FETUIN-A ADSORPTION TO POLYDIMETHYLSILOXANE

FETUIN-A ADSORPTION ON TUNABLE POLYDIMETHYLSILOXANE AND SUBSEQUENT MACROPHAGE RESPONSE

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A Thesis Submitted to the School of Graduate Studies in Partial Fulfillment of the

Requirements for the Degree Master of Applied Sciences

MASTER OF APPLIED SCIENCE (2022) McMaster University

(SCHOOL OF BIOMEDICAL ENGINEERING) Hamilton, Ontario

TITLE: Fetuin-A Adsorption on Tunable Polydimethylsiloxane and Subsequent Macrophage Response

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NUMBER OF PAGES: xiv, 95

Abstract

To date, protein adsorption is an unavoidable response to implanted biomaterials. When proteins interact with materials, adverse biological events such as thrombus formation and inflammation, can occur and challenge device efficacy. Protein adsorption is influenced by various material and surface properties which can be modified in efforts to alter the protein-material interactions and the subsequent cellular response. There is a need for simple modifications of commonly used biomaterials and the effect of these modifications on (1) material properties (2) proteins and (3) cells is important to study.

In this work, the effect of modifying polydimethylsiloxane (PDMS) and its interactions with fetuin-A are studied for potential immunomodulatory properties. PDMS modifications are achieved by altering the ratio of PDMS formulations to simply and effectively control elastic modulus, and by coating PDMS with polydopamine (PDA), a molecule commonly used as a bioglue. Surface characterization confirmed that altering the PDMS formulation changed the elastic modulus without affecting surface wetting properties. Minor changes in surface roughness via atomic force microscopy and surface chemistry via x-ray photoelectron spectroscopy were detected on some samples, and the deposition of PDA was confirmed. Protein adsorption studies provided quantitative and qualitative data on fetuin-A interactions. It was determined that fetuin-A adsorption was influenced by the PDMS formulations, and that the preferential adsorption changed when adsorbed from a competitive environment.

Following modification of samples with adsorbed fetuin-A, the inflammatory effects of fetuin-A were investigated by measuring the concentration of pro- and anti-inflammatory cytokines in response to modified and unmodified samples. Data suggests that elastic modulus influences cytokine secretion at certain timepoints, a result of varied protein adsorption amounts and orientations in response to material stiffness. The addition of a PDA layer demonstrated the potentially cytokine mitigating effect of PDA cell interactions and protein immobilization when compared to unmodified PDMS samples.

Acknowledgments

This thesis would not be complete without acknowledging all the people who have provided me with endless guidance and support throughout this process.

To my supervisor Dr. Kyla Sask, thank you for taking a chance on me and trusting me in the early stages of the lab group. Your guidance has transformed me as a researcher and I'm so grateful I could learn from your expertise. Thank you for creating such a welcoming and encouraging lab environment. You lead with a passion for research and endless compassion, and I couldn't be luckier to have been under your supervision. I would also like the thank my supervisory committee Kathryn Grandfield and Todd Hoare for sharing an interest in my project and their insight and suggestions along the way. Thank you to the School of Biomedical Engineering and the Department of Materials Science and Engineering for being so welcoming and supportive.

To all members of our research group, Jie, Siyuan, Alessandra, and Linan, thank you for your collaboration and support throughout. Jie, thank you for your support and kindness from the very beginning, I feel so lucky to have been able to share the whole journey with another student. Alessandra, I couldn't be more grateful for your positivity and encouragement when times were tough, as well as your drive for research excellence. To all past and present summer and thesis students, it was a pleasure to work alongside you and I look forward to seeing where your research takes you. Thank you to great friends from the Grandfield Research Group who were always quick to offer support inside and

outside the lab. Thank you to members of the Sheardown lab for sharing both space and expertise with our group.

To my friends who are family, I couldn't have imagined that choosing to attend McMaster in 2014 would bring me the best people that I know. Without you this journey would have been impossible, and I can't be thankful enough for the love, support, and laughs that helped me get through these few years. Finally, to my parents. Every step of the way was made easier by your love, support, and every answered phone call. Your belief in me has made what felt impossible attainable, and I can't thank you enough for being my biggest supporters. I couldn't have done it without you.

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List of Abbreviations

AFM	Atomic force microscopy
AHSG	Alpha-2-Heremans-Schmid glycoprotein
APTES	3-Aminopropyl triethoxysilane
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CCK-8	Colourimetric cell counting kit-8
DMEM	Dulbecco's modified eagle medium
EDC	1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
HA	Hydroxyapatite
HSA	Human serum albumin
IFN-γ	Interferon-gamma
IgG	Immunoglobulin G
IL-10	Interleukin-10
IL-12	Interleukin-12
IL-6	Interleukin-6
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LPS	Lipopolysaccharide
PBS	Phosphate buffered saline
PDA	Polydopamine
PDMS	Polydimethylsiloxane
PEG	Polyethylene glycol
QCM	Quartz crystal microbalance
RMS	Root mean square
Sa	Average roughness
SAA	Succinic acid anhydride
SDS	Sodium dodecyl sulfate
SFG	Sum frequency generation
Sq	Root mean square roughness
TGF-β	Transforming growth factor-beta
TMB	3,3',5,5'-Tetramethylbenzidine
TNF-α	Tumor necrosis factor-alpha
XPS	X-ray photoelectron spectroscopy

Declaration of Academic Achievement

The research completed in this thesis was performed by Chelsea Miller with few exceptions. Joseph Deering assisted with compression testing and constructed the stress-strain curves for each sample, while XPS analysis was performed at the BioInterfaces Institute by Zeynel Bayindir. My supervisor, Dr. Kyla Sask, has provided guidance throughout the presented research project.

1. Introduction

1.1 Research Motivation

Protein adsorption is an initial response to biomaterials and a precursor to thrombus formation and inflammation, which can result in significant challenges to device efficacy. Protein-material interactions are crucial in dictating how the body responds to biomaterials (1). The surface chemistry (2) and mechanical properties (3) of biomaterials are known to influence protein adsorption, and modification of these properties provides an opportunity to influence the protein interactions that precede and contribute to adverse biological events. Changing the mechanical properties is of interest as it appears to be a simple and permanent method to alter protein adsorption. The adsorbed protein layer has been shown to sense the surface modulus of the biomaterial and in response adsorbs in different amounts and orientations, inducing altered cellular responses (3),(4). The field of mechanobiology is growing, and there are numerous existing studies exploring the cell-material relationship. Given the importance of protein adsorption in determining device efficacy, there are many questions remaining about material-protein interactions and how they in turn impact the cellular response.

Fetuin-A, or alpha-2-Heremans-Schmid glycoprotein (AHSG), is a plasma protein that upon varying biomaterial stiffness, adsorbed in greater than expected levels from serum (3). Though implicated in a number of processes such as mineralization, insulin receptor inhibition, and inflammation (5), relatively little is known about the function of fetuin-A compared to other plasma proteins. With the rise of mass spectrometry based proteomic techniques, fetuin-A has appeared more often in literature where it adsorbs in high

amounts to biomaterials (6–8), and has been suggested as a potential surface modifier (3). Though many of these studies note the qualitative adsorption of fetuin-A in their proteomic results, it is not often a protein of main interest. In studies investigating fetuin-A properties, researchers have reported both pro- and anti- inflammatory effects (9). Thus, there are gaps in the literature on quantitative and further qualitative studies investigating the adsorption of fetuin-A as it responds to material properties, and subsequent cell effects. In particular, the response of macrophages to fetuin-A modified surfaces will provide insight on the inflammatory response and the potential application of fetuin-A as a surface modifier.

1.2 Objectives

1.2.1 Characterize PDMS substrates of tunable elastic modulus

As proposed by Palchesko et al., Sylgard 184 and Sylgard 527 can be combined in various ratios to create polydimethylsiloxane (PDMS) formulations of varying elastic modulus, without impacting other material properties (10). Here, our aim is to characterize the synthesized PDMS and make notes of any changes in wetting properties, surface morphology, or chemistry. The characterized samples will be used in further studies to investigate the effects of varying modulus on protein adsorption and macrophage response.

1.2.2 Investigate adsorption of fetuin-A from buffer and complex solutions

Fetuin-A is a plasma protein that appears throughout the literature, though not much is known about its adsorption behaviour. As a protein with a variety of functions and a promising biomarker – as well as a proposed surface modifier – we aim to determine

fetuin-A's adsorption behaviour to a range of PDMS samples from buffer and in a competitive environment (plasma). This information is critical to better understand the levels of fetuin-A adsorbed to PDMS alone as well as in response to surface stiffness. Additionally, attaching fetuin-A to the PDMS surface via PDA will allow investigation of its ability to immobilize fetuin-A for potential surface modifications. This analysis is essential to then relate the protein-material interactions to macrophage response – to fully understand the cell-protein-material interactions.

1.2.3 Explore macrophage response to fetuin-A and substrates of varying stiffnesses

Due to fetuin-A's potential for anti- or pro-inflammatory effects, our next goal is to investigate the response of macrophages to fetuin-A adsorbed samples, as well as unmodified samples, of varying stiffnesses and with PDA as a modifier. In these studies, we will determine the number of cells on each sample and the concentration of cytokines released in the cell supernatant. This information will elucidate the initial macrophage polarization in response to fetuin-A as well as samples of varying elastic modulus.

2. Background

2.1 Protein-Material Interactions

2.1.1 Protein Adsorption

In the biomedical field, protein adsorption is a key component of many immunological assays and biosensors, but is also critical in determining the cellular response to

implanted biomaterials (11). As an initial biological event upon material implantation, the adsorbed proteins interact directly with both the material surface and cells – where adsorbed proteins communicate surface characteristics to the surrounding environment (12). In this way, the adsorption of proteins marks the beginning of a complex chain of events that control the relative biocompatibility of biomaterials in particular applications (13). The uncontrolled adsorption of proteins to these implants can lead to undesirable thrombosis, immune response and device failure (11), demonstrating the need for a thorough understanding of protein adsorption, including the composition and characteristics of proteins at the material interface. The order of events following biomaterial implantation is summarized in Figure 1.

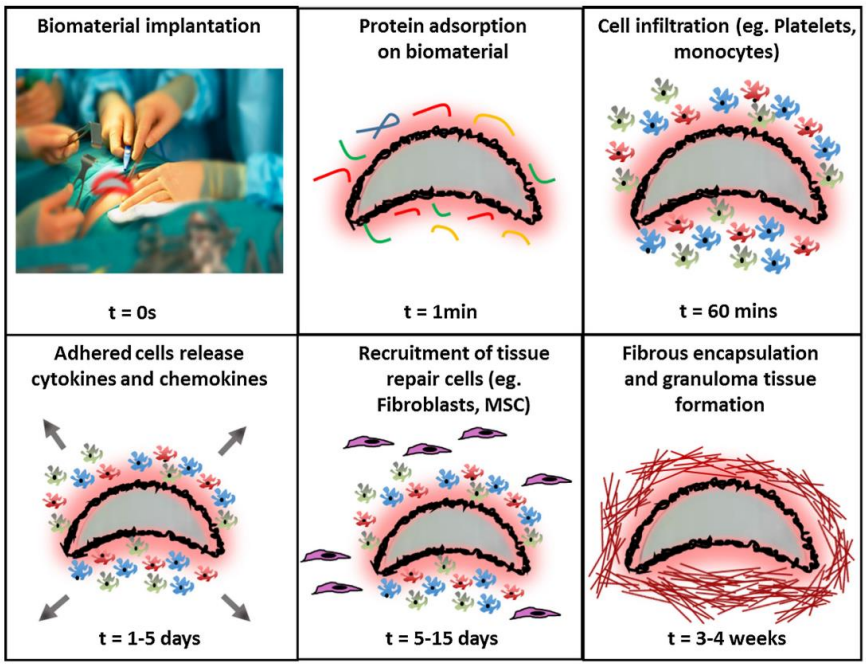


Figure 1. Order of events following implantation of inert biomaterials. Without modification from (14) under Creative Commons CC-BY-NC-ND license.

Adsorbed protein composition does not stay constant, instead over time some adsorbed proteins are replaced by others, a phenomenon known as the Vroman effect. Smaller proteins diffuse more quickly and thus often may make up a large component of the adsorbed protein layer initially, while the increased surface area of larger proteins means they can bind more strongly to the surface (15). During adsorption from blood, the initial protein layer is composed of proteins of a higher concentration and is eventually replaced by less abundant proteins that have greater surface activity (16). Due to this phenomenon, the concentrations of protein in blood are not representative of the adsorbed protein layer. Proteins with relatively low concentrations in blood have been observed to adsorb to biomaterials in high amounts, such as fetuin-A (6) and apolipoprotein-A1 (17).

A relationship between protein concentration and adsorbed surface density is often observed. At low concentrations the surface is covered more slowly, giving proteins the opportunity to pack themselves more efficiently by changing orientation and conformation – covering more surface area than when the concentration is high and the surface is covered very quickly (18). As the density of adsorbed proteins increases, the orientation of proteins goes from being controlled by the protein-material interactions to protein-protein interactions, as a result of charged domains of the proteins interacting with each other (15), as visualized in Figure 2.

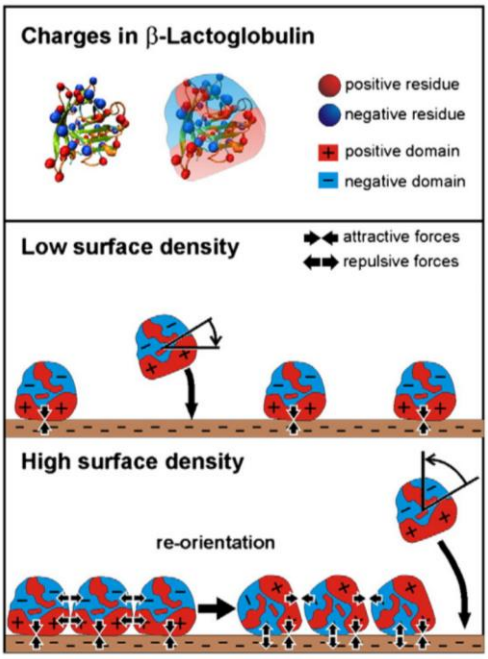


Figure 2. Protein adsorption behaviour of β-Lactoglobulin when surface density is low and material-protein interactions rule orientation, versus under high-density conditions where orientation is influenced by protein-protein interactions as well. With permission from (15).

Protein adsorption is a spontaneous process driven by the increase in entropy from the release of water molecules at the surface of the material (19). Environmental conditions such as temperature and pH can impact adsorption due to an increase in diffusivity to the surface and the relationship between the charge of the protein and the material surface, respectively (15). Ouberai et al. found that changes in surface polarity influenced the adsorbed protein layer of some proteins more than others – based on individual protein packing and orientation in response to surface polarity (20). In addition to polar and non-polar interactions, protein adsorption is impacted by the conformational degrees of freedom of the material surface (21).

As a general rule, proteins bind most strongly to non-polar, high surface tension, and charged surfaces. However, altering the charge of the biomaterial is not a simple solution to deter or promote adsorption. As proteins are complex molecules with domains of different charges, it is not uncommon to see a protein with a net negative charge adsorb to a negatively charged surface, as the orientation of the protein shifts to expose oppositely charged domains (15,22). Hydrophilic surfaces appear to present an opportunity to decrease protein adsorption, as it is less energetically favourable to remove water molecules from a hydrophilic surface. Wilson et al. noted specifically that when proteins adsorb to hydrophilic surfaces the interaction is driven by conformational changes in the proteins and charge interactions (23). A notable exception is glycoproteins, which have hydrophobic areas shielded by glycans, and are found to adsorb in higher amounts to hydrophilic surfaces (15). Studies of non-glycosylated proteins have shown high adsorption to hydrophilic materials (24), some even more so than hydrophobic materials (25), indicating that protein adsorption is driven by more than just the hydrophobicity of surfaces. It has long been suggested that the weaker affinity between proteins and hydrophilic surfaces may not result in less protein adsorption, but instead weaker adsorption (26). Evidence also exists that as adsorption occurs, an irreversibly bound layer is adsorbed to the material surface, while a reversibly adsorbed layer is formed on top (11). As a result, rinsing of hydrophilic materials may remove weakly bound protein.

The one certainty in this field of research is that proteins will adsorb. The amount, orientation, or conformation of the adsorbed protein, however, all rely on a multitude of factors. As Vogler outlines in his 2012 opinion paper, when investigating protein

adsorption the sheer number of experimental variables including but not limited to: protein size, shape, concentration, and charge, alongside the wide range of material surface properties – results in decades of research with evolving experimental design and contradictory results (13). The Langmuir adsorption isotherm is commonly used to model protein adsorption and predict binding affinity. However, Latour finds that the requirements for the application of this method are often unmet – resulting in an inaccurate picture of the protein adsorption process which ignores protein-protein interactions (27). Despite decades of research in the area of protein adsorption, there are still many questions left to be answered, especially when predicting adsorption behaviour and protein layer composition and structure.

2.1.2 Strategies to Control Protein Interactions

When investigating the protein-material interface it is important to consider the effects of both the protein and the material itself. The adsorbed protein layer composition (28) (29), orientation, conformation (30), and total quantity are a result of the material properties of the underlying substrate. Much research has gone into strategies to control protein adsorption, often by reducing non-specific adsorption in general, or targeting the adsorption of proteins of interest. One such strategy attempts to reduce the adsorption of proteins involved in thrombus formation such as fibrinogen and vitronectin, in favour of antithrombin which inhibits coagulation. Unlike some drug delivery or tissue scaffolding applications where controlled degradation of the material is advantageous – there is a need for simple and permanent methods to control protein adsorption. The physical, mechanical, chemical, and biological properties of the material surface should all be

considered when designing materials for implantation in the body as these properties can impact the adsorbed protein layers and thus the subsequent immune and inflammatory response (12).

The “Whitesides design rules” were coined in response to the need for protein resistant surfaces. Ostuni et al. found that material-water interactions were key in determining adsorption. Specifically surfaces that supress protein adsorption should be designed to: be hydrophilic, include hydrogen bond acceptors, lack hydrogen bond donors, and lack a net charge (31). A common surface modification to increase the hydrophilicity of the material is achieved by grafting polyethylene glycol (PEG) to the material surface. Multiple studies have found that upon modification with the hydrophilic PEG there is a decrease in adsorbed proteins (32–34). The increased resistance to protein adsorption is due to the layer of water molecules bound to the hydrophilic PEG surface (35), influencing the thermodynamics of replacing the water molecules with protein. The density of the grafted PEG impacts the amount of protein adsorbed to the material surface, and in a study that used vibrational sum frequency generation (SFG) to probe the surface, it was determined that PEG density influences fibrinogen orientation (33). The modification of gold surfaces by hydrophobic and hydrophilic self assembled monolayers demonstrated a change in the amount and conformation of adsorbed albumin, measured by quartz crystal microbalance (QCM), suggesting that the changing protein conformation relies on the protein-biomaterial interactions (36). These results demonstrate the impact of surface chemistry and surface modifications on not only total protein adsorption, but on protein orientation, and conformation as well.

