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Biocompatible and bioactive surface modifications for prolonged *in vivo* efficacy

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1.0 Introduction

Generally, medical implants, regardless of their construction materials, will become coated in a layer of non-specific proteins mere seconds after implantation. This adsorbed layer activates an irrevocable host defense mechanism, known as the foreign body reaction, which ultimately results in the production of a fibrous avascular capsule that isolates the device from its target tissues, clogs the pores of membranes and sensors, and prevents drug release from a delivery vehicle. Many devices eventually fail because of their inability to effectively communicate with the surrounding tissues.

It is becoming apparent that sub-cellular interactions at the biological-material interface have macroscopic outcomes. The success of the next generation of implants depends on overcoming limitations in biological communication by selective modification of device surfaces. This review focuses on recent advances in increased implant efficacy through selective surface modifications. Several approaches co-opt solutions found in the natural world to create a surface that mimics the properties of the cell membrane, thereby imparting a pseudo-biological character to synthetic materials. We begin with a short description of the foreign body reaction and then examine the biocompatible and bioactive device surface modification advances from the past five years that hold the potential of increased *in vivo* effectiveness.

2.0 Tissue Reaction to Implanted Devices

An understanding of the foreign body reaction, why and how it occurs, is essential for overcoming current device limitations. Ratner, Anderson, and Williams provide an excellent review of the process summarized briefly below.[1]Within seconds of exposure to body fluids, proteins will rapidly coalesce into an adsorbed protein layer on implant surfaces. These proteins are quickly followed by cells of the immune system where macrophages recognize the adsorbed protein layer and attempt to engulf what is presumed to be a foreign invader. However, because the size scale of most implants is orders-of-magnitude larger than that of the cells themselves, the cells become frustrated and fuse to form foreign-body giant cells (FBGC). The FBGC release chemical signals that attract fibroblasts to the region, ultimately sequestering the object in a thin avascular layer of collagen to wall-off its effects from the rest of the body. The FBGC can often remain within the capsule for the lifetime of the patient and continue to invoke a chronic inflammatory response.

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The crux of the foreign body problem lies in the lack of effective signaling between the host and device, resulting in the initial non-specific adsorption of proteins. Biology, on the other hand, utilizes an ordered choreography of specific molecular markers to control interactions between cells and surrounding tissues. The lack of the appropriate markers on an implant ultimately causes the host to treat the implant surface as a foreign body.

Hypothetically, combating the foreign body response requires two approaches that are ideally utilized in tandem (Figure 1). First, the surface must be treated so as to prevent the non-specific protein coat from forming, in effect making the device “stealthy” by producing a non-fouling surface coating. Secondly, the incident cells of the immune system and cells surrounding the site of implantation must be given the proper cues in the form of attached integrin ligands and cytokines (as well as soluble ones) so appropriate biological-material interactions can occur. A coating of such signaling domains on a substrate creates a bioactive surface with which biology has an inherent communicative understanding. Both of these approaches are biomimetic by design, as the surface coatings recreate the natural functions of the biological microscopic environment.

The next generation of medical implants will likely achieve success when techniques utilizing both non-fouling and bioactive strategies are used cooperatively. These multifunctional coatings on the surface of implants should provide the appropriate cues to direct and control wound healing to minimize the fibrous capsule formation while simultaneously promoting tissue regeneration and material-biological interactions.

There are three general routes to modify a surface to introduce functionality as summarized in Table 1. These techniques can be broadly divided into two categories: covalent attachment or adsorption. Adsorption can be further divided into chemisorptive and physisorptive. Each route has specific advantages and disadvantages as listed, and these will become evident in the following discussions.

3.0 Non-fouling Surface Coatings

Historically, a large variety of molecules and macromolecules have been investigated as surface coatings either to reduce or to negate the adsorption of non-specific proteins onto implant surfaces. Molecules and macromolecules of this type have been known for decades (for example, albumin). Current systems under primary investigation for surface passivation are: polysaccharides, or their derivatives, with hyaluronic acid (HA) being a prime example; hydrophilic self assembled monolayers (SAMs); and especially poly(ethylene glycol) (PEG), with the latter macromolecule receiving a majority of the attention and so its use will be addressed later in additional detail (Figure 2).

3.1 Non-fouling Polysaccharide Surface Coatings and SAMs

Polysaccharide coatings on the surface of an implant operate by creating a hydrophilic hydration shell surrounding the device. This shell prevents the adsorption of serum proteins onto the surface, effectively creating a non-fouling surface. For example, Volný et al., reported creating a coating of hyaluronan on flat 316L stainless steel through a process termed “reactive landing,” a modification of traditional plasma deposition which will be discussed in more detail later.[2] The treated stainless steel surfaces were reported to inhibit the aggregation and activation of platelets. Because stainless steel is a preferred material for cardiovascular stenting applications, this coated surface could be used as a non-thrombogenic stent coating. A similar surface coating created by Morra et al., covalently linked hyaluron to titanium (Ti) surfaces. [3] The resulting surface coating was found to be uniform in coverage and showed a marked decrease in fibroblast binding over control Ti surfaces.

The effects of polysaccharides are not limited to metal surfaces alone. Huang et al. adsorbed a coating of a maltose derivative on poly(dimethylsiloxane) (PDMS) to prevent protein attachment.[4] Also, Gupta et al created a dextran modified surfactant polymer on polycarbonate that prevented platelet accumulation.[5] Finally, Zhu and Marchant created a dendrimeric polymer that preferentially adsorbed to octadecyltrichlorosilane (OTS) and presented a high density of maltose terminated brushes to the surroundings, thereby reducing platelet adhesion.[6]

Gold surfaces coated with thiol terminated SAMs were also capable of reducing platelet adhesion by using a surplus of SAMs terminated in hydrophilic groups, often charged functionalities at neutral pH (NH_3^+ and COO^-Na^+), over those terminated in hydrophobic end groups,[7] and through using equal ratios of acid and amine terminated SAMs to produce a neutral charge density.[8] Wyszogrodzka and Haag investigated the effects of branched SAMs as opposed to linear ones using self assembled polyglycerol dendrons (Figure 2).[9] The generation 1, 2, and 3 polyglycerol dendrons showed good resistance to protein adsorption with the smaller generation performing the best. Moreover, similar activity was observed for the OH and OCH_3 derivatives suggesting that the OH derivatives beside being favorable for their high resistance to nonspecific protein adsorption can also be further functionalization with ligands for specific interactions. More recently, they have shown that linear polyglycerol self assembled monolayers adsorb even less proteins from human plasma than a PEG-modified surface as well as prevent cell adhesion.[10] Overall, SAMs provide a useful platform on which to conduct *in vitro* experiments and are easy to modify and assemble on surfaces, so their use will reoccur throughout the remainder of the review.

A new approach to polysaccharide coatings for protein resistance was reported by Guan et al. Instead of using the intact large polysaccharide, a polysaccharide-like polymer was prepared through two polymerization methods (Figure 3). In the first approach, they prepared a diol and diacidchloride based on the reduced sugar, dulcitol, which then underwent a condensation polymerization to afford the desired polymer.[11] A terminal thiol was then introduced to the polymer so that it could be subsequently chemisorbed onto a gold slide. Surface plasmon resonance (SPR) experiments showed the coatings prevented the adsorption of fibrinogen, a clotting protein. In the second approach, a ring-opening polymerization (ROP) of a 7-member dulcitol lactone derivative was used.[12] The results showed that the lipoic acid modified polymer (P(OMe)CL) and the co-polymer with polycaprolactone (P(OMe)CL-*b*-PCL) deposited on gold (Au) exhibited resistance to fibrinogen and lysozyme binding.

As mentioned earlier, the majority of research investigating stealthy biomimetic surface preparation is focused on the use of PEG to form an anti-fouling brush layer on substrate surfaces. A review of the anti-fouling properties of PEG has been reported; see Heuberger et al.[13] A simplified view of PEG's mechanism of action is that the polymeric surface brushes create: 1) steric repulsion preventing proteins from contacting the surface directly, and 2) form a hydration shell around the substrate, thereby preventing the random adsorption and denaturation of proteins characteristic of the foreign body reaction.[13]

Incredible diversity exists among the methods to modify a surface with PEG, but generally these techniques can be broadly divided into two categories: covalent attachment or adsorption, with the latter being further subdivided into chemisorptive and physisorptive (Table 1). Covalent strategies seek to ligate the PEG functionality to the surface and therefore usually involve step-wise treatments to prepare the substrate to accept a modified PEG containing the appropriate reactivity. Additionally, each surface necessitates a different procedure to introduce the suitable reactive functionalities onto the substrate so that it may become receptive of PEGylation. Alternatively, oligo(ethylene oxide)s can be plasma

deposited on surfaces to create a PEG-like surface. Adsorptive strategies are more numerous and rely on chemisorptive or electrostatic methodologies to preferentially attract the PEG macromolecules to the surface. Because of the multitude of research involving PEG, the covalent and adsorptive strategies are examined separately below.

3.2 Covalent Surface PEGylation

Plasma deposition using tri- and tetra ethylene glycol molecules is perhaps the most prevalent method of preparing a PEG-like surface, and this technique has been well-studied since the early 1990s.[14] In this procedure the surface to be coated is placed into a chamber that is evacuated of air. The low-pressure chamber is then filled with a vapor of the molecule to be added to the surface and an electrode in close proximity is charged, thereby bonding the constituents to the surface. However, due to the high energies involved in the process, short polymers with a few repeats are generally used since the larger versions will decompose before they vaporize. Additionally, the high energies can crosslink longer macromolecules so that the resulting films are not characterized by long, straight polymer chains but something else entirely.[14] Recent research by Cao et al., on plasma deposition of tri- and tetraglymes, has shown that the procedure can be performed on the interior of both metallic and plastic tube structures, and that the coatings effectively resist platelet adherence and activation.[15] This possibility is exciting as it holds the potential to develop anti-thrombotic stenting materials and novel catheter coatings to prevent bacterial colonization. The technique has varied applications given that Salim et al., extended this approach to plasma deposition of tetraglyme to create a non-fouling poly(dimethylsiloxane) (PDMS) surface, a favored material for microfluidic applications.[16]

Recently, the groups of Muir, Tobin, and Textor reported a one-step method for the fabrication of poly(ethylene glycol) PEG-like chemical gradients using continuous wave radio frequency glow discharge plasma polymerization of diethylene glycol dimethyl ether.[17] The adsorption of BSA (pI 4.7, 69 kDa), Lys (pI 11.1, 14.6 kDa), and IgG (pI 7.3,159 kDa) was systematically studied. For all three proteins, adsorption increased across the gradients as the amount of carboxylic acid and hydrocarbon species increased. For samples produced at 30W, residual acid groups are present leading to the adsorption of more Lys than BSA due to a contribution from electrostatic attraction and repulsion, respectively. It remains to be seen how plasma deposition techniques such as these could be adapted to coat more complex device designs, but the field is rapidly moving forward. The mesh-like framework of a stent would provide a challenging geometry on which to practice plasma deposition, especially on a pre-formed device.

