Supported lipid bilayer platforms for investigating complement activation, termination and regulation

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NANYANG
TECHNOLOGICAL
UNIVERSITY

**SUPPORTED LIPID BILAYER PLATFORMS FOR
INVESTIGATING COMPLEMENT ACTIVATION,
TERMINATION AND REGULATION**

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SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

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INVESTIGATING COMPLEMENT ACTIVATION,
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SAZIYE YORULMAZ AVSAR

**SCHOOL OF MATERIALS SCIENCE AND
ENGINEERING**

A thesis submitted to the Nanyang Technological University
in partial fulfillment of the requirement for the degree of
Doctor of Philosophy

2017

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research and has not been submitted for a higher degree to any other University or Institution.

11 April 2017

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Date

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Saziye Yorulmaz Avsar

Abstract

The complement system serves as the frontline of host defense for the human body against foreign entities, and is triggered via one or more of several signaling cascades that consist of interactions between soluble and cell surface-bound complement proteins. In this thesis, the objective is to explore how lipid membranes influence the initiation, amplification, termination and regulation of complement activation, utilizing supported lipid bilayer platforms as a model experimental system. The overall hypothesis of this work is that the supported lipid bilayer platform provides a useful model system to recapitulate key protein-lipid and protein-protein interactions involved in complement activation at lipid membrane interfaces, thereby establishing an experimental framework to decipher key trigger factors (*e.g.*, membrane surface charge) as well as evaluate candidate inhibitors acting against complement convertases and membrane attack complex assemblies. Within this scope, four different experimental studies were conducted, leading to new insights into the mechanisms by which lipid membranes influence complement activation. In the first study, the influence of membrane surface charge on the adsorption properties of different pattern recognition proteins involved in initiation of complement activation was investigated. In the second study, the effects of covalent and noncovalent tethering strategies on the self-assembly of the alternative pathway C3 convertase was monitored. It was identified that the C3b immobilization scheme is a critical factor governing convertase assembly, further enabling successful evaluation of a clinically relevant complement inhibitor, compstatin. In the third study, the assembly of the complement-terminating membrane attack complex was monitored onto supported lipid bilayer platforms, with particular attention to the influence of membrane surface charge. It was determined that the membrane attack complex assembly preferentially forms on negatively charged lipid membranes, indicating the importance of membrane electrostatics. In the fourth study, the inhibitory action of vitronectin and clusterin against the membrane attack complex assembly was evaluated, offering insight into the different mechanisms by which these two regulatory proteins inhibit complement activation. In summary, the findings presented in this thesis significantly contribute to the fundamental understanding of complement activation occurring at lipid membrane

interfaces, and offer a new platform to characterize the mechanisms of action for a wide range of complement inhibitors including small molecules, antibodies, and peptides.

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Dedicated to my mother

“ Annem`e ”

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Figure 6.1 Design of charged supported lipid bilayer platforms by the SALB formation method. QCM-D monitoring of the supported lipid bilayer formed on SiO₂ by the SALB formation method. (a) The schematic display charged lipids used to form a planar bilayer as follows: Left) DOPC:POPG (70:30), Middle) DOPC Right) DOPC:DOEPC (70:30). Color key: yellow: DOPC; red: DOEPC; blue: POPG. (b) Changes in frequency (ΔF , blue) and energy dissipation (ΔD , red) were recorded as a function of time at the third overtone, n=3. QCM-D signatures of planar bilayer formation by the SALB formation method were obtained by the following experimental sequence: (Step 1) Add Tris buffer

solution; (Step 2) Inject isopropanol solution; (Step 3) Add 0.5 mg/mL lipid molecules in isopropanol; (Step 4) Exchange isopropanol to Tris buffer solution; (Step 5) add BSA protein (0.1 mg/mL) to check the completeness of the bilayers. (c) Adsorbed lipid mass surface density was calculated by the Sauerbrey equation. (d) Percentage of bilayer completion was calculated based on BSA adsorption to bare SiO₂ substrate (control) and the bilayer after completion of the SALB procedure.

Figure 6.2 Assembly of MAC components on supported lipid bilayer platforms MAC formation is initiated by the incubation of hydrophilic C5b-6 and C7 precursors (1). During the incubation of these two protein precursors, C7 interacts with C5b-6 and causes a hydrophilic-amphiphilic transition to result in precomplex C5b-7 formation and its membrane association. Addition of C8 proteins (2) to membrane-bound C5b-7 precomplex leads both formation of the C5b-8 intermediate complex and its insertion into the hydrophobic core of the membrane. Subsequent introduction of C9 (3) results in an interaction with the membrane-inserted C5b-8 intermediate complex causing significant changes in C9 protein conformation and this makes 12-18 copies of C9 polymerize circularly to generate the final MAC complex(3). Using QCM-D tracking, changes in frequency (a) and dissipation (b) were monitored in order to validate MAC formation as a function of time. Three different kinds of SLB platforms composed of DOPC: POPG (70:30)(black square); DOPC:DOEPC(70:30)(red circle) and DOPC(blue triangle) were used to determine the effects of membrane surface charge on MAC formation. After completion of the SALB procedure and validation of bilayer completeness, the previously incubated C5b-6 and C7 proteins were added in the form of a precomplex C5b-7 to the SLB platform. Then, C8 and C9 were added to the membrane-associated C5b-7 precomplex, respectively. (c) Schematic representation of MAC formation on the negatively charged SALB platform.

Figure 6.3 Complement components C5b-6 and C7 create a C5b-7 precomplex which associates on the negatively charged lipid bilayer in order to initiate MAC formation. Changes in normalized frequency (a) and dissipation (b) are presented as a function of time. The initial MAC precursors including C5b-6 (red circle), C7 (black square), and

C5b-7 (blue up triangle) were individually injected onto the negatively charged bilayers after a stable baseline obtained from BSA addition. C5b-7 was formed at the end of a 30 minute incubation period of C5b-6 and C7. (c) Schematic diagram depicts the interaction of initial MAC precursors with the negatively charged bilayer.

