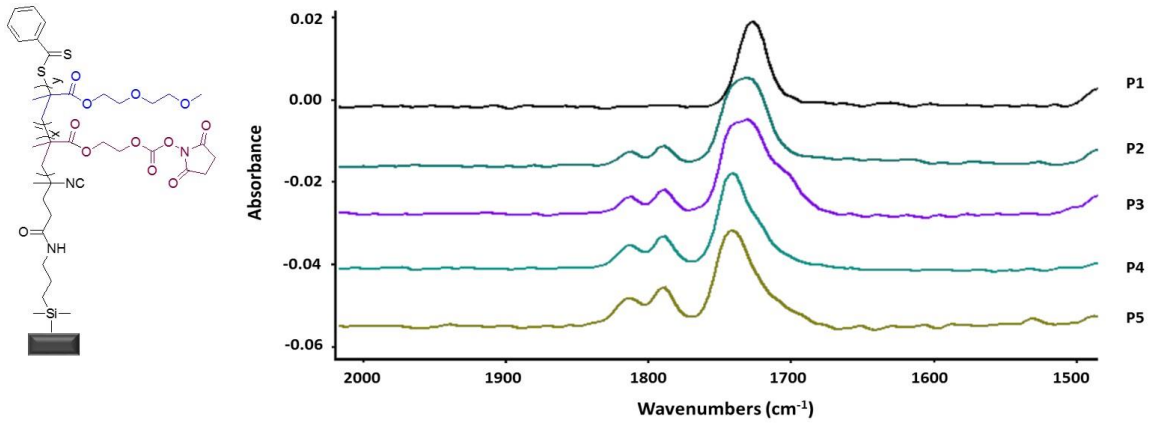
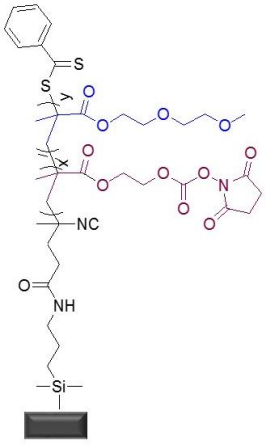
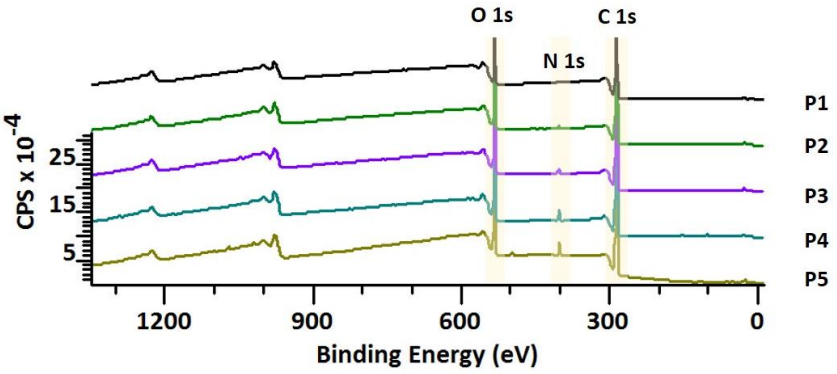



Figure 7.3. ATR-FTIR spectra of polymer brushes P1-P5.

The composition of the P[DEGMEMAy-co-SCEMAx] copolymer brushes was investigated using XPS. Figure 7.4 depicts C1s high resolution scans of a PDEGMEMA homopolymer brush (P1), P[DEGMEMAy-co-SCEMAx] (P2, P3 and P4) as well as of a PSCEMA copolymer brush (P5). The incorporation of SCEMA into the polymer brushes is evident from the nitrogen atom signal around 400 eV in the survey scan of the copolymer brushes. Increasing SCEMA ratio in the polymer composition, an increment of N ratio was observed; while, as expected, no peak corresponding to N atom was observed in the survey and high resolution spectra of PDEGMEMA coated surface (Figure 7.4). Extent of incorporation of monomers in brushes was calculated from XPS. Thicknesses of polymer films were determined by AFM, and it was observed that incorporation of SCEMA resulted in thicker polymer brushes. Additionally, polymer brushes showed an increase in water contact angle values with increasing amount of the hydrophobic SCEMA monomer (Figure 7.4).






| SAMPLE | DEGMEMA/ SCEMA (y/x) (Feed Ratio) | DEGMEMA/ SCEMA (y/x) (Calculated from XPS) | Thickness | Contact Angle |
|---|---|---|---|---|
| P1 | 100/0 | 100/0 | 34.7 ± 0.5 | 58 ± 3 |
| P2 | 80/20 | 81.7/18.3 | 38.8 ± 1.9 | 64 ± 1 |
| P3 | 60/40 | 62.1/37.9 | 42.3 ± 2.6 | 65 ± 1 |
| P4 | 40/60 | 41.2/58.8 | 45.6 ± 1.0 | 69 ± 3 |
| P5 | 0/100 | 0/100 | N.A. | 81 ± 1 |

Figure 7.4. Survey spectra of polymer brush P1-P5 and calculated monomer incorporation from XPS based on N/C ratio.

For representative brushes, C 1s high resolution spectra and their deconvolution results are presented in Figure 7.5. Spectra belonging to the DEGMEMA homopolymer brush (P0) could be deconvoluted into 3 Gaussians at 285, 286.5 and 288.9 eV for C-C and C-H, C-O and O-C=O together with HN-C=O and S=C-S respectively. Incorporation of SCEMA resulted in a new peak at 290.9 eV due to O-(C=O)-O. Intensity of the new peak increases with increasing ratio of reactive monomer in the polymerization mixture, while a significant decrease of peak belonging to C-O is observed.

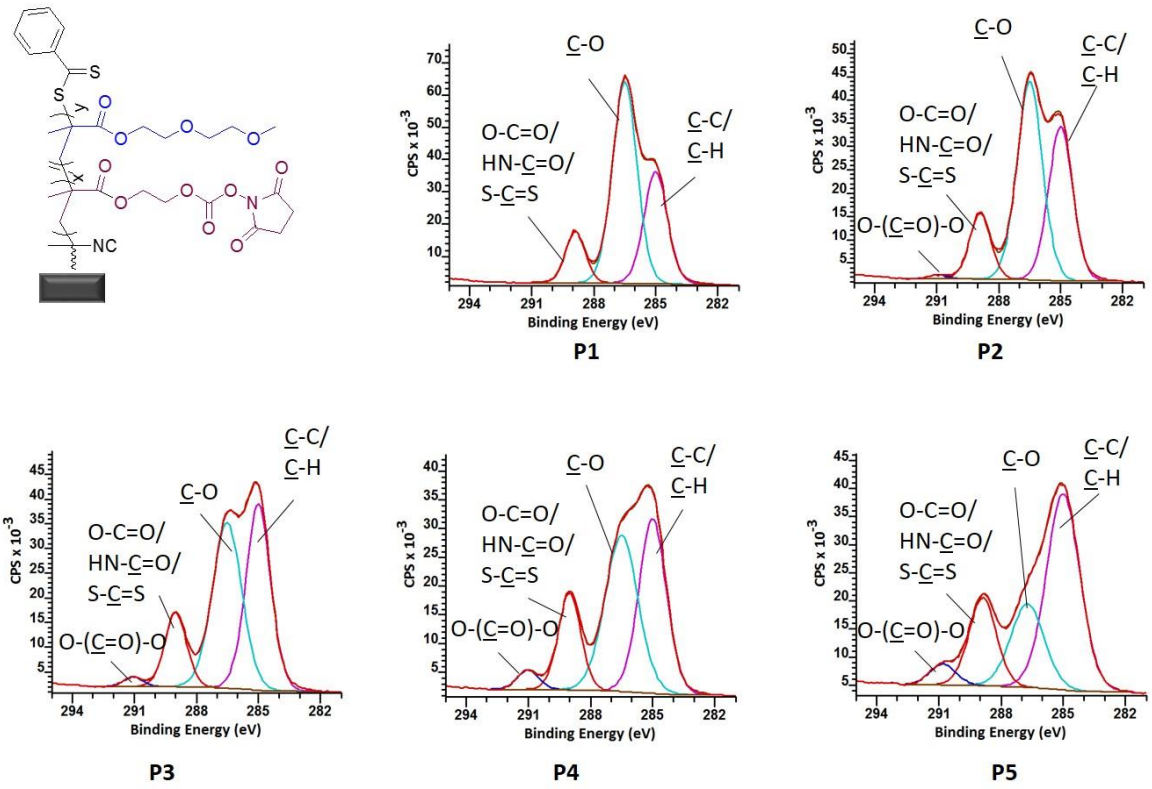



Figure 7.5. High resolution XPS elemental scans of C1s peaks of P1-P5.

Polymer coated surface P1 did not show any N1s peak, thus suggesting that the N atoms of RAFT initiator is buried under DEGMEMA chains. Copolymer coated surfaces P2, P3, P4 and P5 showed N 1s peak at 402 eV which belongs to succinimidyl groups (Figure 7.6). The additional peak at 400 eV can stem from some decomposed products derived from the succinimidyl carbonate group during XPS analysis as Yamaguchi and coworkers observed in succinimidyl carbonate containing monolayer on silicon surface.[273] With increasing ratio of SCEMA monomer in the polymer, composition intensity of N 1s peak increases. The additional peak at 400 eV was very weak in the spectrum for the original compound, perhaps because thick layer of SCEMA monomer is less sensitive to decomposition by X-ray irradiation than polymer brushes (Figure 7.6).

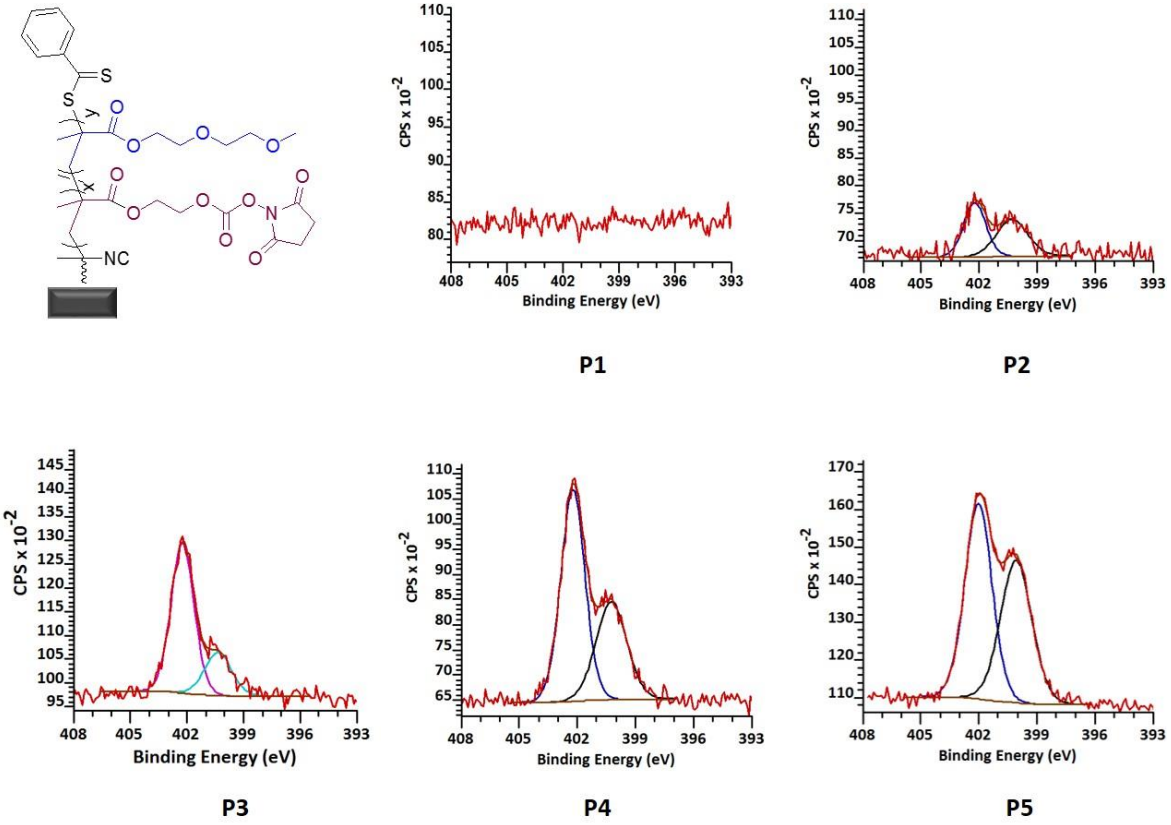



Figure 7.6. High resolution XPS elemental scans of N1s peaks of P1-P5.

In order to confirm that SCEMA monomer does not decompose during polymerization, we compared FTIR and XPS spectra of pure SCEMA monomer and SCEMA homopolymer brushes (Figure 7.7). Other than the C=C unpolymerized methacrylate stretching vibration of double bond of methacrylate units, all stretches were observed at same wavenumbers for both monomer and homopolymer. In the XPS N1s spectra of SCEMA monomer, an additional peak at 400 eV was present, similar to SCEMA homopolymer and other copolymer brushes containing SCEMA. These results show that the additional peak at 400 eV stems from decomposition of succinimidyl carbonate moieties during XPS analysis, as previously reported [272].

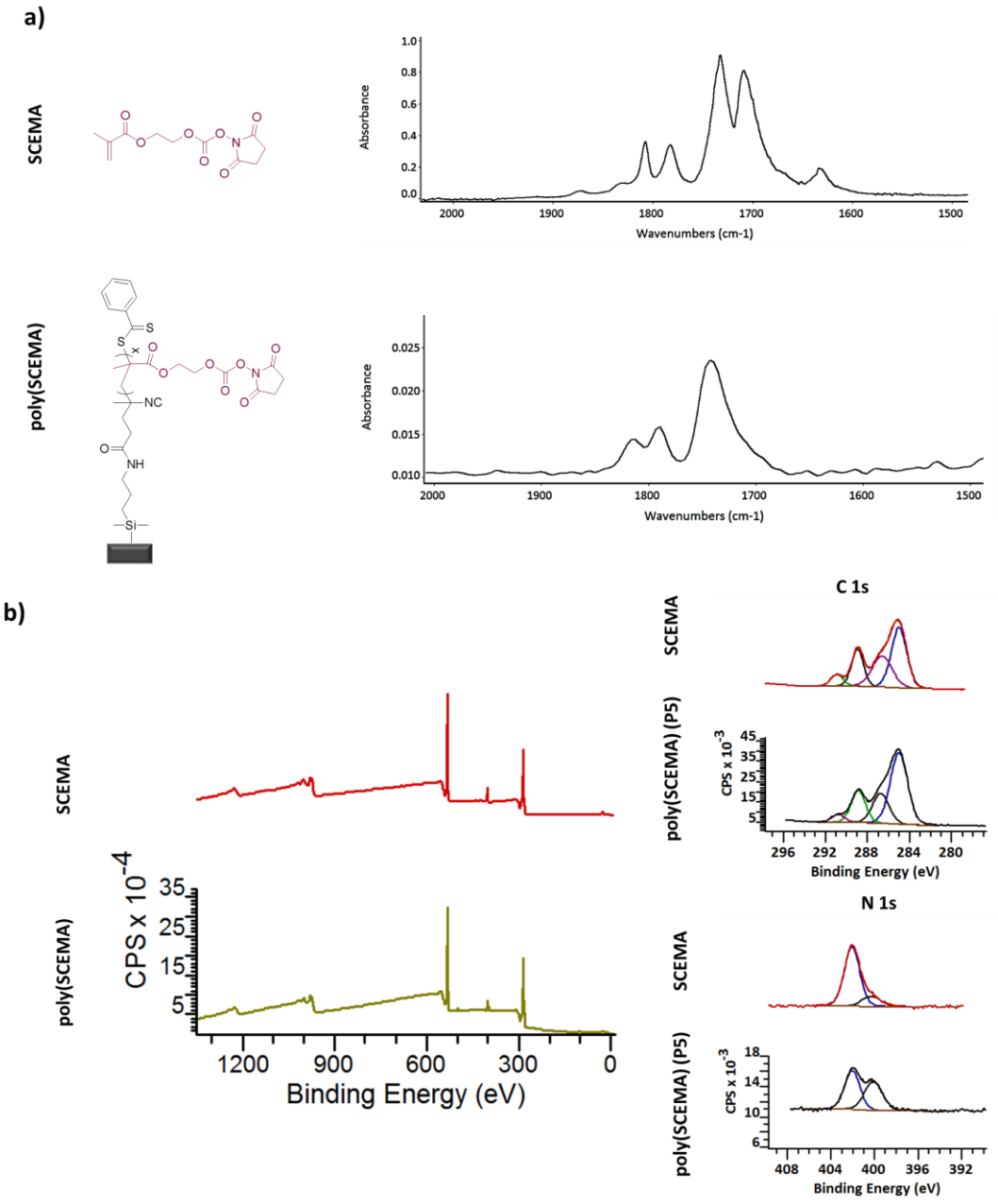



Figure 7.7. Comparasion of ATR-FTIR (a) and XPS spectra (b) of SCEMA and PSCEMA (P5).