Chemical and biological modifications are often used with the goal of selective protein adsorption, or decreased protein adsorption in general. Heparin, a biomolecule that inhibits crucial coagulation factors, is a commonly used surface modifier for catheters and other blood contacting devices to prevent thrombus formation locally, in order to decrease the need for anticoagulant regiments (37). Jahangir et al. observed reduced fibrinogen, fibronectin, and vitronectin adsorption on surfaces modified with fluorinated surface modifying macromolecules, and subsequently less platelet adhesion and activation (38). Massa et al. found that the fibrinogen adsorbed to fluorinated polyurethane seemed to predict platelet adhesion and activation, whereas polyurethane without the fluorinated macromolecules did not direct fibrinogen adsorption and thus showed different platelet adhesion and activation (39). Proteins themselves can be used alongside modifications to induce certain reactions, as discussed above, or occasionally as surface modifiers themselves. Proteins used for surface modification include albumin to improve blood compatibility(40), fibrinogen to induce an inflammatory response (41), and collagen to encourage cell adhesion (42).

Biomaterial mechanical properties have also demonstrated significant impacts on protein adsorption. For polymer samples, the mechanical properties may be described by the elastic modulus, or polymer chain flexibility. Liu et al. studied hydrophobic polymethacrylates with side chains that varied in length and found less bovine serum albumin (BSA) adsorbed to softer polymers with more chain flexibility, a result attributed to the higher activation entropy barrier presented by flexible chains and soft surfaces. They found that these mechanical properties were able to supersede the effect of

increasing hydrophilicity on the polymethacrylates (25). Vyner at al. hypothesized that the observed difference in smooth muscle cell proliferation on acrylated star polymers of various crosslink densities was due to the effect of the chain mobility on protein adsorption (43). They found that fibronectin adsorbed preferentially to surfaces with lower crosslink density, and that the lower crosslinked surface adsorbed more protein overall, while immunoglobulin G (IgG) preferred surfaces with a greater crosslink density.

Also of interest when considering protein-biomaterial interactions is the nanotopography of the surface. When studying cell-material interactions it is common to look at microscale topographies of similar size to cells themselves. Since proteins are in the nanometer range, nanopatterns that may impact protein adsorption are an area of interest for exploration. The adsorption of proteins to nanopatterned surfaces does not always correlate with an increase in surface area (44), as may be expected. Surface curvature also impacts the behaviour of proteins, which is important to consider for protein adsorption to topographical features and nanoparticles. Nanoparticles are used for a number of applications, among them drug delivery, diagnostics, and to model interactions (45). Understanding the adsorbed protein layer on nanoparticles, coined the ‘protein corona’, is crucial as it controls the aggregation and clearance of nanoparticles in the body (46). Vertegel et al. found that the structure and function of adsorbed lysozyme to silica nanoparticles varied with nanoparticle size. The smaller nanoparticles had less interaction with the protein due to the curvature, and the adsorbed protein exhibited structure and function more similar to the native state of lysozyme (47).

Though most proof-of-concept studies will alter only one property at a time, it can be important to consider the effects of combining these modifications. Schulte et al. discovered that through tailoring the topography and stiffness of their substrate, they inadvertently reduced the anti-adhesive properties of PEG (48). Mertgen et al. prepared a thorough review detailing the current literature surrounding combined modification strategies (49).

2.1.3 Downstream Implications of Protein Adsorption

Coagulation, platelet activation, thrombus formation, inflammation, and immune response, are among the many biological events that follow protein adsorption (16), and occur at varying time periods as shown in Figure 3. As protein adsorption occurs immediately, the downstream implications of biological fluid contact and implantation are guided by the adsorbed protein layers (50).

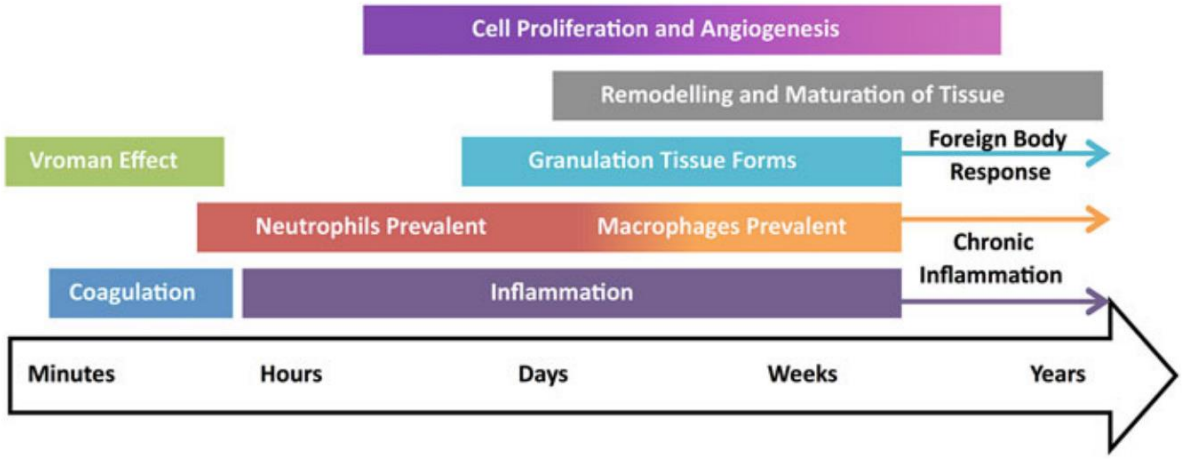


Figure 3. Host response to biomaterial implantation over time. With permission from (51).

Thrombus formation on the biomaterial surface is considered to be a primary limitation of blood contacting medical devices (16). With the buildup of clotting on the surface, device fouling can occur, along with the risk of the clot causing embolism and travelling elsewhere in the body. Due to this risk, patients who receive stents, artificial heart valves, and a number of other medical devices are often on lifelong anticoagulant and antiplatelet regiments (37). Thrombus formation begins with the adsorption of plasma proteins such as fibrinogen, vitronectin, and von Willebrand factor which influence platelet adhesion and activation. Additional platelets are recruited to the surface when the activated platelets release agonists into the environment causing inflammation and activation of the complement system. Over time platelet-fibrin aggregates are formed, resulting in a clot (52). Materials that can prevent the adsorption of platelet activating proteins may reduce the incidence of thrombus formation and provide the field of biomaterials an important tool in designing robust medical devices.

The inflammatory response can be initiated by injury, but is guided by the chemicals released in response to the injury site or to an implanted biomaterial (53). The coagulation enzymes produced after protein adsorption and platelet activation, activates the complement system and induces a local inflammatory response (37). If the subsequent neutrophils and macrophages are unable to eliminate the implanted material, foreign body giant cells are formed and release cytokines which can encourage or discourage further inflammation (12). Macrophages and neutrophils are immune cells and as with other molecules involved in downstream processes, they interact with the adsorbed protein layers, rather than the biomaterial surface. Strategies such as pre-adsorption of albumin to

biomaterial surfaces have shown to reduce phagocytosis and thus reduce immune response (54), providing evidence of successful modification of materials with proteins.

2.2 Fetuin-A

2.2.1 Structure & Function

Fetuin-A, or alpha-2-Heremans Schmid glycoprotein (AHSG), is a plasma protein most frequently expressed in the liver, but is present in all major organs during fetal development (55). The concentration of fetuin-A not only varies person to person, but also throughout the lifecycle, as visualized in Figure 4 . Plasma fetuin-A concentration during fetal development is around 3 mg/mL and subsequently decreases after birth and through childhood before reaching approximately 0.4-0.6 mg/mL in adult plasma (56,57).

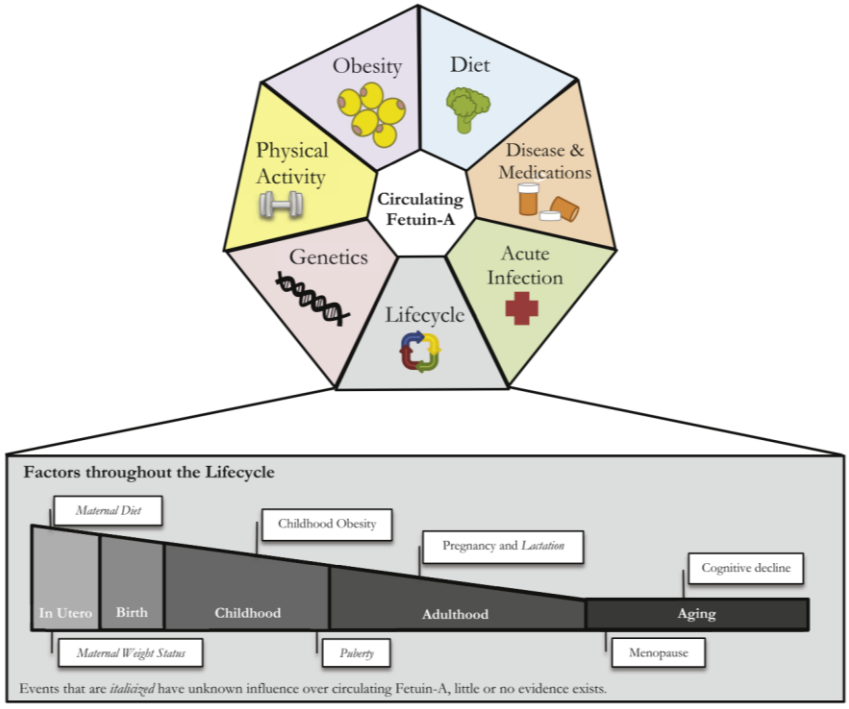


Figure 4. Factors influencing circulating fetuin-A concentrations, and associated trends throughout the lifecycle. With permission from (9).

Fetuin-A begins as a single chain which undergoes post-translational modifications such as glycosylation, phosphorylation, and sulfation (5). The final protein contains three domains over two polypeptide chains, connected by disulfide bonds (9), as visualized in Figure 5. Research has demonstrated the variability in the general structure of human, recombinant human, and bovine fetuin-A – which seems to stem mainly from post-translational modifications (58). It is estimated that 20% of fetuin-A is phosphorylated in at least one site (59), and phosphorylation appears to be essential for activation of fetuin-A (9).

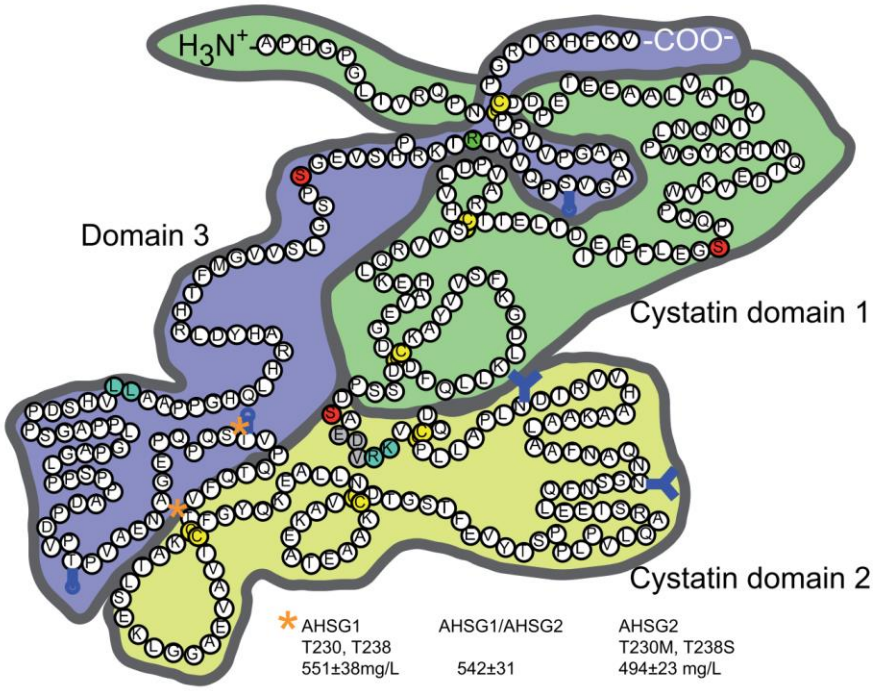


Figure 5. General structure of human fetuin-A, disulfide (C-C) bridges are shown in yellow, Ser-phosphorylation sites (S) in red, N-glycosylation sites and Ser/Thr O-glycosylation sites labelled with blue symbols, cleavage sites in green, and allelic variant labelled with orange asterisks. With permission from (60).

A well researched function of fetuin-A is the regulation of mineralization, due to its many roles in calcium metabolism (55). Fetuin-A acts as an inhibitor of ectopic calcification (61) and aids in the formation of calciprotein particles alongside serum proteins and calcium phosphate, which can be cleared by macrophages (62). The risk of calcification is increased if fetuin-A levels in serum are low under inflammatory conditions (5). Low serum levels of fetuin-A are associated with coronary artery calcification (63), and stroke in haemodialysis patients (64)

As calcium phosphate crystals are considered pro-inflammatory, and often result in undesirable calcification, higher serum levels of fetuin-A seem desirable (65). However, high fetuin-A levels have been noted in patients with Type 2 Diabetes, leading to the discovery that fetuin-A is involved in decreased insulin sensitivity (9). Fetuin-A binds to the insulin receptor and is able to transduce extracellular conditions into the cell. As a result, serum levels of fetuin-A seem to correlate with insulin resistance and a lower tolerance to glucose (66). Given the many supposed functions, it is no surprise that fetuin-A has been proposed as a biomarker for a number of disease states, including postmenopausal osteoporosis (67), multiple sclerosis (68), acute kidney injury (69), and cardiovascular mortality (70).

2.2.2 Appearance of Fetuin-A in Proteomic Studies

With the advanced technical capabilities and accessibility of proteomics, particularly with mass spectrometry, there has been an increase in observations of fetuin-A in various studies in the literature. Many traditional protein quantification methods require the probing of proteins of interest through matched antibodies or radiolabelling, meaning that

to study the adsorbed amount of a protein one must be looking for it. Given the rise of proteomics, fetuin-A has been identified in numerous studies where it may not have been a protein of interest and investigated with traditional methods. Proteomic studies reveal fetuin-A adsorbing in greater than expected amounts to a variety of substrates such as polypeptide nanoparticles (6), PEG hydrogels (71), tissue culture polystyrene (7) and hydroxyapatite (8).

There are a number of serum proteins that are expected to adsorb to materials in high quantities irrespective of the substrate properties, including albumin, which also falls in the 50-80kDa range, and fibrinogen, a larger protein (16). Even on typically protein resistant materials, such as PEG, these proteins continue to adsorb. It appears that due to proteomics studies revealing the adsorption behaviour of relatively understudied proteins, fetuin-A may also belong on this list of high adsorbing proteins. After albumin, fetuin-A adsorbed in the highest amount of any protein from fetal bovine serum on polypeptide nanoparticles (6). Swartzlander et al. used liquid chromatography-tandem mass spectrometry (LC-MS/MS) to identify proteins adsorbed to their hydrogel samples. Over 300 proteins were detected, and fetuin-A was found to be one of the top 20 most identifiable proteins adsorbed to PEG hydrogels (71). Xie et al. observed high amounts of fetuin-A from cell lysates on hydroxyapatite (HA) but not plastic surfaces, where the main source of fetuin-A was the culture media serum (8).

2.2.3 Fetuin-A as a Surface Modifier

In a study by Vyner and Amsden, fetuin-A was determined to be responsible for the difference in cell proliferation on polymers of different stiffnesses, and was suggested as

a potential surface modifier (3). Previous research has noted that locally high levels of fetuin-A and spermine are present for immunosuppression during pregnancy (72). Alongside evidence that fetuin-A enhances phagocytosis of apoptotic cells, Jersmann et al. hypothesize a connection between the high concentration of fetuin-A in fetal blood to the ability of the fetus to seemingly heal without scarring. They go on to propose fetuin-A as a potential therapeutic for inflammatory diseases (73). Fetuin-A has also been suggested as a target for therapeutics that would help manage type 2 diabetes (74). Investigating the role of fetuin-A in modulating cellular adhesion, Nangami et al. proposed that the histones coating cellular exosomes required for the modulation of cellular adhesion are shuttled from elsewhere to cellular exosomes by fetuin-A. They found that in the presence of fetuin-A the adhesion and spreading of breast carcinoma cells increased compared to fibronectin and laminin coated wells (75). A number of these studies suggest that locally high amounts of fetuin-A would result in positive outcomes, though they do not explicitly recommend fetuin-A as a surface modifier. Vyner and Amsden were the first to suggest the potential of fetuin-A as a surface modifier based on the fibroblast response to adsorbed fetuin-A (3).

2.2.4 Potential Immune Implications

In 1979, Lebreton et al. found that serum fetuin-A levels decreased in patients who suffered inflammation in response to a severe bacterial infection, providing evidence that fetuin-A may be a negative acute phase protein (76). Low fetuin-A levels have been detected alongside various inflammatory diseases, and as such has been suggested as a protein biomarker for a number of diagnostic assays outlined thoroughly in the review by

Vashist et al. (65). Reinforcing the idea that with lower levels of fetuin-A an increase in inflammation would be expected, Jersmann et al. found that in the presence of fetuin-A macrophage-mediated phagocytosis was magnified (73).

It has been proposed that macrophages are able to use fetuin-A to determine the levels of extracellular spermine, down regulating the synthesis of pro-inflammatory cytokines and preventing excessive inflammation (77). Further complicating the ability to classify fetuin-A as having pro- or anti- inflammatory effects, Smith et al. found that as fetuin-A binds calcium phosphate and forms calciprotein particles, the uptake of these particles by macrophages resulted in the release of pro-inflammatory cytokines. However, the fetuin-A containing calciprotein particles elicit a lesser inflammatory response than calcium phosphate nanocrystals alone, indicating that the formation of these particles is advantageous and that fetuin-A is somehow able to encourage the clearance of these particles (78). The role of fetuin-A in inflammation and immune response is only beginning to be understood. Investigation into pro- and anti-inflammatory cytokines released in response to the presence of fetuin-A can only help further the understanding of the potential immune implications and potential surface modifying effects of fetuin-A.

2.3 Polydimethylsiloxane (PDMS)

2.3.1 PDMS as a Biomaterial

Polydimethylsiloxane (PDMS) is an organosilicon compound that can be formulated to produce a variety of surface moduli while maintaining surface topography and surface wettability (79). Its relatively low cost alongside the optical clarity, chemical inertness, and ability to mould to submicron features make it a popular choice for biomaterials (80).