Figure 6.4 Electrostatic interaction is essential for the activity of C8 proteins regardless of the presence of the precomplex C5b-7. The changes in frequency (a) and energy dissipation (b) are tracked as a function of time. C8 protein was added right after stabilization of BSA (wine hexagon) and association of the C5b-7 precomplex (green tetragon). The binding of C8 to membrane-associated C5b-7 precomplex creates a C5b-8 complex. (c) Schematic diagram presents the interaction of C8 proteins with a bilayer in the presence or absence of the C5b-7 precomplex.

Figure 6.5 Polymerization of C9 proteins depend on the existence of the membrane-inserted C5b-8 complex in order to form MAC. The changes in frequency (a) and energy dissipation (b) are presented as a function of time. C9 proteins were injected after the stabilization of BSA (purple star) and association of C5b-8 complex (pink left triangle). (c) Schematic representation demonstrates the interaction of C9 with a bilayer and membrane inserted C5b-8.

Figure 7.1 Experimental Strategies to Test Inhibitors of MAC Assembly. Two strategies were employed. In the first strategy, (i) the inhibitor and C5b-7 complex were mixed in solution and then (ii) the mixture was added to the SLB platform, followed by sequential addition of (iii) C8 and (iv) C9 proteins. In the second case, (i) the inhibitor was first added to the SLB platform followed by sequential addition of (ii) C5b-7, (iii) C8 and (iv) C9 proteins to the SLB platform.

Figure 7.2 QCM-D monitoring to identify the effect of glycerol on the SLB. Changes in the frequency (a) and dissipation (b) were recorded as a function of time at the fifth overtone, $n=5$ in order to identify the effect of glycerol. Initial measurement baselines were obtained after the bilayer quality was validated via BSA attachment and then the

experimental sequences were followed: (Step 1) Injection of representative glycerol solution; (Step 2) Inject 50nM Clusterin (Black Square); (Step 3) buffer rinsing.

Figure 7.3 QCM-D Monitoring of MAC Assembly on the SLB Platform (a) Schematic illustration of MAC formation via sequential addition format. Changes in (b) frequency and (c) Sauerbrey mass were monitored as functions of time after sequential addition of C5b-7 complex, C8 protein, and C9 protein onto the 70:30 mol% DOPC:POPG SLB platform. The SLB on silicon oxide was initially formed by the SALB method, and then the measurement time was normalized. At $t = 0$ min, the MAC assay was initiated and the C5b-7 complex was added at $t = 20$ min. Then, C8 protein was added at $t = 80$ min and C9 protein at $t = 150$ min. The adsorbed mass was calculated by the Sauerbrey relationship and the mass sensitivity constant for a 1Hz frequency shift is 17.7 ng/cm^2 .

Figure 7.4 Influence of Vitronectin on Membrane Association of the C5b-7 Complex (a) Schematic illustration shows addition of the C5b-7 complex which was preincubated with vitronectin. Panel (b) presents the QCM-D frequency shift associated with C5b-7 + vitronectin addition to the SLB platform as a function of vitronectin concentration. The C5b-7 complex concentration was fixed at 20 nM. The proteins were added at $t = 20$ min. Panel (c) shows absolute value of the maximum QCM-D frequency shift due to the amount of total adsorbed protein as a function of vitronectin concentration based on the data presented in Panel (b). Each data point is the average + standard deviation of three independent ($n = 3$) measurements. (d-e) Corresponding schematic illustration and QCM-D measurement data for the addition of vitronectin to the SLB platform, followed by addition of C5b-7 complex to the same platform. Panel (f) shows absolute values of the maximum QCM-D frequency shifts due to the amounts of adsorbed vitronectin and C5b-7 complex as a function of vitronectin concentration based on the data presented in Panel (e). Each data point is the average + standard deviation of three independent ($n = 3$) measurements.

Figure 7.5 Effect of Vitronectin on the Sequential Addition of C8 and C9 Proteins to the SLB Platform (a) Schematic illustration of sequential addition of C8 protein to the SLB

platform after vitronectin and C5b-7 addition. (b) QCM-D maximum frequency shift associated with C8 protein addition as a function of vitronectin concentration for each strategy. Each data point is the average + standard deviation of three independent ($n = 3$) measurements. (c-d) Corresponding schematic illustration and QCM-D measurement data for C9 protein addition as a function of vitronectin concentration.

Figure 7.6 Influence of Clusterin on Membrane Association of the C5b-7 Complex (a) Schematic illustration shows addition of the C5b-7 complex which was preincubated with clusterin. Panel (b) presents the QCM-D frequency shift associated with C5b-7 + clusterin addition to the SLB platform as a function of vitronectin concentration. The C5b-7 complex concentration was fixed at 20 nM. The proteins were added at $t = 20$ min. Panel (c) shows absolute value of the maximum QCM-D frequency shift due to the amount of total adsorbed protein as a function of clusterin concentration based on the data presented in Panel (b). Each data point is the average + standard deviation of three independent ($n = 3$) measurements. (d-e) Corresponding schematic illustration and QCM-D measurement data for the addition of clusterin to the SLB platform, followed by addition of C5b-7 complex to the same platform. Panel (f) shows absolute values of the maximum QCM-D frequency shifts due to the amounts of adsorbed clusterin and C5b-7 complex as a function of clusterin concentration based on the data presented in Panel (e). Each data point is the average + standard deviation of three independent ($n = 3$) measurements.

Figure 7.7 Effect of clusterin on the sequential addition of C8 and C9 proteins to the SLB platform (a) Schematic illustration of sequential addition of C8 protein to the SLB platform after clusterin and C5b-7 addition. (b) QCM-D maximum frequency shift associated with C8 protein addition as a function of clusterin concentration for each strategy. Each data point is the average + standard deviation of three independent ($n = 3$) measurements. (c-d) Corresponding schematic illustration and QCM-D measurement data for C9 protein addition as a function of clusterin concentration.

Figure 7.8 Comparison of Inhibition by Vitronectin and Clusterin on Membrane

Association of the C5b-7 Complex. (a) Changes in adsorbed mass of vitronectin and clusterin upon addition to the SLB platform as a function of inhibitor concentration. (b) Changes in adsorbed mass due to inhibitor + C5b-7 complex upon joint addition to the SLB platform as a function of inhibitor concentration. Each data point is the average + standard deviation of three independent ($n = 3$) measurements.