### 7.3.2. Post-Polymerization Functionalization with 4-(Trifluoromethyl)Benzylamine

Reactive polymeric brushes bearing pendant succinimidyl carbonate moieties were functionalized with 4-(trifluoromethyl)benzylamine (TFBA). Conjugated polymer brushes were first analyzed with ATR-FTIR. The C=O stretching vibrations at 1814 and 1790 $cm^{-1}$ belonging to succinimidyl carbonate groups disappeared, while peaks at 1327 and 1067 $cm^{-1}$ were observed. When spectra were normalized to ester carbonyl peak, an increase in intensity of the new peaks with increasing content of reactive group in the polymer brushes was evident (Figure 7.8).

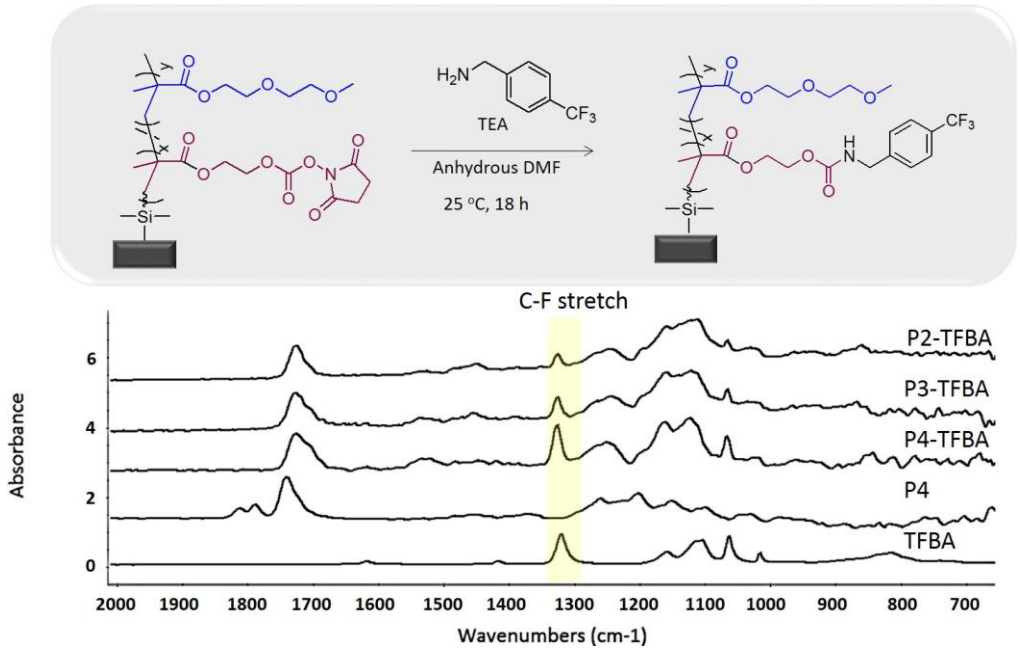



Figure 7.8. a) Scheme of 4-(trifluoromethyl)benzylamine (TFBA) attachment to P2, P3 and P4. b) ATR-FTIR spectra of TFBA immobilized polymer brushes P2, P3 P4 as well as bare P4 and TBFA for comperasion.

The compound TFBA was chosen to analyze the efficiency of functionalization using XPS since it has F atoms which does not exist in the polymer brushes. As expected, XPS analysis of the TFBA conjugated polymer brushes exhibit an increase in the F atom

content in brushes with increasing succinimidyl carbonate content (Figure 7.7). Significant decrease of O-(C=O)-O peak at 290.8 eV while apperance of $CF_3$ was also observed as an additional evidence of conjugation (Figure 7.9-e). The analysis indicates that post-modification reactions proceeded with reasonable conversions of 60.7% for P1, 70.7% for P2 and 83.5 % for P3 as calculated from the [N 1s]/[F 1s] ratio.

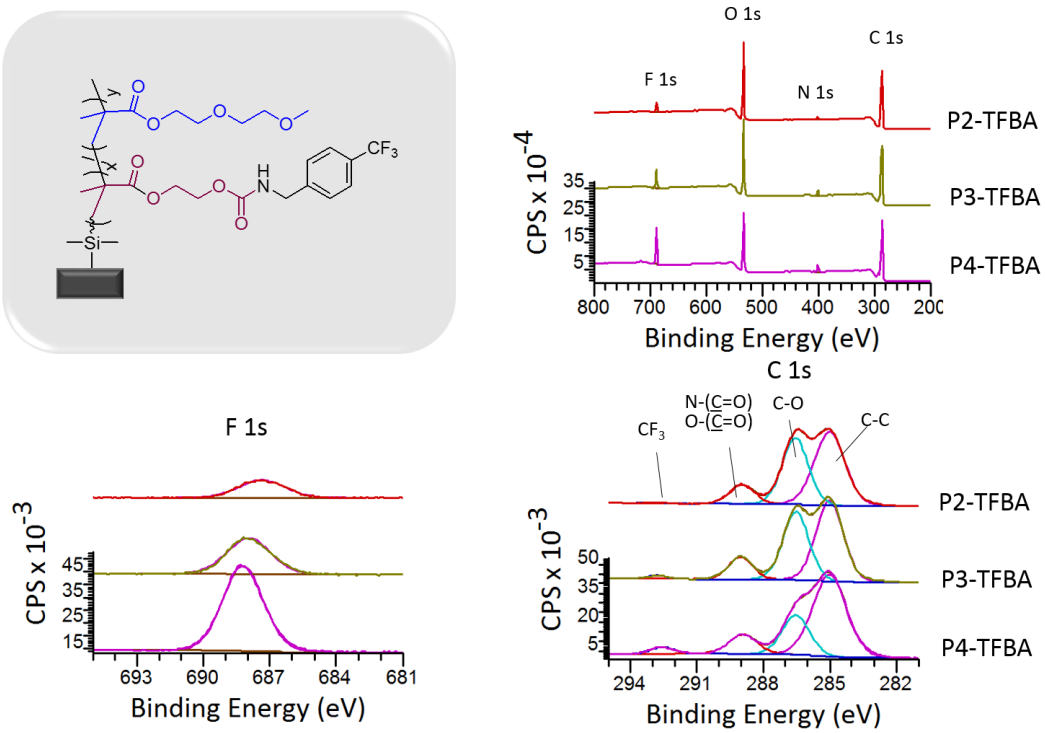



Figure 7.9. a) 4-(Trifluoromethyl)benzylamine (TFBA) attached polymer brush. b) XPS survey spectra of TFBA conjugated polymer brushes P2-P4. c) N 1s high resolution specta. d) C 1s high resolution spectra.

### 7.3.3. Functionalization of Surfaces with Biotin Ligand and Protein Sensing

These reactive polymer brushes were also investigated for ligand-mediated biomolecular sensing. Biotin-streptavidin couple was chosen as a model system due to its known high binding affinity. Biotinylation of these polymer brushes was accomplished through treatment with an amine-containing biotin ligand in anhydrous DMF at room

temperature. After 18 hours, surfaces were washed with copious amounts of DMF and THF, then dried under a gentle stream of $N_2$. Subsequently, biotinylated P3 surface was incubated in streptavidin containing solution in PBS. Figure 7.10 clearly suggests that immobilization of streptavidin took place due to the conjugated biotin ligand since non-biotinylated surface did not show a significant fluorescence intensity. The lack of appreciable fluoresence in the later case also indicates good level of anti-biofouling characteristics of these polymeric surfaces.

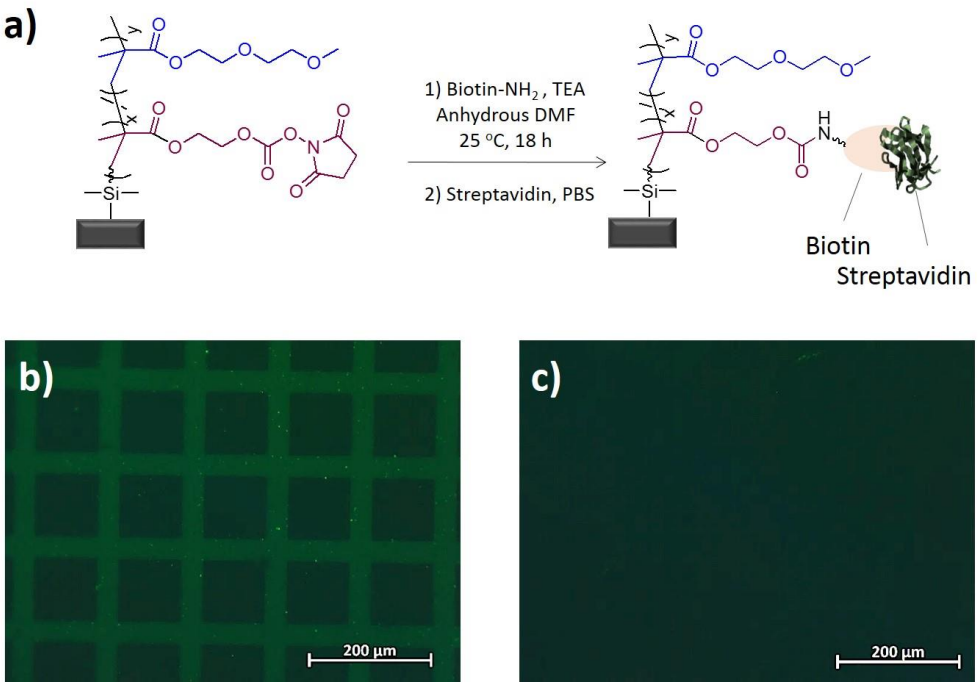



Figure 7.10. a) Biotin-amine immobilization on polymer brushes.

Additionally, biotinlylated copolymers (P2-P4) were immobilized in streptavidin coated Q-Dots. Fluorescence microscopy images indicated that with varying the ratio of succinimidyl carbonate units in the polymer, immobilization of streptavidin coated quantum dots could be tuned (Figure 7.11. d-f). As expected, biotin-amine treated P1 (DEGMEMA homopolymer) and non-biotinylated P3 did not show any significant signal.

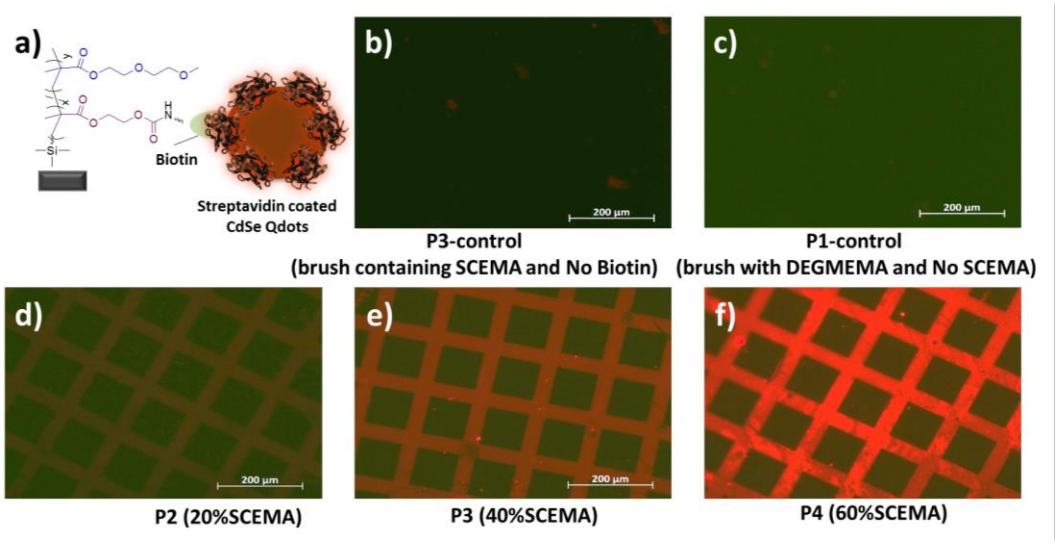



Figure 7.11. a) Scheme of streptavidin coated Q-Dots immobilization on biotinylated reactive brushes. Fluorescent microscopy images of Q-Dots on non-biotinylated P3 (b), and biotin-amine treated P1 (b) as control experiments and biotinylated P2, P3 and P4.

### 7.3.4. End Group Modification Using Orthogonal Reaction

Since these polymer brushes were synthesized via S-RAFT with R group approach, thiocarbonate units are exposed on the surface. The phenyl thioester moieties at the top of polymer brushes opens up possibility for a different post-polymerization modification of polymer films. In the presence of amine units, terminal phenyl carbonothioyl thio units are reduced to thiols which should enable a thiol-maleimide chemistry based orthogonal functionalization of polymer brushes. Firstly, as a model compound, 3-amino-1-propanol was immobilized on P2 polymer brushes. Significant decrease of C=O stretch from succinimidyl carbonate units in the FTIR spectrum suggests the attachment of amine containing molecules. Subsequently, maleimide bearing rhodamine dye was contact printed on the thiol modified P2 surface. Linear patterns were observed under fluorescence microscope while same treatment on the non-reduced P2 brush led to appreciable lower dye conjugation (Figure 7.12). The low amount of conjugation could be due to concurrent hydrolysis of end groups during printing.

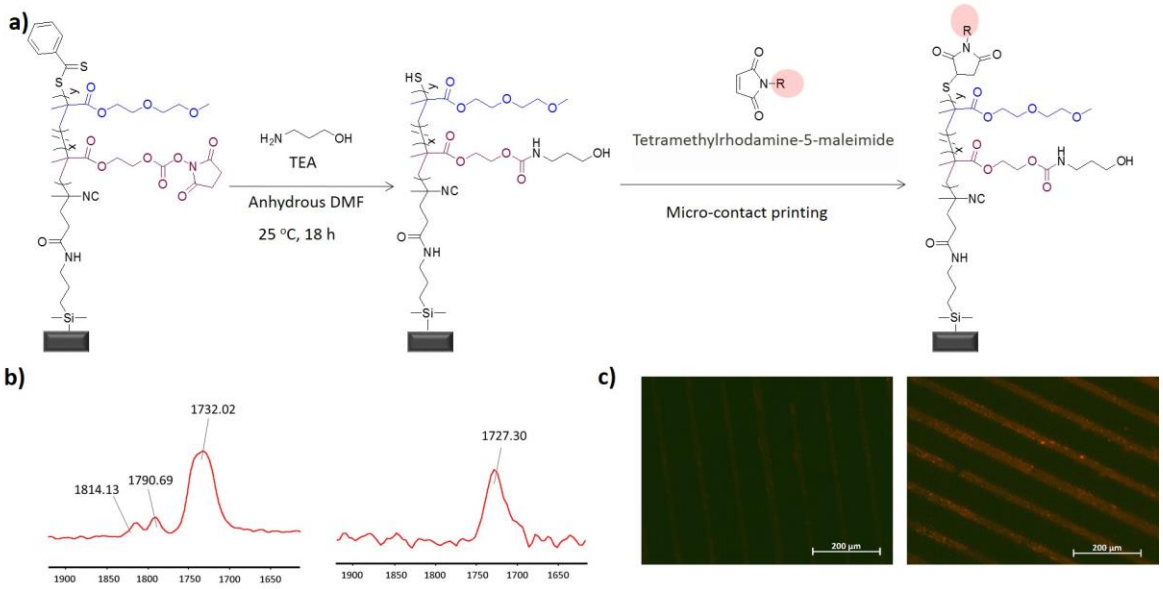



Figure 7.12. a) Immobilization of 3-amino-1-propanol during reduction of dithiocarbonyl units and immobilization of maleimide containing dye. b) ATR-FTIR spectra of surfaces. c) Fluorescence microscope images of maleimide containing dye immobilization.

## 7.4. Conclusion

In conclusion, amine reactive polymer brushes containing succinimidyl carbonate moieties as side chains were synthesized using S-RAFT. We showed that by changing the feed ratio, amount of incorporation of reactive monomer in polymer brushes can be well-tuned. Conjugation with different amine containing molecules were performed under mild conditions and proceeded with moderately good conversions under such mild conditions. Finally, taking advantage of R group approach in S-RAFT, it was observed that the end groups were reduced to thiol units during functionalization of pendant reactive groups. Secondary functionalization after immobilization of amine containing molecules was exhibited by contact printing of maleimide containing dye via the nucleophilic Michael type thiol-maleimide addition.

# 8. FABRICATION AND FUNCTIONALIZATION OF MULTI-CLICKABLE HYDROGELS ON TITANIUM SURFACES

## 8.1. Introduction

Titanium (Ti) and its alloys are commonly used for orthopedic and dental implants or amputation prosthesis due to their stability, excellent corrosion resistance, and good mechanical properties [277]. Although oxide layer on titanium is considered to be biocompatible, it was shown that bare titanium has poor integration with the surrounding bone environment since it adsorbs serum protein upon contact with blood or other body fluids [278-280] which may cause inflammation. Additionally, other implant materials such as *in vivo* sensors and drug delivery devices also require good biocompatibility with the surrounding biological environment. Therefore, modification of titanium based materials for enhancing their long-term clinical performance has been an area of active research in biomaterial science.