Its structure can be seen in Figure 6. Applications include a wide range of biomedical devices such as catheters, membrane oxygenators, and contact lenses. One of the most common PDMS products is Dow's Sylgard 184, used in numerous studies (81–83). Soft lithography and photolithography techniques allow for the production of micron-scale structures (79), while methods have been developed to create thin films via spin coating with thicknesses in the nanometre range (82). A principal limitation of PDMS as a biomaterial is the methyl groups that result in a hydrophobic surface, allowing ample non-specific protein adsorption (84). To combat this, many surface modification methods can be employed.

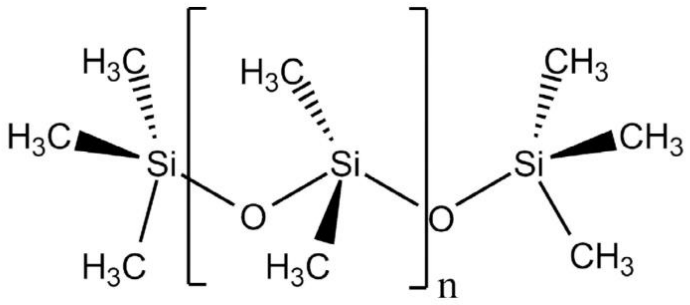


Figure 6. Structure of PDMS. Without modification from (85) under Creative Commons CC-BY-NC-ND license.

2.3.2 Surface Modification of PDMS

The chemical, mechanical, and topographical properties of PDMS can be relatively easily modified when compared to other substrates (79). The simplest modification may be passive adsorption of proteins as a treatment, but covalent immobilization with 3-aminopropyl triethoxysilane (APTES) and glutaraldehyde, as well as 1-ethyl-3- (3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) has demonstrated much

stronger binding interactions (86). The amino groups generated by APTES can be converted to carboxyl groups via succinic acid anhydride (SAA), and can bind proteins via EDC for detection of proteins for enzyme-linked immunosorbent assay (ELISA) applications (81).

Oxygen plasma treatment is a common modification method which increases the hydrophilicity of the surface for a limited time (79). Plasma treatment generates hydroxyl groups which are rearranged away from the substrate surface by uncured chains unless stabilizing treatments are applied (87). Using hydrophilic molecules to prevent non-specific protein adsorption, PDMS microchannels modified with PEG were able to remain hydrophilic over 10 days, and reduced protein adsorption when compared to unmodified PDMS over a period of 28 days (88). Recently developed commercial PDMS-PEG block copolymers can be added to PDMS during the mixing process and modify the surface without extensive surface treatment (2). Other more hydrophilic materials, such as cellulose, have been patterned on the PDMS surface via lithography (82).

2.3.3 Tunable Modulus of PDMS

Commercially available Sylgard 184 and Sylgard 527 can be combined in various ratios to produce substrates with a range of biologically relevant stiffnesses (10). These stiffnesses include the human epidermis (140-600kPa) (79), basement membranes (~1MPa) and arterial walls (~800kPa)(10).Many studies report the tunable elastic properties of PDMS, which is done by either altering the base to curing agent ratio of Sylgard 184 (4,89,90), or mixing Sylgard 184 and Sylgard 527 at different ratios (10).

Modifying the base to curing agent ratio impacts the crosslinking of hydroxyl groups, influencing the surface chemistry as well as the mechanical properties (89). Instead, choosing to combine Sylgard 184 and 527 at different ratios to produce substrates of varying elastic moduli ensures that each mix is stoichiometrically satisfied.

2.4 Polydopamine (PDA) Modification of PDMS

Polydopamine (PDA) can act as a biomimetic surface modification method modelled off of the adhesive byssal proteins found in mussels, as shown in Figure 7. These proteins, which contain high levels of catechol functional groups, facilitate the adhesion of mussels to a variety of materials (91). Catechol functional groups allow PDA to act as a binding agent for other molecules through their amine and thiol groups, or it can act alone as a self-assembling hydrophilic coating (92). PDA is able to assemble onto the material surface under alkaline conditions, at room temperature, and under ambient pressure (92) (93),which provides an advantage over many other aggressive modification techniques. Research has started to explore how solution concentration, pH, and immersion time impact the assembled PDA layer (94)**,** however, there is no agreed upon structure of PDA within literature (92). Traditionally, there have been two main schools of thought on PDA structure as outlined in Delparastan et al. (95). One proposes that PDA coatings are composed of aggregated monomers and oligomers weakly bound through hydrogen bonding and π-π stacking, while the second suggests that PDA coatings consist of polymers due to the covalent linking of dopamine monomers. Delparastan et al. were able to use a PDA coated AFM cantilever to find that PDA layers exhibit polymeric behaviour.

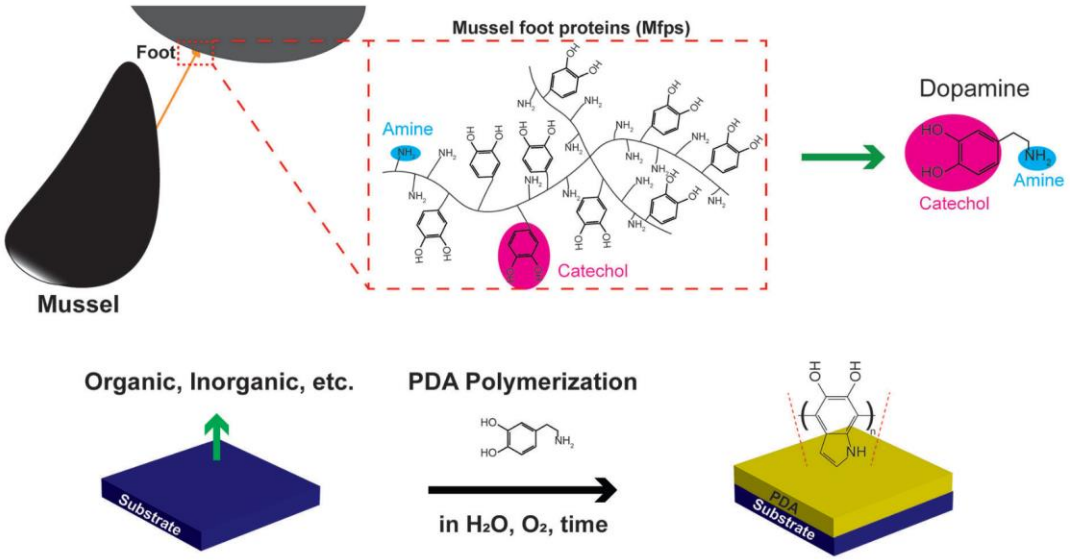


Figure 7. Biomimetic PDA inspired by the catechol and amine chemistry of mussel foot proteins, where polymerization to PDA occurs to form particles or films on the substrate surface. With permission from (96).

PDA films can be applied to polymers, metals, ceramics, and other materials in a single step, with film properties controlled by dopamine solution concentration and incubation time (94). The relative ease of layer formation, universal coverage, and mild polymerization conditions make PDA an enticing surface modification method with applications in energy storage (97), marine antifouling (98), and biomaterial design (96,99), to name a few.

PDA has demonstrated advantages in numerous applications, where it is applied to materials and used directly in biological environments. The modification of QCM sensors by PDA facilitated the investigation of protein-protein interactions, where it proved to be a simple, cheap, and regenerative immobilization method compared to traditional EDC, NHS and ethanolamine protocols (100). Ku et al. were able to selectively deposit PDA on

non-adhesive substrates thus controlling cell patterning, and highlighted the universal nature of PDA as a promising method for patterning cells on non-adhesive materials moving forward (101). PDA coated zirconia demonstrated an increase in human gingival fibroblast adhesion and proliferation, as well as reduced bacterial adhesion when compared to plain zirconia, suggesting PDA modification as a prospective method for dental implant soft-tissue integration (102).

Though there are many applications where PDA coatings alone may provide benefits, further modification may be required for certain antifouling and biomaterial applications. The catechol groups of PDA are readily able to bind with proteins which can result in increased protein adsorption. For applications in which protein adsorption is to be reduced, immobilizing and/or backfilling with proteins or other biomolecules can be employed to provide a passivating layer (103,104). PDA can be used as an intermediate linker to immobilize molecules to the material surface such as growth factors for human neural stem cell differentiation (105), poly(ethylene-oxide) for nonfouling surfaces (106), and heparin and BSA for the improved hemocompatibility of hemodialysis membranes (107).

2.5 Macrophage Interactions with Materials

Along with proteins, macrophages play an important role in the biological response to implanted materials. Macrophages are involved in inflammation, where they recruit fibroblasts, form foreign body giant cells, and clear foreign particles by phagocytosis (108). The implantation of devices triggers the recruitment of macrophages by resident cells, and the biomaterial-macrophage interaction will determine the host response (109).

In addition to their importance in immune response, macrophages perform routine clearance of red blood cells, debris, foreign particles, and dead cells, which is vital to system survival (110).

Upon injury or infection, macrophages resident in local tissue are activated, recruiting classically activated or alternatively activated macrophages to the injury site, often described as M1 or M2 (111). Classically activated or M1 macrophages are necessary to the defense against infection, but in excess can lead to tissue damage (110). The anti-inflammatory cytokines released by M2 macrophages can inhibit the formation of fibrous tissue which can aid in implant integration (14). M1 macrophages promote inflammation and secrete a number of cytokines including interleukin-6 (IL-6), interleukin-12 (IL-12) and tumor necrosis factor-α (TNF-α), while M2 macrophages have anti-inflammatory properties, and secrete cytokines such as interleukin-10 (IL-10) and transforming growth factor-β (TGF-β) (112). Where excess M1 polarization can prolong inflammation, the polarization of M1 macrophages to M2 is characteristic of wound healing and is crucial when considering successful device integration (113). Given their ability to switch between phenotypes, it has been suggested that macrophages are better characterized by specific functions, such as wound healing, host defence, and immune regulation, and can even exist on a spectrum where they contribute to more than one function, as visualized in Figure 8 (110). Despite these complexities associated with macrophage polarization, it is still useful to consider M1 and M2 categorization when investigating the in vitro biomaterial response. Due to the transient nature of macrophage polarization, it is crucial to understand how the surrounding environment, specifically the physical, mechanical,

and chemical properties of the implanted biomaterial, impact polarization for the improved design of immunomodulatory biomaterials (14).

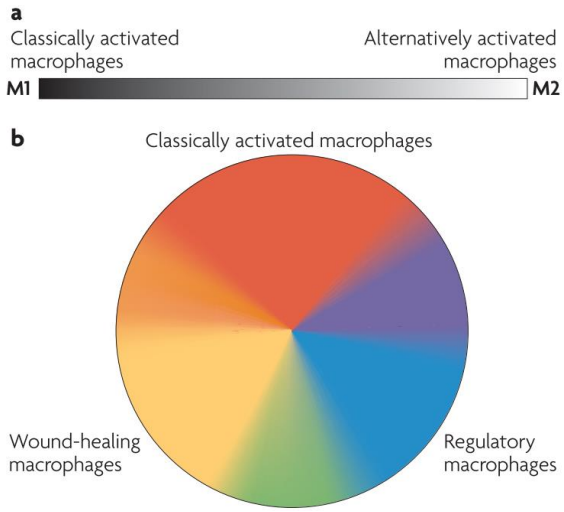


Figure 8. Traditional linear schema of macrophage activation (a) and colour wheel of activation representing three groups of macrophages, where macrophages exist on a spectrum between groups. With permission from (110).

Though full cytokine analysis offers the most information in determining the inflammatory response to biomaterials, it is often necessary to study key cytokines initially. The release of TNF-α triggers the acute inflammatory response and encourages cell recruitment to the site of inflammation, while increased TNF-α levels are associated with various disease states (114) – making it a commonly studied cytokine, alongside IL-10. IL-10 helps supress pro-inflammatory cytokine production by reducing the ability of macrophages to be activated by the classical activator interferon-gamma (IFN-γ) (115).

Given the immediate adsorption of proteins upon biomaterial implantation, and the protein-cell interactions guiding adhesion, the biomaterial properties that affect protein

adsorption in turn impact cellular adhesion and activation (116). Macrophage response is influenced by the mechanical (117–119), topographical (120–122), and chemical properties (123–125) of the biomaterial. Material properties that influence macrophage response can be seen in Figure 9.

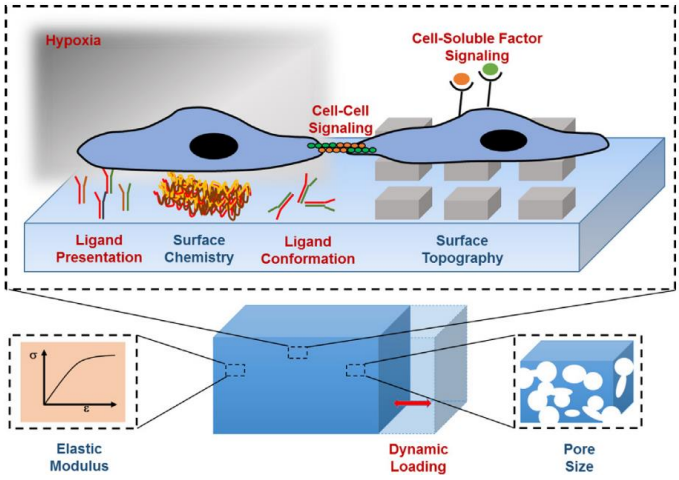


Figure 9. Biomaterial properties that influence macrophage polarization. Without modification from (14) under Creative Commons CC-BY-NC-ND license.

Adlerz et al. found that monocyte-derived macrophages were able to sense and respond to a range of material stiffnesses by modifying their area, and that migration speed and proliferation rate were significantly faster on stiffer substrates (126). Investigating cytokines released in response to material stiffness, Blakney et al. found increased cell spread and classical (M1) activation on stiffer hydrogels (127), which agreed with previous research indicating an increase in cell spread on stiffer substrates (128). In Jain and Vogel's 2018 work, topographical features of PDMS were designed to confine macrophages in micropatterned channels, resulting in supressed pro-inflammatory cytokine release (129). Given these results, studying macrophage response to biomaterials

with a variety of physical, chemical, and material properties may help discover new strategies for designing immunomodulatory biomaterials.

3. Surface Characterization of PDMS Substrates

3.1 Introduction

Material surface characterization plays a crucial role in biomaterials research aiming to understand and predict material-protein interactions. These interactions are complex and are influenced by numerous biomaterial properties, which in turn may impact each other. Thorough surface characterization is required not only to understand what is occurring at the material interface of unmodified materials, but to understand how altering one property may influence others. In the case of PDMS, a commonly used biomaterial, strategies to alter the polymer chain flexibility, and thus the elastic modulus, have been studied (4,130). Palchesko et al. found that by mixing Dow products Sylgard 184 and Sylgard 527 at various ratios they were able to create PDMS substrates that covered a spectrum of biologically relevant stiffnesses without influencing other material properties (10). This work utilizes this method of mechanical property modification to investigate how protein adsorption and macrophage response is influenced by polymer chain flexibility. Modification of the surface of PDMS with PDA is also applied to compare a chemical modification strategy that is expected to increase the amount of protein immobilized and possibly influence its orientation/conformation. To achieve this detailed characterization data, the wetting properties, surface morphology, and surface chemistry

of unmodified and modified substrates are investigated providing insight to how these modifications may impact the material-protein interface.

3.2 Materials and Methods

3.2.1 Sample Preparation

PDMS samples were prepared using Dow's Sylgard 184 and 527 kits (Ellsworth Adhesives, Canada**)**. The Sylgard 184 kit was combined at a 10:1 base to curing agent ratio then mixed by hand for 2 minutes. Sylgard 527 was prepared at a 1:1 ratio between part A and part B, then mixed by hand for 2 minutes. Subsequent mixtures were prepared by combining Sylgard 184 and Sylgard 527 at 1:1 and 1:5 ratios. Mixed PDMS was poured into petri dishes to a thickness of 1 mm, before degassing in a vacuum desiccator for 30 minutes. The samples were left to cure at room temperature for 48 hours before 6 mm samples were punched out.

To form a PDA coating, samples were immersed in a 2 mg/mL dopamine hydrochloride (Sigma, USA) in phosphate buffered saline (PBS) (pH 8.5) solution for 24 hours at room temperature under agitation. Samples were rinsed with PBS (pH 7.4, 3 x 5 minutes). PDA samples for atomic force microscopy (AFM) analysis were additionally dried with N_2 prior to imaging. For compression testing PDMS was mixed as discussed above, before pouring in plastic molds with a diameter of 13.91 mm. The samples were poured to a height of at least 10 mm and were then placed in a vacuum desiccator as usual.

3.2.2 Water Contact Angle

Advancing and receding water contact angles were measured using a goniometer (Ramé-hart, USA). The sessile drop method was performed with MilliQ water at room temperature. Both advancing and receding angles were measured at multiple locations on the substrates. Measurements were taken after changes in droplet baseline as outlined in Huhtamaki et al. (131).

3.2.3 Compression Testing

To determine the elastic modulus of the PDMS samples, all formulations were prepared as described and poured into molds to produce cylindrical samples. Compression testing was performed with a Univert (CellScale, Canada), at a displacement rate of 0.02 mm/s and with a load cell of 200 N. Experiments were continued to a maximum of 50% displacement, and elastic modulus was determined from the slope of the stress strain curve between 30 and 50% strain.

3.2.4 Atomic Force Microscopy

AFM topographical imaging was performed using an Anton-Paar Tosca 400 under ambient conditions in air. Silicon Arrow-NCR cantilevers (NanoWorld, Switzerland) in tapping mode were used to generate 5μm x 5μm images. The Arrow-NCR probes have tetrahedral tips with a tip radius less than 10 nm. Tip resonance frequency is 285 kHz with a force constant of 42 N/m. Tosca Analysis software was used to obtain roughness measurements, profiles, and images. Roughness results were determined after the extraction of the height layer, followed by a first order fit to remove the sample tilt, and

line deletion of severe deviations due to cantilever sticking. The image analysis is outlined in Appendix A1. AFM Image Analysis Protocol.

3.2.5 X-Ray Photoelectron Spectroscopy

X-ray photoelectron spectroscopy (XPS) was performed using a Quantera II XPS (PHI, USA) in spectra mode with a beam size of 50 μm operating at 12.5 W/15 kV. Survey spectra were collected at 3 different points and the C1 peak was set to 284.8 eV for reference (Biointerfaces Institute, Hamilton).

3.2.6 Statistics

All statistical significance in this thesis was determined at levels for p less than 0.05 through Student's t-tests in Microsoft Excel. Data are reported as mean ± standard deviation (SD).