Figure 8.1 Complement targets and inhibitors for pharmaceutical intervention in the complement system. Adapted with the permission from ref. [27]. Copyright 2016 Elsevier B.V.

Figure 8.2 Schematic representation of factor H and mini factor H and their ways of interactions with C3b on host surfaces. Redrawn with the permission from [39] Copyright 2013 The Journal of Immunology

Abbreviations

AFM	Atomic Force Microscopy
AP	Alternative Pathway
APTES	Aminopropyltriethoxysilane
BSA	Bovine Serum Albumin
C1INH	C1 Inhibitor
C4BP	C4 Binding Protein
CARPA	Complement Activation Related Pseudo Allergies
CP	Classical Pathway
CR1	Complement receptor 1
CRP	C reactive Protein
DAF	Decay Accelerating Factor
DCP	Dicetyl phosphate
DLS	Dynamic Light Scattering
DOEPC	1, 2-Dioleoyl-sn-glycero-3ethyl-phosphocholine
DOTAP	1, 2-dioleoyl-3-trimethylammonium-propane
DOPC	1, 2-dioleoyl-3-trimethylammonium-propane
ELISA	Enzyme Linked Immunosorbent Assay
ESR	Electron Spin Resonance
FRAP	Fluorescence Recovery after Photobleaching
GUV	Giant Unilamellar Vesicle
HSA	Human Serum Albumin
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
ITC	Isothermal Titration Calorimetry
LMV	Large Multilamellar Vesicle
LP	Lectin Pathway
LUV	Large Unilamellar Vesicle
MAC	Membrane Attack Complex

MACIF	Membrane Attack Complex Inhibiting Factor
MASP	Mannan Binding Lectin Associated Serine Protease
MBL	Mannan Binding Lectin
MCP	Membrane Cofactor Protein
MLV	Multilamellar Vesicle
MPA	Mercaptopropionic Acid
MPB-PE	1,2-dioleoyl- <i>sn</i> -glycero-3-phosphoethanolamine-N-[4-(p-maleimidophenyl) butyramide]
MG	Mercaptoglycerol
OEO	Oligo ethylene glycol
PA	Phosphatidicacid
PAM	Poly (alkyl methacrylate)
PBMA	Poly (butyl methacrylate)
PC	Phosphorylcholine
PDMS	Poly (dimethylsiloxane)
PE	Polyethylene
PEG	Poly (ethylene glycol)
PET	Poly (ethylene terephthalate)
PG	Phosphatidylglycerol
PHMA	Poly (hexyl methacrylate)
PI	Phosphatidylinositol
PIBMA	Poly (isobutyl methacrylate)
PMMA	Poly (methyl methacrylate)
PMN	Polymorphonuclear Monocytes
POPG	1-palmitoyl-2-oleoyl- <i>sn</i> -glycero-3-phospho-(1'- racglycerol)
POMA	Poly (octadecyl methacrylate)
PS	Phosphatidylserine
PVA	Poly (vinyl alcohol)
PVP	Polymerized vinyl pyrrolidone
QCM-D	Quartz Crystal Microbalance with Dissipation
RES	Reticuloendothelial System

RID	Radial Immunodiffusion
RU	Resonance Unit
SALB	Solvent Assisted Lipid Bilayer
SAM	Self-Assembled Monolayers
SLB	Supported Lipid Bilayer
SPR	Surface Plasmon Resonance
SUV	Small Unilamellar Vesicle
TCC	Terminal Complement Complex
TIRF	Total Internal Reflection Fluorescence Spectroscopy
TP	Terminal Pathway
UV	Ultraviolet

Chapter 1

Introduction

The complement system serves as the frontline defense for the human body against foreign invaders and is triggered via one or more of several signaling cascades that consist of interactions between soluble and cell surface bound complement proteins. Detailed knowledge about the corresponding protein-protein interactions on foreign surfaces is crucial for understanding how materials interact with components of the innate immune system as well as for the development of complement regulating therapeutics. This chapter presents an overview of the complement system and its role in recognition between host and non-host surfaces which lead to innate immune responses.

1.1. Problem of Statement

As the frontline defense of innate immune system in humans, the complement system responds quickly to pathogenic microorganisms (*e.g.* bacteria, parasites, or viruses) and altered host cells (*e.g.* apoptotic or necrotic cells) that come into contact with body fluid (*e.g.* blood), resulting in their clearance before substantial harm is done to the host [1, 2]. This system is comprised of a variety of soluble serum and membrane bound proteins that act synergistically in a sequential and cascade-like manner to remove life-threatening foreign entities. As such, the versatile nature of the complement system links the innate immune response with the adaptive immune response that establishes an integrated and powerful immune response against foreign and pathogenic entities. In their natural state, the complement proteins are inactive and once a foreign surface comes into contact with the blood, the inactive complement proteins interact with the foreign matter and subsequently initiate complement activation.

Depending on the specific complement proteins that recognize the foreign surfaces to mediate an immune response, the initiation of complement activation can differ and there are three different activation pathways: classical, lectin, and alternative [3-13]. After the complement activation is initiated, three main consequences emerge in order to make up an immunological response. The first is to mark or label the complement activating surface by opsonic fragments (*e.g.* C3b, iC3b, and C1q) which stimulate phagocytic recruitment and engulfment of activating surface. The second is the generation of anaphylatoxins or chemoattractants (*e.g.* C3a and C5a), leading to the translation of danger information from fluid phase into defined cellular responses (in other words, acting as a cell activator). The last is the formation of the membrane attack complex (MAC or C5b-9 or terminal complement complex, TCC) which eliminates the complement activating entity through the cytolytic activity. Moreover, these three consequences upon complement activation can also create stress onto host cells and tissues and can even lead to cellular damage. Thus, in order to diminish these adverse effects of complement activation, there are regulatory proteins in the blood, which distinguish and to differentiate the host cells from pathogenic and foreign entities and

they further downregulate the immune response against the host cells in a feedback loop manner.