Lately, modification of surfaces with polymeric materials has taken inspiration from biological organisms [204]. Especially, mimicking of mussel adhesive proteins by using dopamine based building blocks has attracted significant interest [205]. Mussels adhere to practically all types of inorganic and organic surfaces (Figure 8.1) [206], including adhesion-resistant substrates such as poly(tetrafluoroethylene) (PTFE) [205]. Mussels' adhesive property stems from the amino acid composition of proteins found near the plaque-substrate interface, which are rich in dihydroxyphenylalanine (DOPA) and lysine amino acids [207]. In addition to participating in reactions leading to bulk solidification of the adhesive [208,209], DOPA forms strong covalent and noncovalent interactions with various substrates [210]. It is well established that the catechol based subunit of this adhesive protein binds strongly to a variety of metal/metal oxide surfaces [211-215].

Messersmith and coworkers were the first to utilize DOPA as an anchoring group for the surface immobilization of poly(ethylene glycol). They showed that DOPA containing polymers were adsorbed to the $TiO_2$ surfaces in a robust manner when at least two catechol units were present in the polymer chain [213,216]. The adlayer stability further increased when three repeating DOPA units were used as a multiple adhesion foot and such adlayers were capable of imparting non-fouling character to the surfaces. As an alternative to antibiofouling linear polyethylene glycol, Textor and coworkers reported fabrication of a non-fouling coating by modifying titanium surface with hydrophilic dendrons, containing catechol groups in their core and polyethylene glycol arms on the periphery. They showed that the number of the catechol units in the core should be at least three to enable irreversible attachment to the surface. When branched polymer coated antifouling surface was compared with the linear PEG coating, dendritic adlayers were found to possess lower hydration and much lower dissipation. Despite different mechanical properties, they showed that both dendritic adlayer and linear PEG coating imparted excellent non-biofouling property to titanium substrates [217].

Lee and coworkers designed catechol-grafted poly(ethylene glycol) to prepare a surface-independent interfacial modifier to impart anti-biofouling property to various surfaces including titanium. Multiple catechol units tethered on the backbone of PEG provided significant PEGylation of the surfaces. They demonstrated the attachment of the pendant catechol bearing material onto various metallic surface such as gold, silicon, titanium, as well as some polymeric surfaces including polycarbonate and polytetrafluoroethylene. They confirmed that PEGylation of those surfaces make them quite resistant to protein and cell adhesion, which suggests that the surfaces were effectively coated by the PEG-based modifier [281].

Messersmith and coworkers demonstrated the synthesis of hydrogels using DOPA-functionalized PEG-PLA-MA block copolymer [282]. It was observed that the wet adhesion of the hydrogels to titanium surfaces improved upon incorporation of DOPA-containing peptides. Obtained hydrogels exhibited moduli of 30−40 kPa which is similar to that of soft tissues. Additionally, they showed that with oxidation of the DOPA units,

adhesion strength of hydrogel to titanium decreases which shows that DOPA needs to be in its reduced form for the strong water-resistant adhesion to titanium surface.

The bioinspired approach has also been utilized to obtain reactive polymeric coatings for surface functionalization. A well-defined bifunctional poly(dopamine acrylamide-co-propargyl acrylamide) copolymer was synthesized by sequential post-polymerization modification of reactive poly(pentafluorophenyl acrylate) homopolymer with dopamine and propargyl amine. Thus obtained copolymers were coated onto titanium surfaces. Subsequent functionalization of these surfaces was demonstrated through attachment of various fluorescent dye molecules through click chemistry. Moreover, after modifying the polymeric surface by using appropriate linkers, they demonstrated successful immobilization of biomolecules such as avidin and concanavalin using the copper-catalyzed azide-alkyne 1,3-dipolar cycloaddition (CuAAC) [283].

Herein, we report the preparation of a PEG based multifunctional hydrogel layer that is attached to titanium surfaces in a robust manner using a dopamine methacrylamide based surface modifier (Figure 8.1). Furan protected maleimide containing monomer was used as a multi-reactive monomer and PEGMEMA was used to create the anti-biofouling matrix of the hydrogel. These monomers were crosslinked on top of the methacrylate bearing monolayer on titanium surfaces in the presence of a PEG-based cross-linker and a photo-initiator. Thus fabricated hydrogel layer could undergo radical thiol-ene and ieDDA reactions with the strained oxanorbornene unit in the furan-maleimide cycloadduct. Additionally, after removal of furan units from maleimide by simple heating under vacuum, the hydrogel surfaces gain nucleophilic thiol-ene, as well as the reversible Diels-Alder cycloaddition reactivity.

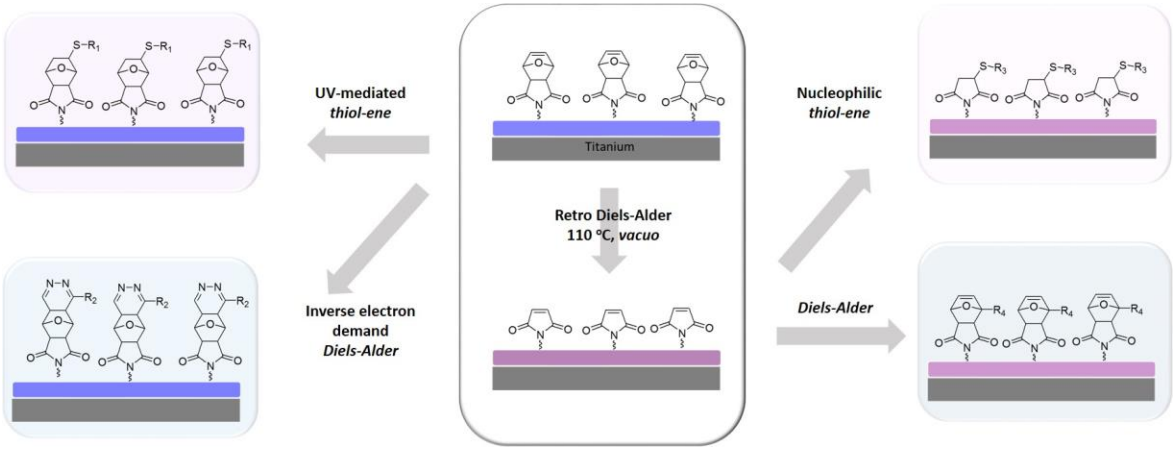



Figure 8.1. Multi-functionalizable hydrogel layer on titanium substrates.

## 8.2. Experimental

### 8.2.1. Materials

Poly(ethylene glycol)methyl ether methacrylate (PEGMEMA, average $M_n$ 300), poly(ethylene glycol) dimethacrylate (PEGDMA, average $M_n$ 550), 2,2-dimethoxy-2-phenylacetophenone (DMPA), RGDC (H-Arg-Gly-Asp-Cys-OH), 4′,6-diamidino-2-phenylindole (DAPI) and *N*,*N*,*N*',*N*'',*N*''-pentamethyldiethylenetriamine (PMDETA), biotin-benzyl-tetrazine and TRITC-conjugated extravidin were purchased from Sigma-Aldrich. Sodium hydride, furfuryl alcohol and propargyl bromide was purchased from Alfa Aesar. Dopamine methacrylamide (DMA) [225], furan-protected maleimide methacrylate (FuMaMA) monomer [189], BODIPY-SH [154] and BODIPY-furan [284] were synthesized according to literature procedures. All organic solvents were used as received without further purification. Silicon surfaces were coated with Ti through electron-beam evaporation. For cell culture studies titanium foil was obtained from Aldrich (thickness 0.25 mm, 99.7% trace metal basis). Alexa Fluor 488 phalloidin was obtained from Invitrogen.

### 8.2.2. Methods

Prior to immobilization of DMA, titanium substrates were cleaned using a Novascan PSD Series UV/Digital Ozone System for 30 minutes. Attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy was performed on a Thermo Scientific Nicolet 380 FTIR spectrophotometer. Fluorescence microscopy images of samples on titanium surfaces were recorded at room temperature on a Zeiss Observer.Z1 fluorescent microscope (ZEISS Fluorescence Microscopy, Carl Zeiss Canada Ltd, Canada). BODIPY derivatives and Alexa Fluor 488 were visualized by filter set 38, TRIT-C conjugated extravidin was visualized by filter set 43, and 4′,6-diamidino-2-phenylindole (DAPI) was visualized by filter set 49.

### 8.2.3. Modification of Titanium with DMA

Ozone plasma-cleaned titanium substrates were immersed in the solution of DMA in methanol (1 mM, 100 mL) and left 18 hours at room temperature to obtain a reactive surface coating. Then, they were washed with methanol and dried under stream of nitrogen.

### 8.2.4. Synthesis of Hydrogels

10% FuMaMA containing hydrogel H1 was synthesized as follows: FuMaMA (20 mg, 68.69x$10^{-3}$ mmol), PEGDMA (37.78mg, 68.69x $10^{-3}$ mmol), PEGMEMA (164.7 mg, 549.93x $10^{-3}$ mmol) and DMPA (8.8 mg, 34.35x$10^{-3}$ mmol) were dissolved in DMF (250 µL). 10 µL of hydrogel precursor solution was spread onto 1x1 $cm^2$ titanium surface via spin-coating at 500 rpm for 18 seconds. Then, the solution was covered with a microscope cover glass and placed under UV light for 30 minutes. Lastly, the cover glass was taken off and hydrogel layer on surfaces was washed with copious amount of DMF and THF, and dried under a gentle stream of nitrogen. Increasing FuMaMA monomer ratio to 30% and

50% and keeping cross-linker, DMPA and molar concentration of the solution as constant, H2 and H3 were synthesized with the same method.

### 8.2.5. Functionalization of Hydrogels with Radical Thiol-Ene Reaction

BODIPY-SH (1 mg, $2.38x10^{-3}$ mmol) and DMPA (0.12 mg, $0.476x10^{-3}$) were dissolved in THF. Hydrogel layer on titanium was soaked with 10 µL of the dye solution and surface was exposed to UV light for 5 min through a photomask placed on the hydrogel. Subsequently, the hydrogel layer was washed with copious amount of THF to remove all unbound materials.

### 8.2.6. Functionalization of Hydrogels with Inverse Electron Demand Diels-Alder Reaction

Hydrogel coated substrate was incubated in a solution of Biotin-benzyl-tetrazine in 1:1 THF/ MeOH mixture (0.5 mg/mL). After 18 hours, the samples were washed with THF and MeOH and dried under a gentle stream of $N_2$. A solution of TRITC-ExtrAvidin (20 µL, 0.1 mg/mL in PBS) was dropped on the biotinylated hydrogel samples. The samples were placed 30 minutes in a dark place, and then they were gently rinsed with copious amounts of PBS and water.

### 8.2.7. Activation of Maleimide Functional Groups

Furan-protected maleimide-containing hydrogel layers on titanium surfaces were placed in preheated vacuum oven at 120 $^{o}$C for 30 minutes. Then, heating was switched off and surfaces were kept under vacuum for 120 minutes to cool down to room temperature before exposing to ambient atmosphere.

### 8.2.8. Functionalization of Hydrogels Containing Thiol-Reactive Maleimide Groups

Hydrogel coated surfaces were incubated in solution of BODIPY-furan (0.1 mg/mL in toluene). Hydrogels were incubated in the solution for 18 h in dark. Subsequently, non-reacted dyes were rinsed off using toluene and THF.

8.2.8.1. Functionalization with Nucleophilic Thiol-ene. Hydrogel coated surfaces were placed in a vial containing BODIPY-SH solution (1 mg/mL in DMF) for 18 h in dark. After conjugation, dye solution was removed and the hydrogel sample was gently rinsed with copious amounts of DMF.

8.2.8.2. Functionalization with RDG-SH. Hydrogel coated titanium surfaces were placed into a solution of RGDC (1 mg/mL in DMF. After incubation for 18 hours, peptide solution was removed, and substrates were washed with DMF, and dried under a gentle flow of $N_2$ gas.

8.2.8.3. Cell culture on hydrogels and staining. Mouse fibroblasts L929 were seeded onto hydrogel coated slides with a density of $2 \times 104$ cells/cm$^2$ using a stock solution $2 \times 105$ cells/mL. Then cell suspensions were dropped onto the substrates, and incubated at 37 °C in 5% $CO_2$ containing atmosphere for 3 h. Afterwards cell media (1 mL) was added into each well containing hydrogel coated titanium substrates. After incubation (24 or 48 h), cell media was removed, substrates were washed with 1× PBS. Cells were then fixed with 3.7% formaldehyde solution at room temperature. Filamentous actins (F-actins) were stained according to following protocol: Cells were incubated in 0.1% Triton X-100 in PBS for 5 minutes at room temperature. After washing with 1× PBS, they were incubated in Alexa Fluor 488 phalloidin solution (5 units/mL concentration containing 1% bovine serum albumin (BSA) in 1× PBS) for 20 min at room temperature. Lastly cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (1 μg mL−1 in MilliQ water) for 10

minutes at room temperature. Stained cells adhered to hydrogels were visualized using fluorescence microscopy.

## 8.3. Results and Discussion

### 8.3.1. Synthesis of Reactive Hydrogel Coating on Titanium Surfaces

Multi-functionalizable reactive hydrogels on titanium were synthesized from furan-protected maleimide methacrylate (FuMaMA) monomer and poly(ethylene glycol)methyl ether methacrylate (PEGMEMA) via photopolymerization in the presence of poly(ethylene glycol) dimethacrylate (PEGDMA) as a crosslinker and 2,2-dimethoxy-2-phenylacetophenone (DMPA) as a photoinitiator. Furan protected maleimide methacrylate (FuMaMA) units provide multi-functionalizable property to the hydrogel coatings since the bicyclic strained alkene in protected form as well as the active maleimide group in deprotected form are reactive.

Prior to coating the titanium surfaces with hydrogel layer, they were modified by dopamine methacrylamide (DMA) to provide a monolayer to make hydrogel layer be covalently attached to the titanium surface. Monomers, crosslinker and photoinitiator were dissolved in methanol. Hydrogel precursor was spin-coated on the surface and covered with a microscope cover glass and irradiated with ultraviolet light for 30 minutes. Keeping crosslinker ratio as constant and altering monomers' ratio as 10%, 30% and 50% FuMaMA in the precursor, hydrogels with different functional group density were synthesized on modified titanium surfaces and abbreviated as H1, H2 and H3, respectively. Thickness of hydrogel coating H1 was determined using ESEM as 20 μm.

Incorporation and control over density of FuMaMA units with changing feed ratio of monomers in the precursor solution was confirmed using ATR-FTIR analysis. The FTIR

spectra showed C=O stretching band belonging to ester groups at ~1727 cm-1 for all surfaces. In addition, the spectra revealed the presence of the out-of-phase C=O stretching vibration at 1704.85, 1702.39 and 1700.97 $cm^{-1}$ corresponding to furan-maleimide cycloadduct units of hydrogel H1, H2 and H3, respectively. In addition, weak bands at 1765.60, 1770.84, 1772.50 for H1, H2 and H3 were assigned to the in-phase C=O stretching vibration of maleimide units (Figure 8.2). As expected, the FTIR spectra showed an increase in the intensity of those stretching vibrations with an increase in the amount of FuMaMA monomer in gelation feed.

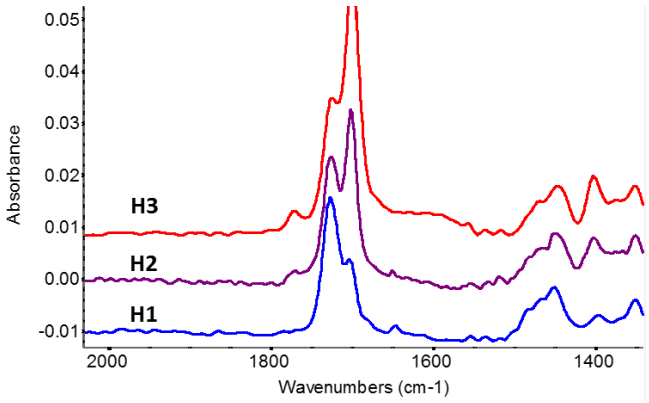



Figure 8.2 ATR-FTIR spectra of the hydrogels on titanium a) H1, b) H2 and c) H3.

### 8.3.2. Functionalization of Hydrogel Coatings Using Radical Thiol-Ene Reaction

The double bond of the oxanorbornene is functionalizable via photochemical radical thiol-ene reaction which enables site specific manipulation. Since this reaction requires UV light to occur, patterned immobilization of molecules of interest can be achieved when a photomask is used. To show this is applicable to the prepared hydrogel layers, a patterned immobilization of fluorescence dye molecule BODIPY was performed (Figure 8.3-a). A dye solution of BODIPY-SH and DMPA was prepared in THF. Hydrogel layer on titanium was soaked with dye solution and a photomask (Figure 8.3-b) was placed onto the hydrogel. After exposing the surface to the UV light for 5 minutes, photomask was removed and excess dye was washed off using copious amounts of THF. Fluorescence

microscopy images reveal that dye molecules were attached to oxanorbornene units on the exposed part of the surface while covered parts stayed unreacted (Figure 8.3-c).