In addition to the grafting method of plasma deposition, another common method to PEGylate device surfaces involves multi-step chemical syntheses that takes advantage of surface groups present on the material to form covalent bonds with PEG. The three most common conjugation strategies involve the reaction of an amine with an epoxide, NHS activated functionalities with an amine or thiol, or maleimide with a thiol, as shown in Figure 4. Since the first report describing amide bond formation using N-hydroxysuccinimide ester chemistry,[18] this coupling reaction has become commonplace because it is highly reliable and facile to perform. Moreover the reaction can be performed in aqueous or nonaqueous solutions with a range of small molecule and macromolecular succinimidyl esters. More recently, Kang and Lee reported a method to bond PEG to stainless steel (SS) by first electropolishing, then treating the surface with a piranha solution.[19] This method exposes hydroxyl groups on the SS, which in a solvent environment can be reacted with a trimethoxysilane conjugated to an epoxy, followed by an aminated PEG species, which reacts with the epoxy functionalized SS. As expected, the treated stainless steel surface resists protein fouling. Techniques for titanium (Ti) surface modification have also been developed. Fan et al., reported a surface initiated atom transfer

radical polymerization that coats Ti substrates with PEG oligomers. [20] After reacting overnight, the surfaces were exposed to fresh fibroblast cells twice weekly and cultured for extended periods. The modified surfaces prolonged the time to cell confluence to 11 weeks, as compared to bare Ti where cells were able to attach immediately, a marked improvement in non-fouling outcomes. Finally, Chen et al., created an anti-fouling PDMS surface by the introduction of Si-H groups on the surface of the siloxane, followed by reaction with bis-allyl-PEG for 15 hours.[21] Again, the surface-modified PDMS showed a reduction of protein adhesion. Within the last decade, azide-alkyne [3 + 2] cycloaddition reactions[22] have garnered much attention and success for surface modification.[23] Like the other reactions, this reaction is high yielding, thermodynamically favored, and user-friendly; and such types of coupling reactions have been recently classified as click reactions.[22] For example, Yagci et al., have used this coupling reaction to modify a UV-cured epoxy network with PEG to increase its hydrophilicity.[24]

Hubbell and Klok recently reported controlling the creation of PEG-brush surface coatings on polyethylene (PE) using a two-step reaction strategy that first photobrominated the PE surface and then created the polymer brushes by using a surface-initiated atom transfer radical polymerization (SI-ATRP).[25] They were able to show that the amount of PEGylation of the surface was dependent on the amount of time that the ATRP reaction occurred, with saturation being reached at approximately 10-15 hours after commencement. The developed coatings are quite stable and remain unaffected by *in vivo* implantation in a rat model even after 10 days of incubation. Similarly Zheng et al., has preparednonfouling poly(HPMA) brushes on gold surfaces using a surface-grafted and ATRP strategy.[26] The polmer coated surfaces exhibited high protein resistance to blood plasma and serum as well as prevented fibroblast adhesion. PEG-brushes can also be further functionalized to contain bioactivate groups to create a combination bioactive/cytophobic coating of the type discussed later in this review. Chilkoti and Textor studied SI-ATRP kinetics for the closely related poly(oligoethylene glycol methylmethacrylate) (OEGMA) and determined similar results using a quartz crystal microbalance with dissipation (QCM-D). Their results showed that the degree of surface polymerization was dependent on both the initiator density and the time of reaction, and that these kinetic responses could be predicted through the use of a continuum model. [27] Further wetlab work with this system by Chilkoti and Zauscher in collaboration with Ratner has shown that SI-ATRP can be used to pattern non-fouling regions on surfaces that are resistant to protein and cellular adherence and absorption.[28]

Healy and coworkers grafted interpenetrating networks (IPNs) comprised of PEG and poly(acrylamide) (P(AAm-co-EG)) to create a non-fouling surface on a variety of materials.[29] The polymers are cross-linked to the surface creating a thin “hydrogel” layer on the substrate that resists protein adhesion (Figure 5). These hydrogels are often combined with a cell-interacting motif, a technique that will be discussed further in the dual-therapy biomimetic section later. Granger and van der Mei report using a similar approach to reduce the adhesion, and more importantly, biofilm formation of *Staphylococcus epidermidi* and studied how these coatings and biofilms affected the subsequent binding of osteoblasts in a co-culture experiment.[30] In the presence of adhering *staphylococci*, the PEG-based coatings lost the ability to bind osteoblasts. Similar to this approach, Revzin et al. created PEG based hydrogels on silanized glass substrates using photolithography to create patterned sections that inhibited cell adhesion.[31] A photolithographic approach has been used also by Kim et al., to create microfluidic coatings comprised of PEG to create non-fouling channels within PDMS.[32]

Thierry et al., combined both PEG and HA to create a metal surface coating resistant to platelet binding where short PEG chains were used to bridge the HA to a surface.[33] Specifically, the carboxylic acid terminated PEGs of the PEG-HA coating were coupled,

using carbodiimide chemistry, to surface amines of a plasma treated NiTi alloy surface. The HA-PEG coating displayed increased hydrophilicity and reduced human platelet adhesion compared to bare NiTi surfaces. This method afforded a smooth and highly hydrated surface with the HA fully exposed.

The group at Michigan Molecular Institute created a non-fouling coating from crosslinking of amine-functional polyamidoamine-polyethylene glycol (PAMAM-PEG) multi-arm stars with difunctional PEG crosslinkers.[34] The resulting hydrophilic coatings inhibited salt transport as well as prevented biofouling. Although these coatings were designed for water purification, the teachings may be applicable to the biomedical arena, especially drug delivery from implanted devices.

Larger polymer aggregates or microgels have been used to form a thin PEG hydrogel coating on surfaces, as described by Elbert and coworkers. A surface incubated with this microgel, via single treatment, reduced non-specific cell adhesion to an extent that was much greater than 20 layers of PEG applied via a covalent layer-by-layer method. [35]

As seen in the above approaches, the procedures to create a non-fouling surface can be quite caustic or require specialized and expensive equipment to manufacture the appropriate surface chemistries. Additionally, because the coating procedure is often done in solvent, is UV-activated, and/or utilizes other high energy processes, the techniques are not easily extended to other sensitive molecular species (e.g., growth factors, cytokines, enzymes, proteins) that have clinically important biological effects. These current modification processes may limit the surface coatings that can be utilized to non-bioactive ones, such as creating stealthy surfaces with PEG brushes. As will be shown in the bioactive coating section in this review, these covalent techniques are not readily adaptable to creating coatings of protein fragments or short peptide sequences necessary for an effective bioactive coating (though it can be done). However, the benefits of these covalent strategies are pronounced in the production of non-fouling, stealthy surfaces as the PEG compounds are often irreversibly bound to the surface. Work remains to be done in examining the *in vivo* benefits of such an approach. Most likely, these approaches will prolong the stealthy temporal window, but as shown with the *in vitro* research, eventually these coatings will foul as well. The use of PEG alone as a surface coating can be seen to prolong the inevitable occurrence of biofouling and encapsulation. This certainly would have pronounced benefits for short-term implants (days to weeks), such as drug delivery devices; however, this approach would be suboptimal for implants that would remain *in vivo* for longer periods of time (months to years).

3.3 Chemisorptive Surface PEGylation

Contrary to covalent bonding, the adsorptive strategies in both this section and the next easily lend themselves to creating biomimetic coatings, as the procedures often rely on application through adsorption from an aqueous buffer. Because these coating strategies can be applied in comparatively mild conditions, it becomes somewhat trivial to adapt them to coating biologically sensitive compounds to device surfaces, as we will see later. It is important to keep in mind the bio-conducive properties of these methodologies when reading the next and following sections and to realize that most of these techniques are not solely limited to the creation of anti-fouling surfaces (as was generally the case with the covalent strategies).

PEG-modified alkanethiolate self-assembled monolayers (SAMs) on gold (Au) surfaces have been widely utilized to create an adsorptive non-fouling coating through the formation of a strong S-Au bond.[36] However, these bonds necessitate a defined surface crystalline repeat for complete coverage of the substrate, a physical order generally typified by metallic

surfaces, primarily gold and silver. Additionally, the extent of protein resistance is governed by the surface packing of the PEG functionalities, which is a function of deposition time, chain length, surface chemistry, and solvent used for application. The coating density and orientation of these molecules is therefore somewhat finicky, with Li et al., reporting that a "delivery" mixture of 95 %/5 % ethanol/water works best for protein resistance, and that the traditional application method in 100 % ethanol fails to provide effective resistance.[37] Further examination of mixed SAM monolayers by Arima and Iwata used varying ratios of methyl, hydroxyl, acid, and amine terminated thiol SAMs to coat chromium surfaces and found that eventual cell binding to treated surfaces was primarily a function of surface hydrophobicity. Surfaces displaying primarily methyl functionalities resisted adhesion by a wide variety of human cells.[38] Singh et al., used $(BrC(CH_3)_2COO(CH_2)_{10}S)_2$ to create a chemisorbed layer on gold substrates and subsequently used this as a scaffold on which to build PEG brushes through surface-initiated atom transfer radical polymerization, with the resultant coatings resistant to both protein and cell biofouling.[39] The *in vivo* value of SAMs remains to be fully answered, but initial work looking at the stability of the monolayers in biologic mediums (saline, serum) shows good stability for upwards of 3 weeks with oxidation being the most likely culprit for the observed degradation.[40]

A unique chemisorptive approach was reported by Messersmith and coworkers.[41] Their approach is based upon biomimicry of the mechanism behind the binding of bivalves (mussels) to substrates. Specifically, a catecholic amino acid, L-3,4-dihydroxyphenylalanine (DOPA), was identified as one of the major contributors to the adhesive and cohesive properties of mussel adhesive proteins. In one of their first reports, they linked this amino acid or a decapeptide analogue of a protein found in *Mytilus edulis* adhesive plaques (Ala-Lys-Pro-Ser-Tyr-Hyp-Hyp-Thr-DOPALys) to a monomethoxy-terminated PEG to create a non-fouling coating (Figure 6).[41a] When this polymer was coated on Au or Ti surfaces, it significantly prevented fibroblast attachment for up to two weeks compared to untreated surfaces or control surfaces treated with PEG alone or PEG-Tyr. Based on these results, biomimetic PEG conjugates containing 1, 2, or 3 repeats of DOPA (mPEG-$DOPA_n$) were synthesized, and the mPEG-$DOPA_3$ was found to be the best performing polymer coating.[41b] Time-dependent adsorption measurements showed that it rapidly bound to Ti in an essentially irreversible process forming a coating of about 30 Å. Resistance to serum protein adsorption was achieved on TiO_2 when the polymer was applied under cloud point conditions.