Although the complement proteins are capable of recognizing a wide range of pathogenic-biological entities, it is not surprising that they also react to tailored surfaces, such as biomaterials (*e.g.* implants), medical devices, (*e.g.* drug-eluting stents, haemofiltration and haemodialysis devices) and therapeutics or colloidal drug delivery systems (*e.g.* liposomes, micelles, and nanoparticles). These surfaces have been utilized in many medical and pharmaceutical applications. Upon their contacts with the complement proteins the complement activation is triggered, and then results in inflammation and undesired damage to a host if the activation is not properly regulated. Additionally, since innate immune system reacts to any integrated surface, efficiency of the tailored entities- implant, drug, or device- is also significantly reduced. Moreover, if the regulation of complement activation is not well controlled, this can cause an immune imbalance and establishes an autoattack against the host cells, which has been also associated with serious tissue and cellular damages, principally complement related diseases. Taken together, the interactions of complement proteins with any integrated surfaces are essential in the development of new immunocompetent biomaterials and therapeutics because it determines whether or not such surfaces activate the complement system and induce any adverse immune responses.

Recent studies have indicated that the recognition of biomaterial surfaces mainly occurs through complement proteins that specifically initiate the alternative pathway. Among the other complement activation pathways, the alternative pathway plays a critical role in complement activation since it can be activated by its own product, C3b, which is produced by the cleavage of C3 by C3 convertases. This event results in the amplification of the complement activation through alternative pathway and is the primary mechanism of the innate immune response in regardless of initial complement activation pathways [14]. Moreover, after multiple experimental and clinical studies with biomaterials, it was found out that the efficacy of biomaterials is heavily influenced by innate immune response [15-17]. Therefore complement testing or evaluation has become a requirement for the assessment of the hemocompatibility of a biomaterial before its usage, which has been clearly defined in ISO standards (ISO-10993-4) [18-20].

Over the last decade, obtaining the biophysical profiles of individual protein interactions in the complement system has been a major focus for many researchers. Notably, the complement protein-target surface and further protein-protein interactions has been mapped on different model surfaces including inorganic materials, protein coatings, polymers, and self-assembled monolayers. Such information can be further applied towards the design of immunocompetent or immunologically safer biomaterials or biomedical devices (see **Chapter 2**). A wealth of knowledge has been generated by applying surface-sensitive measurement techniques to investigate this subject. So far, the interactions of complement proteins on a material surface have been dictated by the physicochemical properties of the surface (*e.g.*, morphology, composition, functional groups, charge, area, and size), therefore in this context there is significant potential for exploring complement activation on lipid membrane interfaces, which constitute a key architectural element of biological systems spanning from many viruses and bacteria to mammalian cells and organelles thereof along with other emerging subjects such as exosomes.

Towards this goal, there are still many technical difficulties and limitations in the material design and surface engineering methods for generating immunocompetent materials in the context of lipid-based systems. Indeed, biological membranes are a major component of cells in the human body, and membrane mimetics have been extensively used in liposome-based drug delivery systems over the past few decades because the liposomal systems enhance the pharmacological and therapeutic activity of medicines and help to protect hosts from toxic effects of encapsulated medicines. Additionally, it has been also considered that the liposomes are normally non-immunogenic entities since they do not contain proteins. However, it is now known that liposomes are recognized by innate immune system through complement proteins because

- (i) Sizes and shapes of the liposomes mimic the pathogenic entities such as virus, microbes or bacteria which are in nano size range [21].
- (ii) Liposomes mimic the membrane vesicles (*e.g.* ectosomes) apart from the host cells.
- (iii) Liposomes lack from the self-discriminatory molecules or regulators (*e.g.* CR1, DAF, CD59, and see **chapter 2**) on their surface

Therefore, the innate immune system cannot distinguish whether the liposomes are friend or foe. **Table 1.1** summarizes the common effects and consequences of interactions between liposomes and the immune system.

Table 1.1. Interaction between liposomes and immune system-common effects and consequences. Adapted with the permission from [22]. Copyright 2011 Elsevier B.V

Effects of Immune System on Liposomes		Effects of Liposomes on Immune System	
Effects	Consequences	Effects	Consequences
Binding	Change of pharmacokinetics and pharmacodynamics	Activation of inflammatory cells	Inflammation
Uptake	Interference with efficacy of liposomal drugs	Activation of allergy mediating cells	Pseudoallergy
Destruction	Leakage of drugs	Activation of T/B lymphocytes	Immunogenicity
		Suppression of phagocytic cells	Immunosuppression

Moreover, the recognition of liposomes by the innate immune system is a result of complement activation and it has been associated with complement related-adverse biological responses such as hypersensitivity or infusion reactions, named as complement activation related pseudo allergies (CARPA). The consistency of complement activation differs based on the physicochemical properties of the liposomes (*e.g.* size, shape, charge and composition). However, to date the underlying mechanisms of recognition and activation of lipid based assemblies (*e.g.* liposomes or host cells) by complement system at molecular level are not fully understood and still need to be elucidated. Therefore, one of the biggest opportunities lies in studying complement activation at lipid membrane interfaces by employing surface-sensitive measurement techniques and by taking advantage of recent innovations in model membrane platforms to dissect the underlying protein-lipid and protein-protein interactions.

Therefore, the purposes of the studies conducted in this dissertation are to delineate the interaction of complement proteins with biological membranes in the form of supported lipid bilayers, which represent one of the most popular model systems for mimicking cell membranes. Through engineering principles, the findings lead to a fundamental understanding of how complement proteins recognize lipid bilayers and

subsequently trigger the protein-protein interactions for complement activation, amplification, regulation, and termination, respectively.

1.2. Objective and Scope

Understanding of the interaction between biomaterials and the innate immune system is necessary step for designing the immunocompetent interfaces. Therefore, there is a significant interest in developing strategies to regulate complement-mediated immune responses to foreign biomaterials such as liposomal-therapeutics and medical device coatings. One major challenge in the field is the lack of biomimetic platforms to support the direct characterization of interactions between biointerfaces and complement proteins, which is a key step that can trigger the complement activation.