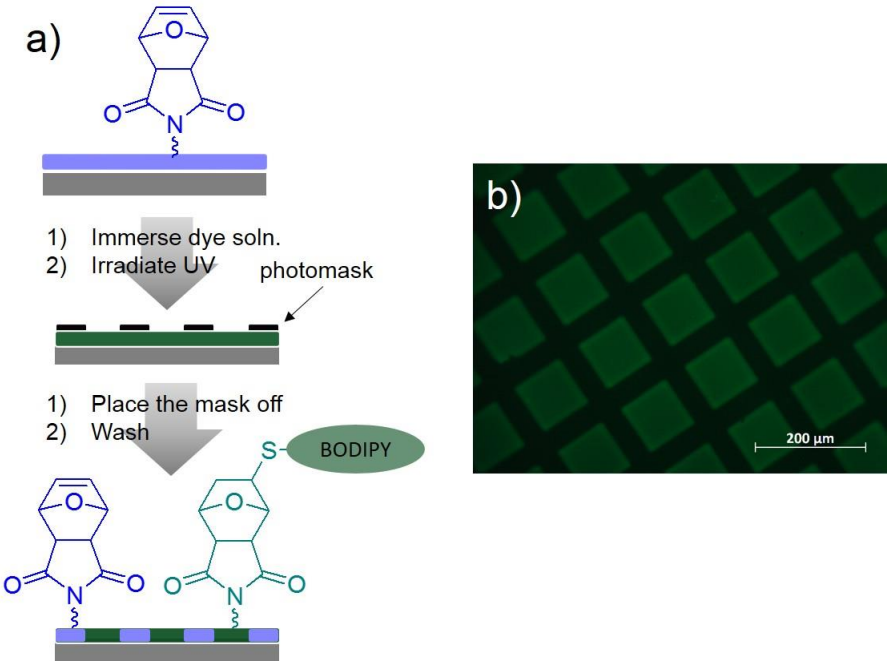



Figure 8.3. a) Schematic illustration of patterned immobilization via thiol-ene reaction. b) Fluorescence microscope image of BODIPY patterns on H1.

### 8.3.3. Functionalization of Hydrogel Coatings Using Inverse-Electron-Demand Diels-Alder Reaction

The inverse-electron-demand Diels–Alder (iEDDA) reaction involves a cyclo-addition reaction of an electron-rich dienophile with an electron-poor diene. It has been considered as click chemistry due to its fast and efficient nature. Being an oxanorbornene, furan-maleimide cycloadduct react irreversibly with the tetrazine to yield dihydropyrazine products. Furan protected maleimide which is a strained electron rich dienophile, a very suitable functional group for iEDDA reaction with molecules with tetrazine functionality. Incubation of extravidin on biotin-benzyl-tetrazine immobilized surfaces resulted in controlled immobilization of enzyme. It was observed that fluorescence intensities of the protein appended hydrogels increased with increasing amount of oxanorbornene (Figure

8.4). This experiment clearly demonstrates that the extent of functionalization can be controlled by tuning the amount of the reactive monomer in the gel.

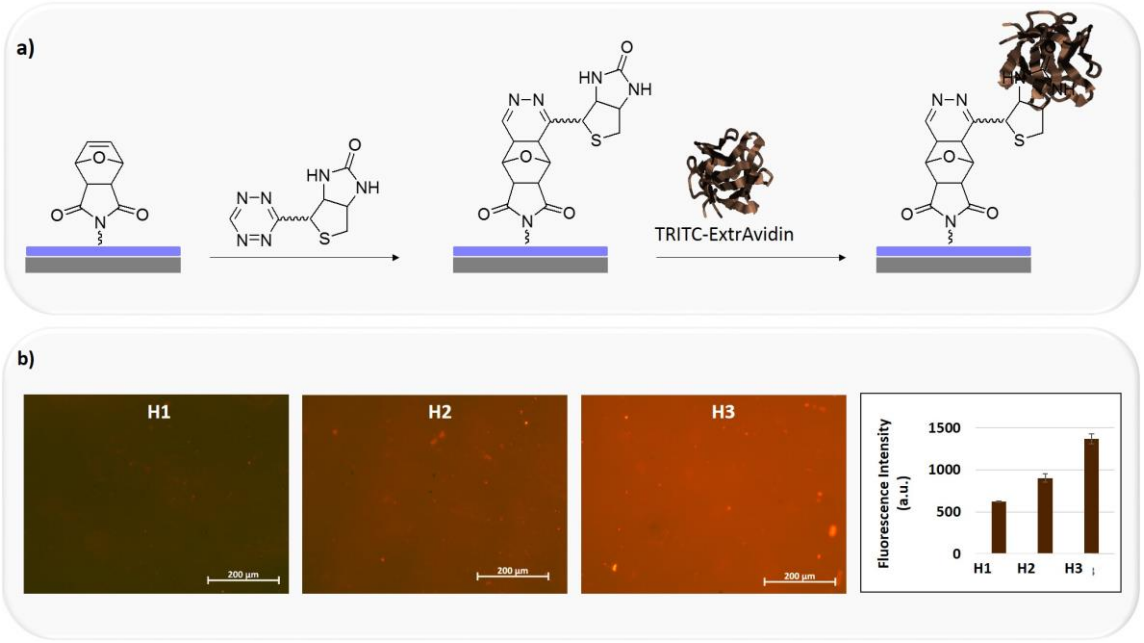



Figure 8.4. a) Scheme of conjugation of oxanorbornene containing hydrogels with biotin-benzyl-tetrazin and following ExtrAvidin immobilization, b) Fluorescence microscope images of ExtrAvidin immobilized hydrogels and relative fluorescence intensity graph.

As a control experiment non-biotinylated hydrogel H2 was incubated in ExtrAvidin solution. As seen in Figure 8.5, fluorescence microscope image did not show significant fluorescence which is due to antibiofouling property of PEG side chains of the cross-linked polymer on the surface.

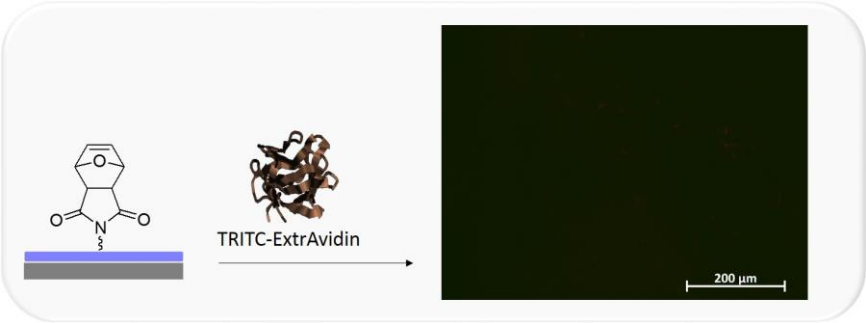



Figure 8.5. a) Control experiment with non-biotinylated H2 and related fluorescence microscope image.

### 8.3.4. Activation of Maleimide Groups in Hydrogels via Retro Diels-Alder Reaction

Hydrogel layer on titanium surfaces was heated to 120 °C *in vacuo* for 30 minutes to remove the furan protection group from the maleimide based cycloadduct via the retro-Diels-Alder reaction. Substrates were slowly cooled down to room temperature under vacuum for an additional 120 minutes before being removed from the oven. This thermal treatment unmasks the maleimide groups to their thiol- and diene- reactive form and yields hydrogels ready for functionalization with nucleophilic thiol-ene and Diels-Alder reactions (Figure 8.7).


Figure 8.6. Removal of furan groups via retro Diels-Alder reaction.

Successful conversion to maleimide functional groups on the surface was confirmed using ATR-FTIR. Retro Diels-Alder reaction to unmask maleimides was confirmed from the shift of C=O stretching vibration band to higher wavenumber for each hydrogel coating (Figure 8.7).

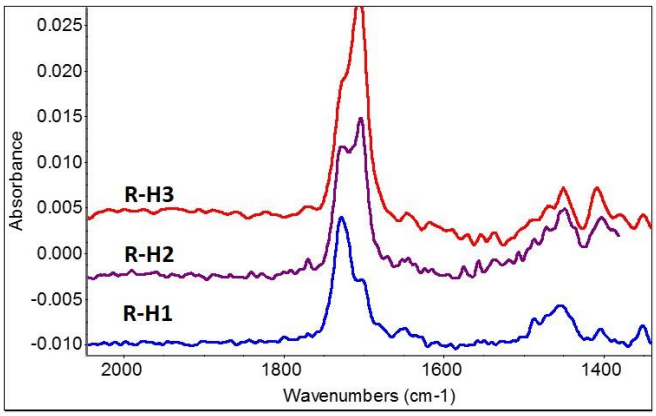



Figure 8.7. ATR-FTIR spectra of the hydrogels after retro Diels-Alder reaction on titanium a) R-H1, b) R-H2 and c) R-H3.

### 8.3.5. Modification of Hydrogel Coating with the Diels-Alder Reaction

Functionalization of hydrogels through Diels-Alder reaction with diene bearing molecules was evaluated using a furan containing BODIPY dye (Figure 8.8). Hydrogel H1 obtained after retro Diels-Alder reaction, namely R-H1, was immersed in BODIPY-furan solution in toluene (0.1 mg/mL) for 18 hours. After washing with THF to remove unbound dye, bright green fluorescence was observed when the dye conjugated surface was analyzed using fluorescence microscopy. When hydrogel H1 where the maleimide units have not been unmasked was used instead of hydrogel R-H1, no significant fluorescence signal was detected which suggests functionalization was realized through Diels-Alder reaction (Figure 8.8-d). It is well known that the Diels-Alder linkages are thermoreversible, hence to observe that the BODIPY-furan conjugated hydrogel surfaces were heated up to 110 $^{o}$C for 18 hours. Lack of any appreciable fluorescence from the surface indicated successful release of the dye from the surface (Figure 8.8-e). In order to demonstrate that absence of fluorescence intensity stems from removal of the furan containing dye and not dye decomposition, we incubated the obtained hydrogel layer in dye solution and showed re-functionalization of the same surface (Figure 8.8-f). This supports the fact that indeed the removal of dye proceeds through retro Diels-Alder reaction which simultaneously creates the active maleimide units in the hydrogel, which can be used for subsequent re-functionalization.

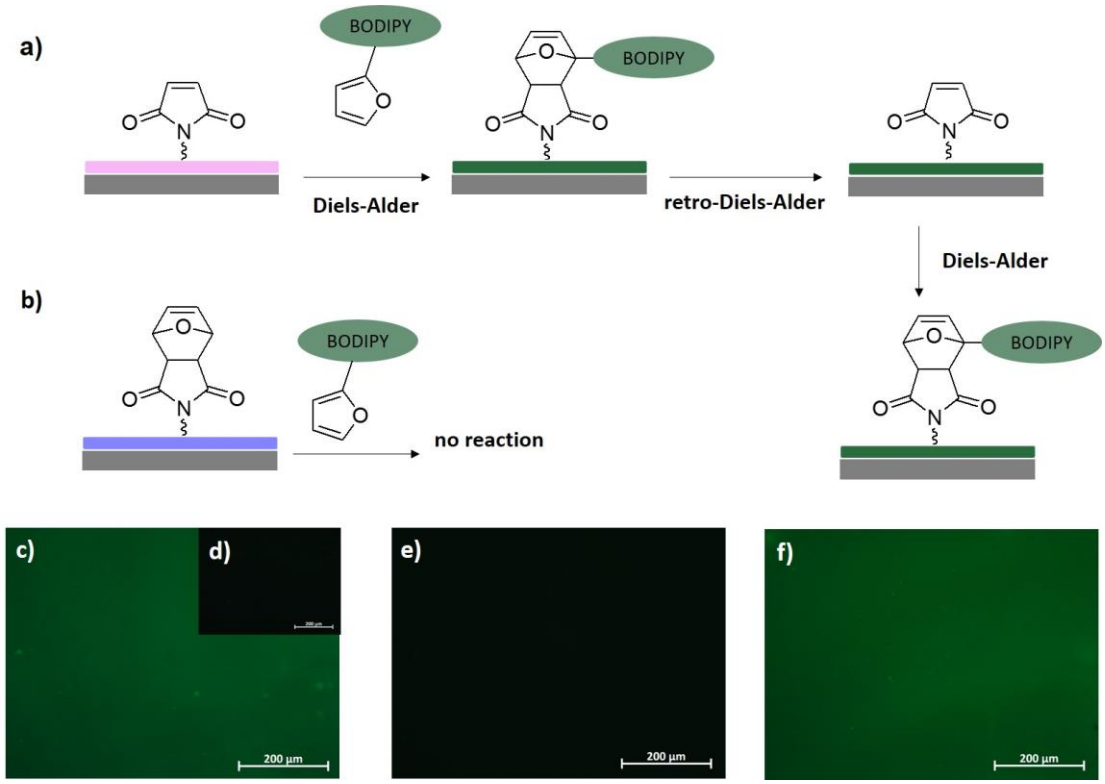



Figure 8.8. (a) BODIPY-furan reversible conjugation sequence, (b) control reaction. Fluorescence microscopy images of (c) dye conjugated hydrogel, (d) control experiment, (e) hydrogel after dye de-conjugation and (f) hydrogel after dye re-conjugation.

### 8.3.6. Functionalization of Hydrogel Coating with Michael Addition Reaction

After deprotection of maleimide units via retro Diels-Alder reaction, surfaces were also functionalized via BODIPY-SH to show functionalization using nucleophilic thiol-ene reaction. Thiol containing dye solution was prepared in DMF and activated and non-activated surfaces were incubated in this solution for 18 hours. Then they were washed with DMF and THF and visualized via fluorescence microscope. Since thiol does not undergo nucleophilic attack to furan protected maleimide, no fluorescence is observed (Figure 8.9-c inset). Successful conjugation of fluorescent dye to deprotected H1 was visualized via fluorescent microscopy as a green image (Figure 8.9-c).

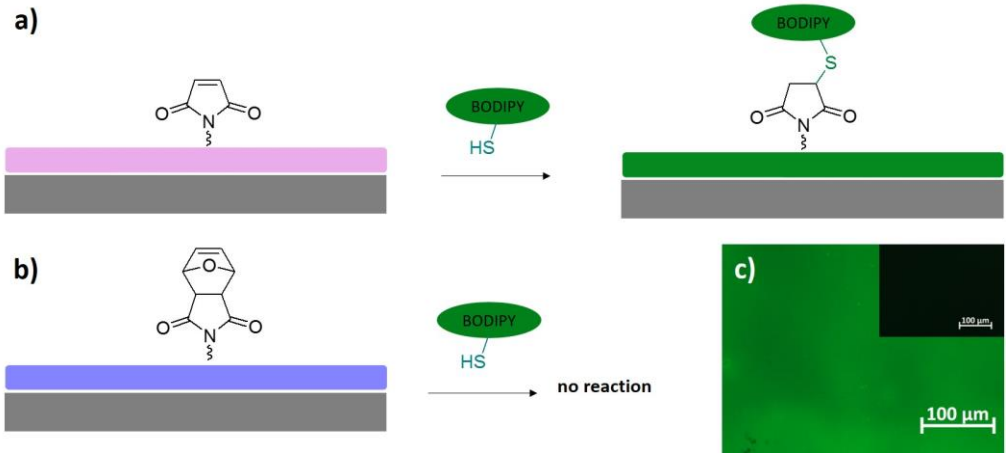



Figure 8.9. Scheme of immobilization of BODIPY-SH to R-H1 (a) and control experiment with protected maleimide containing surface (b) and fluorescence microscope images of dye immobilization (inset: control reaction)

### 8.3.7. Assessment of Cytocompatibility of Native and Peptide Modified Hydrogel Coating

Cellular adhesion on surfaces can be controlled by modifying them with specific peptides that have affinity for the receptors on cells. It is well established that RGD sequences are recognized by integrin receptors and promote attachment of cells (Figure 8.10). We attached thiol containing RGD on R-H1 surfaces via Michael addition to prepare a platform for cell growth. Mouse fibroblast L929 cells which are known to have affinity for this peptide, were seeded onto these modified surfaces and also unmodified but reactivated hydrogel surfaces (R-H1). After incubation for the specified time (24 and 48 h), the actins and nuclei of the cells were stained. A significant difference was observed between the RGD immobilized and control hydrogels. After the first 24 h due to antifouling character of PEG based hydrogels, there were only few cells adhered on the hydrogel which had not been modified via RGD, while more cells adhered to the peptide conjugated hydrogel. From the green fluorescence of the stained actin filament it was clear that the cells on peptide conjugated surfaces were spreading on the substrate. On the second day, cells had spread more and proliferated on the RGD immobilized surface while the non-functionalized hydrogel did not exhibit such characteristics. These results shows

that the maleimide bearing PEG based anti-biofouling hydrogel coatings can be easily modified with peptides and thus modified hydrogel surfaces are suitable substrates for cell growth.