X-ray photoelectron spectroscopy studies revealed that DOPA binds to the TiO_2 surface via its catecholic oxygens. Further analysis of the adhesive mussel proteins revealed an abundance of lysine and 3,4-dihydroxyphenylalanine (DOPA) residues, which led to the design of a synthetic mimic YKYKY (Y = DOPA; Figure 6) by the group.[26] These substrate adhesive residues were then grafted with a PEG functionality and adhered to various surfaces, including titanium. On Ti, the PEGylated surface had both cell and protein repellent behaviors. Recently, Messersmith and Lee have reported a facile and general method for modification of a range of materials including metal oxides, semiconductors, noble metals, and synthetic polymers.[42] Their approach entailed the oxidative polymerization of norepinephrine, a small catecholamine molecule, in alkaline aqueous media to afford a coating which contains hydroxyl groups for subsequent modification.

3.4 Physisorptive Surface PEGylation

In the next section, we focus on methodologies that rely on creating surface coatings through the use of non-covalent interactions. Several of the approaches have selected to use electrostatic, H-bonding, and hydrophobic forces like those found in protein-protein interactions through the use of polypeptide sequences. For example, poly(L-lysine) (PLL)

grafted to poly(ethylene glycol) (PLL-g-PEG) readily and facilely adsorbs on negatively charged surfaces through electrostatic interactions, a material characteristic that includes most metals due to their oxide layers (Figure 7). These molecules orient themselves with the cationic lysine repeats resting on the anionic surface resulting in full hydration of the PEG functionalities and are designed to prevent protein adsorption on the surface. Similar to the results seen earlier, the packing density of the PEG chains is important in determining the amount of surface fouling that occurs. Textor and coworkers created PLL-g-PEG surface coatings on niobium pentoxide-coated silicon wafers that contained varied levels of PLL-g-PEG grafting efficiencies showing that, as expected, the 100 % grafting ratios functioned best in resisting protein adsorption.[43] Voros and coworkers have reported a method to extend this coating technique to hydrophobic PDMS surfaces by the introduction of negatively charged groups on the substrate followed by the introduction of the PLL-g-PEG.[44] Importantly, this amphiphilic coating technique has begun preclinical evaluation in an *in vivo* porcine stenting model. Stainless steel stents were left bare or dip-coated into a PLL-g-PEG solution for a mere 30 seconds before implantation. Using this methodology Billinger et al., reported significant reductions, on the order of 50 %, in the amount of restenosis--re-occlusion of the vessel lumen--in the coated stents 6-weeks post-implantation when compared to the bare metal stent controls.[45] These results are promising as restenosis is a continuing problem in cardiovascular device applications. Unlike the approach used by drug eluting stents, this adsorptive technique does not release a cytotoxic drug that delays the healing process but instead reduces inflammation through the creation of an inert surface that is stable in physiological conditions for upwards of a few weeks; however, the degradation method is still unknown.

The PLL-g-PEG adsorption is stable for *in vivo* applications; nevertheless in low pH or high pH solutions, the surface and lysines lose sufficient charge density to effectively bind, thereby reducing the coating density and leading to surface fouling. It is unlikely that these conditions would occur in a physiological environment, but to circumvent this limitation, Blättler et al. covalently bound PLL-g-PEG to inorganic and polymeric surfaces. They reported sustained protein repellent behavior even after exposure to high ionic buffer strengths and pHs while adsorbed PLL-g-PEG coatings were no longer capable of binding.[46]

Grinstaff and Kenan et al., screened combinatorial peptide libraries by phage display (see reviews of phage display by Smith and Petrenko[47]) to discover peptides that bound strongly to surfaces such as polystyrene (PS), and then used these peptides, coupled to PEG to modify the substrate(Figure 7).[48] The phage display technique selects peptides that have a defined affinity for a particular surface, however, the mechanism for the non-covalent binding (be it a combination of hydrophobic, H-bonding, or electrostatic) is not elucidated to the experimenter until further experiments or modeling are performed after the selection. In regards to the polystyrene binders, the authors reported that the predominant mechanism for the substrate-peptide interaction is through the plurality of hydrophobic residues but that this explanation alone did not completely explain the obtained results. In an experiment to show the pronounced selectivity of the phage technique, these peptides were characterized on both polystyrene and tissue-culture polystyrene with the peptides found to interact with the polystyrene surface on which they were selected 24x more strongly. In biological use, the coating demonstrated a reduction in both bacterial colonization and mammalian cell adhesion to the treated materials when compared to controls of uncoated surfaces, surfaces with the peptide sequence alone without the PEG terminator, or surfaces treated with PEG alone. The combination of the two components is essential for the proper functioning of the coatings.

Based on the above results, they then used phage display to identify a peptide that bound titanium oxide in order to prepare a similar PEGylated-peptide coating.[49] A high affinity Ti-

binding 22-mer peptide containing three repeats of the selected HKH sequence was prepared based on the results of phage display and amino acid substitution experiments. This peptide selectively binds Ti over SS, Au, PS, or SiO_2 and has sub-micromolar binding affinities. QCM-D analysis of the binding behavior at various ionic strengths and pH showed that electrostatic interactions play a major role. Covalent attachment of a PEG3400 to the peptide terminus afforded a PEGylated-peptide. This PEGylated-peptide coating on Ti efficiently blocked the adsorption of fibronectin and significantly reduced the extent of *Staphylococcus aureus* attachment and biofilm formation *in vitro*. Although this PEGylated-peptide coating can reduce non-specific protein adsorption and inhibit bacterial colonization, the limited stability of the coating (≈50% loss within 2 hours) was a potential problem.

Consequently, multivalency was examined as a means to increase coating stability by preparation of PEGylated peptides containing one, two, or four titanium binding peptides.[50] The mono, di, and tetravalent peptides were synthesized using a convergent approach with the branched peptide being synthesized first followed by attachment of the PEG in the final step (Figure 8). The binding affinities increased ten fold on going from the mono to the tetravalent peptide with a final binding affinity of 16.5 nM for the tetravalent peptide. Coating stability was then evaluated in 100% serum. After 2 hours of exposure to serum, only 50% of the monovalent coating remained on the surface, and by the end of 2 weeks, less than 5% remained. The dimeric showed improved stability, with approximately 50% remaining at 2 weeks. In contrast, more than 90% of the tetrameric coating remained after two weeks. As expected the PEGylated tetravalent peptide prevented S. aureus colonization and subsequent biofilm formation. The above approaches are attractive for metal or plastic surfaces since the surfaces can be easily coated by a facile immersion one-step modification process that does not require surface pretreatments or harsh reaction conditions.

The final physisorptive approach uses multilayer films formed by the layer-by-layer (LbL) deposition of polyelectrolyte films of alternating charge. Thin polyelectrolyte films on silica surfaces were prepared by sequential electrostatic deposition of PLL and poly(L-glutamic acid)-grafted PEG (PGA-g-PEG).[51] Multilayers topped with three PLL/PGA-g-PEG bilayers exhibited a 92% reduction in *E. coli* adhesion. In another natural polymer design, hyaluronan/chitosan (HA/CH) polysaccharide multilayers afforded an 80% reduction in the number of adherent *E. coli* on glass surfaces.[52] One advantage to using multilayers is that the structures can be further functionalized. For example, a potent antimicrobial peptide can be added to the mulitlayer and this resulting coating inhibited the growth of several infectious pathogens.[53] Leachable bactericides and silver nanoparticles have also been added to these multilayers to further enhance their antibacterial efficacy.[54]

3.5 Non-fouling Conclusions

Degradation and/or desorption of all these non-covalent coatings is a concern when considering the possibility of prolonged *in vivo* use. Depending on its application, the coating material may need its anti-fouling capabilities for the lifetime of the implant. Covalent strategies certainly can accomplish this requirement, but necessitate attachment methodologies that can be both caustic and time-consuming. Adsorptive strategies are potentially superior because of rapid application, biocompatibility, and adaptability to working with a variety of biologics. However, it is still not certain for how long a surface needs to be passivated to achieve the desired effect. The results of the *in vivo* stent experiment point to the fact that an adsorptive PLL-g-PEG coating can have pronounced clinical effects, though more work needs to be done to determine just how long the coating was active and adhered.[45] Additionally, although the adhesion forces of individual interactions for adsorptive methodologies are weaker, the multivalency of adsorptive coatings produces cumulative adherence forces only 3-4x less than those of covalent

attachments. Separate molecular pull-off experiments on single molecule adsorption by Kenan et al., with their phage display identified peptide-PEG conjugate, and Lee et al., with the DOPA-PEG macromolecule, showed that their respective adsorptive molecules had attachment strengths of 675 pN and 800 pN, respectively.[48,55] The force required to break covalent bonds is reported to be a few nanonewtons, while that to break hydrogen bonds is only a few piconewtons. Admittedly, these adsorptive coatings accomplish their attachment strengths through the use of multiple attachment domains, compared to a single covalent bond, but this highlights how strong the cooperative multivalent attachment strategy can be in the establishment of seemingly irreversible binders with strengths that lie somewhere between ideal covalent and non-covalent bonds. The possibility of oxidation of the coatings also needs to be considered when these coated devices are implanted. The question of whether desorption or oxidation is predominant is yet to be fully examined for *in vivo* usage but more experiments on the subject would be beneficial, as the research to date has focused on increasing the adsorption strength of the peptide with minimal consideration given to the possibility of oxidation or other degradation mechanism.

Covalent strategies, while certainly beneficial for short-term drug delivery applications to create stealthy particles, may not be desirable for use as a long-term implant coating. In fact, initial work with degradable (e.g., poly(caprolactone) (PCL), poly(glycolic acid)/poly(lactic acid) (PGLA)) and non-degradable (e.g., poly(urethane), poly(ethylene terephthalate) (PET)) polymeric stent coatings showed a detrimental effect where the coatings invoked an inflammatory response and promoted thrombosis.[56] Though the size scale of these coatings (100 μm) were orders-of-magnitude beyond those for even the thickest non-fouling coatings described above, the importance of possible deleterious effects from any surface modification cannot be overstated. Most likely the ideal solution to the dilemma will be a short term desorbable or degradable coating that provides an initial window of non-fouling behavior during the body's peak inflammatory response (a coating that ideally lasts weeks but not years). This coating then also contains a second functionality, such as the bioactive moiety identified in the following sections, which would use this time period to catalyze appropriate integration and regeneration of the surrounding tissues.

4.0 Bioactive Surface Coatings

Besides surface passivation, the second approach to modulating cell interaction with an implanted surface is through providing appropriate signals that direct the biological activities at the interface. As mentioned earlier, integrins and other receptors expressed on the external membranes of individual cells are responsible for mediating binding and interactions with the proteins adsorbed on an implant. Integrins are dimeric receptors comprised of α and β subunits that span the cell membrane.[57] Using combinations of the two subunit types, cells are able to recognize these integrins and bind to distinct bioactive regions on structural proteins of the extracellular matrix (ECM) in order to provide mechanical stability to the cytoskeleton as well as to initiate cytoplasmic signaling cascades.