Along this line, the main objective of this thesis is to dissect key protein-lipid and protein—protein interactions involved in complement activation occurring at lipid membrane interfaces. Currently, research on complement activation at lipid membrane interfaces is mainly focused on liposomes as a model system. To date, the liposomal systems have been used within the hemolytic or immunoblot assays. Hemolytic assays assess the reduction of the complement proteins (principally MAC components) in the liposome-serum mixture whereas immunoblot assays examine the presence of complement proteins on the liposome surface after the liposomes are incubated in serum. However, since these two assays require serum samples, it makes difficult to maintain the liposome integrity (due to nonspecific protein adsorption) as well as to determine the initial complement proteins that bind to the liposomes. Moreover, real-time measurement of protein deposition onto liposome surface at the label free format is also still challenging. To address these challenges supported lipid bilayers (SLBs) have been used to perform the complement related studies due to the fact that the SLB platforms provide a two-dimensional model membrane interface that is capable of being integrated with a wide range of surface-sensitive measurement techniques to study interaction kinetics, including determination of protein uptake and resultant conformational changes—all of which are purported to be closely related to triggering complement activation to varying extents. Therefore, in the context of answering outstanding questions relating to

complement activation at lipid membrane interfaces, several experimental studies are proposed in this dissertation.

First of all, it is known that the complement activation by liposomes differs in terms of initiation pathways. Depending on the surface charge of liposomes, the complement activation is triggered by either classical or alternative pathways. It has been depicted that negatively charged liposomes activates the classical pathway while the positively charged liposomes activates the alternative pathway [23]. It is also noted that the classical pathway is triggered on liposomes in an antibody independent manner and through direct binding of C1q proteins. However, although the pattern recognition proteins of alternative pathway- properdin, C3b and C3- are identified, their interactions with lipid membrane surface and its role in complement activation remain unclear. Therefore, supported lipid bilayers along with the QCM-D measurement technique are proposed to employ for the investigation of how membrane surface charge influences the adsorption properties of different pattern recognition proteins involved in alternative pathway-complement activation.

Secondly, after all complement activation pathways are triggered from different sets of initiators, they converge at the C3 convertase assembly although the compositions of the C3 convertases are different. The C3 convertases particularly cleave the C3 proteins into its fragments, C3b and C3a. The generated new C3b proteins play a key role in the amplification of the complement activation-mediated immune responses when they bind to target surfaces. The way of C3b binding to surface can contribute to the fate of sequential cascade-like protein-protein interactions. However, there has been limited information what extent C3b attachment strategies affect the further protein-protein interactions to amplify the complement activation. Thus, supported lipid bilayers containing maleimide functionalized or negatively charge lipids are proposed to use for C3b attachment to lipid membrane surface through covalent or noncovalent interactions, respectively. The contributions of both covalent and noncovalent interactions to C3b attachment as well as further protein-protein interactions to assemble biomacromolecular complexes are also discussed.

Thirdly, the complement activation is terminated by the assembly of membrane attack complex (MAC) on target surface irrespective of complement activation pathways.

If the MAC forms on the liposome surface, it can lyse the liposome and lead to the content release. The MAC assembly is started from the generation of C5b from the C5 convertases and then C5b sequentially binds to C6, C7, C8 and C9, yielding to either cytolytic MAC or C5b-9 complex on the surface. The step-wise assemblies of MAC components onto the lipid membrane surfaces and the role of membrane surface charge have not been clearly investigated. Consequently, it is proposed to use the QCM-D measurement technique to monitor the formation of supported lipid bilayer platforms as well as the assembly of MAC components depending on membrane surface charge.

Lastly, vitronectin and clusterin are known to be regulators of MAC assembly and they inhibit the MAC assembly at two stages where the C5b-7 complex inserts into the lipid membranes and the C9 proteins bind to surface bound C5b-8 and C5b-9 complexes. The existing assays are not designed for the investigation of mode of actions of complement inhibitors. Therefore, the supported lipid bilayers along with the QCM-D measurement technique offer an excellent platform to monitor the impacts of vitronectin and clusterin on the assembly of MAC components.

1.3. Hypothesis

The overall hypothesis of this dissertation is that the supported lipid bilayer platform provides a useful model system to recapitulate key protein-lipid and protein-protein interactions involved in complement activation at lipid membrane interfaces. It also offers an experimental framework to decipher key trigger factors (*e.g.* membrane surface charge) as well as to evaluate candidate inhibitors of both complement convertases and MAC assemblies. The SLBs are formed on inorganic substrates either by the vesicle fusion or solvent-assisted lipid bilayer (SALB) methods. Since the SLB structure mimics the surface of a membrane, it allows setting of physiological conditions to study the binding interactions that occur between membrane associated and soluble proteins. More specifically, the SLBs offer two main benefits. First, it involves the precise control of membrane characteristics (*e.g.* composition, charge, fluidity) by changing lipid composition [24]. Second is compatible with a variety of surface sensitive analytical tools including quartz crystal microbalance with dissipation (QCM-D), surface

plasmon resonance (SPR), atomic force microscopy (AFM), and fluorescence recovery after photobleaching (FRAP) [25-30]. Moreover, SLB platforms can be also decorated with functional proteins through covalent and noncovalent interactions which changes depending on the nature of proteins. Hydrophilic proteins are linked to lipid membrane interfaces through functional groups that allow covalent attachment (*e.g.* thiol to maleimide) or through ligands that bind to specific targets (*e.g.* streptavidin to biotin) in the bilayers. Alternatively, membrane association of proteins may be influenced by noncovalent electrostatic and Van der Waals interactions. Therefore, I have formulated the following testable hypotheses to serve as the overarching motivation of this work:

- The surface charge of lipid membranes regulates adsorption of complement proteins through noncovalent interactions, and the degree of adsorption and corresponding functional properties of membrane-associated proteins influences downstream, complement-related processes.
- The type of attachment chemistry (covalent *versus* noncovalent) modulates complement protein immobilization on lipid membrane surfaces and influences functional properties of the membrane-associated proteins.
- The mechanism of action of complement inhibitors targeting membrane-associated processes (*e.g.*, recognition, MAC assembly) can be deciphered using the SLB platform.