3.3 Results and Discussion

3.3.1 Contact Angle

Water contact angle measurements were used to test for surface energy changes across the PDMS formulations and results are displayed in Figure 10. There were no significant differences found when comparing the measured contact angles of the Sylgard 184, 1:1, and 1:5 samples, and advancing angles were consistent with those found in literature for PDMS (79,83,103). As expected, the receding contact angle values were lower than the advancing angles, due to hysteresis. The lack of significant difference in contact angle measurements between formulations indicates that changing the ratio of Sylgard 184 to Sylgard 527 doesn't significantly alter the hydrophobicity of the material, an important

consideration when modifying biomaterials and investigating protein adsorption. Protein adsorption is a spontaneous process driven by the increase in entropy from the release of water molecules at the surface of the material (19), as well as other electrostatic and hydrophobic interactions (11). A generalization is often made that proteins adsorb in higher amounts and more strongly to hydrophobic surfaces due to the interactions between hydrophobic domains on the protein and material releasing water from the surface, although this is not always the case (25). For this reason, when comparing adsorption between samples it is important to characterize the surface wetting properties of the material. A consistent contact angle measurement across the PDMS samples indicates that any significant protein adsorption differences are not due to changes in surface wettability.

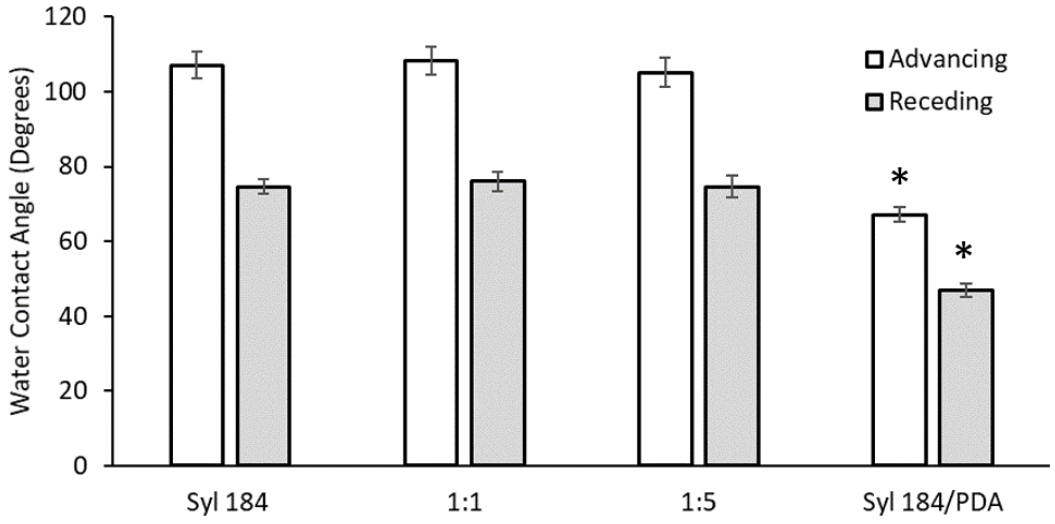


Figure 10. Advancing and receding contact angle of Syl 184, 1:1, 1:5 and Syl 184/PDA samples. PDMS formulations determined manually using the sessile drop method. Data are mean ± SD, n = 6. Statistical significance ($p < 0.05$) indicated by (*).

Sylgard 184 modified with PDA demonstrated a significant decrease in contact angle when compared to the unmodified PDMS formulations. The addition of PDA increases the hydrophilicity of the surface, as expected due to the catechol and amino functional groups present in PDA (132). A complete PDA layer has been found to have a contact angle between 50 and 60° regardless of underlying substrate (133), indicating that a fully homogeneous layer may not have been formed on the surface of the PDMS. It is thermodynamically favourable for water molecules to spread on the surface of hydrophilic materials, thus providing a larger barrier to protein adsorption when compared to hydrophobic materials (35). Notably, PDA is often used as an adhesive modification where the catechol functional groups are readily able to bind proteins (104,107), despite the increase in hydrophilicity.

3.3.2 Compression Testing

To determine how the mechanical properties of PDMS samples changed as the amount of Sylgard 527 increased, compression testing was performed on the Sylgard 184, 1:1, and 1:5 samples. The measured elastic modulus values are shown in Figure 11. The intentional variation of the mechanical properties by changing the formulation ratio resulted in the modulus values increasing significantly with an increase in Sylgard 527, as expected. Statistical significance ($p < 0.05$) was found between Syl 184, 1:1, and 1:5 formulations.

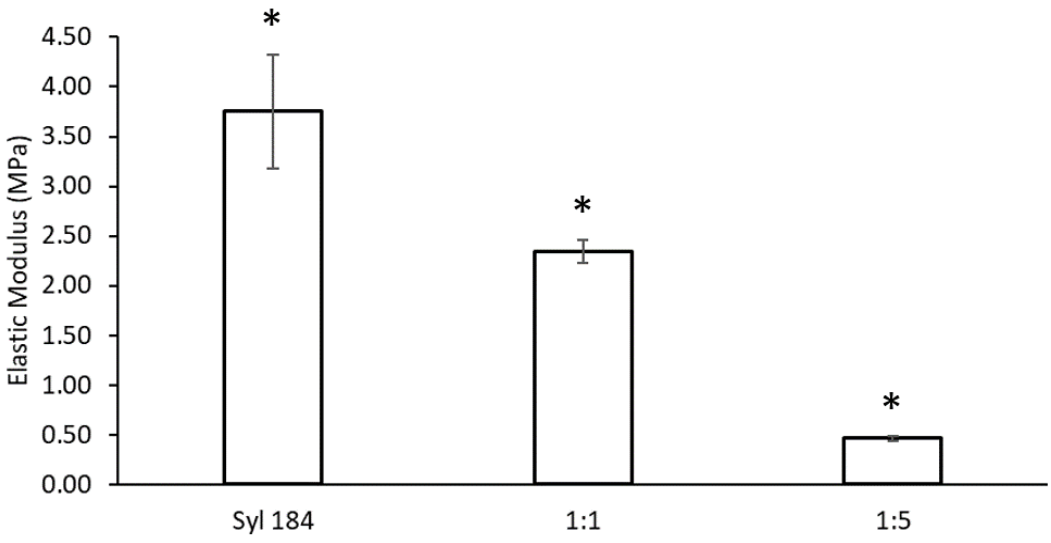


Figure 11. Elastic modulus generated from the elastic region of the stress-strain curve obtained by compression testing. Data are mean ± SD, n = 3. Statistical significance ($p < 0.05$) indicated by (*).

As discussed by Palchesko et al. these moduli values cover a range of biologically relevant stiffnesses including arterial walls (10), fibronectin fibre, and adipose tissue

(134). The biological relevance of these stiffnesses is key when considering PDMS for biomedical devices. Depending on the location of the device and its intended application, mimicking the mechanical properties of the surrounding or replaced tissue may be advantageous in directing the subsequent cell response, for example reducing undesired inflammation. Likewise, it is of interest to investigate how fetuin-A, as a surface modifier with potential cell instructive and immunomodulatory properties, adsorbs to PDMS of varying stiffnesses. Determining protein interactions to materials of varying stiffnesses provides an opportunity to better understand any direct impacts on protein binding and may also allow us to tailor the protein layer simply by altering the mechanical properties.

3.3.3 Atomic Force Microscopy (AFM)

AFM was employed to investigate the surface topography of the PDMS formulations. As previously mentioned, Sylgard 184 contains silica nanoparticles, but these are absent in the softer Sylgard 527 formulation. Topographical imaging and surface roughness measurements were required to characterize any surface morphology changes at the air-PDMS interface. Representative topographical images can be seen in Figure 12.

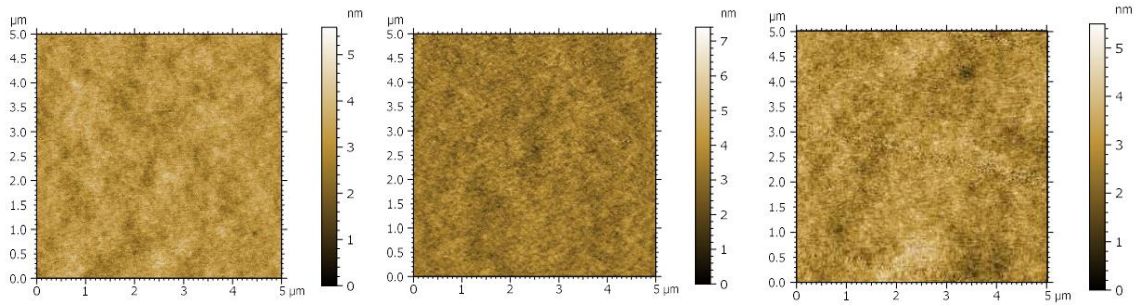


Figure 12. Representative AFM images of Syl 184, 1:1, and 1:5 surface topography.

Comparing the images for each formulation, there are no extreme differences in surface morphology. Of note, the height scale of the image for the 1:1 formulation is greater than the images for the Syl 184 and 1:5 samples. Surface roughness and root mean square roughness (RMS) values are presented in Figure 13.

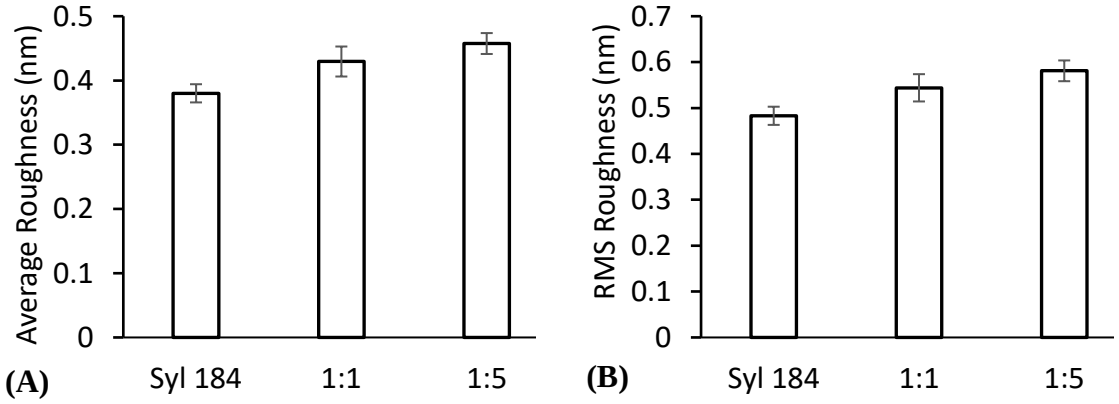


Figure 13. Plotted (A) average roughness and (B) root mean square roughness data of Syl 184, 1:1 and 1:5 samples. Results obtained using Tosca Analysis software on gathered AFM images. Data are mean ± SD, n=2.

With increasing Sylgard 527 content, thus a decrease in elastic modulus, there seems to be an increase in average surface roughness and root mean square roughness. The largest

difference in average surface roughness is between the Syl 184 and 1:5 samples, which has a difference in average roughness of 0.0775nm which even on a biological scale is quite small. These data are contrary to the results found in Palchesko et al. where RMS roughness increased with increasing elastic modulus. In both their work and here, all roughness values are less than 1 nm which is considered to be below what cells can detect and therefore the differences should not have an influence on biological response (10). It is worth noting in their study, topography was measured after spin coating on glass slides, where here PDMS was cured in petri dishes. An additional important consideration is the scale of the instrument noise. It is most easily visualized in Figure 14 where the profile of a line in the previous image of Syl 184 is displayed.

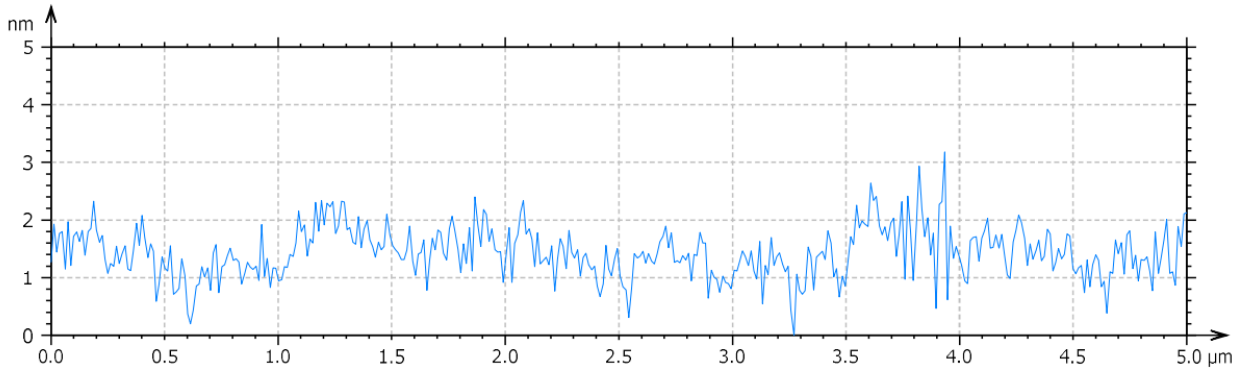
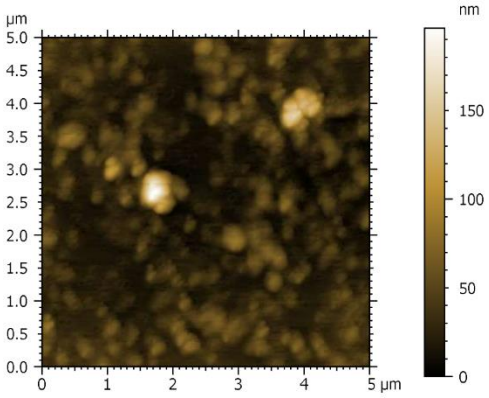


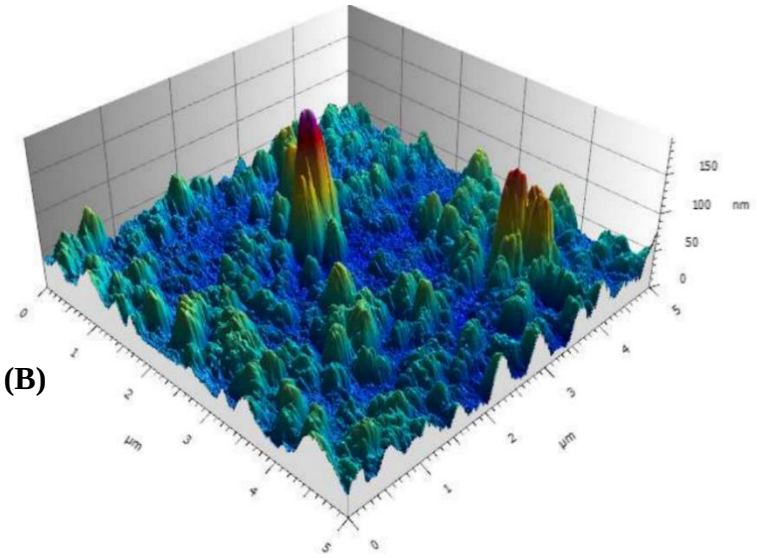
Figure 14. Profile of Syl 184 sample generated using Tosca Analysis software.

It is evident that based on the instrument noise, the roughness results may reflect overlap of noise and surface roughness. AFM is a robust method for imaging surface features, however here where the sample surface lacks features – it is more difficult to obtain clear images and precise, accurate, roughness values.

Syl 184/PDA samples were imaged, as shown in Figure 15. The scale of surface roughness is significantly greater due to the PDA layer, as noted in other works (103,107). The 3D view of the sample surface indicates the somewhat inhomogeneous PDA coverage, which aligns with the slightly higher than expected contact angle values obtained previously. Though roughness values are greater than the unmodified PDMS, it is expected that the interactions between proteins and the catechol and amine groups of the PDA dominate the adsorption interactions, as has been seen in other work.



(A)



(B)

Height Parameters	
Sq	19.4nm
Sa	13.5nm

(C)

Figure 15. Representative (A) 2D and (B) 3D image of the sample surface. Tosca Analysis generated height parameters (C) root mean square roughness (Sq) and average surface roughness (Sa).

3.3.4 X-Ray Photoelectron Spectroscopy (XPS)

Analysis of surface elemental composition was performed with XPS. The Sylgard 184 and 1:1 formulations showed no significant differences between levels of carbon, oxygen,

and silicon as seen in Figure 16. The concentrations of C, O, and Si of Syl 184 and 1:1 samples are consistent with values found in literature (103). The 1:5 formulation demonstrated a slight increase in the atomic concentration of carbon and a decrease in oxygen compared to the other two PDMS formulations.

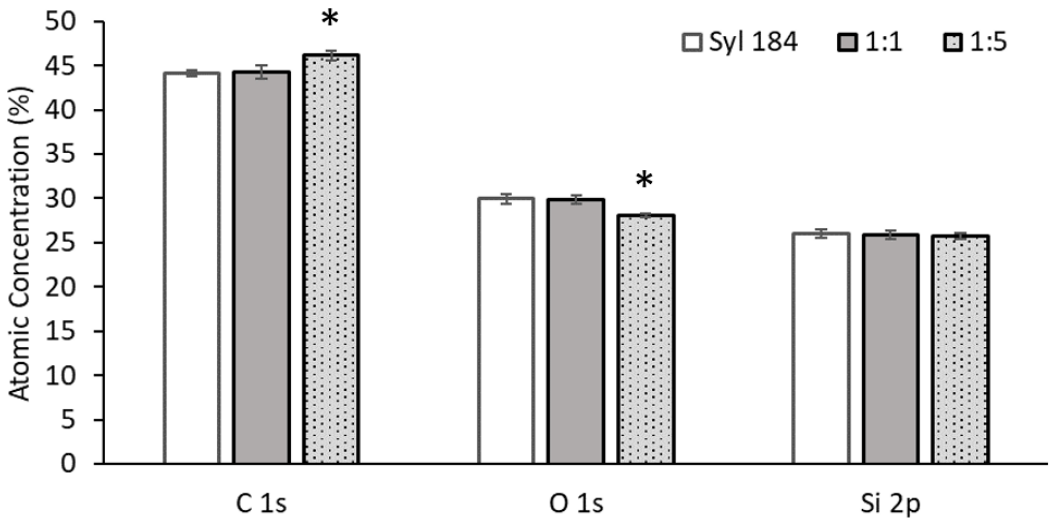


Figure 16. Surface elemental composition of Syl 184, 1:1, and 1:5 samples obtained by XPS. Data are mean ± SD, n = 3 independent locations. Statistical significance ($p < 0.05$) indicated by (*).