Early work into bioactive surface conjugation sought to graft the entirety of common ECM proteins (e.g., fibronectin, collagen, laminin, and osteopontin) onto substrates to impart the desired-biological characteristics. However, as will be seen later, controlling the conformation and orientation of these grafted and adsorbed proteins as well as obtaining sufficient quantities for an effective coating proves to be difficult. To overcome the low concentration of displayed active sites, the use of short bioactive peptide sequences, as opposed to the protein in its entirety, is preferable. Currently, dozens of short peptide sequences exist that have interactions and known effects on a variety of cell lines. A majority of the known sequences up until 2003 and their effects and target cells was reviewed by Shin et al.[58] Perhaps the most ubiquitous and well studied of these sequences is

the arginine-glycine-aspartic acid (RGD) motif found in most ECM proteins (such as fibronectin, laminin, and vitronectin) where it influences cell adhesion, mobility, proliferation, and survival.[59] The particular mode of action is well understood and RGD has been shown to bind approximately half of the 24 known human integrins.[59c] The problem arises that many of these ECM proteins have multiple active sites, not just RGD, and the presence and presentational spacing among these sites is proving to be just as important as their surface densities. Current research continues to examine the effects of these surface presented short recognition sequences, but research has also recently shifted back to using large ECM proteins or fragments to control cellular interactions. These strategies are somewhat different in their requirements and, as such, will be covered separately in the following sections.

4.1 Large ECM Protein/Fragment Bioactive Coatings

The investigations in this area seek to preserve the tertiary structure of the ECM proteins upon surface adsorption, a problem which hindered earlier developments of these systems. A secondary goal is to ensure that the macromolecules orient themselves so the active site, typically RGD, is presented towards the surrounding biology and away from the material surface. As large ECM proteins exhibit multiple integrin interaction domains, the spacing between these domains affects cell binding and migration events. Full protein presentation often has the best binding capabilities, most likely because the inter-domain spacing is consistent with what the cells encounter *in vivo*.[60] The most common approach to study and control the orientation of ECM proteins is the use of thiol SAMs of differing terminal functionalities to create surface coatings which are variable in their hydrophobic and electrostatic properties. Ratner, Jiang, and coworkers used these surfaces to investigate surface adsorption of complete osteopontin (OPN), which contains RGD among other domains,[61] and a fibronectin (FN) fragment $FNIII_{7\text{-}10}$.[62] This fragment contains both the RGD motif and proline-histidine-serine-arginine-asparagine (PHSRN) that together have been shown to work synergistically to promote cell attachment. Both proteins were allowed to adsorb on gold surfaces containing NH_2 and COOH terminated SAMs, before antibody and cell binding experiments were performed. The results showed that both OPN and $FNIII_{7\text{-}10}$ performed significantly better on the positively charged amine surface because the proteins oriented to present more cell binding domains to their surroundings.

Further research by the same group showed that a coating of collagen I on a polystyrene substrate increased the presentation efficiency of the RGD motif on OPN when compared to uncollagenized controls.[63] Petrie et al., utilized a similar approach to present $FNIII_{7\text{-}10}$ on gold surfaces coated with SAMs; however, the FN was crosslinked to the COOH-SAMs using N-hydroxysuccinimide (NHS) chemistry.[64] Again, the cells adhered more strongly and proliferated more rapidly on this bioactive surface in comparison to surfaces presenting RGD alone or RGD-PHSRN separated by a polyglycine linker epitomizing the importance of the proper spacing and presentation. A similar study conducted by Keselowsky et al., showed comparable results for preosteoblastic cells binding to whole FN coated SAM surfaces.[65] They report that these cells differentiated best and accumulated the most matrix on NH_2 and OH terminated surfaces.

Instead of relying on surface adsorption Vallières et al., bound complete FN to an ammonia plasma treated poly(tetrafluoroethylene) (PTFE) surface using two separate covalent crosslinking strategies.[66] The results from both antibody and cell experiments showed that the RGD moiety was displayed optimally when the FN was bound using glutaric anhydride as opposed to a sulfosuccinimidyl-4-(*p*-maleimidophenyl)butyrate linker (though both performed better than a surface without FN).

A closely related field seeks to immobilize growth factors on the surface of substrates through a variety of covalent and adsorptive mechanisms.[67] The “solid phase” presentation of these growth factors can be just as effective as delivery in solution with regards to directing cellular migration, differentiation, and other biological events. A complete treatment of this area of research would be beyond the scope of this bioactive section as we are highlighting advances in promoting integration with surrounding tissues and cells through integrin interactions. It is, however, important to keep in mind that the surface presentation of growth factors would have significant implications in directing the biological activity and patterning of the adhered cells and that many of the techniques identified here would be applicable to this field as well.

4.2 Covalently Bound Short Signaling Domains

The majority of the attention in bioactive coatings research is focused on using short peptide sequences in ECM proteins that have been identified as ligand domains. These short motifs offer the following advantages compared to whole proteins: they are easier to manufacture, easier to orient on surfaces, easier to pattern, and can be presented in a higher density. Researchers have again utilized covalent, chemisorptive and physisorptive techniques to retain and present the appropriate signaling domains to the adjacent biology. *In vitro* work by both Santiago et al., and Gauvreau and Laroche used a similar approach whereby various peptide fragments were covalently bound to PTFE[68] or poly(caprolactone) (PCL)[69] surfaces that were previously plasma treated with ammonia to produce amine functionalities. These amines were then conjugated to the bioactive domains through carbodiimide chemistry or an N-hydroxysuccinimide–maleimide crosslinker. For example, Santiago et al., grafted three domains from the ECM protein laminin: RGD, YIGSR, and IKVAV onto the aminated PCL surface. Human adipose derived stem cells were introduced to the surface revealing that the IKVAV sequence promoted the best cellular adhesion. Gauvreau and Laroche used their aminated PTFE surface to print different combinations of CRGD, CGRGDS, and CWQPPRARI all derived from FN. The cysteine residue on the N-terminus of the peptides was used to link to the surface-bound maleimide. They found that a combination of GRGDS and WQPPRARI worked best in promoting attachment, spreading, and proliferation of human umbilical vein endothelial cells (HUVECs). Meinhart et al., examined endothelial cell binding to PTFE vascular devices containing RGD coated with a commercially available fibrin gel.[70] Endothelial cells were sensitive to the density of the RGD motif with the densest amounts unexpectedly increasing cell detachment under flow conditions when compared to blank controls. However, an optimal concentration was observed with RGD concentrations that promoted endothelial cell binding and stability under flow, hinting at possible improvements that can be made to current vascular grafts. Morra et al., treated titanium implants to create an acrylic acid layer that was subsequently crosslinked to collagen.[71] The treated titanium surfaces showed increased mesenchymal stem cell adhesion *in vitro*, but more importantly, treated orthopedic Ti screws showed increased *in vivo* osseointegration over plain controls. Osseointegration is an important end point when dealing with load bearing orthopedic implants, thus increased binding indicates that the device is less likely to loosen and require subsequent surgical revision.

To look at changes in gene expression when cells are in the presence of RGD or fibronectin covalently attached to titanium, Abiko et al., first prepared the modified Ti surface using the tresyl chloride method. [72] Once prepared, MC3T3 E1 osteoblastic cells were bound to the Ti surfaces. After 15 days, the cells were analyzed using an Affymetrix gene chip. The gene expression patterns between the two groups were similar except that the gene for the discoidin domain receptor 1 (DDR-1) was upregulated (3-fold) for the Fn sample compared to the RGD sample. DDR-1 has been implicated to play a role in osteoblastic differentiation, and, thus, its activation may be important for proper osseointegration and healing at the

implant site. These results suggest that a single cue, such as RGD, may not be sufficient for optimal results.

Again, all the treatments identified in this section necessitate multi-step preparation to ensure the substrate is properly receptive for crosslinking with the biological moiety. Often, the time requirements or preparatory techniques are prohibitive and do not allow for these procedures to be widely utilized. Adsorptive strategies are therefore preferable to allow for rapid and efficient bioactive coatings, though it is still unclear whether these techniques have enough temporal stability to allow for proper integration, or if this stability is even a necessity.

4.3 Chemisorptive Short Signaling Domains

As before with the non-fouling strategies, a second method for creating bioactive surfaces is to use the inherent strength of the metal-ligand bond typically created through a thiol terminated molecule. Elmengaard et al., created a thiol terminated cyclic RGD conjugate (RGDfK(-beta-mercaptopropionyl)) and exposed it to unmodified Ti implants that were inserted *in vivo* into a canine orthopedic model with a millimeter sized gap between the bone and implant.[73] The implants were left for 4 weeks and the histology and strength of the implant-tissue integration were examined. Coatings of chemisorbed RGD resulted in a doubling of the bone ongrowth, which translated into increased integration between the device and tissue. Additionally, in all cases, the mechanical fixation was increased in the RGD system when compared to the uncoated control implanted in the same animal, showing that this chemisorptive approach can yield *in vivo* success.

4.4 Physisorptive Short Signaling Domains

A unique approach to this technique was undertaken by Reyes et al., involving the creation of collagen triple-helical mimics which were passively adsorbed onto substrates (Figure 9).[74] The synthetic collagen macromolecule was synthesized to have the $\alpha_2\beta_1$ recognition sequence, GFOGER (where O=hydroxyproline) found on collagen I, surrounded by repeats of GPP among other residues. These peptidomimetics imitated collagen both in the formation of a triple-helical structure and through the promotion of cell spreading and adhesion. *In vivo* implantation of an orthopedic Ti rod coated with the peptide demonstrated increased osseointegration and regeneration, even when compared to a coating of native collagen alone, showing the possibility of synthetic materials having increased efficacy over their natural analogs. Fischer et al., reported a method to make surface adsorbed hydrogels using triblock amphiphilic peptide chains of 190 residues in length that were manufactured in bacteria (Figure 9).[75] The end blocks were synthesized using a hydrophobic leucine zipper domain repeat with both blocks adjoining a center hydrophilic block supporting an RGDS sequence. The leucine zipper domains preferentially associated with the surface of the material, and amongst themselves, forming trimeric aggregates that created a gel structure. Surface hydrogel coatings of this material on plastics and glass in serum-free media supported the adhesion and spreading of cells.