1.4. Specific Aims

In order to test the aforementioned hypotheses, this dissertation aims to provide a comprehensive understanding of the cascade of complement protein interactions with supported lipid bilayers that are involved in the alternative pathway of the complement system. The interactions of complement proteins with supported lipid bilayer platforms were investigated with surface-sensitive measurement techniques in order to characterize protein-lipid and protein-protein interactions. Therefore, the following specific aims will be achieved:

1. To determine how the complement proteins, C3, C3b and properdin initiate the alternative pathway of complement activation when interacting with lipid membrane targets with different membrane surface charges (*e.g.* negative, positive and neutral).
2. To investigate the influence of inhibitors (compstatin) and C3b protein-immobilization on different lipid membrane interfaces and how that correlates to the real time enzymatic activation and regulation of the alternative pathway C3 convertase assembly.
3. To examine the real-time assembly of the step-wise formation of the membrane attack complex on supported lipid bilayers depending on the membrane surface charge.
4. To quantitate the stoichiometry of the membrane attack complex assembly and determine how two known complement inhibitors -clusterin and vitronectin-function as MAC antagonists in the context of lipid membranes.

1.5. Dissertation Organization

The dissertation has been organized into the following chapters:

Chapter 1: “Introduction” presents the background of the research field and outlines the significance and objectives of the Ph.D. research.

Chapter 2: “Literature Review” discusses the recent advances in investigation of the complement system on various model interfaces along with a detailed review of conventional and surface-sensitive measurement approaches to study the complement system. A future perspective with respect to applications is also briefly discussed.

Chapter 3: “Experimental Methodology” lists the materials, materials preparation, and details the experimental techniques used throughout this dissertation.

Chapter 4: “Recognition of Membrane Surface by Complement Proteins” introduces membrane recognition capabilities of complement proteins, C3, C3b and properdin that initiate complement activation through the alternative pathway depending on the membrane surface charge.

Chapter 5: “Convertase Assembly on Membrane Surface” presents the influence of immobilization strategies of C3b on the assembly of the functional complement convertase.

Chapter 6: “MAC Assembly on Membrane Surface” discusses the real-time assembly of the membrane attack complex (MAC) on SLBs while altering the surface charge of the membrane. Using the SALB method, the lipid composition of the membrane was tailored to form neutral, negative, and positive charged membrane surface respectively. MAC components were then added in sequence, starting with the C5b-7 pre-complex, C8, and C9. The binding of each MAC component was tracked as a function of time by QCM-D.

Chapter 7: “Inhibition of the MAC Assembly on Membrane surface” introduces the inhibitory effects of two well-known complement inhibitors, vitronectin and clusterin, on the MAC assembly. The modes of action of inhibitors were monitored using the QCM-D measurement technique in real-time and a label free format.

Chapter 8: “Conclusion and Future Outlook” summarizes concluding remarks of the above chapters and makes recommendations for the future work based on the findings in this dissertation.

1.6. Main Contributions of the Thesis

This dissertation led to several novel outcomes by:

1. Correlating the recognition of lipid membrane interfaces by complement proteins with the design of synthetic lipid bilayers with immunocompetent features.

2. Offering guidance for design and evaluation of membrane associated biomacromolecular complex while introducing attachment schemes for immobilization of complement protein at lipid membrane interfaces.
3. Providing insights into molecular assembly of biomacromolecular complexes on lipid membrane interfaces in real time using the quartz crystal microbalance dissipation measurement technique
4. Establishing a supported lipid bilayer platform to test inhibitors of complement activation, leading to generate critical mechanistic information (mode of actions) to guide drug discovery and development

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Chapter 2

Literature Review

The complement system is a key component of innate immune surveillance in the human body. It is associated with many adverse side effects when activated by foreign biological or engineered particulates and interfaces. In this chapter, an overview of the complement system is presented along with a detailed review of conventional and surface-sensitive measurement approaches to study the complement system. Then, a critical discussion of previous studies conducted on various model interfaces is presented, including current gaps in scientific knowledge and open questions about the interactions between complement proteins and lipid bilayer interfaces. At the end, the objective of the PhD work is established within the context of the field.

2.1. Overview of the Complement System

The complement system is the first response of the innate immune system against pathogenic invaders such as microorganisms (*e.g.* bacteria, parasites, or viruses) or damaged host cells (*e.g.* apoptotic or necrotic cells) as they enter the body. It refers to a complex network of a group of proteins that react with each other in a sequential-cascade manner and work together with antibodies to clear the pathogenic entities from the host (see **Table 2.1** for complement proteins). Although it was initially thought that the complement system is triggered by the interactions of the antibody with certain antigens on the target surface, the activation of the complement system is now shown that it is not all the time antigen specific. The earliest observations regarding the complement system were by Dr. Jules Bordet as he described that serum has an ability to lyse certain bacteria. Subsequently, Dr. Buchner named the observed activity of the serum against bacteria as “*alexin*” [1, 2]. Furthermore, in order to explain the details of the observed activity, Dr. Jules Bordet conducted versatile experiments and he found out that the heat-labile components-known as complement proteins now- of the serum are required for the red blood cell lysis while the heat-stable components of serum-known as antibodies now- are ineffective on their own. Later, he revealed that, antibodies are sufficient to mediate an immune response against the certain microbes, while complement proteins are primarily responsible for nonspecific antimicrobial activity. Moreover, since Dr. Buchner’s *alexin* was identical to “*complement*” as described by Ehrlich and Morgenroth, the nomenclature has been set as “*complement*” [3].

Today, it is known that complement proteins are naturally inactive until a pathogenic invader comes in contact with the blood. After interactions of inactive complement proteins with an unknown entity, the complement proteins identify the unknown entity as a potential harm to host, and therefore, they promote the elimination of unknown entity through phagocytosis or membrane lysis. Since the complement proteins are capable of recognizing a wide range of biological entities, it is not surprising that they also recognize the most of the biomaterials and therapeutics that have been utilized in nanomedicines. Their interaction with complement protein in blood causes permanent or transient activation of the complement system and then it is followed by their immune

clearance. As such, when these materials are introduced into the body, complement activation cause an adverse inflammatory response that can either be harmful for the patient or reduce the efficiency of the integrated implant, drug, or device. Therefore, for the development of new immunocompetent biomaterials and therapeutics it is crucial to assess whether or not such materials activate the complement system and establish adverse immunogenic responses.