Sylgard 184 and Sylgard 527 are similar but not identical in composition, with the addition of silica nanoparticles in Sylgard 184 as described in the safety data sheets. However, the full composition of each product is not publicly available. These slight differences in product composition may be responsible for the small inconsistencies in surface chemistry seen with XPS. The sensitivity of XPS is important to consider, as the numerical differences are quite small – but may register as statistically significant in part

due to the high surface sensitivity of the XPS results and potential influences of surface contaminants. The small increase in carbon and decrease in oxygen for the 1:5 ratio sample is not expected to influence surface properties or protein adsorption, especially when considered in tandem with contact angle results. The large decrease in elastic modulus, demonstrated by compression testing, would likely provide a dominating effect.

3.4 Conclusions

The three investigated formulations of PDMS (Syl 184, 1:1, and 1:5) demonstrated a distinct increase in elastic modulus as the ratio of Sylgard 184 : Sylgard 527 was decreased. The wetting properties remained constant across all unmodified PDMS samples, while the addition of PDA increased the hydrophilicity of the surface. AFM images show a qualitative lack of morphological differences, while roughness calculations indicate a slight increase in surface roughness with decreasing elastic modulus. The surface chemistry of Syl 184 and 1:1 were similar, while 1:5 had slightly higher concentrations of carbon and lower concentrations of oxygen than the other two samples. A summary of these findings can be found in Table 1. The characterization of the Syl 184, 1:1, and 1:5 samples aimed to investigate the mechanical properties of PDMS along with the physical and chemical properties, to determine if there were also significant changes in these. It is evident that the Syl 184 and 1:1 samples only differ in their elastic modulus and not surface chemistry. The results obtained demonstrate that while some differences are observed with respect to surface roughness and chemistry on the 1:5 formulation, no significant trends of these properties with elastic modulus were evident across all three samples. The change in modulus as a result of Sylgard 527

addition did not influence contact angles in these studies, and so we would not expect changes in protein adsorption due to the wetting properties. In many studies surface chemistry is the main factor explored without considering mechanical properties, and this work highlights the importance of examining multiple types of characterization data. When considering just the contact angle results, less protein adsorption from PDA coated samples may be expected due to the decreased hydrophobicity. As discussed previously, it can be more energetically favourable to displace water and adsorb to hydrophobic surfaces than hydrophilic ones. However, this is not the case for PDA coated surfaces, as seen in our work and in the literature. There are other more dominant effects to consider including the interaction of catechol groups on PDA with the amine groups of proteins, that can increase adsorption over unmodified surfaces (103).

Table 1. Summary of significant ($p < 0.05$) surface characterization findings.

	Unmodified PDMS	***Syl 184/PDA***
Contact Angle	No significant differences	↓ in hydrophobicity when compared to unmodified PDMS
Elastic Modulus	All samples significantly different	N/A
AFM	↑ roughness with ↓ elastic modulus (apparent, n =2)	↑ roughness compared to unmodified PDMS
XPS	↑ C1s ↓O1s on 1:5 sample compared to 1:1 and Syl 184	N/A

4. Protein Adsorption Studies

4.1 Introduction

Prior to cellular interactions with implanted biomaterials, proteins adsorb rapidly and the quantity, composition, and orientation/conformation at the surface influences the cellular response and thus the host response. Due to this, understanding protein adsorption and how it is influenced by the material and chemical properties is crucial in designing biomaterials with controlled immune responses. With this goal in mind, one strategy is to reduce non-specific protein adsorption through surface modifications, which can assist in decreasing thrombus formation and inflammation (11). It is important to understand which proteins are advantageous to the desired immune outcome, and how the adsorption of these proteins is influenced by the material properties. Changes in adsorption amounts or orientation due to material properties is not universal to all proteins, which is why studies of specific proteins on their own and from complex solutions are essential in fully understanding material-protein interactions. Here, the principal focus is on the adsorption behaviour of the plasma protein fetuin-A. In proteomic studies fetuin-A has appeared to adsorb in greater than expected amounts, and to our knowledge only one study which quantifies the amounts of adsorbed fetuin-A to particular materials exists (3). Investigating the adsorption of fetuin-A both quantitatively and qualitatively from single and multi-protein solutions, as well as to chemically modified PDMS-PDA surfaces, provides novel insight into the adsorption behaviour of this protein.

4.2 Materials and Methods

4.2.1 Sample Preparation

PDMS samples for protein adsorption experiments were prepared following the same protocol as described previously for surface characterization. Sylgard 184 and 527 were prepared independently following the manufacturer's instructions before they were combined at 1:1 and 1:5 ratios, respectively. After mixing by hand for 2 minutes, formulations were poured into petri dishes and degassed for 30 minutes, then left to cure at room temperature for 48 hours. Samples 6 mm in diameter were punched out and used in adsorption experiments.

4.2.2 Polydopamine (PDA) Surface Coating

To immobilize fetuin-A and investigate its potential as a bioactive surface modifier, Sylgard 184 samples were coated with PDA. A 2 mg/mL solution of dopamine hydrochloride (Sigma, USA) in PBS (pH 8.5) was prepared. The PDMS samples were immersed in solution for 24 hours at room temperature and agitated. Samples were then rinsed three times with PBS (pH 7.4) prior to protein adsorption for 1 hour in a 0.15 mg/mL human fetuin-A (Sigma, SRP6517) solution in PBS.

4.2.3 Bicinchoninic Acid (BCA) Total Protein Assay

The Pierce micro bicinchoninic acid (BCA) assay was used to detect and quantify total protein adsorbed to the PDMS samples. The BCA assay was performed on the eluent from 6 mm PDMS discs approximately 1 mm thick. The samples were incubated in either 0.15 mg/mL human serum albumin (HSA) or fetuin-A solutions in PBS for 2 hours. In a separate experiment, samples were incubated in a 2 mg/mL fibrinogen solution in PBS

(MilliporeSigma, USA). Samples were rinsed with PBS (pH 7.4) 3 times for 5 minutes each time to remove loosely bound protein. After rinsing, elution occurred overnight in 2% sodium dodecyl sulfate (SDS). Protein standards were prepared to the specifications included in the Pierce protocol. The absorbance was read at 562 nm on a SpectraMax ABS Plus (Molecular Devices, USA).

4.2.4 Enzyme Immunoassay (EIA)

An enzyme immunoassay (EIA) was used to determine the total amount of protein available for functional binding. A 2 mg/mL BSA blocking solution was used to block wells for 1 hour prior to the start of the assay. 6 mm PDMS discs were placed individually in the wells of a 96-well plate. Protein adsorption was carried out from a 0.15 mg/mL fetuin-A solution for 2 hours, followed by a 1-hour incubation with mouse anti-fetuin A antibody (Sigma, SAB4200820) and then a goat anti-mouse secondary antibody (Sigma, AP308P). In between each adsorption step samples were rinsed with PBS (pH 7.4) three times for 5 minutes each. Samples were moved to a new well for each step of the assay. Adsorption was determined based on colourimetric change following the addition of 3,3’,5,5’-Tetramethylbenzidine (TMB) and sulfuric acid stopping solution, then read on a SpectraMax ABS Plus (Molecular Devices, USA) plate reader at a wavelength of 450 nm.

4.3 Results and Discussion

4.3.1 Adsorption from Single Protein Solutions

Adsorption from a single protein solution in buffer was performed to determine if altering the elastic modulus influenced the total adsorption of protein. Both fetuin-A and human

serum albumin (HSA) were investigated. Aside from being the most abundant protein in human plasma, the globular shape of HSA and a molecular weight similar to fetuin-A make it a good candidate for comparison. The amount of protein adsorbed to sample surfaces from single solutions of both fetuin-A and HSA in PBS (pH 7.4) at 0.15 mg/mL is shown in Figure 17.

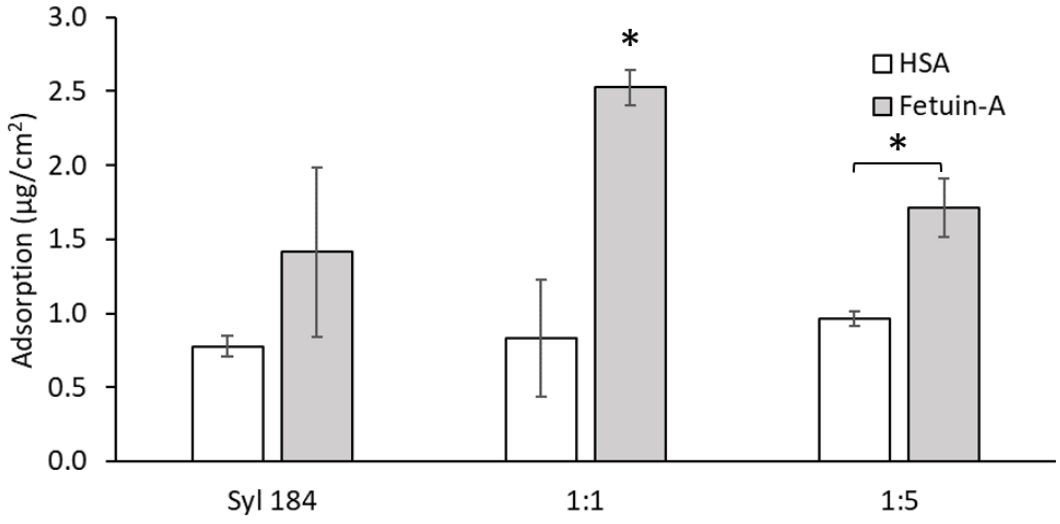


Figure 17. Comparison of the amount of adsorbed human serum albumin (HSA) and fetuin-A on PDMS of various formulations. Data are mean ± SD, n = 4. Statistical significance ($p < 0.05$) indicated by (*).

Across all samples there appears to be more fetuin-A adsorbed than HSA, with the 1:1 and 1:5 samples showing a statistically significant increase. In adult human plasma the concentration of albumin is between 35-50 mg/mL (135) while fetuin-A levels are in the range of 0.4-0.6 mg/mL (56,57). Notably, fetuin-A and HSA are mid-sized proteins with molecular weights of 49 kDa and 67 kDa, respectively. Given that fetuin-A and albumin

are among the highest adsorbed proteins to biomaterials in various proteomic studies (3,6,7,71,136,137), it is interesting to find that from solutions of the same concentration fetuin-A appears to adsorb in greater amounts.

A separate experiment was performed where fibrinogen was adsorbed to Syl 184, 1:1, and 1:5 samples and data is shown in Figure 18. Fibrinogen is also a protein that is abundant in human plasma and is known to readily adsorb to biomaterial surfaces. Since proteins are complex macromolecules with varying hydrophobic and electrostatic regions, it is of interest to compare how material properties may influence the adsorption of various different proteins on the same samples.

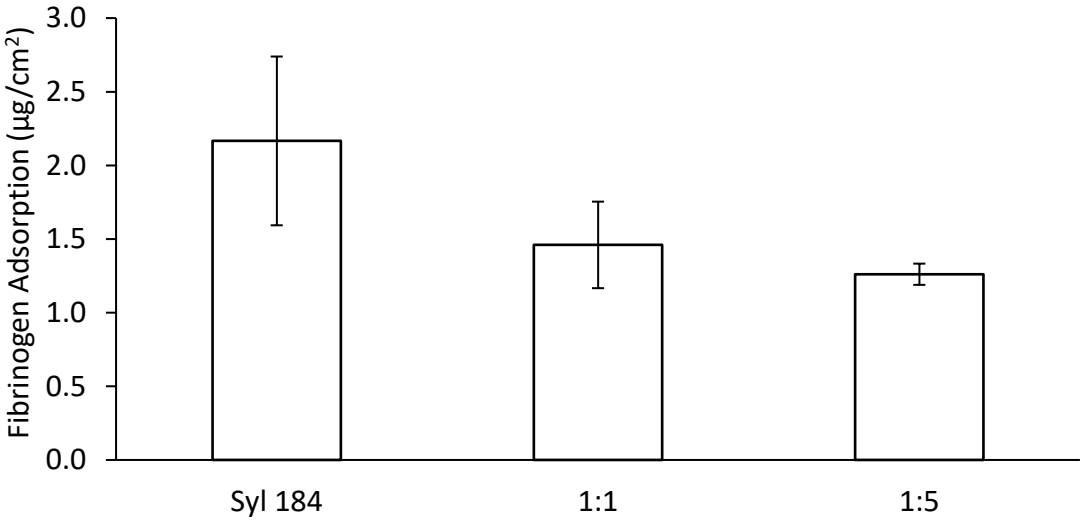


Figure 18. Adsorbed fibrinogen on Syl 184, 1:1, and 1:5 samples after 2 hours of adsorption from 2 mg/mL fibrinogen in PBS (pH 7.4). Data are mean ± SD, n = 3.

While there is no significant difference in the amount of HSA or fibrinogen adsorbed across the PDMS formulations, significant differences were found for fetuin-A

adsorption. The 1:1 samples, with a measured elastic modulus of 2.34 ± 0.12 MPa, adsorbed significantly more fetuin-A than the stiffer and softer substrates. Although not statistically significant due to variability, there may be a trend towards more HSA adsorption on the 1:5 substrates, while fibrinogen appears to adsorb in greater amounts to the stiffer Syl 184 samples. This indicates that while elastic modulus may impact the adsorption of fetuin-A, it does not necessarily have the same effect – or any effect at all on others. Differences in protein adsorption on substrates with varying mechanical properties has been observed previously in the literature, often attributed to differences in polymer chain flexibility (25,43)(138). Berglin et al. found that fibrinogen had greater adsorbed mass and less change in conformation to samples with more flexible polymer chains, while albumin did not seem to be impacted at all (139).

To date the literature on fetuin-A adsorbed to biomaterials is only from qualitative proteomic studies, except for one study following up on its adsorption to copolymer elastomers (3). Measuring and comparing protein adsorption using the BCA assay provides a way to gather quantitative results on another polymer, adding valuable information to the limited studies of fetuin-A adsorption to biomaterials. Due to limitations of the BCA assay and its indirect nature, including the necessity for eluting the adsorbed protein off the surface, we also performed a direct EIA to compare methods and gain additional insight (Figure 19). With the EIA, the adsorbed protein remains on the sample surface, providing a more direct method for comparison between samples without elution. Specifically, the EIA assesses the functionality of the adsorbed proteins through its ability to bind to corresponding antibodies. Comparisons are made based on

absorbance values between samples, rather than the adsorbed protein per surface area data obtained through the BCA assay. Since the assay uses antibodies specific to fetuin-A, the absorbance values are a direct indication of the amount of protein exposed on the outer surface layer and available for binding, most important to subsequent cell response.

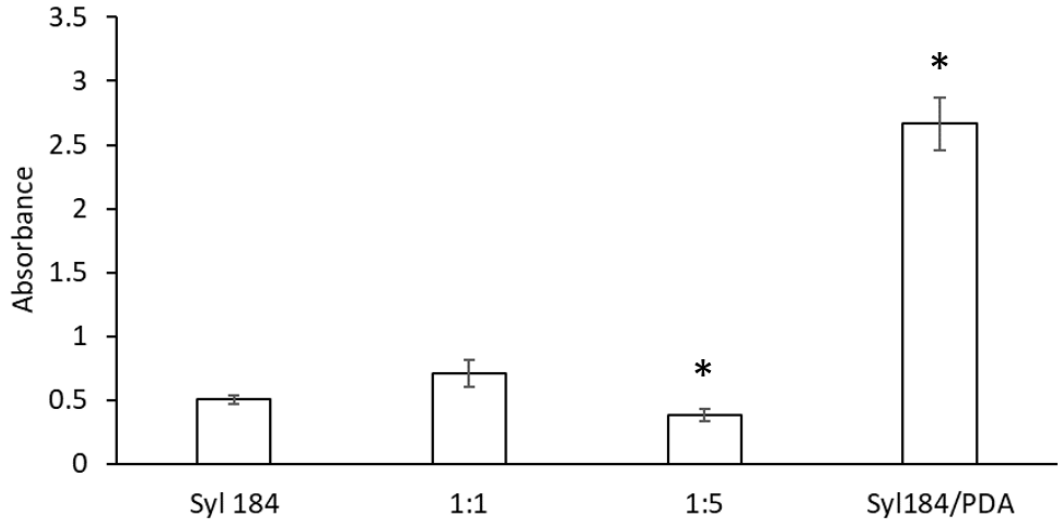


Figure 19. Comparison of fetuin-A available for functional binding from a 0.15 mg/mL fetuin-A solution in PBS, detected by an enzyme immunoassay (EIA). Absorbance read at 450nm. Data are mean ± SD, n = 3. Statistical significance ($p < 0.05$) indicated by (*).

From a 0.15 mg/mL fetuin-A solution in PBS, Syl 184 adsorbed significantly more fetuin-A than the 1:5 samples. Consistent with the BCA assay results, the 1:1 samples adsorbed significantly more than the 1:5 samples and appeared to adsorb more protein than Syl 184 though this difference was not statistically significant. Since no protein elution is required for the EIA, it is important to consider the effect of the elution of

proteins by SDS during the BCA assay, and how this may impact the results and comparisons.

The ability of SDS to elute proteins has been observed to change based on incubation time and protein concentration, as well as the substrate material (140). All loosely bound protein is likely desorbed from the surface, but it is possible that some strongly bound protein will remain. This demonstrates that caution should be taken in determining protein adsorption from BCA assay results alone and additional methods should be considered. The direct EIA allows the observation of protein adsorption trends while avoiding the potential implications of the surface properties on the ability of SDS to completely elute adsorbed fetuin-A. It also allows the comparison of samples that are designed to strongly attach proteins, including PDA coated materials. Due to this, careful comparison between the BCA and EIA results provides some insight into potential differences in protein binding strength to the various samples. The BCA assay measures the total protein eluted from the material surface after rinsing with SDS, while the EIA relies on antibody detection methods that will bind to the outermost proteins adsorbed to the surface with domains available for binding (4). Here, we see similar trends in the results of both experiments despite differences in measurement methods. With absorbance rather than surface concentration values for the EIA, we are unable to compare absolute adsorption values, but can compare trends. To determine if SDS elution conditions are adequate when compared to EIA results, or if the eluted protein is in similar quantities to the outermost adsorbed fetuin-A detected by the EIA, surface concentration results for both methods would be needed.

An additional sample of Sylgard 184 PDMS was coated with PDA (2 mg/mL, PBS pH 8.5), to establish a method to immobilize fetuin-A, increase its amount, and possibly control its orientation/conformation on the surface. As expected, the PDA modified PDMS resulted in significantly greater fetuin-A adsorption when compared to all other samples. The protein likely covalently binds with the available catechol functional groups of the PDA layer, resulting in stronger adsorption. A change in total fetuin-A adsorption with varying mechanical properties was not necessarily expected from a single protein solution, but it is interesting to note that both the BCA assay and EIA results demonstrate an increase in adsorption to the 1:1 samples. The BCA assay showed no significant changes in adsorption amounts of HSA and fibrinogen, though EIA experiments were not carried out for these proteins. As discussed, not all proteins are expected to react equally to substrates of varying stiffnesses – or polymer chain flexibility (139). This indicates the importance of investigating adsorption behaviour of the protein of interest from a single protein solution, prior to complex solutions, as the effect of mechanical properties on adsorption is not universal.