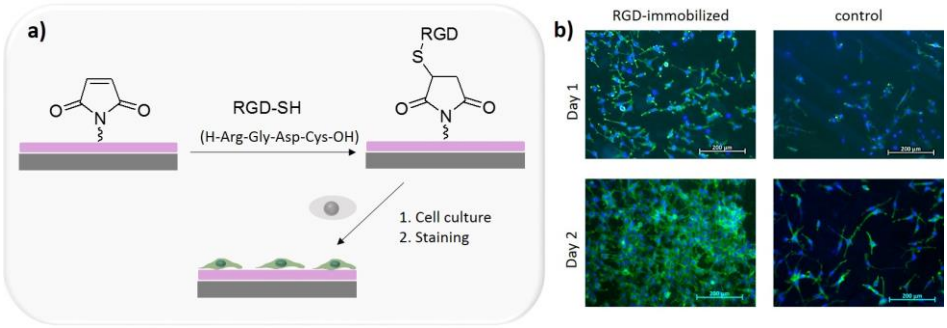



Figure 8.10. a) Scheme of peptide conjugation on hydrogels for cell growth and b) fluorescence images of hydrogel H1 with and without RGD conjugation upon Alexa Fluor 488 phalloidin (green) and DAPI (blue) staining of L929 mouse fibroblasts after 1 and 2 days.

## 8.4. Conclusions

In conclusion, furan-protected maleimide-containing multi-clickable biocompatible hydrogel layer on titanium surfaces were fabricated. To preserve the maleimide functional group during polymerization, it needs to be protected with furan as a thermoreversible cycloadduct. Hydrogels could be functionalized using the furan-protected maleimide units due to their reactivity towards radical thiol-ene and inverse-electron-demand Diels-Alder reactions. We demonstrated that the extent of functionalization on hydrogels can be controlled by attachment of biotin-benzyl-tetrazine followed by immobilization of TRITC-labelled streptavidin. Additionally, taking advantage of site-specific UV thiol-ene click reaction, we showed patterned immobilization of the fluorescent dye BODIPY-SH through a photomask. Further, after removal of furan units, facile functionalization of hydrogels was demonstrated using Diels-Alder and nucleophilic thiol-ene reactions. Enhanced cellular adhesion on RGD immobilized hydrogel revealed that prepared hydrogel network on titanium is highly cyto-compatible.

# 9. CONCLUSIONS

In this dissertation, we investigated the design and fabrication of reactive polymeric coatings on solid substrates to render them suitable for various biomedical applications. Polymer coatings were prepared as thin polymeric layers, polymer brushes and thin hydrogel layers. A progression in thickness of the functionalizable coating material has been targeted to increase the loading efficiency of the interface. While the thin polymeric coatings were fabricated from well-defined polymers using a 'graft to' approach, polymer brushes and thin hydrogel coatings were made using a 'graft from' approach using combination of monomers. The thiol- and amine- reactive polymer brushes were obtained using controlled polymerization techniques SI-ATRP and S-RAFT, respectively. The hydrogel based coatings were obtained using photo-polymerization of combination of monomers. Solid substrates such as glass, $Si/SiO_2$ or titanium was used as underlying surface. Based on the choice of the underlying substrate, polymeric materials were attached strongly to the surface with either silyl ethereal bonding or catechol ligands to afford robust coatings that do not peel off in aqueous media. Work undertaken in the thesis demonstrates that a variety of different conjugation reactions such as nucleophilic reactions of amine with activated carbonates, nucleophilic and radical thiol-ene reactions and the Diels-Alder cycloaddition reactions allow efficient functionalization of polymeric surfaces with molecules of interests.

In the first chapter of the thesis, background information about polymeric coatings of solid substrates and a summary of various coating methods were provided. Additionally, the chapter provides an overview of the state-of-the-art applications of such materials for biosensing and implant coating through selected literature examples. In the second chapter, aim of the research undertaken to culminate this thesis was described in brief. The following three chapters report novel functionalizable thin polymeric coatings that were anchored onto $Si/SiO_2$ and titanium surfaces that were evaluated as protein sensing platforms and anti-bacterial coating, respectively. The third chapter described the synthesis

of reversibly functionalizable polymeric coatings on glass and $Si/SiO_2$ surface. Side chain furan containing surface reactive polymers were synthesized and coated onto the surface. Facile functionalization of these polymer coated surfaces was demonstrated through conjugation of a maleimide containing fluorescent dye via Diels-Alder chemistry. It was also shown that these surfaces were renewable i.e. the conjugated molecule could be released from the surface through heating, and the regenerated surface could again undergo functionalization. Additionally, it was demonstrated that bioactive ligands can be localized on the surface to enable ligand-directed protein immobilization. In the fourth chapter, a novel methodology towards preparation of maleimide-containing thiol-reactive thin polymeric films anchored to $Si/SiO_2$ surfaces is reported. The thin films undergo facile functionalization with thiol-containing small molecules and ligands under mild conditions. Importantly, it was demonstrated that the extent of functionalization on the surface can be tailored by adjusting the amount of reactive functional group on the polymeric precursor. Lastly, ligand directed protein sensing and biomolecular immobilization in a tailored fashion was realized on these polymer coated surfaces. The fifth chapter extends the chemistry developed in the preceding chapter to metal/metal oxide surfaces. Thiol-reactive thin polymeric coating on titanium surfaces was obtained using copolymers bearing maleimide group and chelating catechol units as side chain functionalities. Polymers were anchored onto titanium surfaces via mussel inspired chemistry which involves surface adhesion mediated through catechol units. Polymer coated substrates were functionalized with antibacterial peptides and bactericidal activities were tested against gram positive and gram negative bacteria.

Thicker polymeric coatings in tens of nanometers were targeted in the next two chapters. The sixth chapter reports the fabrication and functionalization of polymeric brushes containing thiol-reactive maleimide groups using ‘graft from’ approach via SI-ATRP. Polymer brushes with varying amounts of a furan-protected maleimide groups were synthesized. After unmasking the maleimide units, the polymer brushes undergo facile functionalization with thiol-containing molecules via the nucleophilic thiol-ene addition reaction as deduced via XPS and fluorescence microscopy. Additionally, after attaching thiol-containing bioactive ligand, the brushes could be used to attach streptavidin coated quantum dots. The following chapter presented the synthesis and characterization of

amine-reactive polymer brushes containing pendant succinimidyl carbonate groups. In this case, S-RAFT was utilized to provide a series of brushes with varying amount of the activated carbonate group. Efficient modification of these polymer brushes with amine containing molecules was demonstrated. Bioactive ligands could be easily tethered to provide a platform for protein sensing and immobilization. Lastly, orthogonal functionalization of side chain and chain-end was accomplished by attachment of a maleimide-containing molecule to the thiol functional group installed at the chain-end of the polymer brushes which forms during the amine-based conjugation along the side chains.

Lastly, in the final chapter of this thesis, fabrication of thicker polymeric coatings were realized using a surface bound hydrogel layer. It was also demonstrated that coatings containing the furan-protected maleimide functional group can be used as multi-functionalizable materials. Modification of titanium surface with hydrogel layer was accomplished using the catechol group as an anchoring group. Functionalization of this parent material and its derivative with four different types of chemistry were described. First, the strained alkene units on the masked maleimide group were used as a handle for functionalization of hydrogels with UV-mediated radical thiol-ene reaction. Alternatively, an inverse electron demand Diels-Alder reaction between the oxanorbornene unit and tetrazine-containing molecules could be also employed for facile modification of hydrogels. After unmasking of the maleimide units, functionalization of the hydrogel based coating could be achieved with either normal Diels-Alder cycloaddition reaction or a Michael-type nucleophilic thiol-ene reaction. It was also demonstrated that while surface modification of titanium by PEG-based coating reduces their ability to sufficiently interact with cells, it could be considerably increased through functionalization with a cell adhesive peptide.

In summary, in this dissertation functional and antifouling surface coatings for biomedical applications were prepared using novel reactive copolymers. Modification of polymer coated surfaces were performed using various types of efficient conjugation techniques. Density of reactive group on surfaces could be tuned by changing the amount

of reactive monomer in the parent polymer or coating precursor. Presented reactive surfaces bear the potential to serve as good candidates for biosensing or implant coatings applications due to simplicity of their fabrication and efficient functionalization.

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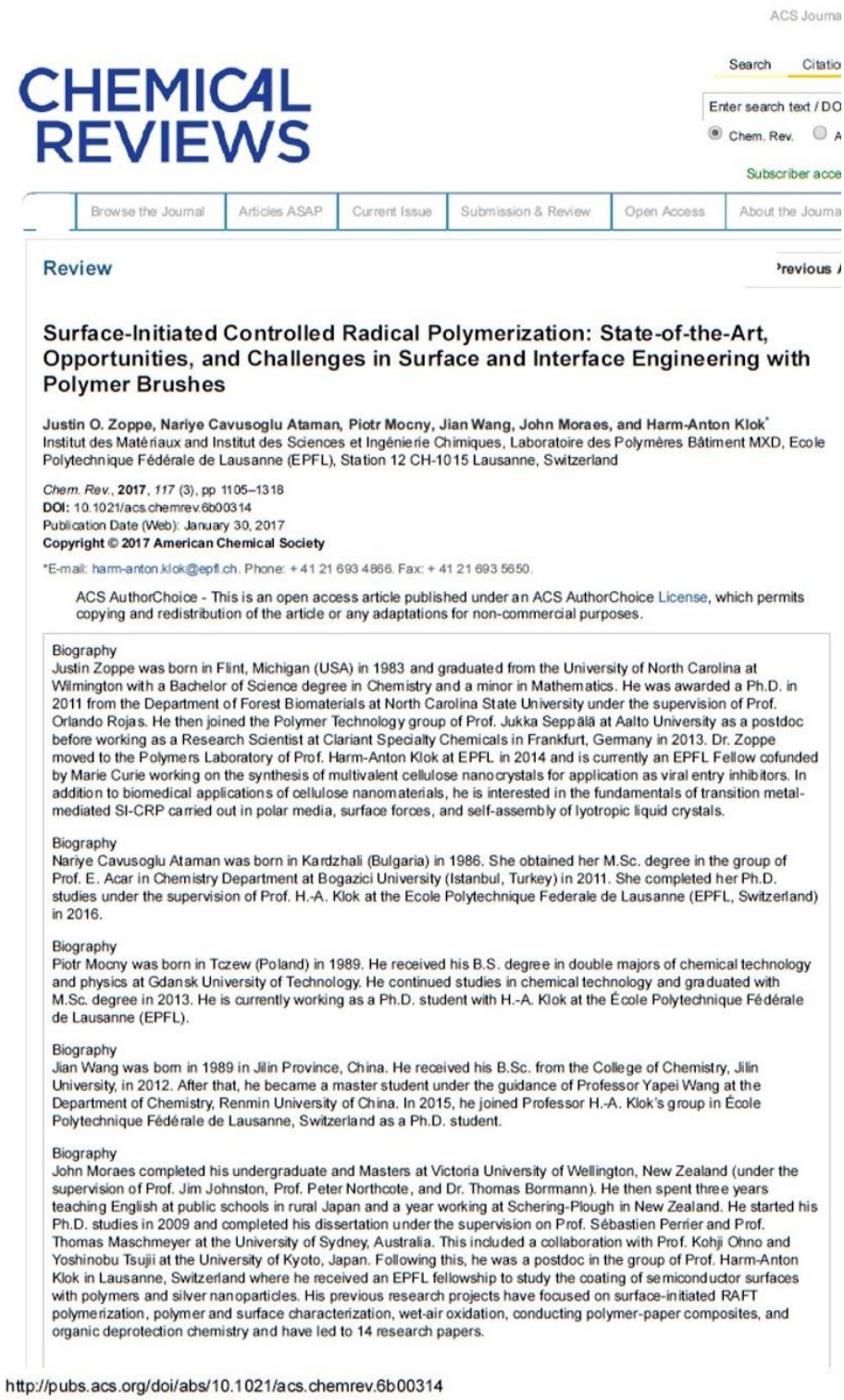

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## Surface-Initiated Controlled Radical Polymerization: State-of-the-Art, Opportunities, and Challenges in Surface and Interface Engineering with Polymer Brushes

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Publication Date (Web): January 30, 2017


*E-mail: harm-anton.klok@epfl.ch. Phone: + 41 21 693 4866. Fax: + 41 21 693 5650.



Biography
Justin Zoppe was born in Flint, Michigan (USA) in 1983 and graduated from the University of North Carolina at Wilmington with a Bachelor of Science degree in Chemistry and a minor in Mathematics. He was awarded a Ph.D. in 2011 from the Department of Forest Biomaterials at North Carolina State University under the supervision of Prof. Orlando Rojas. He then joined the Polymer Technology group of Prof. Jukka Seppälä at Aalto University as a postdoc before working as a Research Scientist at Clariant Specialty Chemicals in Frankfurt, Germany in 2013. Dr. Zoppe moved to the Polymers Laboratory of Prof. Harm-Anton Klok at EPFL in 2014 and is currently an EPFL Fellow cofunded by Marie Curie working on the synthesis of multivalent cellulose nanocrystals for application as viral entry inhibitors. In addition to biomedical applications of cellulose nanomaterials, he is interested in the fundamentals of transition metal-mediated SI-CRP carried out in polar media, surface forces, and self-assembly of lyotropic liquid crystals.

Biography
Nariye Cavusoglu Ataman was born in Kardzhali (Bulgaria) in 1986. She obtained her M.Sc. degree in the group of Prof. E. Acar in Chemistry Department at Bogazici University (Istanbul, Turkey) in 2011. She completed her Ph.D. studies under the supervision of Prof. H.-A. Klok at the Ecole Polytechnique Federale de Lausanne (EPFL, Switzerland) in 2016.

Biography
Piotr Mocny was born in Tczew (Poland) in 1989. He received his B.S. degree in double majors of chemical technology and physics at Gdansk University of Technology. He continued studies in chemical technology and graduated with M.Sc. degree in 2013. He is currently working as a Ph.D. student with H.-A. Klok at the École Polytechnique Fédérale de Lausanne (EPFL).

Biography
Jian Wang was born in 1989 in Jilin Province, China. He received his B.Sc. from the College of Chemistry, Jilin University, in 2012. After that, he became a master student under the guidance of Professor Yapei Wang at the Department of Chemistry, Renmin University of China. In 2015, he joined Professor H.-A. Klok's group in École Polytechnique Fédérale de Lausanne, Switzerland as a Ph.D. student.

Biography
John Moraes completed his undergraduate and Masters at Victoria University of Wellington, New Zealand (under the supervision of Prof. Jim Johnston, Prof. Peter Northcote, and Dr. Thomas Bormann). He then spent three years teaching English at public schools in rural Japan and a year working at Schering-Plough in New Zealand. He started his Ph.D. studies in 2009 and completed his dissertation under the supervision on Prof. Sébastien Perrier and Prof. Thomas Maschmeyer at the University of Sydney, Australia. This included a collaboration with Prof. Kohji Ohno and Yoshinobu Tsujii at the University of Kyoto, Japan. Following this, he was a postdoc in the group of Prof. Harm-Anton Klok in Lausanne, Switzerland where he received an EPFL fellowship to study the coating of semiconductor surfaces with polymers and silver nanoparticles. His previous research projects have focused on surface-initiated RAFT polymerization, polymer and surface characterization, wet-air oxidation, conducting polymer-paper composites, and organic deprotection chemistry and have led to 14 research papers.