Table 2.1. Complement proteins and their biological functions

Complement proteins	Molecular Weight (kDa)	Serum Concentration (µg/mL)	Function
Classical Pathway			
C1q	410	70	Binds to IgG or CRP to initiate the activation of CP
C1r	170	34	Cleaves C1s
C1s	85	31	Cleaves C4 and C2
C2	117	25	Catalytic subunit of the CP C3 convertase and part of CP C5 convertase
C4	206	600	Activated C4 (C4b) binds to the activating surface and C4b is part of CP C3 and C5 convertases
Lectin Pathway			
MBL	600	1-4	Binds to microbial and apoptotic cells and initiates the activation of the LP
MASP-1	83	6-11	May be involved in direct cleavage of C3
MASP-2	76	0.5	Cleaves C2 and C4
MASP-3	95	5	Product of alternative splicing of MASP1 and possible role in AP activation
Ficolin-1	35	0.06	Recognizes carbohydrate patterns, initiates LP
Ficolin-2	35	4	Recognizes carbohydrate patterns, initiates LP
Ficolin-3	34	7-23	Recognizes carbohydrate patterns, initiates LP
Alternative Pathway			
C3	185	1300	Activated C3 (C3b or C3 (H ₂ O)) binds to target surface and mediates phagocytosis and cytolysis. C3b is the part of C3 and C5 convertases while C3a is an inflammatory peptide
Factor B	93	200	Catalytic subunit of AP C3 convertase and a part of AP C5 convertase
Factor D	24	1	Cleaves factor B that is bound to C3b or C3(H ₂ O)
Properdin (factor P)	53	20	Stabilizes AP C3 convertase and initiate the activation of AP
Terminal Pathway			
C5	180	75-80	C5b initiates the assembly of MAC while C5a is an inflammatory peptide
C6	128	45-60	Participates in MAC assembly
C7	120	55	Participates in MAC assembly
C8	150	65-80	Participates in MAC assembly
C9	79	60	Participates in MAC and pore assembly

2.1.1. Initiation of Complement Activation

The complement system consists of three different pathways (classical, lectin, and alternative) that are activated in a cascade-like manner as seen in **Figure 2.1**. The key differences of the three pathways are the initiating protein reactions that occur at target surfaces.

2.1.1.1. Classical Pathway

The classical pathway is initiated after deposition of C1q to the activator surfaces. It is well known fact that C1q can recognize the antibody (e.g. immunoglobulin G or M) bound –antigenic surfaces. However, in the absence of antibodies C1q can still recognize to foreign surfaces through a number of charged entities including serum amyloid P (SAP)[4], C-reactive protein (CRP)[5], β amyloid fibrils[6], pentraxin 3 (PTX3)[7], annexins A2 and A5[8], DNA[9], urate crystals[10], heparin protamine[11], and cardiolipin containing –liposomes[12]. After C1q binding to activator surface, two proteases, C1r and C1s, bind to surface bound C1q in the presence of Ca^{2+} and generate a C1 complex in the form of $\text{C1qC1r}_2\text{C1s}_2$ [13]. The activated C1s protease is capable of cleaving C4 proteins into C4a and C4b. Then, the C4b can then bind to functionalized surfaces due to its internal thioester bond, where this binding event is known as opsonization. Additionally, activated C1s proteases also split C2 into the fragments, C2a and C2b. The released C2a then binds to the surface bound C4b to form a proteolytic enzyme or enzymatic protein complex, C4b2a, which cleaves one of the most abundant complement proteins in serum, C3, into its fragments, C3a and C3b.

2.1.1.2. Lectin Pathway

The lectin pathway is initiated by the interaction of pattern recognition proteins (e.g. mannan binding lectin (MBL) and ficolins (L-ficolin, H-ficolin, M-ficolin) with the so-called pathogen-associated molecular patterns-PAMPs (e.g. D-mannose, N-acetyl-D-glucosamine, or acetyl groups) which are expressed on the surface of microorganisms

[14-19]. After the initiation, each pattern recognition protein subsequently binds to a MBL-associated serine protease (MASP1, MASP2, or MASP3) to create a MBL (or ficolin)-MASPs complex on the activating surface. This binding event reestablishes a protein-cascade similar to the classical pathway, where the MBL-MASP-2 complex cleaves both C2 and C4 into their smaller fragments to form a C3 convertase with the protein composition of C4b2a. Eventually the MBL-MASP-2 complex functions as the aforementioned enzyme $C1qC1r_2C1s_2$ and causes the lectin pathway to continue to C3 cleavage in the same manner as the classical pathway.

2.1.1.3. Alternative Pathway

It is interesting to note that the alternative pathway of complement activation is not referred to as “*alternative*” because of the way it activates the complement system. Instead it is called “*alternative*” because it was discovered as the second pathway after the classical pathway. The alternative pathway is spontaneously initiated by hydrolysis of C3 proteins in the blood without requirement of any specific activator surface[20]. Once the C3 proteins are hydrolyzed, the C3 (H_2O) proteins are formed and then bind to factor B in the presence of Mg^{2+} which are further cleaved by the factor D, resulting in a solvent-based C3 convertase known as C3(H_2O)Bb. After that, this convertase cleave the native C3 proteins into the nascent C3b which further bind to amino or hydroxyl groups of foreign surfaces and reinitiates the alternative pathway on the target surface [21-23]. The C3b proteins onto target surface interact with factor B and factor D again to form surface bound C3 convertase (C3bBb) in order to promote the complement cascade[24-26]. Other than C3 and C3b proteins, properdin can also initiate the alternative pathway because properdin can either recognize specific entities on the apoptotic T cells, necrotic cells or glycosaminoglycan (GAG) chains on surface proteoglycans[27]. After properdin binding, the C3b binds to properdin to promote the further protein-protein interactions to generate convertases on the target surface. It has been also shown that positively charged liposomes initiate the alternative pathway although which protein exactly initiates the activation is not known.