Substrate characterization results are important to consider when interpreting this data. Specifically, the 1:1 samples showed a significant increase in fetuin-A adsorption over 1:5 samples and an apparent increase over Syl 184. For these 1:1 samples, there were no large differences during characterization, other than their distinct elastic modulus. Contact angle and surface roughness results showed no significance when compared to Syl 184 and 1:5 samples. Syl 184 and 1:1 showed statistically similar surface chemistry results, while the 1:5 samples had increased C 1s and decreased O 1s atomic concentration. Based

on this characterization data, the small changes in surface chemistry of 1:5 when compared to Syl 184 and 1:1 should be considered alongside the very large change in elastic modulus between each sample set. Thus, the decrease in elastic modulus for the 1:1 sample over Syl 184 most likely plays the dominant role in the increase of fetuin-A adsorption from buffer.

Altering the material stiffness has been found to influence the adsorbed protein composition from a multi-protein environment (3). Adsorption from a single protein solution is not representative of the complex solutions biomaterials are exposed to during cell culture or with implanted devices. As such, these preliminary results are not representative of changes in adsorption in competitive environments but provide important insight into fundamental protein-specific interactions, as well as the comparison to immobilized fetuin-A levels via PDA surface modification. Differences detected in cell studies may be due to the change in protein composition in a competitive environment and/or the change in adsorbed protein orientation or conformation. Although the BCA assay cannot be used for multi-protein solutions as it only indicates the presence of total protein, the EIA provides a means to detect protein in these competitive environments.

4.3.2 Adsorption from Multi Protein Solutions

Protein adsorption from a competitive environment, in this case human plasma, was investigated to determine the effects of material surface stiffness in a biologically relevant fluid, and to assess the amount of fetuin-A adsorbed and present at the outermost surface. The PDA coating provides an additional method to immobilize and alter the material

surface in a relatively simple way, when compared to other conjugation methods (100). Figure 20 shows fetuin-A adsorption results from 100% human plasma measured by an EIA.

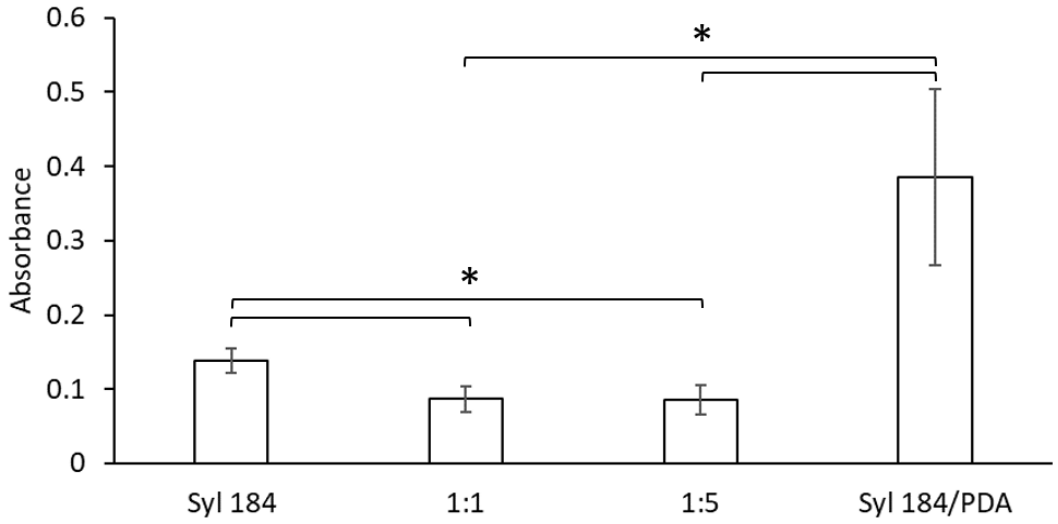


Figure 20. Comparison of fetuin-A available for functional binding from 100% plasma detected by enzyme immunoassay (EIA). Absorbance read at 450nm. Data are mean ± SD, n = 3. Statistical significance ($p < 0.05$) indicated by (*).

Unlike the EIA results from buffer, no significant differences were found between the 1:1 and 1:5 samples when fetuin-A adsorption was measured from plasma. Here, results demonstrate the preferential adsorption of fetuin-A to the stiffer Syl 184 samples, as there is statistically significant adsorption over the 1:1 and 1:5 formulations. This is in contrast to Vyner's 2016 work where fetuin-A appears to adsorb in greater amounts to polymers with greater chain flexibility (3), more similar to the results obtained from fetuin-A in buffer. It should be noted that the modulus values of the observed polymers were reported

in their 2013 work as 7.8 MPa and 1.2 MPa (43), whereas the samples presented in this work cover a range from 0.46 -3.8 MPa. The stiffest sample in their work has twice the elastic modulus of the Syl 184 sample here, where the modulus of their softer sample falls somewhere between the presented 1:1 and 1:5 sample.

Fromell et al. investigated the proteins human complement factor 3 and human factor XII, and found that more flexible polymer chains seemed to increase protein packing and encourage adsorption of proteins in their native conformation, while stiffer chains encouraged a change in conformation (141). In this work our samples have been prepared with varying ratios of Sylgard 184 : Sylgard 527, where Sylgard 527 is composed of chains with more repeat units than Sylgard 184 (130), explaining the decrease in elastic modulus seen with increasing Sylgard 527.

It is important to consider that this method of detection using the EIA determines the exposure of proteins for antibody binding, and these proteins could be hidden on certain substrates due to polymer chain directed changes in conformation or protein-protein interactions when adsorption is measured in a competitive environment. With the addition of the PDA layer, there is a significant increase in fetuin-A adsorption over the 1:1 and 1:5 samples. Though not statistically significant, there appears to be an increase in fetuin-A adsorption on the Syl 184/PDA samples over the Syl 184 samples as well. Given the required exposure of the antibody binding domain for detection via EIA, it is likely that protein immobilization through PDA results in fetuin-A oriented in such a way that the antibody binding domain is exposed and thus also more accessible for cell interactions.

In experiments where adsorption occurs from a competitive environment, the PDA layer provides a coating method with not only increased protein binding, but more strongly bound fetuin-A. The interactions between the functional groups of the PDA surface and protein result in adsorbed protein that is less likely to exchange with other proteins in the plasma. In this way PDA modification offers not only a more permanent protein immobilization method, but a snapshot into what the initially adsorbed levels of fetuin-A may be, as the strong binding and irreversibility would likely interfere with the typical Vroman effect. This also allows important comparisons to be made with these bioactive modified materials when studying subsequent cellular interactions.

4.4 Conclusions

On unmodified PDMS samples the highest levels of fetuin-A adsorption from buffer appear to occur on the Syl 184 and 1:1 samples. From plasma, the stiffer Syl 184 shows higher amounts of fetuin-A adsorption. PDA coated Syl 184 appears to have the highest amount of fetuin-A adsorption over all samples, with the Syl 184 slightly lower, but not significant. The results are summarized in Table 2. The significantly greater adsorption to Syl 184/PDA samples from buffer and slightly greater adsorption from plasma demonstrates the dominating effect of the PDA chemical modification over elastic modulus. Despite differences in measurement method, the BCA assay and EIA from buffer results appear to agree when looking at the trends, based on the relative differences achieved through absorbance readings with the EIA. This work demonstrates the importance of determining protein adsorption in both single and competitive protein environments.

Table 2. Summary of significant ($p < 0.05$) protein adsorption results obtained from BCA assay and EIA.

	Unmodified PDMS	***Syl 184/PDA***
BCA Assay	1:1 ↑ adsorption over 1:5	N/A
EIA (fetuin-A in buffer)	1:1 ↑ adsorption over 1:5 Syl 184 ↑ adsorption over 1:5	Greatest adsorption
EIA (100% plasma)	Syl 184 ↑ adsorption over 1:5	↑ adsorption over 1:1 and 1:5

5. Macrophage Studies

5.1 Introduction

Following protein adsorption to an implanted material, macrophages are recruited to the surrounding area. Here, the cells phagocytose foreign particles and can secrete cytokines that further recruit and activate immune cells. The macrophage response, and thus the cytokines secreted, dictate whether the response to injury leads to wound healing or a continued pro-inflammatory response (109). Traditionally, macrophage polarization has been characterized as either M1 or M2, which release pro- and anti-inflammatory cytokines, respectively (111). Over the years, research has shifted to view macrophage polarization on a spectrum, dictated by the function of the macrophage (110). With either viewpoint, the activation of macrophages in response to the implanted material, and any further polarization, is crucial in determining the host response to the biomaterial. The inflammatory response can be investigated by evaluation of macrophage activation and polarization, specifically the release of cytokine markers including interleukin-10 (IL-10) and tumour necrosis factor-α (TNF-α). IL-10 shows anti-inflammatory effects by suppressing the production of TNF-α from macrophages activated by IFN-γ (115). TNF-α induces the acute inflammatory response by recruiting pro-inflammatory cells to the site

of injury (114). Initial pro-inflammatory polarization is required to fight off infection, but further anti-inflammatory polarization is required for wound healing (113). In this work, the macrophage response to PDMS substrates with various surface modifications is investigated by measuring the concentration of IL-10 and TNF-α released by the attached macrophages. These studies are important in the fundamental understanding of the influence of fetuin-A on macrophages and in the pursuit for discovery of biomaterial modifications with immunomodulatory effects.

5.2 Materials and Methods

5.2.1 Sample Preparation

In efforts to minimize sample handling and best control the surface area of the samples, Syl 184, 1:1, and 1:5 formulations were prepared as described in previous chapters and poured directly into the wells of a 96-well plate to cure. After 30 minutes in a vacuum desiccator the samples were left to cure for 48 hours. Plates were placed under UV light for 30 minutes before samples were rinsed 3 times for 5 minutes each with sterile, low endotoxin PBS (pH 7.4, < 0.05 EU/mL). 200μL of a 2 mg/mL dopamine hydrochloride solution in PBS (pH 8.5) was deposited onto Syl 184 samples to prepare Syl 184/PDA samples. All other samples in the plate were hydrated in PBS (pH 7.4) and plates were left covered overnight on a plate shake to facilitate PDA layer formation.

Prior to protein adsorption the solution in all wells was aspirated off, and Syl 184/PDA samples were rinsed (3 x 5 min, PBS pH 7.4). 100μL of fetuin-A in buffer (sterile PBS, pH 7.4, < 0.05 EU/mL) was added to half of the wells and left to adsorb for 1 hour at room temperature. The 96-well plate set up for cell culture studies is shown in Figure 21.

Prior to cell studies, samples with protein adsorbed were rinsed (3 x 5 min, PBS pH 7.4, < 0.05 EU/mL) to remove any loosely bound or non-adsorbed protein.

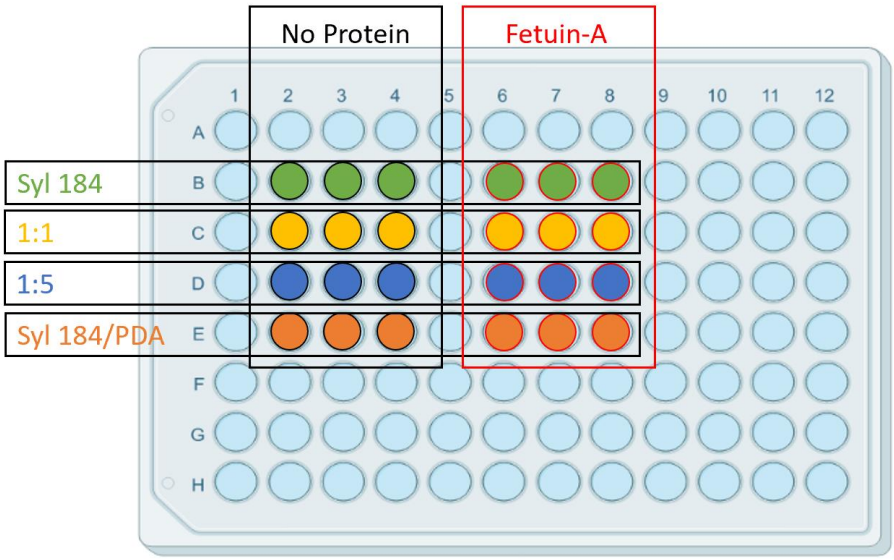


Figure 21. Sample distribution in the 96-well plate for cell culture studies.

5.2.2 Cell Culture

RAW 264.7 macrophages (Bowdish Lab, McMaster University) were cultured in Dulbecco's Modified Eagle Medium (DMEM, Wisent, 319-007-CL) containing 10% fetal bovine serum (FBS) with less than 0.2 EU/mL (Wisent, 090-150) and 1% penicillin-streptomycin (Wisent, 450-201-EL). The cells were maintained in a 5% CO_2 incubator and passaged using TrypLE™ Express Enzyme (ThermoFisher, 12604021). Once cells reached 70% confluence after 3 days they were counted with a hemocytometer. After counting, cells were re-suspended in media and seeded on PDMS samples at $1.0x10^4$ cells per well in a 96-well plate.

5.2.3 Cell Proliferation

The colourimetric cell counting kit-8 assay (CCK-8, Sigma, 96992, USA) was used to quantify the RAW 264.7 macrophages adhered to the sample. Cells were seeded onto samples in wells of a 96-well plate at $1.0x10^4$ cells per well. The plates were left to incubate (5% CO_2 at 37°C) for 24 hours and 48 hours where they were rinsed 3 x 5 minutes with sterile PBS (pH 7.4, < 0.05 EU/mL) prior to the addition of 100 μL of fresh cell culture media and 10 μL of CCK-8. After a 2.5-hour incubation, the cell supernatant was transferred to a new well for absorbance reading at 450nm with a SpectraMax ABS Plus (Molecular Devices, USA). Standards were seeded in a 96 well plate and left to adhere for 4 hours in an incubator at 37°C before 10 μL of CCK-8 solution was added per well. After an additional 1-hour incubation, absorbance of the standard wells was read at 450nm.

5.2.4 Cytokine Quantification (ELISAs)

The secretion of TNF-α and IL-10 in response to substrate stiffness and fetuin-A pre-adsorption was investigated with IL-10 (Mabtech, 3432-1H, Sweden) and TNF-α (Mabtech, 3511-1H, Sweden) ELISAs. 24 and 48 hours after cell seeding, cell supernatant was extracted via centrifugation (Allegra X-12 R, Beckman Coulter, USA). The protocol provided by the manufacturer was followed for the assays. Cytokine standards were prepared from the known concentrations provided in the respective ELISA kits. Final steps involved 15 minute incubation with TMB followed by the addition of a 0.2 M sulfuric acid stop solution. Absorbance was measured at 450 nm (SpectraMax ABS Plus, Molecular Devices, USA) within 15 minutes of adding the stop

solution. A reading at the reference wavelength of 600 nm was also taken and was subtracted from the 450 nm data.

5.3 Results and Discussion

5.3.1 Cell Quantification Assay

In other works, altering properties such as surface chemistry (93), topography (142), and mechanical properties (143) have yielded changes in cellular activation and proliferation. Since adsorbed proteins act as a bridge between the material surface and attached cells, it is important to consider the previous protein adsorption results when analyzing macrophage data. In these experiments half of the samples had fetuin-A pre-adsorbed prior to cell culture. This pre-adsorption step allowed exploration of fetuin-A's role in biomaterial interactions, along with its potential as a surface modifier, through both physical adsorption and modification with PDA to facilitate greater immobilization. Previous BCA assay and EIA data are important when considering adsorbed fetuin-A amounts prior to cell culture, as samples with adsorbed fetuin-A from a single protein solution and from plasma are both represented here.

To investigate cell attachment and proliferation, the CCK-8 assay was performed at two time points. As used in other studies (4,102,107), this assay allows for the comparison of living cells at longer timepoints due to its relatively low toxicity when compared to the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay and other cell counting methods. The absorbance results 24 hours after cell seeding can be seen in Figure 22. Establishing the absolute number of cells across the samples requires a standard curve that was not possible with these samples. However, the absorbance values

are still able to provide relative comparisons and allow the normalization of cytokine release data based on absorbance.

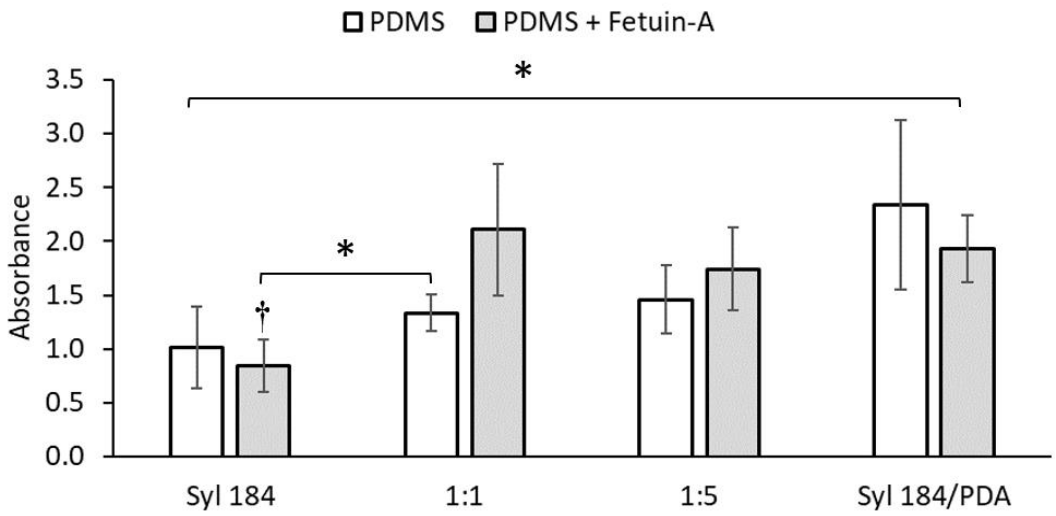


Figure 22. Comparison of the number of adhered cells to samples 24 hours after cell seeding, as measured by absorbance. Data are mean ± SD, n = 3. Statistical significance ($p < 0.05$) indicated by (*) between samples indicated, and (†) when compared to all other PDMS + fetuin-A samples.