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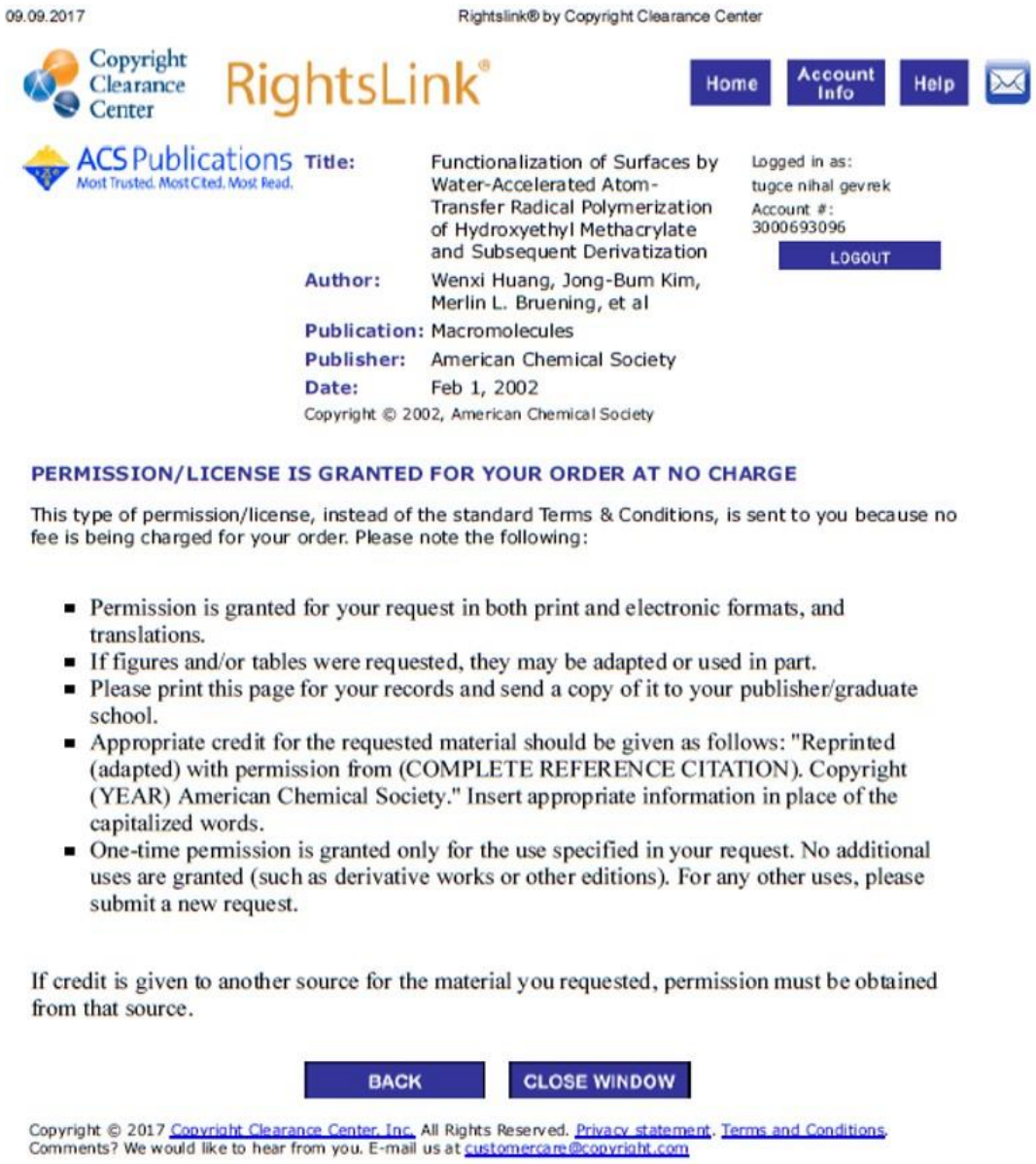

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Author: Wenxi Huang, Jong-Bum Kim, Merlin L. Bruening, et al

Publication: Macromolecules

Publisher: American Chemical Society

Date: Feb 1, 2002

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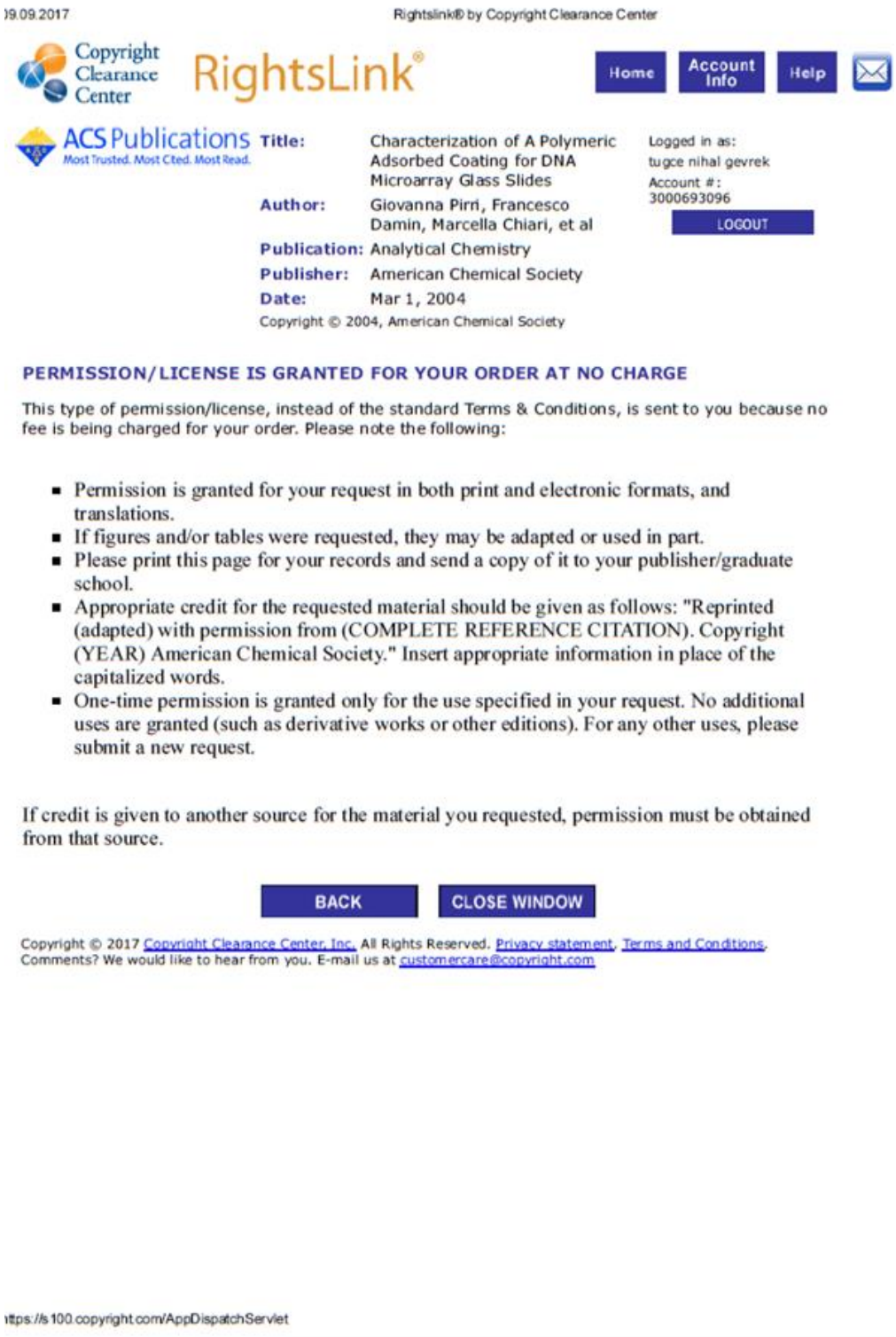

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Author: Giovanna Pirri, Francesco Damin, Marcella Chiari, et al

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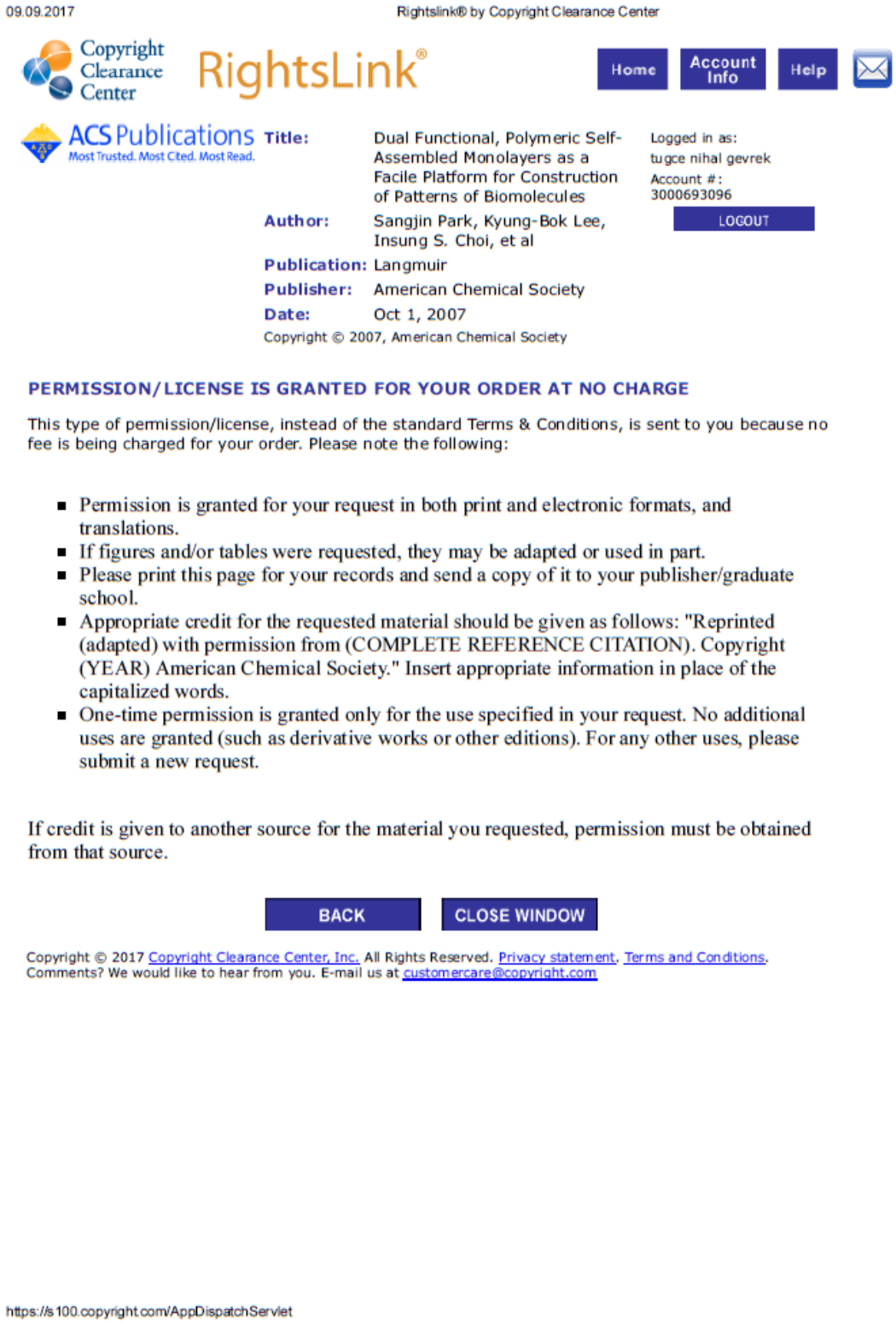

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Author: Sangjin Park, Kyung-Bok Lee, Insung S. Choi, et al

Publication: Langmuir

Publisher: American Chemical Society

Date: Oct 1, 2007

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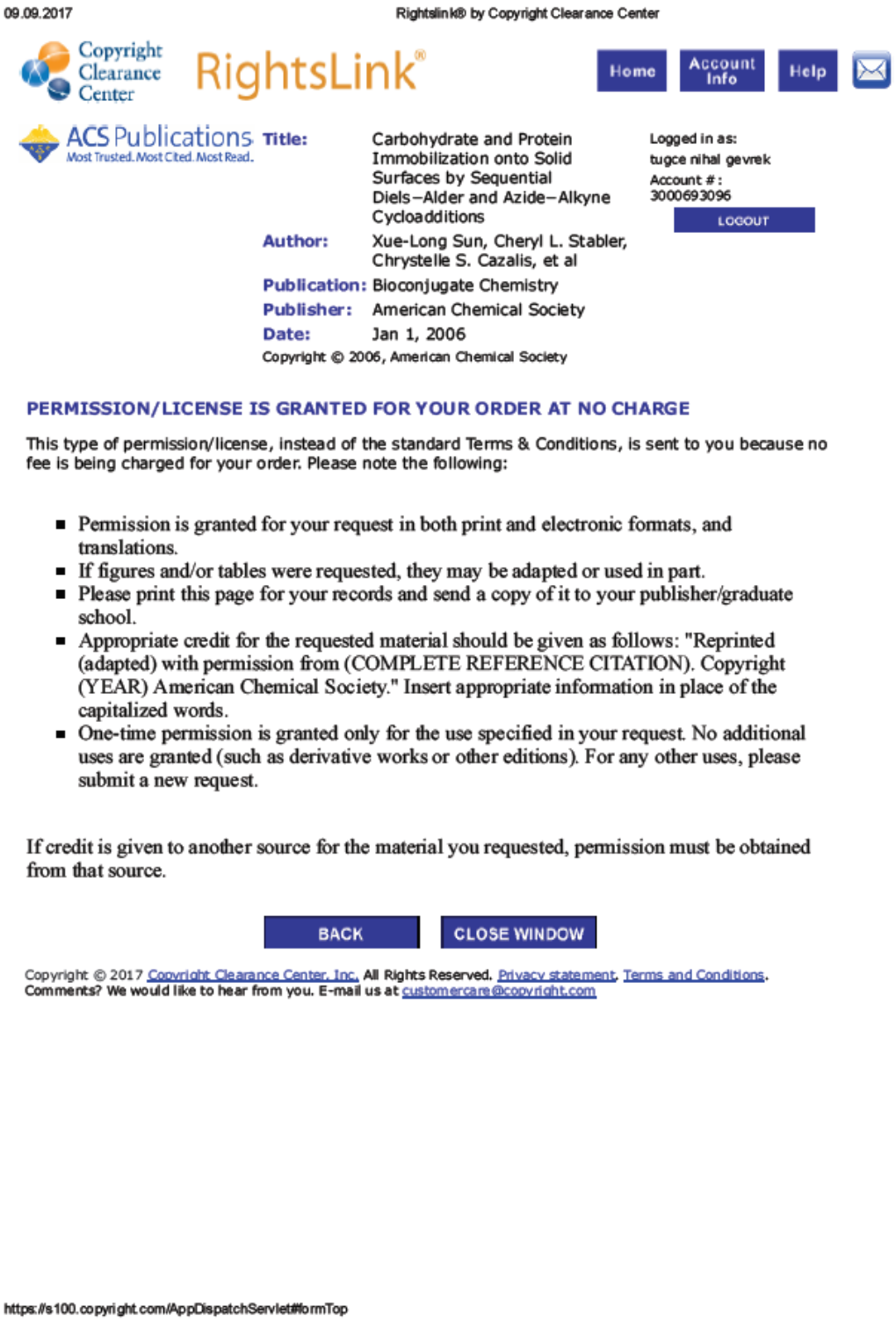

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Publication: Bioconjugate Chemistry

Publisher: American Chemical Society

Date: Jan 1, 2006

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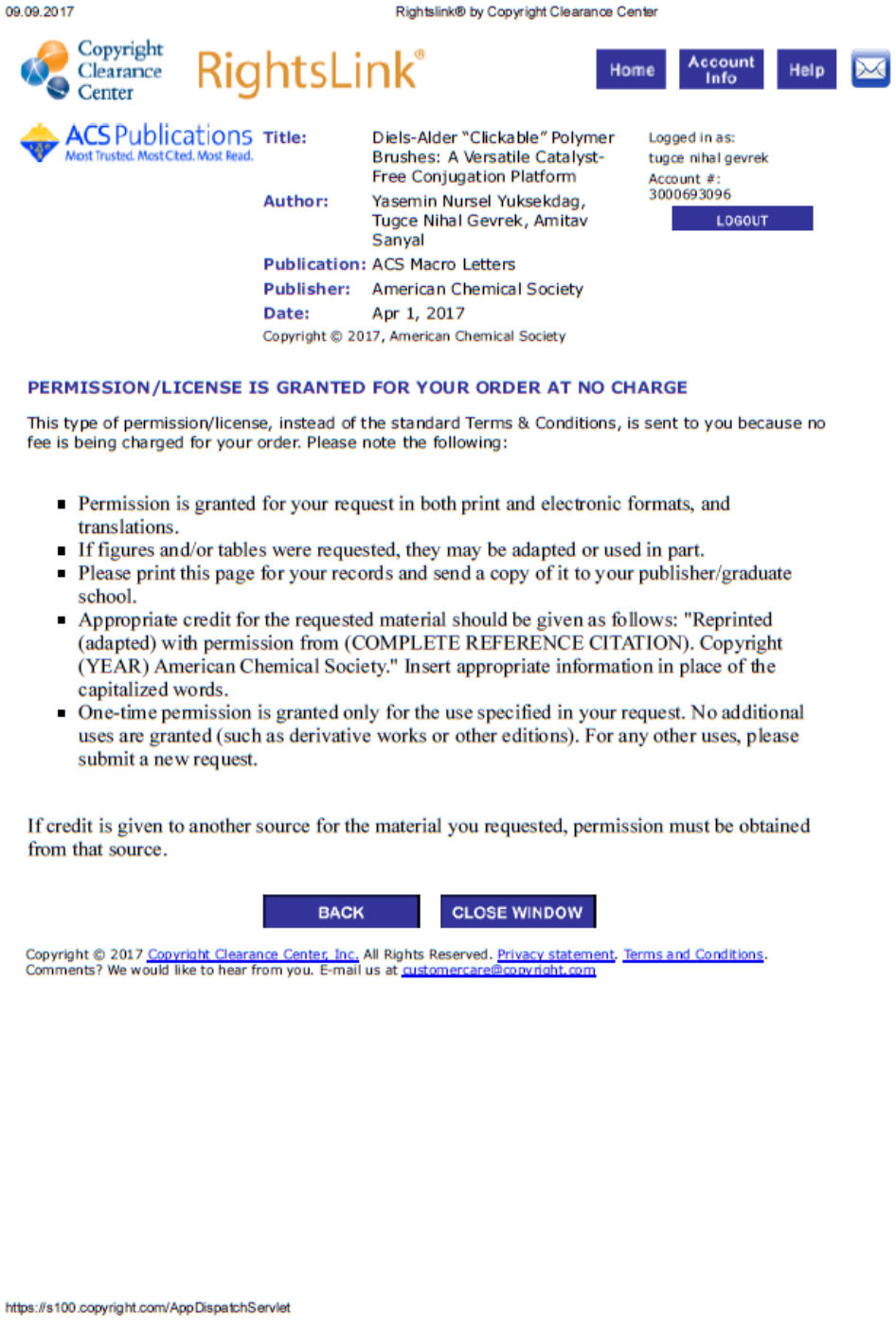