Kottke-Marchant, Marchant, and coworkers have developed surfactant polymers that preferentially adsorb onto surfaces due to hydrophobic interactions while still presenting signaling motifs to the surroundings (Figure 9).[76] These comb polymers consist of a poly(vinyl amine) backbone with pendant groups consisting of bioactive hydrophilic domains alternated with perfluorocarbon or alkyl side chains depending on the substrate to be used. Signaling domains with the alkyl version were varied among the combinations of RGD and two heparin sulfate proteoglycan (HSP) binding domains identified from FN: WQPPRARI and SPPRRARVT. The inclusion of the HSP binding domains by themselves on the adsorbed polymer was sufficient to bind cells, but long-term stability was

compromised without the additional inclusion of RGD. The combination of both the HSP domain and RGD resulted in a coating that performed as well as or better than native FN. Coatings for PTFE were accomplished by switching the hydrophobic alkyl side-chain to perfluorocarbons and using only the RGD motif. Experiments with the coating demonstrated reduced endothelial cell attachment compared to FN alone, but the cells showed an increased growth rate. Additionally, the coating performed significantly better than uncoated materials.

Upon recognizing that the RGD sequence is not ideal for cardiovascular applications, since it binds both endothelial cells and platelets[57b] and thus carries the deleterious risk of thrombus formation, the Marchant group investigated the CRRETAWAC sequence.[77] This RRETAWA motif has been shown to bind the $\alpha_5\beta_1$ integrin that is present on the surface of endothelial cells but lacking on platelets. Even though the RRETAWA does possess some affinity for the $\alpha_v\beta_3$ integrin that is present on endothelial cells and platelets, the interaction is significantly weaker than the binding to $\alpha_5\beta_1$ and so platelet adherence will be reduced. The CRRETAWAC sequence was grafted on to poly(vinyl amine) which also possessed the perfluorocarbon chains. When coated onto ePTFE, endothelial cells attached and proliferated. Moreover, the endothelial cells remained attached to the surface under flow, and there was no significant cell loss after 4 hours of 47.8 dynes/cm^2 applied shear stress. At the same time, platelet adhesion was significantly diminished.

Lin et al., created a triblock amphiphilic laminin mimic by creating a peptide that contained branched IKVAV separated from a heparin sulfate binding motif RKRKLERIAR by hydrophobic repeats of aminohexanoic acid.[78] These peptides would adsorb on the surface so as to present both signaling domains to the environment while the alkyl chains remained surface bound due to hydrophobic interactions. The peptide was used to coat both metal and plastic surfaces and was shown to increase endothelial, smooth muscle, epithelial, progenitor, osteoblast, and myoblast cell attachment to both substrates. In a rat PTFE subcutaneous implant model, increased tissue integration over an uncoated control was observed.

A branched chain polypeptide of poly[Lys(DL-Ala)] containing multipled copies of cyclic RGD was reported by Markó et al., for coating polystyrene or glass surfaces.[79] The branched polymer was synthesized by first ring opening the cyclic anhydride of lysine followed by grafting of the Ala oligomers. Finally, chloroacetylation followed by coupling to cyclo[RXDfC] or linear H-RGDfC-NH_2 peptides via formation of a thioether linkage was performed. The resulting polymers supported the attachment and spreading of several cell lines (MDCK, GENC, NE-4C, mesenchymal, astrocyte, and HUVEC).

Mardilovich et al., synthesized a fibronectin mimic that was used to coat mica surfaces.[80] An amphiphilic molecule consisting of an alkyl chain followed by RGD separated from PHSRN by a quintuple repeat of SG was coated onto the surface and compared to a FN coating. Endothelial cells were shown to adhere, spread, and function just as well on the FN-mimic as they did on native FN.

The final adsorptive approach utilized phage display to select sequences that adhere to a variety of surfaces and then cap these peptide sequences with an appropriate signaling domain. Grinstaff, Kenan, and coworkers have utilized the phage display approach to identify peptide sequences with affinity for polymer and metal substrates.[81] The surface binding sequences FFPSSWYSHLGVL and SCSDCLKSVDFIPSSLASS for polystryene (PS) and Ti respectively, were both capped with an RGD moiety and used to coat their respective substrates (Figure 10). As these peptides contain at least one material binding sequence and at least one biologically active sequence, they have been termed Interfacial

Biomaterials.[82] When the PS-RGD peptide was coated on PS, endothelial cells attached and spread significantly compared to untreated PS surfaces or PS surfaces treated with a PS-RGE peptide. The binding was, as expected, integrin dependent. In addition, higher surface concentrations of the PS-RGD resulted in increased viability under a constant apoptotic challenge of TNF-α, demonstrating that these cell–coating interactions are not limited to simple adhesion, but rather are capable of directing important downstream biological events such as cell survival. Next, the Ti-RGD peptide, SCSDCLKSVDFIPSSLASS-SSG-RGDSP, coating on Ti was investigated under both static and flow conditions.[81a] Under static conditions, the coating promoted attachment and increased spreading of HUVEC significantly over uncoated or RGE terminated controls. These results were even more pronounced when examining attachment under flow where the RGD coating produced 3-4x increase in cell attachment when compared to either control surface (Figure 10).

To determine if this approach would translate to coating more complex polymeric material geometries, the team of Truskey, Reichert, Kenan and Grinstaff investigated polyglycolic acid (PGA) meshes.[83] PGA was selected because this biodegradable polymer has been used as a scaffolding material to engineer vascular grafts.[84] However, re-endothelialization of the inner PGA lining of these engineered vascular grafts remains a significant challenge. To address this need, they used phage display to identify a short, selective, and high affinity peptide to PGA, and then coupled the RGD peptide to this sequence in order to mediate interactions between the PGA and endothelial cells. First, quartz crystal microbalance with dissipation monitoring (QCMD) was used to determine the association constant ($K_A = 1 \times 10^7\ M^{-1}$) and surface thickness (~3.5 nm) of this peptide coating on PGA. Endothelial cell binding studies indicated that the peptide coating efficiently mediated adhesion, spreading, and cytoskeletal organization of endothelial cells on PGA in an integrin-dependent manner. The coating promoted a 200% increase in endothelial cell binding to PGA as well as 70-120% increase in cell spreading from 30-60 minutes after plating compared to native PGA.

The above two results were encouraging with the RGD peptide; however, as stated earlier, this tri-peptide is not ideal for cardiovascular applications. Consequently, the RGD group was exchanged for the CRRETAWAC sequence. Specifically, a bifunctional 28-mer peptide consisting of a polystyrene binding domain and a CRRETAWAC domain was synthesized. Results showed that the peptide bound endothelial cells quantitatively as well as the common RGD motif, but unlike RGD, it did not show any preference for platelet adherence. The positive *in vitro* results observed by both Marchant[77] and Grinstaff[85], particularly in regard to endothelial cell over platelet binding, encourage further investigation of these approaches and coating materials.

Similarly, Sanghvi et al., used phage display to select a peptide sequence, THRTSTLDYFVI, that expressed preferential binding to chlorine-doped polypyrrole (PPyCl), a conducting polymer material.[86] This binding sequence was terminated with a motif of GRGDS and coated onto PPyCl allowing cells to adhere in significantly higher numbers than on an uncoated control. Taken as a whole, all of the above studies highlight the fact that these mimetic strategies capture the essential portions of the endogenous proteins and create short, easy to obtain fragments that can work as well as the native molecules they are imitating.

4.5 Bioactive Surface Conclusions

Significant *in vivo* results have been obtained using these methodologies, most notably in regards to improved osseointegration of orthopedic implants. The most common cause of revision surgery in orthopedic implants is due to mechanical loosening of the device within the bone, requiring a new larger device to be friction fit into the bone cavity. Though the

numbers are relatively small, only 10 % of hip prosthesis patients or ≈20,000 patients/year need revisions after the first 10 years, improvements in osseointegration most certainly would reduce these amounts, thereby lessening the burden to the healthcare industry and the patients themselves.[87] Even well-tolerated implants have room for improvements in their integration both in regards to the strength of the tissue-material bond and how quickly full integration is achieved. The *in vivo* work identified above using bioactive coatings shows the promise of realizing these goals. Other areas of medicine would benefit from this approach to achieve increased device integration with the tissue or to promote degradation and remodeling of an implanted polymeric scaffold. The ability to turn on or turn off cell binding could also advance the area, and such systems have been recently reported using light and temperature by Jiang[88] and Haag,[89] respectively. Additional bioactive domains, beyond RGD and the others listed herein, as well as multi-domains are still needed to meet the requirements of selective biological interactions for many of these clinical applications. Questions still remain regarding the nature of the coating type, and whether a more permanent covalent linkage is superior to a shorter-term adsorptive bond. Certainly, there has been success using both techniques and further experiments will need to be done to determine if the solution is application or material specific or both.

5.0 Biomimetic combined approach

Combining both the non-fouling PEG moiety listed in the initial sections with the biomimetic oligopeptide sequences may further improve the outcomes of implanted materials (Figure 11). Initial forays into these mixed coatings have shown some exciting and promising results in creating controlled surfaces for examining specific cell-ligand interactions; however, the *in vivo* application studies accomplished to date have shown mixed results. All of the following approaches selected PEG as the non-fouling portion of the stealthy-bioactive mixture owing to the favorable properties of this well-studied macromolecule. In terms of the surface application, all of the previous surface modification strategies (i.e., covalent, ligand-metal, hydrophobic, electrostatic) discussed earlier are still applicable for applying this dual coating. However, due to the importance that protein and peptide confirmations have in the overall functioning of the final product, these combination approaches are more likely to use the gentler methodologies of adsorption over those of covalent bond formation. By far the most common technique is to create an adsorbed PEG hydrogel or layer on the surface of a substrate followed by bioactivation with an appropriate signaling domain. Exceptions to this approach are provided by covalent or metal-ligand conjugation and PLL-g-PEG methods.

Patel et al., grafted a dual PEG-RGD brush coating onto the surface of poly(methyl methacrylate) (PMMA) using a two step conjugation strategy.[90] First, the PMMA surface was functionalized and conjugated to a PEG. An unreacted functionality on the PEG was then linked to an RGD sequence. The resulting surface possessed the hydration shell typical of the PEG brush coating, but also presented signaling domains that allowed for the controlled adherence and spreading of cells in an attempt to create a keratoprosthetic implant. Tugulu et al., used surface-initiated radical polymerization to prepare PEG-RGD brushes of varied chain length on glass and silicon substrates.[91] After growing the chains on the substrate surface, the PEG was activated and reacted with the RGD containing peptides. HUVEC were cultured on the surfaces and the cells were able to spread and adhere to only those surfaces bioactivated with a peptide sequence containing RGD. In contrast, scrambled sequences (i.e., RDG) provided very little adherence. Groll et al., reacted modified titanium surfaces with a spin-coated dendrimeric star shaped polymer of PEG chains. The star polymers were capped by RGD short peptides introduced in varied amounts.[92] As expected, surfaces with the RGD functionality allowed cell adhesion by a variety of cell types

(fibroblast, osteogenic sarcoma (SaOS), and mesenchymal stem cells) with the amount of adhesion dependent on the PEG:RGD ratio used.