Syl 184 samples with pre-adsorbed fetuin-A show a significantly smaller ($p < 0.05$) absorbance value than all other samples pre-adsorbed with fetuin-A, indicating less adhered cells. Our previous EIA data on fetuin-A available for functional binding found significantly greater amounts of fetuin-A from buffer on Syl 184 and 1:1 when compared to 1:5, along with overall greater adsorption to Syl 184/PDA samples. Given the previous data, and evidence of fetuin-A influencing cell attachment, we expected that fetuin-A may also increase macrophage adhesion. At this time point, we were unable to distinguish between fetuin-A modified 1:1, 1:5, and Syl 184/PDA data, and supernatant collected

from Syl 184 + fetuin-A samples demonstrated a significantly lower absorbance. On the samples without pre-adsorbed fetuin-A, no distinct trends are seen between Syl 184, 1:1 and 1:5. There is significantly less cell adhesion on the unmodified Syl 184 when compared to Syl 184/PDA modified with fetuin-A, which would be expected if fetuin-A plays a role in encouraging cell attachment – as protein adsorption experiments demonstrated the most fetuin-A adsorption to Syl 184/PDA samples.

The same measurement for cell adhesion values was also taken 48 hours after seeding, as seen in Figure 23. Results 48 hours after seeding no longer show a significant decrease in absorbance of Syl 184 modified with fetuin-A compared to all other modified samples, only a difference when compared to the softer 1:5 + fetuin-A remains. At this time point there is a significant decrease in fetuin-A modified Syl 184 absorbance when compared to the unmodified Syl 184 sample which was not significant after 24 hours. Syl 184/PDA has a significantly greater absorbance value than all other samples excluding Syl 184/PDA modified with fetuin-A. The Syl 184/PDA unmodified sample will adsorb various serum proteins from the cell media, and may interact directly with the cells, likely attributing to the increased cell adhesion (144).

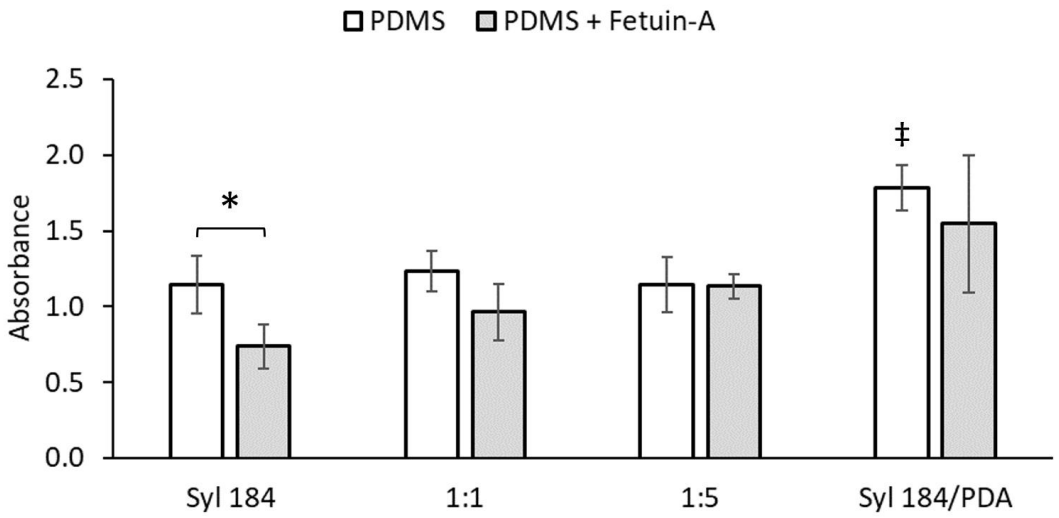


Figure 23. Comparison of number of adhered cells to samples 48 hours after cell seeding, as measured by absorbance. Data are mean ± SD, n = 3. Statistical significance ($p < 0.05$) indicated by (*) between samples indicated, and (‡) when compared to all non-PDA samples.

Vyner et al. found that with fetuin-A and plasma supplemented media there was an increase in fibroblast attachment to elastomers of two different molecular weights, when compared to non-supplemented media after 1 day (3). Interestingly, they did not see this increase in attachment when plasma was not present, suggesting that fetuin-A responds to particular material properties when in a complex plasma environment, and is able to influence fibroblast response. Comparing our data on fetuin-A adsorption from buffer (Figure 17 and Figure 19), there is an increased fetuin-A adsorption on the 1:1 samples over the 1:5 samples. Given evidence of fetuin-A's involvement in the attachment and growth of other cells, including carcinoma cells (145), it would not be surprising to see increased cell proliferation on samples which demonstrated high levels of fetuin-A

adsorption. In contrast, Siegel-Axel et al. studied the effects of fetuin-A on the proliferation of various cells and found that the proliferation of endothelial cells and perivascular fat cells after 72 hours was reduced upon 1 hour exposure to a 300μg/mL dose of fetuin-A, when compared to controls (146). There is also evidence that fetuin-A may act as an opsonin increasing interactions with macrophages (77). This research suggests that the influence of fetuin-A on cell attachment and proliferation is dependent on the cells studied and is a dynamic process, with the concentration of fetuin-A and the cell environment also playing a role.

The fetuin-A data obtained from the EIA is important and unique since it provides an indication of not just the quantity of total fetuin-A, but the fetuin-A that is available for functional antibody binding. This functional binding amount provides a direct indication of the fetuin-A that would be available to interact with cell receptors. After 24 hours there is evidence of elevated cell attachment on the 1:1 sample modified with fetuin-A, however at 48 hours this is no longer the case. This suggests that the trends of fetuin-A adsorbed to PDMS of varying elastic modulus observed from buffer does not necessarily translate to altered cell attachment over time. Adsorption data for fetuin-A from plasma (Figure 20) demonstrated an increase in adsorption to the stiffer Syl 184 over 1:1 and 1:5 samples. After 24 hours, the number of cells adhered based on absorbance values for Syl 184 modified with fetuin-A were significantly lower than all other fetuin-A modified samples. When exposed to a competitive environment, pre-adsorbed fetuin-A on the stiffer polymer chains may be replaced by other serum proteins as suggested by Vyner et

al. (3), and this may explain the lower absorbance indicating less adhered cells to Syl 184 + fetuin-A samples.

From data presented in Figure 19 the greatest amount of fetuin-A available for antibody binding is evident on Syl 184/PDA samples. Comparing adhered cells after 48 hours on fetuin-A modified and unmodified Syl 184/PDA (Figure 23), there is no significant difference in absorbance. The large variability and error bars on modified Syl 184/PDA may in part be due to inhomogeneous PDA coverage across samples, as discussed previously. The human fetuin-A levels on the pre-adsorbed Syl 184/PDA are evident from Figure 19, however, for the samples in cell culture we would expect bovine fetuin-A and other proteins to be present on unmodified Syl 184/PDA due to the FBS supplemented media, which may explain the lack of significant difference between these two PDA samples. Additionally, interactions between the cells and the PDA molecules themselves may play a role. It is evident that there is an early influence of elastic modulus on cell adhesion, but this changes throughout the time points investigated. The use of PDA coatings on PDMS increases and maintains cell adhesion likely through the binding of various proteins including fetuin-A. The level of macrophage adhesion is important to consider, but the immune response will be influenced by many factors such as cytokine secretion.

5.3.2 Cytokine Assays

There are numerous reports of the anti-inflammatory effects of fetuin-A and an influence of material stiffness on macrophage response. Dziegielewska et al. found that fetuin-A was able to attenuate pro-inflammatory macrophage response to lipopolysaccharide (LPS)

(147). Similar effects showing a reduction in macrophage activation were observed by Blakney et al. upon culture on softer substrates, without the explicit addition of fetuin-A, though media was supplemented with fetal bovine serum (127). In fact, Adlerz et al. found that macrophages were able to sense and respond to material stiffness within 1 hour of exposure, and that migration speed and proliferation rate were increased significantly on stiffer substrates (126). Here we aim to investigate the effects of surface stiffness and fetuin-A modification on RAW 264.7 macrophage response. Given the dynamic change in macrophages over time, we sought to investigate cytokine release at two time points. The data presented at each time point has been normalized over the absorbance values gathered from the CCK-8 assay. Normalized IL-10 concentration data after 24 hours of cell culture can be seen in Figure 24.

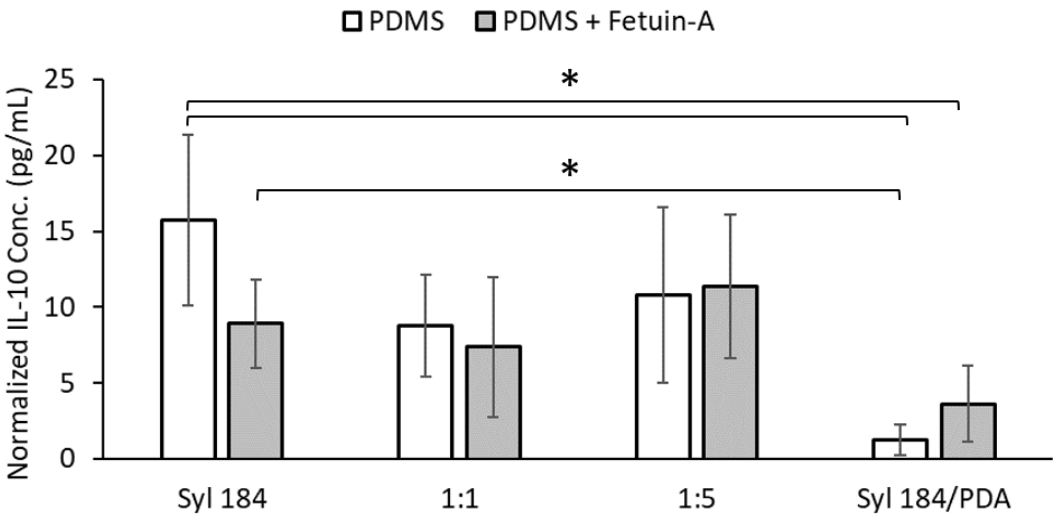


Figure 24. IL-10 concentration after 24 hours normalized over average absorbance value from CCK-8 assay for relative number of adhered cells. Data are n = 3 ± SD. Statistical significance ($p < 0.05$) indicated by (*).

After 24 hours, there is large variation in IL-10 data, and no trends can be seen between samples of varying stiffness, regardless of fetuin-A modification. Of note, the cell supernatant from cells cultured on Syl 184/PDA shows a significantly lower concentration of IL-10 when compared to both fetuin-A modified and unmodified Syl 184. This demonstrates that in response to PDMS modified with PDA, both with and without fetuin-A, the macrophages secrete less IL-10. This suggests that PDA itself is able to contribute to this decrease, but likely does so through the many proteins that will attach from serum in the cell media. Based on EIA data of PDA samples assessing binding in plasma, it is likely that this protein layer also contains some fetuin-A along with other plasma proteins. It is also possible that despite fetuin-A being immobilized on the PDA surface, other proteins may also bind, influencing the response. Thus, the PDA layer and its adhesiveness for a variety of proteins is likely contributing to this lower amount of IL-10 secretion. The data after 48 hours demonstrates this more significantly, as seen in Figure 25.

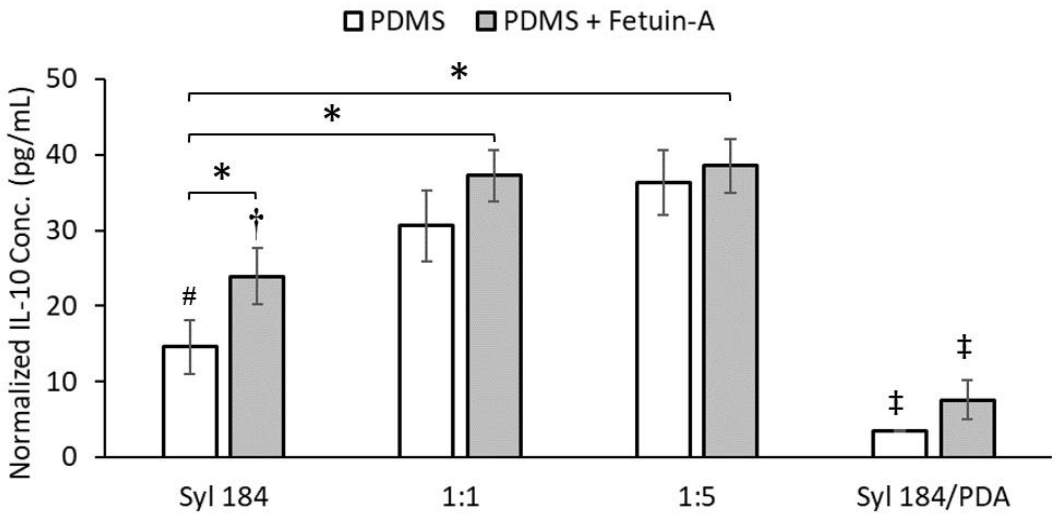


Figure 25. IL-10 concentration after 48 hours normalized over average absorbance value from CCK-8 assay for relative number of adhered cells. Data are n = 3 ± SD. Statistical significance ($p < 0.05$) indicated by (*) between samples, (#) when compared to all PDMS samples, (†) when compared to all PDMS + fetuin-A samples, and (‡) when compared to all non-PDA coated samples.

After 48 hours, IL-10 secretion in response to Syl 184/PDA samples is lower than all other unmodified PDMS samples. The collected supernatant from Syl 184/PDA samples modified with fetuin-A also shows a reduced IL-10 concentration when compared to all other fetuin-A modified samples. Based on previous protein adsorption experiments we would expect Syl 184/PDA samples to have the greatest amount of protein attached from FBS. The substantially lower IL-10 concentration from both Syl 184/PDA samples at both time points, and the lack of significant difference between the fetuin-A modified and plain Syl 184/PDA sample, suggests once again that the PDA influences cell adhesion and the reduced IL-10 concentration, likely through the proteins that attach. To our

knowledge, data on IL-10 expression in response to planar PDA coated polymer samples in FBS supplemented media does not yet exist in the literature.

Both fetuin-A modified and unmodified Syl 184 resulted in less IL-10 than their 1:1 and 1:5 counterparts at this time point, suggesting that stiffness or the adsorbed protein layer in response to stiffness, plays a role in the amount of IL-10 secreted. Based on the protein adsorption data, from buffer the EIA demonstrated significantly greater adsorption of fetuin-A with exposed binding domains on Syl 184 than the 1:5 samples, and from plasma significantly more from Syl 184 than 1:1 and 1:5 samples. The most adsorption occurred on Syl 184/PDA samples in both cases. Given these results it appears that after 48 hours the samples that have the greatest amount of fetuin-A adsorbed and available for binding, based on previous experiments, show the least amount of secreted IL-10.

Cell supernatant concentrations of the pro-inflammatory cytokine TNF-α were measured for comparison using the same batch of cells as the IL-10 results. After 24 hours the TNF-α results can be seen in Figure 26. The greatest TNF-α concentration was found in response to unmodified and fetuin-A modified Syl 184 samples. For the samples that did not have fetuin-A pre-adsorbed, ELISA results showed significantly increased TNF-α concentration in response to Syl 184 over 1:1, 1:5, and Syl 184/PDA samples. In earlier adsorption studies, fetuin-A was adsorbed from plasma to the same substrate surfaces, which would be similar to what is occurring here from cell media prior to macrophage attachment. The EIA results indicated more fetuin-A adsorption from plasma on Syl 184 when compared to 1:1 and 1:5 samples, but the most adsorption occurred to Syl 184/PDA samples. Here there is a similar trend between the Syl 184, 1:1, and 1:5 samples, where

the stiffest sample results in the most TNF-α production – results that are also found elsewhere in the literature. Investigating cytokines released in response to material stiffness, Blakney et al. found increased cell spread and classical (M1) activation, with increased TNF-α expression on stiffer hydrogels (127), which agreed with previous research from another group that demonstrated an increase in cell spread on stiffer substrates (128).

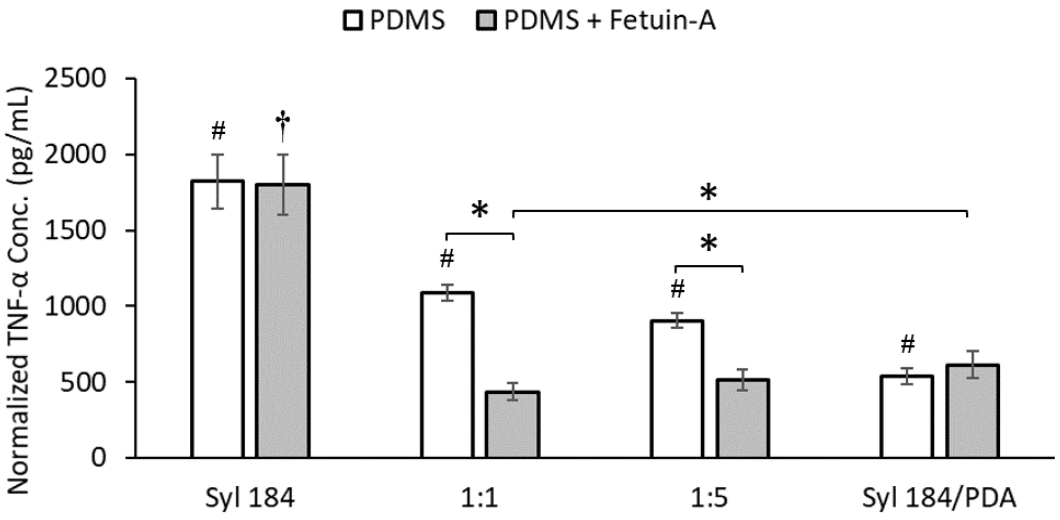


Figure 26. TNF-α concentration after 24 hours normalized over average absorbance value from CCK-8 assay for relative number of adhered cells. Data are n = 3 ± SD. Statistical significance ($p < 0.05$) indicated by (*) between samples, (#) when compared to all PDMS samples, and (†) when compared to all PDMS + fetuin-A samples.

Since there is evidence that the stiffest samples adsorb the most functionally available fetuin-A and result in a higher concentration of TNF-α, it is possible that there is a correlation between these. However, our EIA results from plasma indicate that the greatest amount of functionally available fetuin-A occurs on the Syl 184/PDA sample,

while there is the lowest amount of TNF-α in response to the same sample. Xue et al. found that TNF-α production was decreased on PDMS surfaces modified with PDA and hyaluronic acid (HA), when compared to PDMS alone and PDMS + HA (93). Jin et al found a decrease in TNF-α released by lipopolysaccharide-induced macrophages in response to PDA degradation products, demonstrating its ability to mediate the inflammatory response (148).