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Publication: ACS Macro Letters

Publisher: American Chemical Society

Date: Apr 1, 2017

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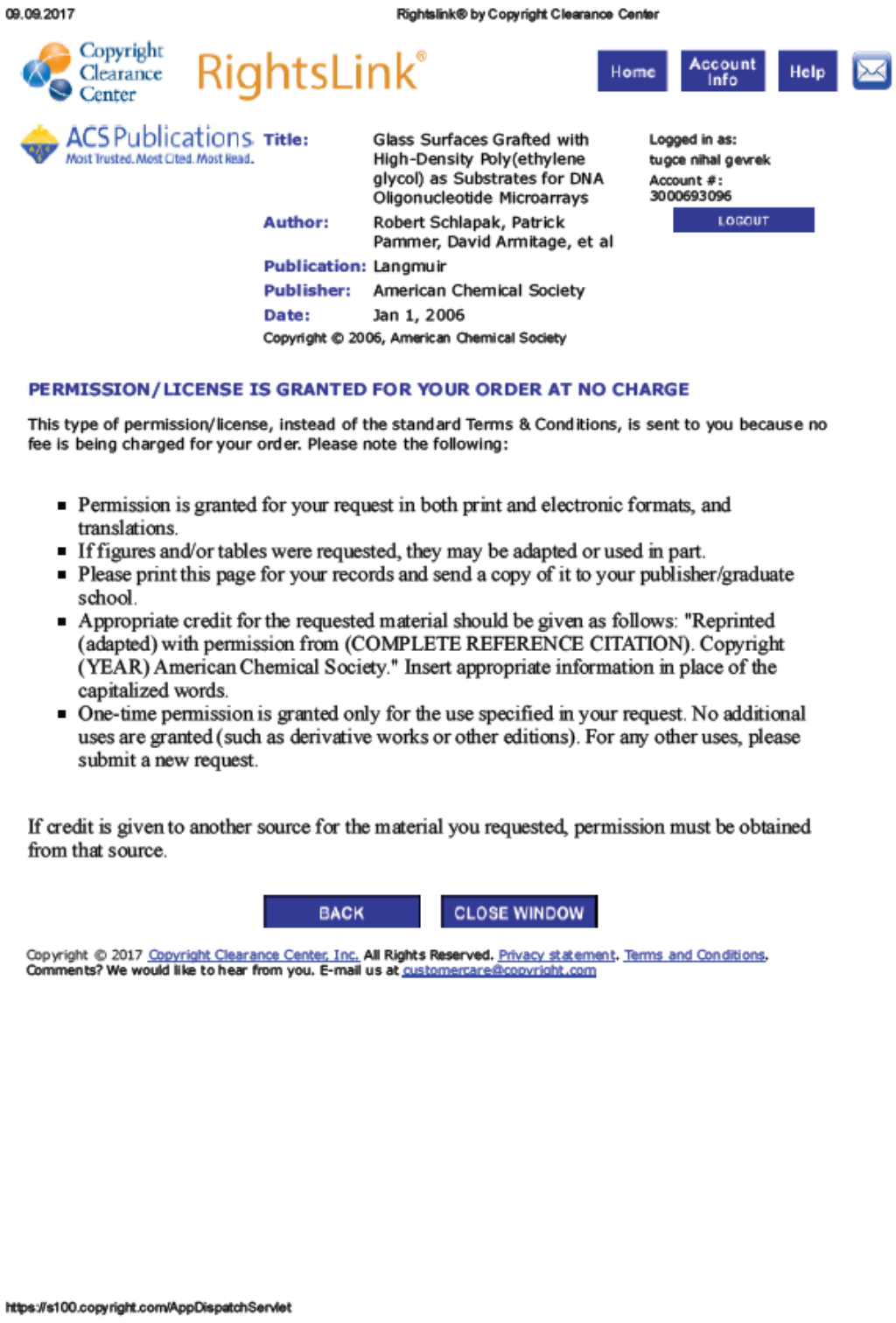

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Date: Jan 1, 2006

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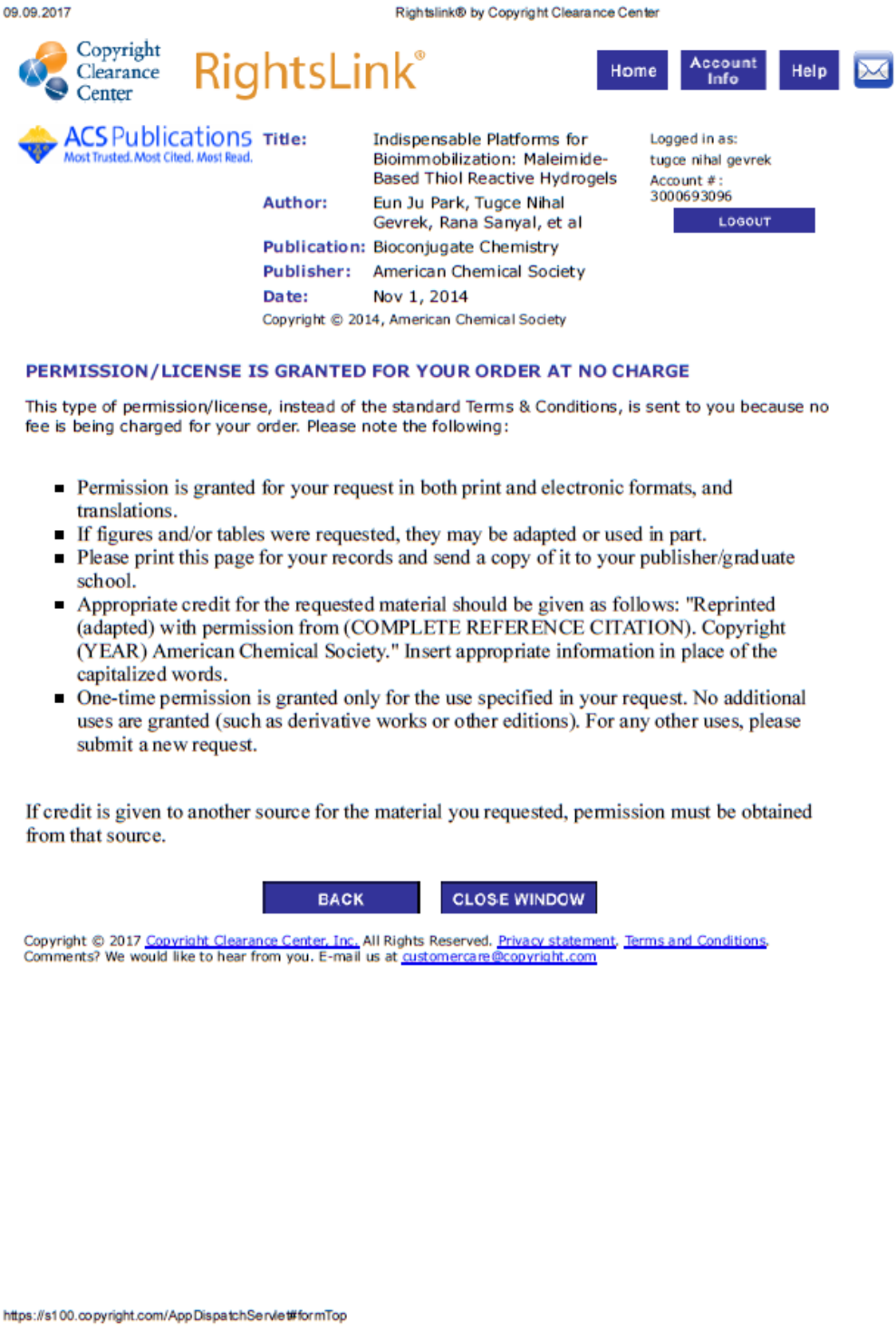

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Int J Mol Sci. 2014 Aug; 15(8): 13849–13880. PMCID: PMC4159828
Published online 2014 Aug 11. doi: 10.3390/ijms150813849

# Antibacterial Surface Treatment for Orthopaedic Implants

Jiri Gallo,[1,*] Martin Holinka,[1] and Calin S. Moucha[2]

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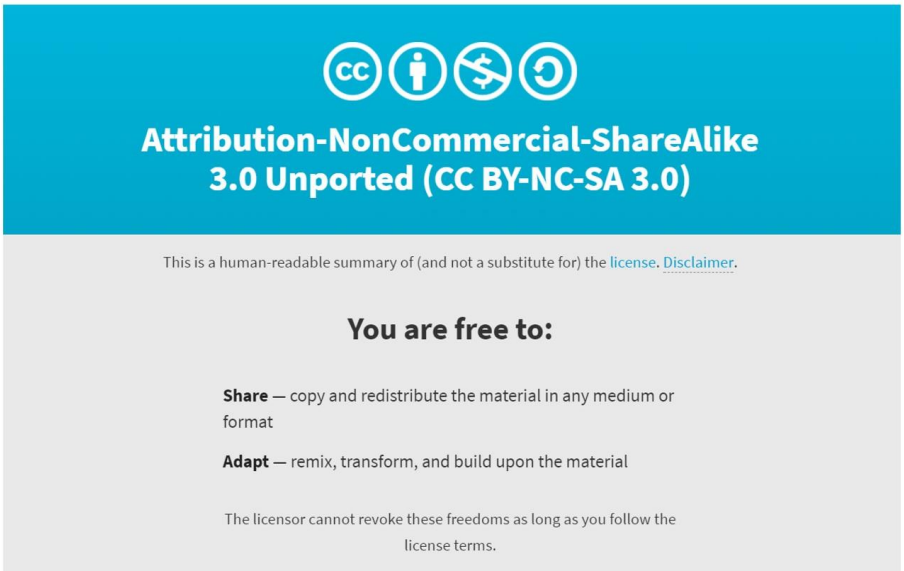
Received 2013 Dec 30; Revised 2014 Jun 6; Accepted 2014 Jun 13.



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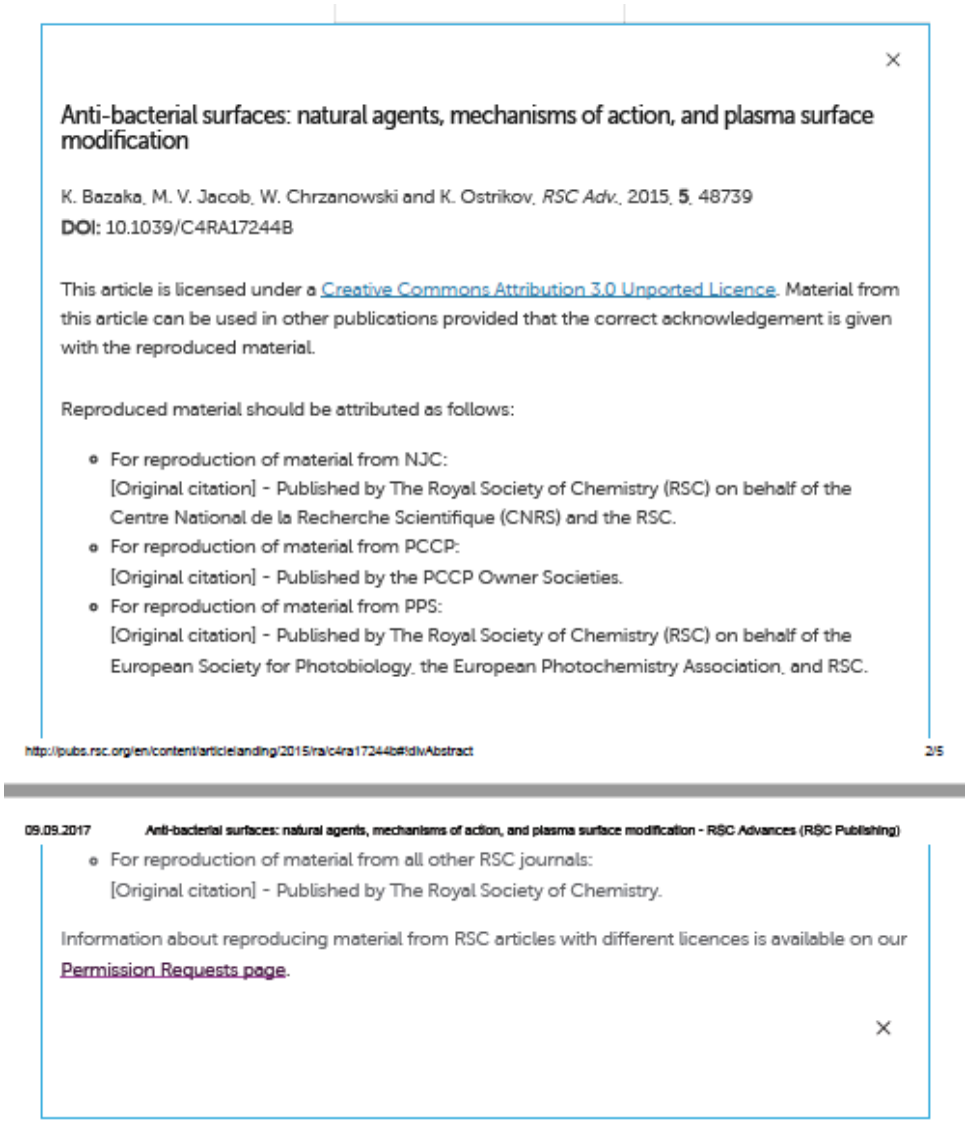

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K. Bazaka, M. V. Jacob, W. Chrzanowski and K. Ostrikov, *RSC Adv.*, 2015, **5**, 48739
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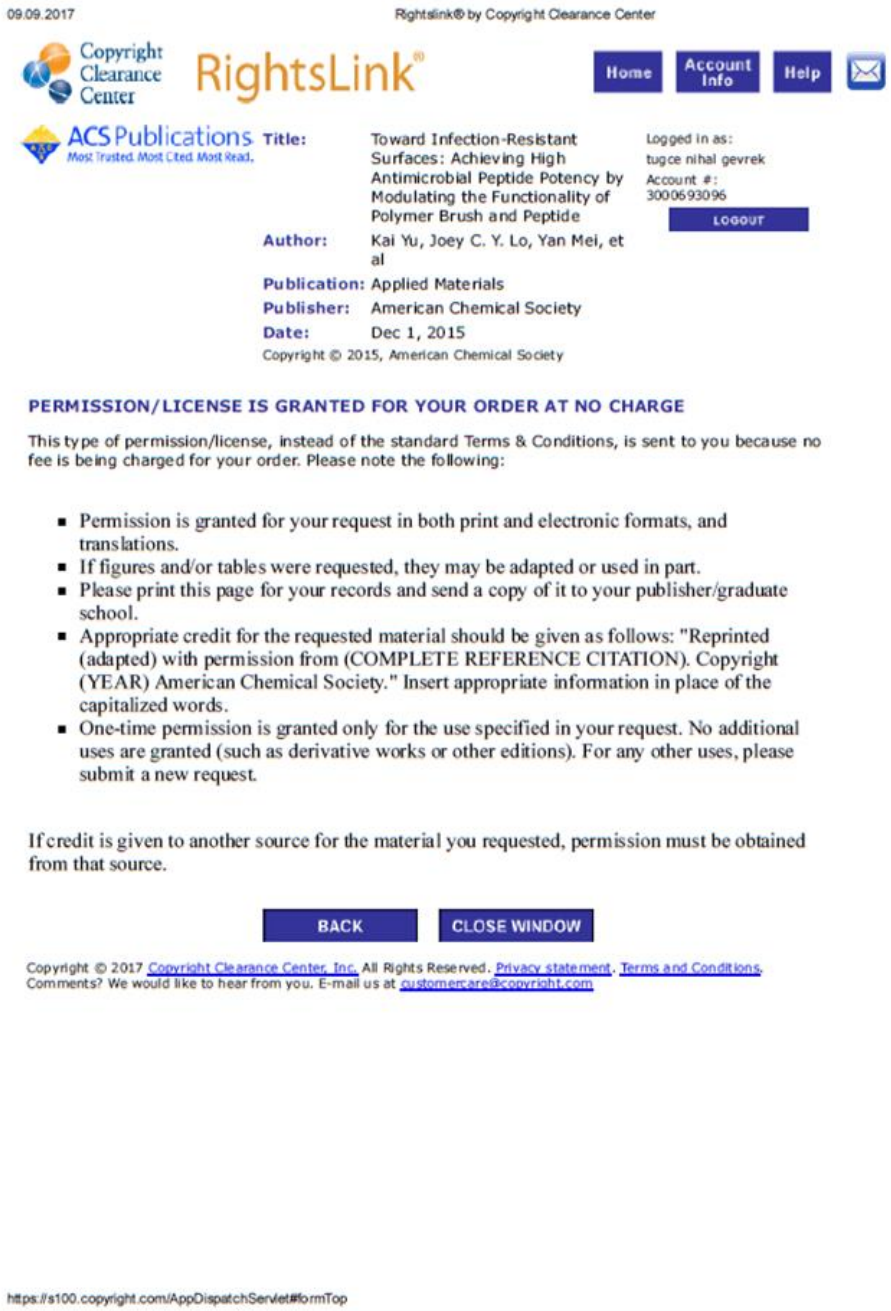