Raynor et al., reported modifying the surface of titanium with SAM-PEG brushes terminated with the aforementioned GFOGER collagen mimetic sequence.[93] The dual-purpose surface provided excellent resistance to non-specific cell and protein adhesion for upwards of one month while the GFOGER sequence promoted the adhesion of mouse osteoblast-like cells whereas the control surface did not.

Tosatti, Textor, and coworkers used their PLL-g-PEG electrostatic adhesion system to attach RGD motifs through a vinyl sulfone–cysteine crosslink.[94] The resultant films were investigated for platelet adhesion and activation in addition to epithelial cell attachment. The authors report increased adherence and spreading of the cells on the PEG-RGD coated surfaces compared to scrambled or plain surfaces. Although the platelets did bind to the RGD domain, little or no activation was observed. This result is not surprising as the RGD signaling domain binds platelets in addition to many other cell types. This nonselective property of the motif limits its potential uses to non-blood contacting devices, and hence would be suboptimal for vascular implants, as discussed earlier.

Building upon the above results, they investigated the apparent conflicting issues of preventing protein adsorption and bacterial adhesion while encouraging cell attachment on Ti oxide surfaces. For these experiments, the investigators again attached the RGD sequence to PLL-g-PEG coating.[95] In the first study, the PLL-g-PEG/PEG-RGD coating was shown to reduce bacterial adhesion in the absence of mammalian cells.[95a] However, the effect of mammalian cells on the anti-bacteria performance of the film could not be determined due to the experimental set-up. To overcome this limitation, van der Mei and Textor recently reported the performance of this coating in a co-culture of both bacterial and mammalian cells. *S. epidermidis* biofilms grown on either PLL-g-PEG or PLL-g-PEG/PEG-RGD were not tightly secured and detached, whereas, as expected, U2OS osteosarcoma cells neither adhered nor spread on PLL-g-PEG coating but adhered and spread on the PLL-g-PEG/PEG-RGD coating.[95b]

Recently, Garcia et al., have reported the covalent attachment of the fibronectin (FN) fragment $FNIII_{7\text{-}10}$ to an oligo(ethylene glycol) brush grafted onto Ti metal for improved osseointegration (Figure 12).[96] The non-fouling poly(OEGMA) brush system was prepared by surface-initiated atom-transfer radical polymerization of poly(oligo(ethylene glycol) methacrylate). The resulting poly(OEGMA) brushes were then activated with 4-nitrophenyl chloroformate and coupled to either $FNIII_{7\text{-}10}$ or RGD to afford a system which exhibits non-fouling characteristics as well as the ability to tune the ligand density. The coating presenting the $FNIII_{7\text{-}10}$, as opposed to RGD or the unmodified titanium, supported significant osteoblastic differentiation of bone marrow stromal cells. This result was a consequence of enhanced $\alpha5\beta1$ integrin binding compared to $\alpha v\beta3$ binding. The $FNIII_{7\text{-}10}$ and RGD coated Ti plugs were then implanted *in vivo* and evaluated in a rat tibia cortical bone model. The $FNIII_{7\text{-}10}$ coating showed enhanced bone tissue formation and functional osseointegration, as determined through histological analysis and mechanical testing, compared to the controls: RGD coating, poly(OEGMA) brushes alone, or bare, untreated Ti metal (Figure 12). While $FNIII_{7\text{-}10}$ showed an increase, RGD did not belying the importance of selecting the proper bioactive motif dependent upon the specific usage application.

The final method to make a biomimetic surface involves the creation of a crosslinked hydrogel on a material surface both to resist the non-specific adsorption of proteins and cells and also to provide appropriate bioactive signals and specific binding sites for desired cell types. Healy and coworkers have conducted studies using a covalently attached

interpenetrating network (IPN) of poly(acrylamide-*co*-ethylene glycol/acrylic acid) (p(AAm-*co*-EG/AAc)) modified to display bioactive signaling domains. In earlier work, five different RGD containing sequences were incorporated into an IPN hydrogel and compared to binding sequences taken from heparin or collagen. The results showed that all the RGD modified IPNs functioned far better in promoting osteoblast adhesion and subsequent mineralization than the collagen or heparin sequences.[97] An additional study used a 15 residue sequence from bone sialoprotein (BSP) to coat titanium surfaces to determine the effects on osteoblast function.[98] The IPN-BSP combination promoted the osteoblast phenotype and supported better mineralization compared to plain Ti. Additionally, these experiments studied how varying the amount of signaling ligand present in the PEG hydrogel affected cell interactions and demonstrated that a critical RGD concentration was necessary to support the osteoblastic phenotype. The BSP-IPN system was coated onto a murine orthopedic implant and implanted into the animal with the device subsequently examined for osseointegration and mechanical stability over time.[99] Surprisingly, the results of this study showed that the IPN-BSP system did not perform any better *in vivo* than the uncoated control systems that were used. Additionally, the results determined that the surface topography of the implant proved to be more important for fixation strength than the coating. One rationale for the observed results was that the IPN-surface conjugation was too permanent and did not allow for essential matrix remodeling by the impinging cells, thereby preventing the formation of an environment conducive to their growth and adhesion. The system was redesigned to overcome this limitation by incorporating an engineered matrix metalloprotease cleavable sequence to allow for the degradation of the IPN dependent on cellular enzymatic factors.[100] Implantation of these new coatings into the murine orthopedic model produced results similar to those observed with the stable IPN coating where the coating did not significantly affect the outcome. While not a direct dual approach, Grinstaff et al., has also reported a similar degradation technique whereby the phage-selected peptide coatings have an incorporated enzyme recognition sequence that allows for therapeutic release from a surface, a formulation that can be easily incorporated into the peptide toolkit.[101]

Taken together, these studies indicate that coatings can provide favorable substrates on which to culture various cell types and examine exactly what morphologic changes can be correlated to specific bioactive motifs. These surface coatings function well in this role as they resist the non-specific biofouling of serum protein while only presenting the designated signaling domains to the cells in the desired contexts. So far, this combined non-fouling/ bioactive technique has shown both significant and non-significant *in vivo* improvement in function and the outcomes most likely depend on the temporal degradation, bioactive sequence used, presentation density, etc on a per-application basis. However, research into these synergistic device coatings is still in its infancy. The *in vivo* studies highlighted earlier using only PEG or RGD alone have shown beneficial outcomes revealing the potential that this dual therapy may yet hold. A need, therefore, exists for the creation of *in vitro* models that more closely match the environments found *in vivo* or earlier preclinical screening of these coatings, since, as to date, there appears to be little correlation between the observed *in* vitro and *in vivo* results owing to the more complicated physiological environment that these coatings face upon implantation.

6.0 Conclusions and Future Directions

A wide range of methodologies exist to create biomimetic materials for implantation. This review describes both the covalent and adsorptive strategies for device modification and their respective pros and cons. The steps towards creating a non-fouling surface for *in vivo* use have been reasonably successful; however, the longevity of such devices is still unknown and requires further investigation. What is certain is that even under carefully

controlled *in vitro* conditions, non-fouling coatings lose their efficacy after a few months at best—a process that will be more rapidly completed *in vivo*. We believe it is critical that future devices use this initial window of anti-fouling capability to properly integrate with the adjacent tissues so that by the time the stealthy coatings lose their potency the device will already be partially or fully integrated. Unquestionably, herein lie the majority of the challenges remaining to be solved. The solutions are just beginning to become apparent, and significant work remains to be done. For example, what is the optimal performance lifetime of the non-fouling coating, and closely related to that, what attachment methodology (covalent, adsorptive) should be used to match this time scale? What is the mechanism for degradation and removal from the surface (oxidation, desorption, yet unknown mechanism)? How much does performance lifetime vary with implantation site, surgical procedure, and usage application?

At the forefront of the biomimetic approach is the exploration of combined non-fouling and bioactive surfaces. The few experiments that have used this technique *in vivo* found mixed results. Experiments still need to be done involving this nascent synergistic approach, and these initial results must be interpreted cautiously. Optimistically, the results suggest that other strategies to afford non-fouling surfaces which are enzymatically degradable, or loosely bound through non-covalent methodologies, might ensure that the cells remodel up to and properly integrate with the device surface, replacing the synthetic coatings with natural ECM components. However, a number of research questions remain: What are the optimal concentrations and ratios of the “non-fouling’ and “signaling” components? What are the best signaling domains? Should fragments, or small peptides be used? What density and spacing is required on the surface? What additional role should growth factors play, and what concentrations and types would be ideal for which applications? How can the surface be experimentally coated using procedures that are mild and non-damaging to biologics? Finally, is presentational patterning of the functionalities important, and if so, how can geometries and spacings that are important for beneficial outcomes be elucidated?

From a commercialization perspective, there exists another set of questions and challenges. Manufacturing issues including synthesis, coating approach (covalent vs. adsorptive), sterilization, packaging, and shelf-life are all important. Small peptides can now be prepared on the kilogram scale cost-effectively, and many proteins can be expressed in cell culture for commercial applications. With a coating identified, does the final device come pre-coated or is the coating applied at the time of surgery? Both approaches offer benefits and limitations. For example, for application at the point-of-care, the coating can be stored in a powder form, dissolved and applied to the device, but is the application of the coating reproducible between the clinicians prior to the operation? As for the other alternative, if the device is precoated will the biologic remain active after sterilization or three-years of shelf-storage? Again, a number of scientific opportunities are present for investigation that are critical for the ultimate use of these coatings in a clinical setting.

Continued development of new device coating materials, procedures, and subsequent evaluation with *in vitro* and *in vivo* models will provide the necessary knowledge to move this field forward. This is critical since the opportunities are significant if successful. The majority of research has focused on orthopedic applications with some encouraging leads. Areas such as cardiology, neurology, and ophthalmology are under-explored. In the cardiovascular area, for example, coatings that would improve small vascular graft integration while preventing occlusion would have a significant clinical impact. Likewise, moving from a drug eluting stent to a pro-healing stent that integrates appropriately with both endothelial cells and smooth muscle while preventing platelet and leukocyte adhesion would be of interest. Such ideas are already being explored. Implants that would guide neural growth over large separation distances for spinal cord injuries are needed as current

procedures are less than ideal. In ophthalmology, synthetic corneas that integrate appropriately with host tissue affording long-term viability with transparency and refractive power are desired.

Many of the approaches described herein use biomimicry for the modification of a device surface so that it effectively communicates with the surrounding cells and proteins. This signaling interaction at the tissue-implant interface is missing in currently used medical implants, reducing the ultimate efficacy of these devices by forcing biology to treat it as a foreign entity. It is only after overcoming this limitation that the next generation of devices may realize their full potential and achieve optimal performance with prolonged *in vivo* efficacy.

The purpose of this review is to stimulate discussions, highlight recent approaches and success, and provide further motivation for the design, synthesis, and development of new coatings for medical implants that properly integrate with the biological surroundings to give native-like performance.