It is also important to consider that the results of the EIA from plasma are not an exact representation of what is occurring during the cell studies since the supplemented cell media will contain some different molecules than the human plasma, but provides similarities to evaluate an environment with multiple plasma proteins present. Upon modification of 1:1 and 1:5 samples with fetuin-A there is a significant decrease in TNF-α. It is typically expected that a decrease in TNF-α corresponds to a greater secretion of IL-10 in response to materials based on IL-10's ability to down regulate TNF-α production (115). For these samples the IL-10 data is variable and does not show a significant increase. However, the reduction in TNF-α expression in the presence of these fetuin-A modified samples may also be due to the protein's role in inhibiting TNF-α production in the presence of cationic molecules (72).

After 48 hours, there was more TNF-α production in response to Syl 184 + fetuin-A samples than on the unmodified Syl 184, 1:5 + fetuin-A, Syl 184/PDA, and Syl 184/PDA + fetuin-A, as seen in Figure 27. It is expected, based on previous protein data, that the Syl 184 samples have increased levels of fetuin-A over 1:1 and 1:5 samples and the Syl 184/PDA samples have the highest levels. It is important to note the differences in protein

interactions on the Syl 184, 1:1, and 1:5 samples compared to the Syl 184/PDA samples. With physical adsorption to the material, proteins that adsorb are likely to exchange with other proteins in the serum/plasma, and they may also unfold and change conformation. On PDA modified materials the initially adsorbed proteins will likely remain due to stronger binding, and may be less likely to change conformation, however this is not yet known. It was hypothesized by Vyner et al. that on stiffer polymers, it is more likely that pre-adsorbed protein is exchanged with other proteins over time (3). It is possible that the Syl 184 samples do not maintain the pre-adsorbed level of fetuin-A that is initially present. Instead, it may be that inflammatory proteins such as fibrinogen, which appear to preferentially adsorb to stiffer substrates, will replace some of the pre-adsorbed fetuin-A. The exchange of pre-adsorbed fetuin-A on stiffer samples may explain why at 24 hours the difference between fetuin-A modified and unmodified 1:1 and 1:5 samples is not seen with the Syl 184 samples.

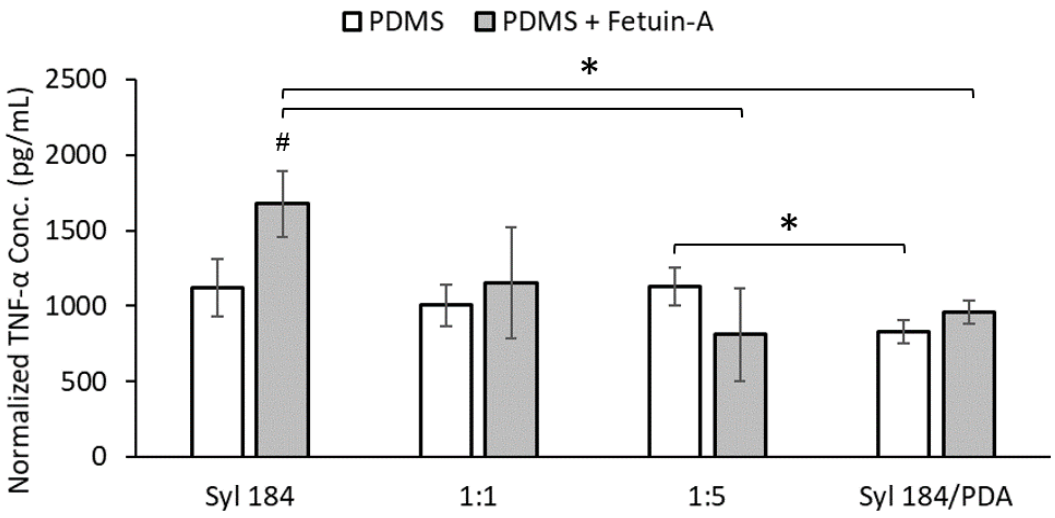


Figure 27. TNF-α concentration after 48 hours normalized over average absorbance value from CCK-8 assay for relative number of adhered cells. Data are n = 3 ± SD. Statistical significance ($p < 0.05$) indicated by (*) between samples, and (#) when compared to all PDMS samples.

Although the fetuin-A modified 1:1 and 1:5 samples initially reduced TNF-α after 24 hours, this is no longer the case after 48 hours. This does not appear to be a strictly pro-inflammatory effect, as after 48 hours the IL-10 concentration from 1:1 and 1:5 samples are increased over Syl 184 samples as well. This is surprising based on IL-10's role in down regulating M1 polarization. Similarly, after 48 hours the TNF-α concentration in response to both Syl 184/PDA samples increases from the 24-hour time point. It is likely that there are numerous complex cytokine and cell interactions involved and additional studies with various other cytokines and indicators of cell response are needed to fully understand these processes.

5.4 Conclusions

Based on cell attachment and proliferation studies, measured by absorbance values with the CCK-8 cell counting assay, there were no significant effects found for fetuin-A pre-adsorption compared to unmodified samples. In most cases, the cell counts appeared to be influenced by the stiffness of the substrates and the presence of a PDA coating. At both 24 and 48 hours, fetuin-A modified Syl 184 had a significantly lower absorbance value than 1:5 + fetuin-A. At 24 hours, cell adhesion based on absorbance for the Syl 184/PDA + fetuin-A sample cell supernatant was greater than Syl 184 and at 48 hours Syl 184/PDA was increased over all other samples except Syl 184/PDA + fetuin-A. Significant results are summarized in Table 3.

Investigation into macrophage response through secreted cytokines demonstrated a reduction in IL-10 concentration on Syl 184/PDA when compared to fetuin-A modified and unmodified Syl 184 at 24 and 48 hours. The same was found for TNF-α concentration, except at the 48-hour time point, unmodified Syl 184 was no longer significantly less than Syl 184/PDA samples. These results indicate the ability of PDA to reduce both IL-10 and TNF-α cytokine secretion. This may be due to the adhesive layer of PDA and its ability to bind multiple proteins from plasma influencing interactions with macrophages. Previous protein adsorption data showed the greatest fetuin-A adsorption from buffer and plasma on Syl 184/PDA samples, and fetuin-A appeared to preferentially adsorb to unmodified Syl 184 over softer samples. This suggests that fetuin-A behaves differently when adsorbed to Syl 184, than when immobilized on Syl 184/PDA, and the cytokine secretion results are influenced by this. This is likely due to various factors

including exchange of proteins within the complex environment after physical adsorption, alteration of structure on the surface through changes in orientation and conformation, and the influence of adhesive PDA interactions with proteins and cells.

At 24 hours, TNF-α secretion appeared to be influenced by fetuin-A modified 1:1 and 1:5 samples, as they showed significantly lower TNF-α concentrations over their unmodified counterparts. The fact that this did not occur on the Syl 184 samples may provide further evidence that there is an exchange of fetuin-A proteins pre-adsorbed to these stiffer samples. This phenomenon is not seen at 48 hours, which may be due to the loss of the fetuin-A adsorbed layer over time or structural changes of the fetuin-A over time. PDA was not able to facilitate the fetuin-A modification to this effect, likely due to PDA also interacting with other proteins and the fetuin-A structural properties influencing cell activation. It is evident that controlling the elastic modulus can influence the adsorption of fetuin-A such that it is in a configuration to influence macrophage activation at particular time points. If this configuration of fetuin-A can be achieved through tailoring mechanical properties and/or stabilizing fetuin-A over time it may be possible to obtain fetuin-A modified materials with immunomodulatory properties.

Table 3. Summary of significant ($p < 0.05$) cell proliferation and cytokine concentration data collected from the CCK-8 assay and IL-10 and TNF-α ELISA experiments.

	24hrs	***48hrs***
CCK-8	• Syl 184 + fetuin-A ↓ all other fetuin-A modified samples • Syl 184 ↓ Syl 184/PDA + fetuin-A	• Syl 184 + fetuin-A ↓ 1:5 + fetuin-A and Syl 184 • Syl 184/PDA ↑ all other samples except Syl 184/PDA + fetuin-A
IL-10	• Syl 184/PDA ↓ Syl 184 + fetuin-A, Syl 184 • Syl 184/PDA + fetuin-A ↓ Syl 184	• Syl 184/PDA ↓ all samples except Syl 184/PDA + fetuin-A • Syl 184/PDA + fetuin-A ↓ all fetuin-A modified samples • Syl 184 ↓ 1:1, 1:5 • Syl 184 + fetuin-A ↓ 1:1 + fetuin-A and 1:5 + fetuin-A
TNF-α	• Syl 184 and Syl 184 + fetuin-A ↑ all samples except each other • All samples without fetuin-A are distinct from each other (Syl 184 > 1:1 > 1:5 > Syl 184/PDA) • 1:1 ↑ 1:1 + fetuin-A • 1:5 ↑ 1:5 + fetuin-A • Syl 184/PDA + fetuin-A ↑ 1:1 + fetuin-A	• Syl 184 + fetuin-A ↑ all samples except 1:1 + fetuin-A • 1:5 ↑ Syl 184/PDA

6. Conclusions

6.1 Thesis Summary and Key Findings

A variety of PDMS samples were successfully prepared by mixing Sylgard 184 and Sylgard 527 at different ratios to achieve materials with distinct elastic moduli. Characterization results for these samples indicate that there was no change in surface wetting properties for the samples of varied moduli and no changes in surface roughness and surface chemistry between the higher modulus Sylgard 184 samples and the lower modulus 1:1 ratio samples. For the lowest modulus 1:5 samples, minor changes in surface roughness and surface chemistry were evident, likely due to the slight differences in

Sylgard 184 and Sylgard 527 formula. This method of PDMS sample preparation is an advantageous option for studying impacts of elastic modulus without substantially altering other material properties. The addition of a PDA coating on cured Sylgard 184 samples resulted in a decrease in hydrophobicity and increase in surface roughness, as would be expected with the deposition of this polymer film. It is expected that the catechol and amine chemistry of the PDA-protein interactions are the major driving force for the ability of these modified materials to play a role in the adsorption process.

Fetuin-A adsorption to the PDMS substrates was investigated and 1:1 samples had greater total fetuin-A adsorption than 1:5 samples from buffer, while both Syl 184 and 1:1 showed increased amounts of fetuin-A exposed for functional binding over 1:5 samples. From plasma, fetuin-A adsorption was highest on the stiffer Syl 184 samples. Based on these results it is clear that fetuin-A adsorption can be influenced by controlling the elastic modulus of polymer samples and in turn the polymer chain flexibility. Fetuin-A appears to preferentially adsorb to the stiffer substrates over the softest. When Syl 184 samples were coated with PDA to provide a bioadhesive coating, an increase in fetuin-A adsorption was achieved indicating that chemical modification can also be used to immobilize proteins and study the effects of cell response.

Cell attachment data indicated an early influence of elastic modulus, observed through decreased absorbance from stiffer Syl 184 samples. PDA coatings were able to increase cell adhesion, likely through the increased binding of proteins to the sample surface. Comparable cell attachment was expected on Syl 184 and Syl 184/PDA due to their similar trends from adsorption experiments. Given the opposite was observed, it indicates

that the adsorption orientation or conformation of fetuin-A and other proteins to these two functionally different sample surfaces may alter cell attachment. Normalized cytokine data for IL-10 and TNF-α secretion also suggests that fetuin-A behaves differently when adsorbed to Syl 184 versus when immobilized to Syl 184/PDA, likely due to protein exchange that will occur more readily on Syl 184 samples, adsorbed orientation and conformation, and interactions between proteins, cells, and PDA. This work shows evidence that at specific time points macrophage activation is influenced by elastic modulus, likely due to altered protein adsorption amounts and configurations in response to materials of varying stiffnesses. Based on this, it may be possible to simultaneously tailor mechanical properties and bioactive properties by attaching proteins such as fetuin-A to achieve materials for immunomodulation.

6.2 Future Directions

6.2.1 Surface Characterization of PDMS Substrates

In this work we have investigated how decreasing the ratio of Sylgard 184 : Sylgard 527 impacts the surface wetting, surface chemistry, and morphology alongside the changing bulk elastic modulus. Previous work by Palchesko et al. described this method of preparing PDMS samples of varying elastic moduli and stated that other material properties remained constant across the samples (10). Here, we discovered that changes in surface chemistry and surface roughness existed between some PDMS samples, particularly at the lowest elastic modulus. Further experimentation could add to the suite of characterization techniques performed in this work. XPS measurements provide surface sensitive information on elemental and chemical surface changes. However, due

to this surface sensitivity, samples can readily contaminate in typical lab environments and variability in data can result. Further XPS studies with angle resolved methods would provide additional data on surface chemistry. Surface stiffness measurements of these substrates by AFM would also be valuable to be studied and compared to the bulk stiffness values obtained. To reduce the influence of varying surface roughness, PDMS samples could be cured on mica to create atomically flat surfaces for adsorption and cell studies rather than exposing them to rougher PDMS cured in air.

6.2.2 Protein Adsorption Studies

In this work, the BCA assay was used as an indirect method for measuring total protein adsorption, and an EIA used to determine the binding availability of adsorbed fetuin-A. Neither method provides a direct quantitative measurement of total protein adsorption. Methods that could accomplish this, such as radiolabelling, should be performed to determine the amount of fetuin-A adsorbed to the material surface regardless of adsorbed orientation and conformation to address insufficiencies in BCA assay and to complement EIA methods. It is well known that the material properties influence total protein adsorption, along with the composition of the protein layer, and both of these were addressed in this work. The orientation and conformation of adsorbed proteins also impacts the subsequent biological response and should be studied further in response to varying elastic moduli and surface modification. Through the EIA, the availability of adsorbed fetuin-A for binding was able to be assessed, but more thorough studies on fetuin-A should be performed to determine the native orientation and conformation of the adsorbed protein, potentially using monoclonal antibodies to specific sites on fetuin-A.

Orientation of adsorbed proteins has been investigated through AFM imaging (149), quartz crystal microbalance with dissipation (QCM-D) and dual polarization interferometry (DPI) (20).Researchers have paired methods such as QCM-D and circular dichroism (150), as well as QCM-D and surface plasmon resonance (SPR) (139) to infer conformational changes. To date there is no single method to directly determine conformation of adsorbed proteins on planar polymer surfaces. It would also be beneficial to obtain dynamic protein adsorption data over just end point studies. Methods including QCM-D and SPR would also allow these real-time in-situ measurements to be made. To investigate the polymeric materials and surface modifications used in this work, spin coating or drop coating onto the QCM or SPR sensors is a necessary first step. Following this sample preparation and appropriate characterizations, dynamic measurements of protein adsorption can then be obtained.

6.2.3 Macrophage Studies

In this work, the concentration of IL-10 and TNF-α in cell supernatant was investigated to determine the polarization of RAW 264.7 macrophages in response to PDMS samples of varying elastic moduli and PDMS modified with PDA both with and without pre-adsorbed fetuin-A. As IL-10 and TNF-α are only two of the many cytokines released, a full profile of cytokine secretions would be valuable to understand the macrophage response. Here, we investigated macrophage response at 24 and 48 hours as macrophage polarization changes over time. Additional time points could be added to further investigate the dynamic macrophage response. Imaging of the cells with scanning electron microscopy (SEM) would also be of interest to determine any resulting changes

in cell morphology. There are various unique aspects of fetuin-A that could be further investigated to enhance the understanding of its role in adsorption to biomaterials and subsequent cell interactions. Since fetuin-A binds to calcium, it is possible that this may influence the surrounding environment and play a role in of the response of various types of cells. Studies to determine changes in calcium concentration at the surface would be valuable to track and relate to cell adhesion and activation. Cell studies could also be broadened to include platelets to better understand fetuin-A's role upon biomaterial contact with blood. Currently, any influence of fetuin-A on thrombus formation is unknown, but due to the many links between thrombosis and the immune response this may provide valuable insight. Finally, as discussed, there is an indication that both modifications to mechanical properties and bioactive properties through protein attachment may influence macrophage response and these effects should be further studied with these and other polymers and proteins.

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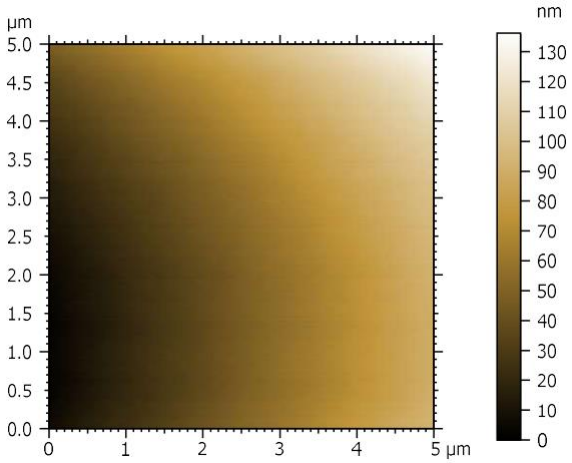
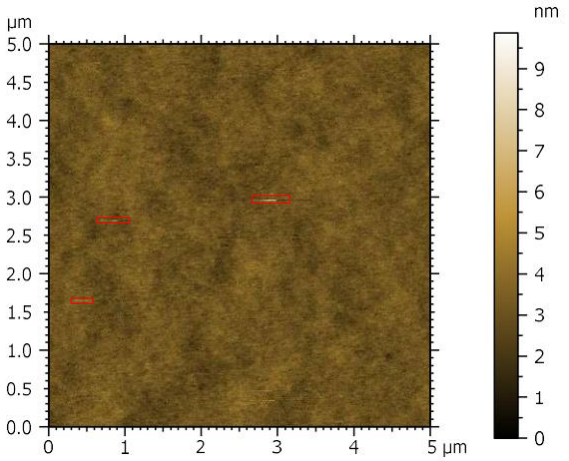
Appendix A

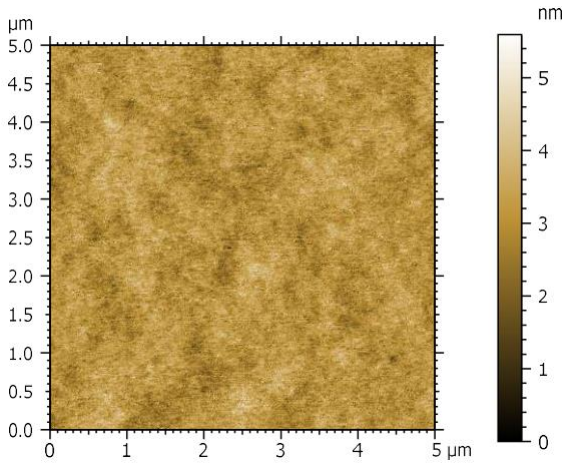
A1. AFM Image Analysis Protocol



Extracted height layer showing significant tilt in the sample. Often due to uneven sample height (bulk).



The image after first order flattening. Topography of the surface starts to emerge.
Areas targeted for removal can be seen surrounded by a red box.



After the removal of select lines that showed extreme peaks likely due to the tip skipping over the surface or springing back from being stuck in the substrate.