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Publication: Applied Materials

Publisher: American Chemical Society

Date: Dec 1, 2015

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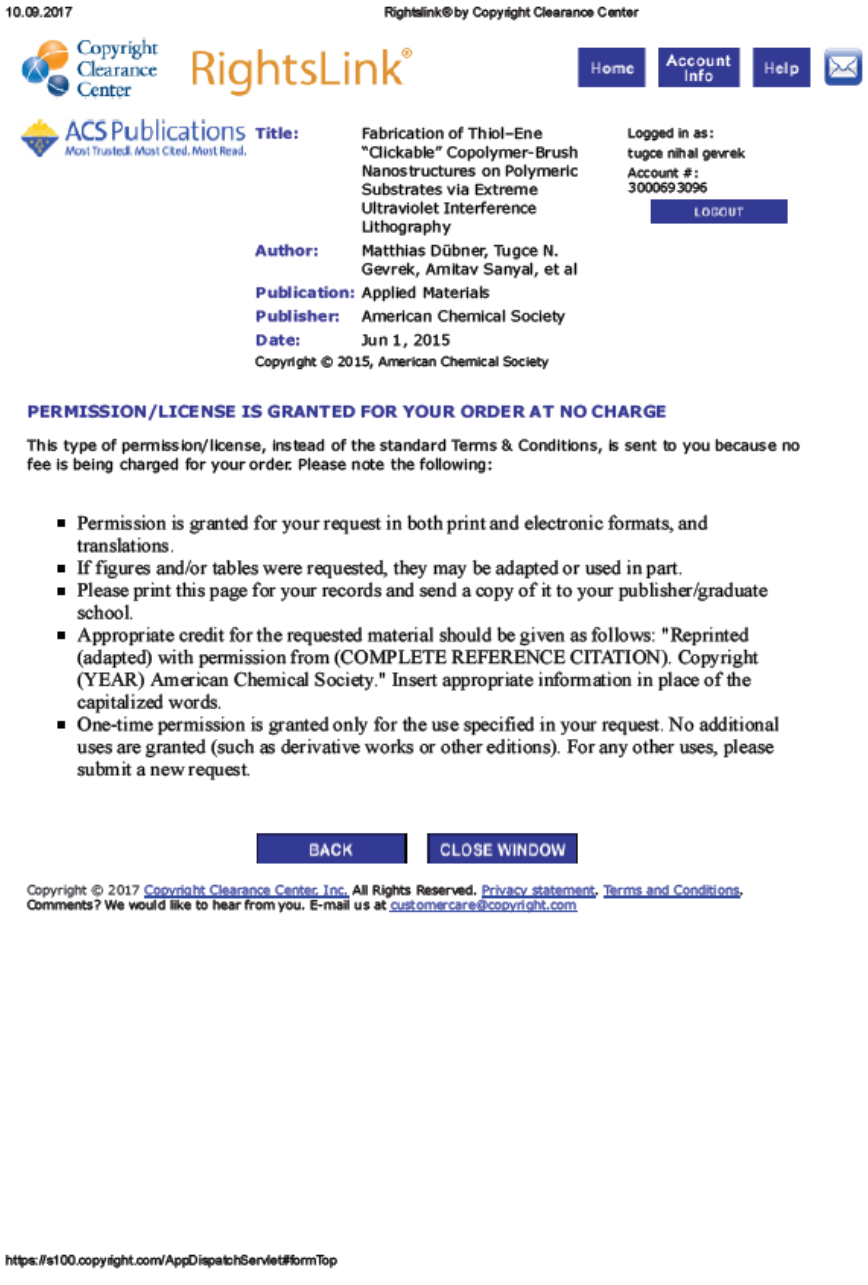

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Title: Fabrication of Thiol–Ene "Clickable" Copolymer-Brush Nanostructures on Polymeric Substrates via Extreme Ultraviolet Interference Lithography

Author: Matthias Dübner, Tugce N. Gevrek, Amitav Sanyal, et al

Publication: Applied Materials

Publisher: American Chemical Society

Date: Jun 1, 2015

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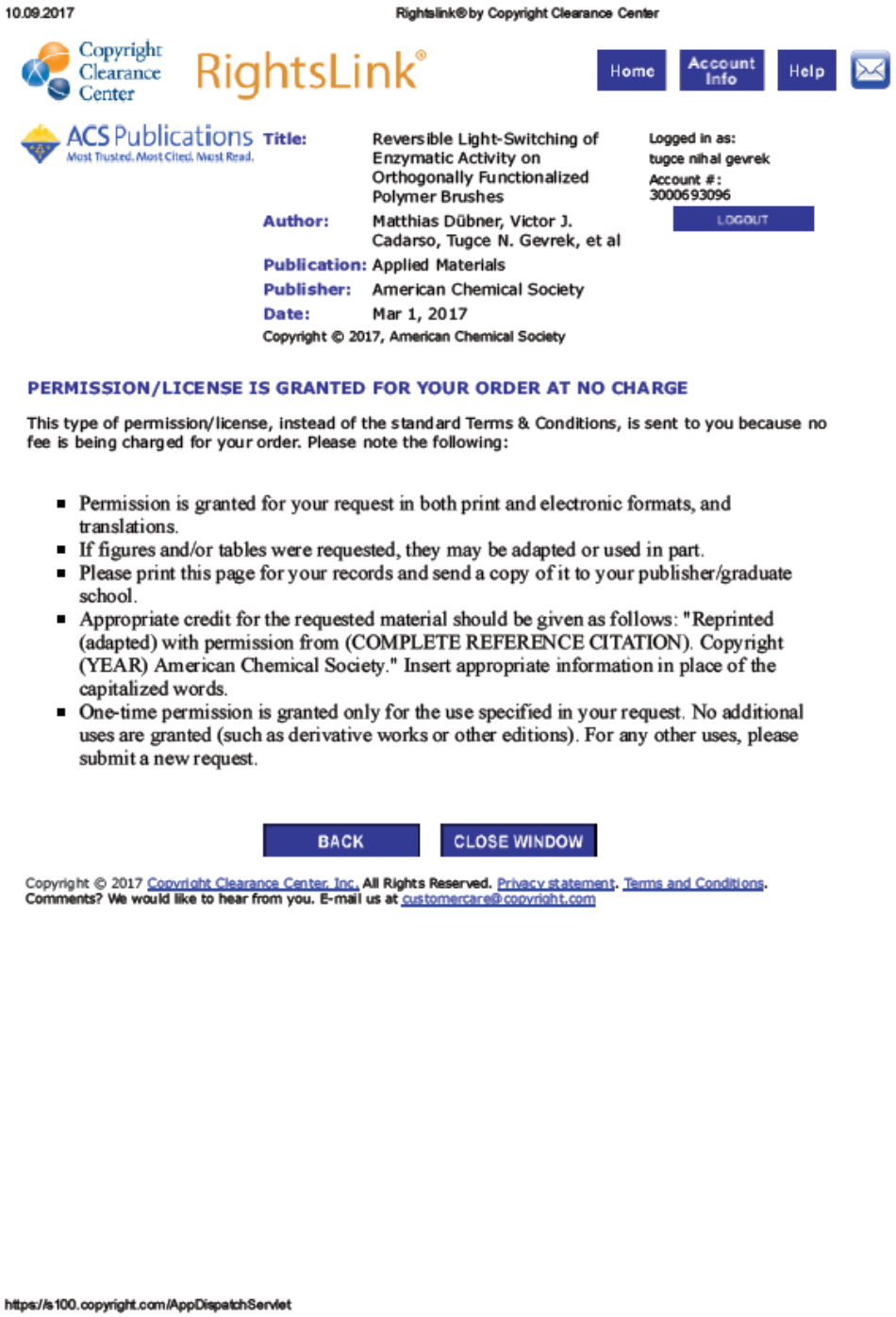

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Title: Reversible Light-Switching of Enzymatic Activity on Orthogonally Functionalized Polymer Brushes

Author: Matthias Dübner, Victor J. Cadarso, Tugce N. Gevrek, et al

Publication: Applied Materials

Publisher: American Chemical Society

Date: Mar 1, 2017

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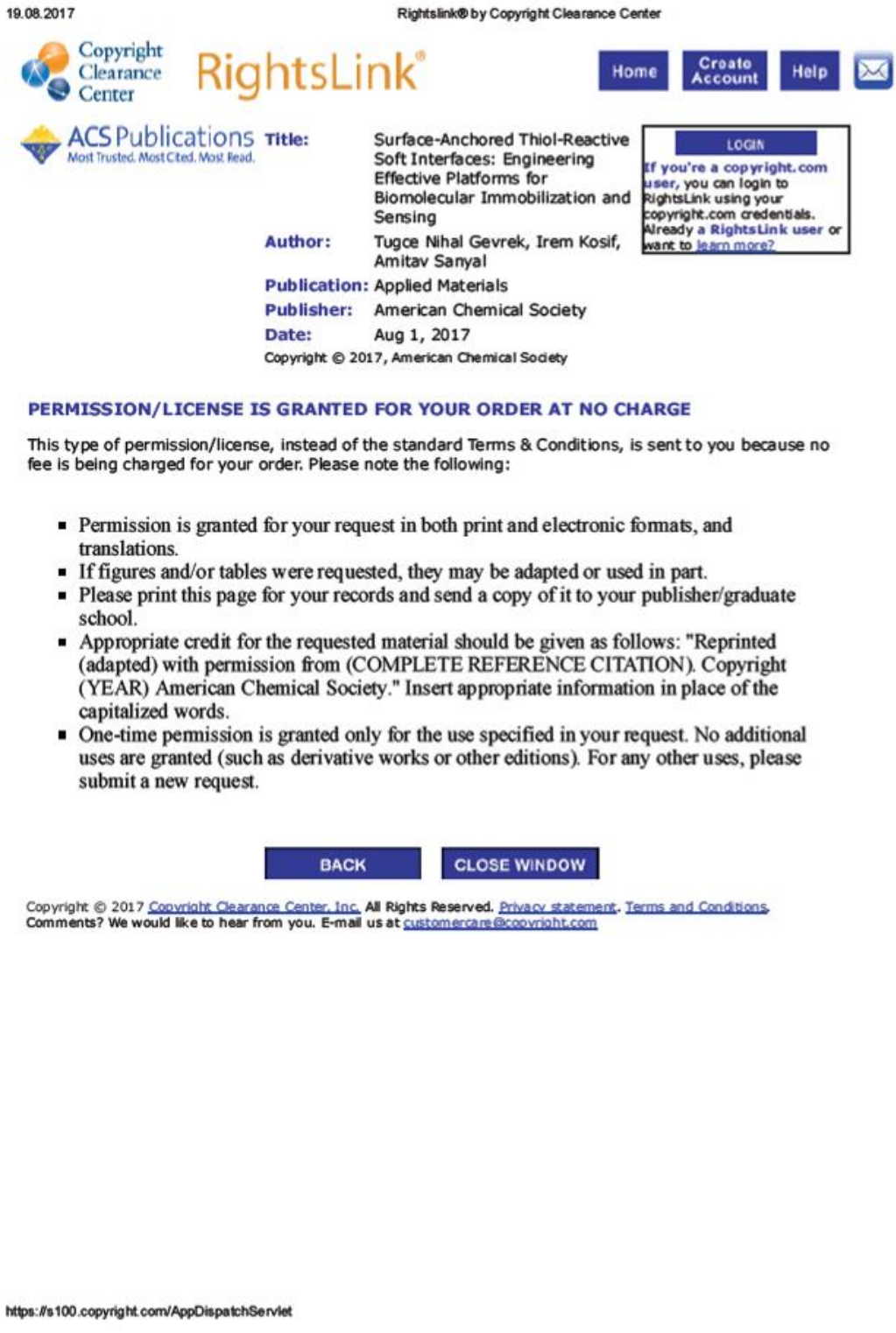

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Author: Tugce Nihal Gevrek, Irem Kosif, Amitav Sanyal

Publication: Applied Materials

Publisher: American Chemical Society

Date: Aug 1, 2017

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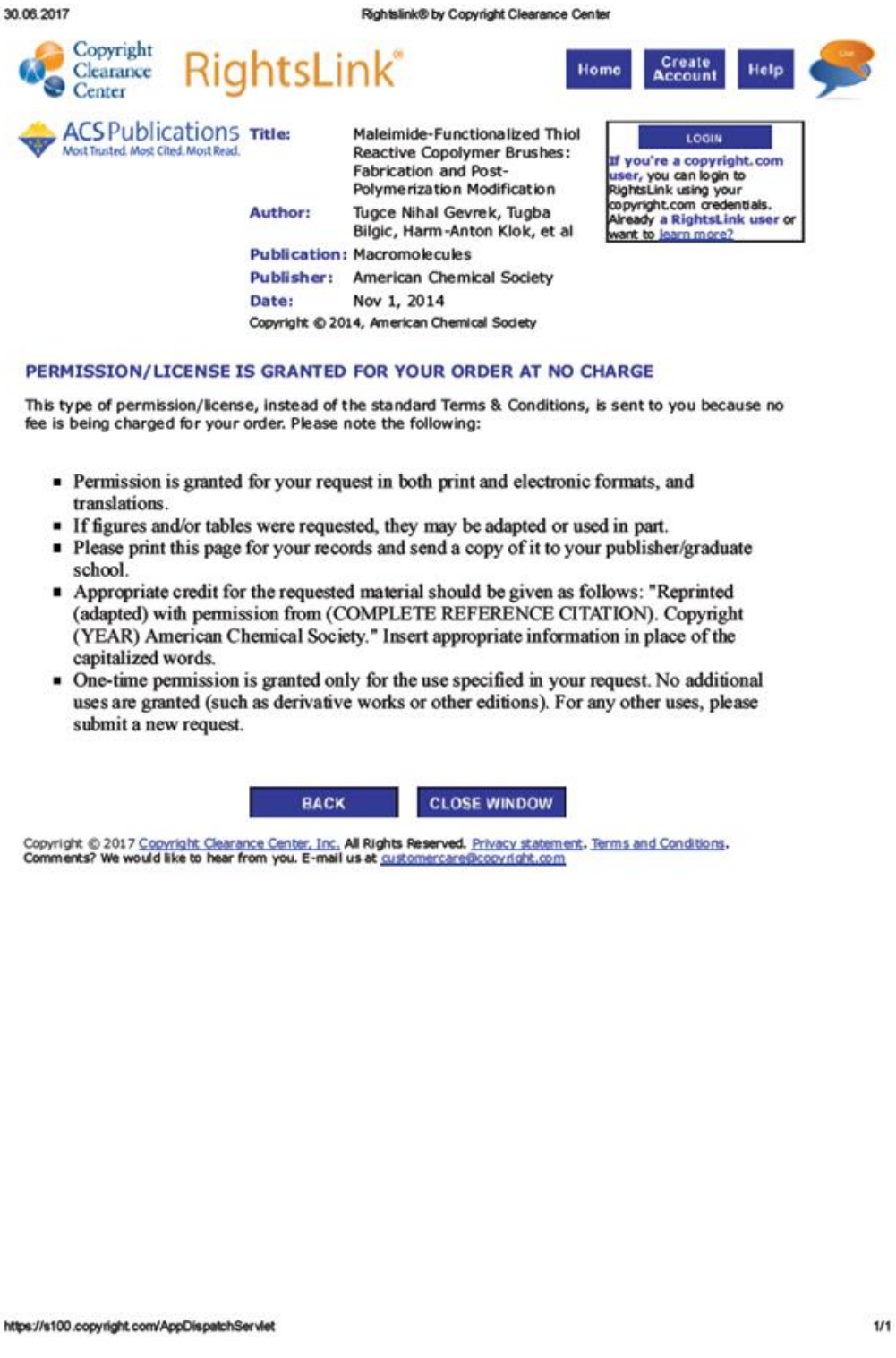

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Author: Tugce Nihal Gevrek, Tugba Bilgic, Harm-Anton Klok, et al

Publication: Macromolecules

Publisher: American Chemical Society

Date: Nov 1, 2014

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