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9.0 Biographies

Mark W. Grinstaff was born in 1965 and attended Occidental college where he received his undergraduate A.B. degree in Chemistry with Honors. Mark received his Ph.D. from the University of Illinois under the mentorship of Professor Kenneth S. Suslick and was an NIH postdoctoral fellow at the California Institute of Technology with Professor Harry B. Gray. He is currently a Professor of Biomedical Engineering and Chemistry at Boston University and a College of Engineering Distinguished Faculty Fellow. Mark's awards include the ACS Nobel Laureate Signature Award, NSF Career Award, Pew Scholar in the Biomedical Sciences, Camille Dreyfus Teacher-Scholar, Alfred P. Sloan Research Fellowship, and the Edward M. Kennedy Award for Health Care Innovation. His current research activities involve the synthesis of new macromolecules and materials for biomedical applications such as tissue engineering, drug delivery, and wound repair with the goal of relating molecular structure and properties to function and performance.

Steven R. Meyers was born in 1980 and attended Duke University where he earned a B.S. degree in Biomedical Engineering with distinction and a B.A. in Computer Science. He then attended Boston University and worked on the Interfacial Biomaterials research project graduating in 2008. Dr. Meyers is currently working at the American Chemical Society managing the society's international activities.

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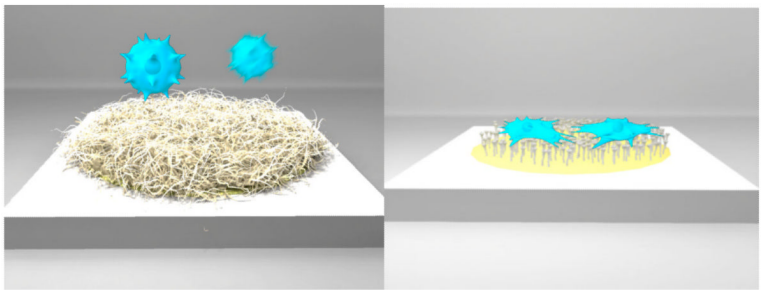

Figure 1.
Schematic representation of the two main traditional approaches for combating the foreign body response. (Left) Either the device is made stealthy through the use of a polymer (off-white) to avoid the development of an adsorbed protein coating thereby hiding it from the cells of the body (blue), (Right) or the device is modified with short bioactive peptide motifs (off-white) to imitate the signaling properties of the cell membrane. Future approaches will likely need to use a combination approach to achieve successful *in vivo* efficacy (Not to scale).

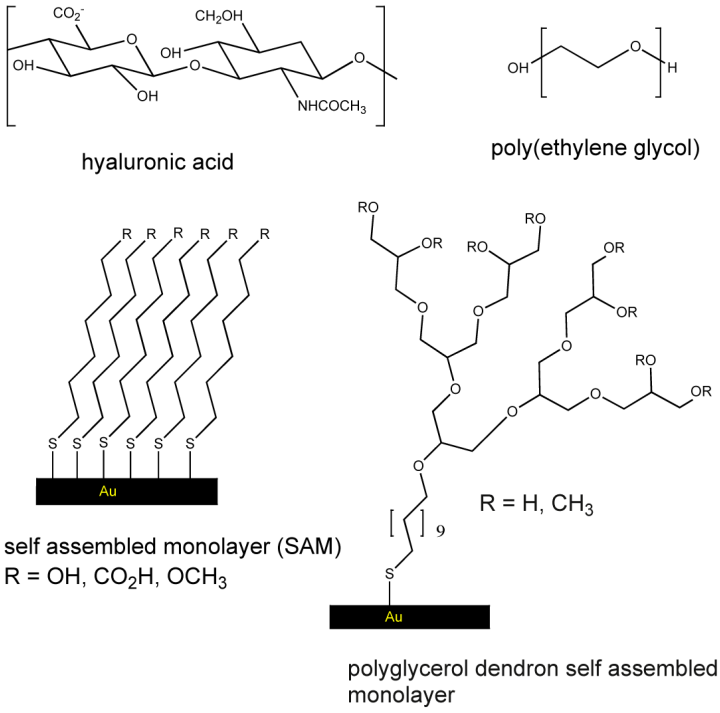

Figure 2.
Molecules under investigation for the creation of a non-fouling surface. Poly(ethylene glycol) (PEG) has received a majority of the attention.

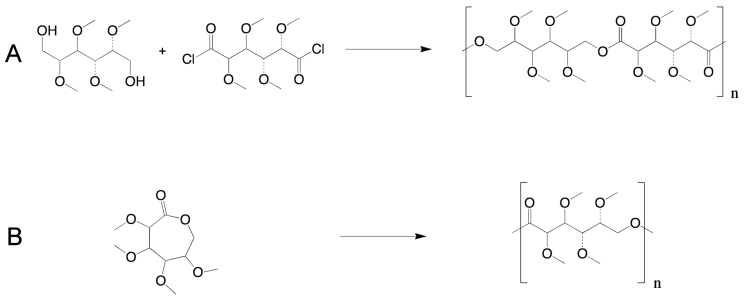

Figure 3.
Two synthetic routes to prepare polysaccaride-like polymers: (A) step-growth polymerization,[9] and (B) living ring-opening polymerization.[10]

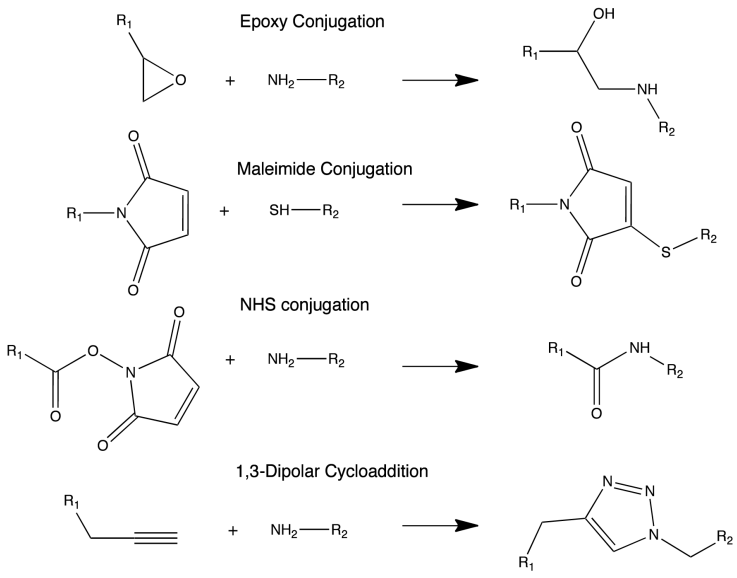

Figure 4.
Typical ligation reactions for modifying surfaces. The maleimide and N-hydroxysuccinimide (NHS) conjugation methods are used extensively by researchers identified throughout the remainder of this review.

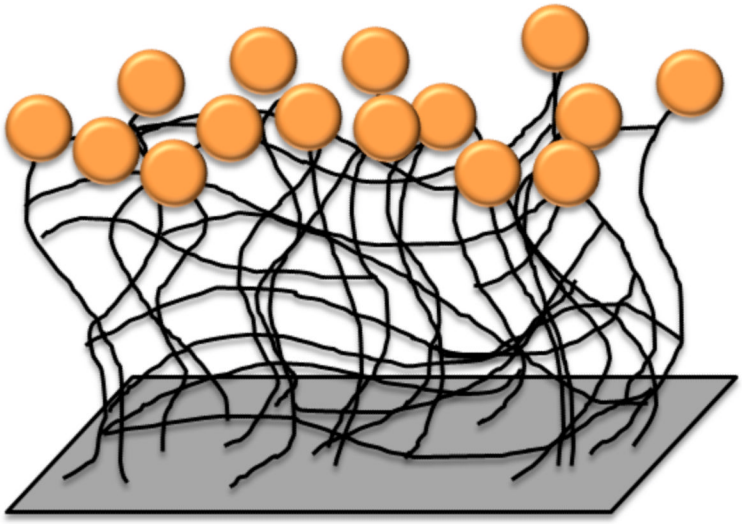

Figure 5.
Schematic of an interpenetrating network (IPN) grafted to a surface. The IPN can itself be comprised of PEG to form an anti-fouling layer, or the IPN can provide a scaffold for subsequent pendant modification of PEG with a bioactive modality (shown in orange).

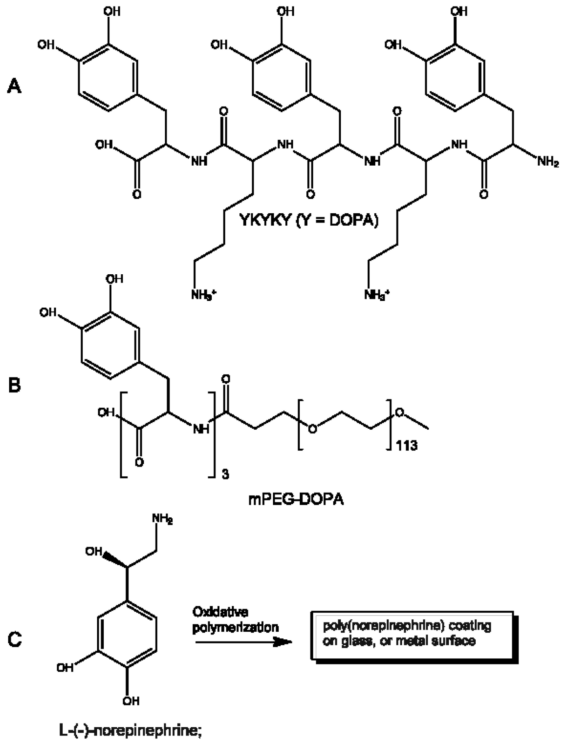

Figure 6.
Chemical structures of three catecol based coatings prepared by Messersmith et al.,: (A) DOPA peptide,[38a] (B) PEGylated DOPA,[38b] and (C) poly(norepinephrine). [39]

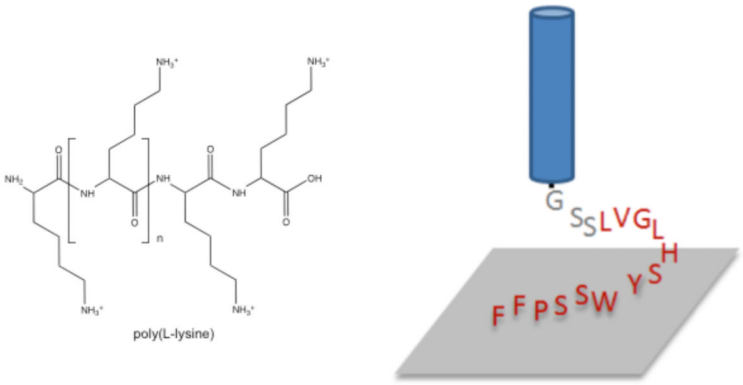

Figure 7.
(left) Poly(L-lysine) and (right) phage identified residue sequence (FFPSSWYSHLGVL) schematic with short SSG linker and PEG (blue) functionality.[45]

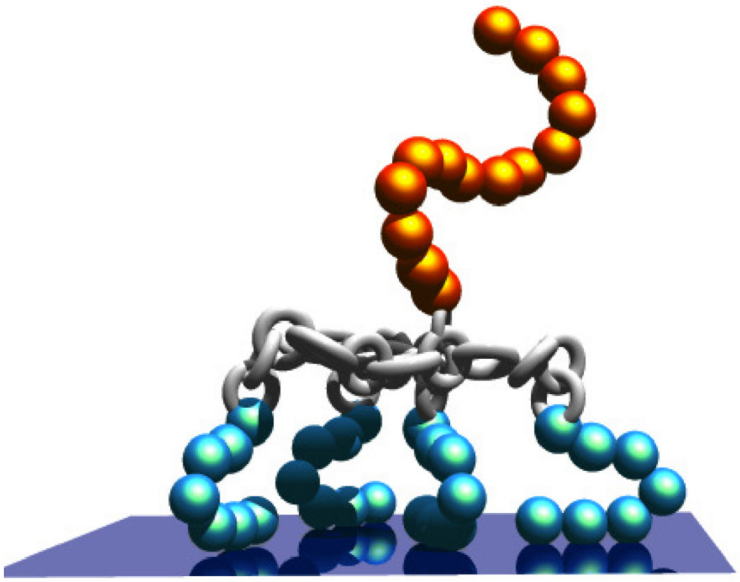

Figure 8.
Structure of a multivalent PEGylated peptide coating used to prevent *S. aureus* colonization and biofilm formation. The top PEG domain is shown in gold, the linker in silver, and the multivalent titatnium binding peptides in blue.[47]

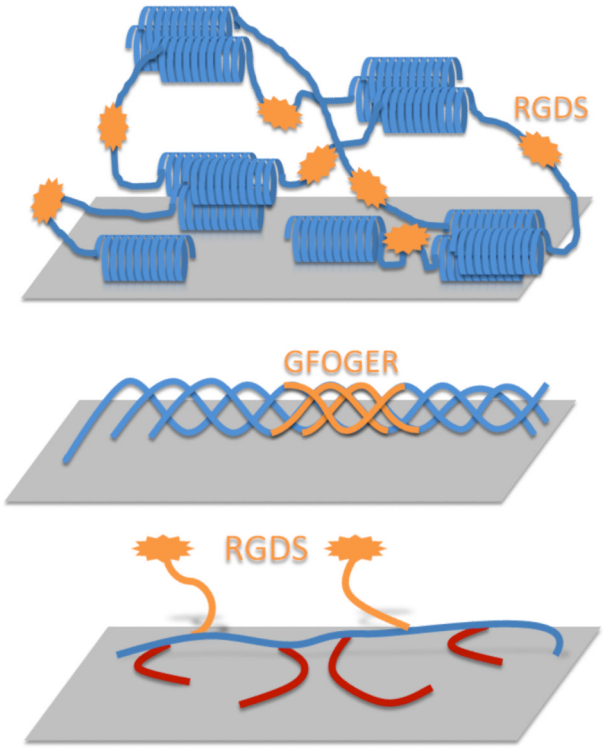

Figure 9.
(Top) Amphiphilic triblock of Fischer et al. which forms a hydrogel due to helical preferential interactions,[72] (Middle) collagen mimic of Reyes et al bound to a surface,[71] and (Bottom) Marchant and co-worker's fluorosurfactant polymer containing a vinyl backbone and pendant groups containing the bioactive peptide (orange) and perfluorocarbons (red).[73]

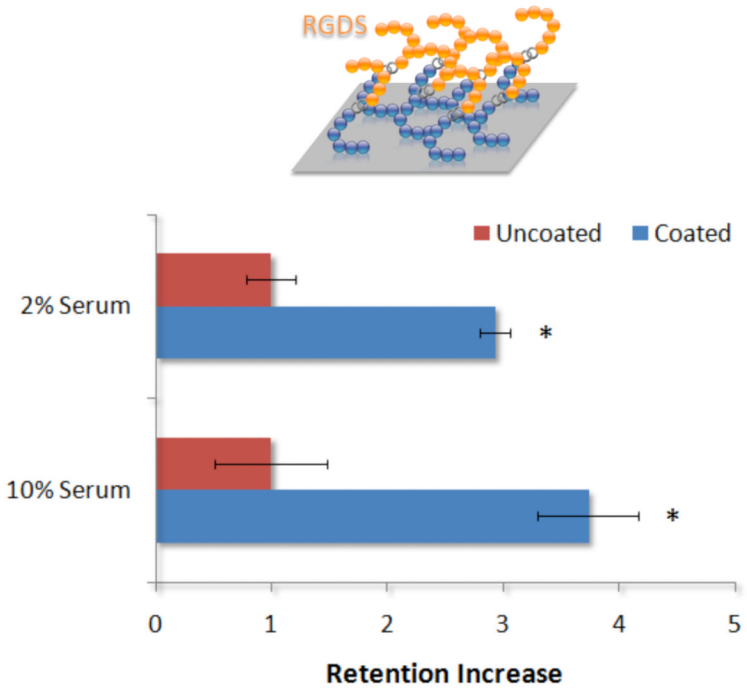

Figure 10.
(Top) schematic of Grinstaff and Kenan's adsorptive peptide with a material adsorptive domain (blue), a short linker (grey), and a bioactive motif (orange); (Bottom) cellular retention increase provided on a Ti surface under dynamic flow conditions. (* $p < 0.0001$; n=4; error bars are SD). [78a]

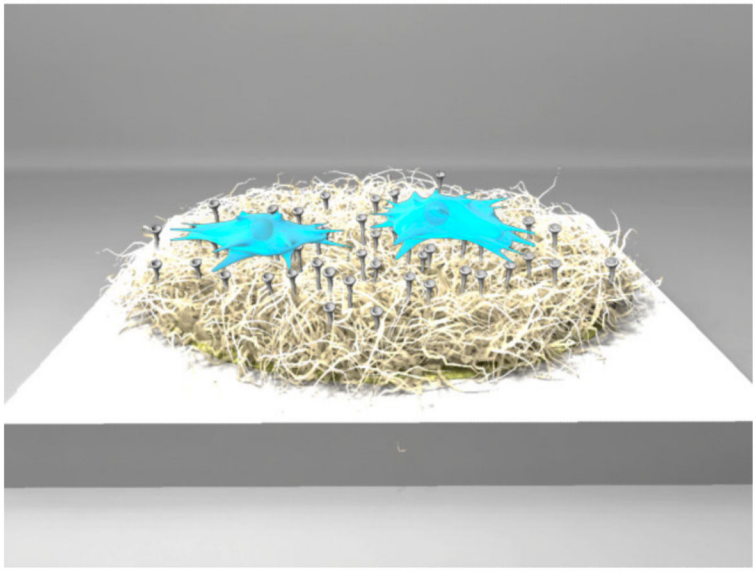

Figure 11.
Combined biomimetic approach whereby non-specific protein and cell adhesion is prevented with an anti-fouling layer (off white) allowing only the selected bioactive signaling motifs (dark gray) to be displayed on the surface for interacting with the cells (blue).

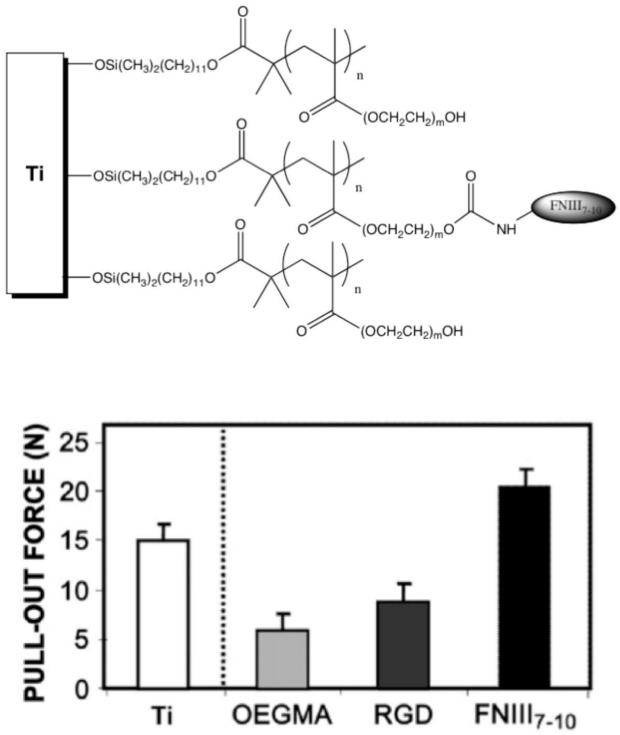

Figure 12.
(top) Chemical stucture of the poly(OEGMA) brushes on Ti metal where some of the brushes possess the $FNIII_{7-10}$ fragement at the terminus. (bottom) Pull-out force measurements of the control (Ti) and functionalized Ti (OEGMA, RGD, & $FNIII_{7-10}$) metal implants from the tibia to assess for functional osseointegration. The $FNIII_{7-10}$ coating had a significantly higher pull-out force than the other groups. Reprinted with permission from Reference 96.

Table 1

Comparison of the characteristics for surface modification techniques.

	Covalent	Chemisorptive	Physiosorptive
Interaction Type	Covalent bonds	Thiol-Metal/weak molecular interactions	Electrostatic, hydrophobic interactions
Examples	Plasma deposition, covalent crosslinking	Self assembled monolayers (SAMS)	Peptides, fluorosurfactants
Interaction Strength	Strong nN forces; non-desorbable	Weak pN forces for single interaction; plurality of interactions builds strength	Weak pN forces for single interaction; plurality of interactions builds strength
Coating Procedure	Step-wise chemical preparations or high-energy processing	Desorption from solution (Dip coating)	Desorption from solution (Dip coating)
Surfaces that can be Modified	Most surfaces with prior preparation and known processing; different techniques for each surface	Usually metal surfaces with crystallographic structure to get complete coating	Metals with oxide layers (electrostatic); polymers (hydrophobic)
Use with Delicate Biologics	Usually not, treatments are too caustic or too much energy	Yes, adsorption from aqueous solution	Yes, adsorption from aqueous solution
Degradation Mechanism	Little to no desorption; most likely oxidation, hydrolysis, and other unknown mechanisms	Desorption a concern in addition to hydrolysis, oxidation, and other mechanisms	Desorption a concern in addition to hydrolysis, oxidation, and other mechanisms
Non-fouling/Bioactive Versatility	Mostly useful for the creation of non-fouling coatings, generally not for bioactives	Non-fouling and bioactive	Non-fouling and bioactive