Moreover, alternative pathway C3 convertases are homologous to the one produced by classical and lectin pathway in terms of their function. However, C3 convertase formation has been shown to be more pronounced through the alternative pathway where surface bound C3 convertase generation has been reported to create more than 10 billion C3b fragments within the first 10 to 15 minutes of activation [28]. In the case where the alternative pathway activation is directed by properdin, surface bound properdin interacts with C3b allowing the C3 convertase assembly in the presence of factor B and factor D [29, 30]. Similar to C4b, C3b interacts with functionalized surfaces through its thioester moiety and is also an opsonizing agent. The surface bound C3b can then be recognized by the complement receptor 1 (CR1) that is present on red blood cells, stimulating immunogenic responses and phagocytic clearance.

2.1.2. Common cascade steps for convertase assembly and amplification loop

Irrespective of the initiation pathway of complement activation, all pathways converge to the C3 convertase assembly (**Figure 2.1.**). Although the protein compositions of C3 convertases (C4b2a from both lectin and classical pathways or C3bBb from alternative pathway) are different, C3 convertases cleave native C3 protein into C3a and C3b. Newly generated C3a is a potent anaphylatoxin which causes inflammation, whereas nascent C3b plays an important role in the re-initiation of the alternative pathway. In other words, C3b ultimately participates into an amplification feedback loop driven by the alternative pathway activation. Moreover, the C3b can also bind to the surface bound C3 convertases, leading to form a new enzymatic protein complex-C5 convertase- (C2a4bC3b from classical and lectin pathway or C3bBbC3b from alternative pathway) which specifically targets the C5 protein [17, 31]. This protein complex cleaves C5 into its smaller fragments, C5a and C5b. Similar to C3a, the C5a fragment is an anaphylatoxin that causes an inflammatory response [32], whereas the C5b initiates to terminal pathway of the complement system [33] that is described in the relevant section below.

Figure 2.1. Schematic illustration summarizing three activation pathways of the complement system

2.1.3. Terminal Pathway

As a downstream of the C5 convertases, the C5b interacts with C6 in fluid phase, resulting into a stable C5b-6 complex. Then, the C5b-6 complex interacts with C7 in fluid phase and forms C5b-7 complex which can insert into target membrane through a hydrophobic domain of C7 protein. Thereafter, C8 protein binds to the membrane associated C5b-7 complex and forms a C5b-8 complex. Furthermore, C9 protein binds to C5b-8 complex to create C5b-9 complex which acts as a core for the subsequent binding of C9 proteins. Binding of multiple copies of C9 proteins to the C5b-9 complex leads to polymerize the C9 proteins barrel-like structure yielding a transmembrane channel with diameter of 10 nm on the membrane of the target cells [34, 35]. This final complex is also known as membrane attack complex or terminal complement complex (MAC or TCC, respectively) as shown in **Figure 2.1**.

2.1.4. Regulation of Complement Activation

After the complement activation is triggered by the non-self-surfaces, it should be tightly regulated; otherwise the complement activation can attack to the host cells and destroy them. This improper event has been also associated with different pathologic diseases today. Therefore, in order to keep the complement activation under control, our own body generates soluble and host cell surface regulatory proteins which are listed in **Table 2.2**.

More specifically, the host cells express versatile regulatory proteins including decay accelerating factor (DAF or also known as CD55), membrane cofactor protein (MCP or also known as CD46) and complement receptor 1 (CR1 or also known as CD35). The DAF proteins exist on the all peripheral blood cells and accelerate the decay of C3 and C5 convertases by promoting the dissociation of Bb or C2a from the convertases [36]. The MCPs are expressed on both leukocytes and platelets and serve as a cofactor for the cleavage of surface bound C3b and C4b by factor I. Moreover, the CR1 presents on all blood cells excluding platelets and it binds to C3b and C4b to inhibit the formation of the C3 alternative and classical pathway convertases, respectively. Moreover, it also

accelerates the decay of C3 and C5 convertases by acting as a cofactor for the inactivation of C3b and C4b by factor I.

The regulation is not strictly restricted by the cell surface proteins. A number of soluble proteins also work for the regulation of the complement activation. C1 inhibitor (C1INH) is one of the most specific regulatory proteins that inhibit the complement activation initiated by classical and lectin pathways [37]. C1INH binds to activated sites of C1r and C1s to inactivate them to assemble into the C1 complex (C1qC1r₂C1s₂) as well as it binds to MASP2 to block its further activity [38]. Factor H and C4b binding protein (C4BP) regulates the activity of C3b and C4b, respectively [39, 40]. C4BP binds to the C4b and serves as cofactor for inactivation of C4b (iC4b) by factor I. It also accelerates the decay of C3 and C5 convertases of classical pathway. Factor H works like C4bBP to more specifically regulate the alternative pathway, and it acts as cofactor for factor I to mediate the C3b inactivation (iC3b) and decay acceleration of C3 and C5 convertases. As mentioned earlier, factor I is an active serine protease that inactivates both C3b and C4b proteins in the presence of factor H and C4BP, respectively. It also further breaks C3b to its fragments (*e.g.* C3c, C3dg, and C3d) [41] and C4b to its fragments (C4c and C4d) [42]. Moreover, upon complement activation anaphylatoxins are also generated and they are simultaneously regulated by the anaphylatoxin inhibitor, serum carboxypeptidase N which more specifically splits anaphylatoxins at the C-terminal arginine domain.

The membrane attack complex assembly is a signature for the termination of complement activation and it is regulated by both cell surface and soluble proteins. The CD59 (protectin) and MAC inhibiting factor (MACIF or C8 binding protein) proteins are expressed on the cell surfaces such as blood cells, hepatocytes and epithelial cells. The CD59 proteins bind to C8 and C9 and thus prevent the polymerization of C9 proteins to create a cytolytic pore [43]. There is not enough information about the MACIF but it has been assumed that it binds to C8 and C9 to protect the host from reactive cell lysis. The soluble proteins are vitronectin (known as serum spreading protein or S protein) and clusterin (called as apolipoprotein or SP40, 40). The vitronectin interact with the membrane association site of the C5b-7 complex and prevent the insertion of the C5b-7 complex into cell membranes [44, 45]. Clusterin also conducts similar functions with vitronectin

[46]. After association of clusterin and vitronectin with C5b-7 complexes, the C8 and C9 binding might still occur, but the formed protein complexes are not cytolytic. Regulatory proteins of the complement activation are summarized in the **Table 2.2**.

Table 2.2 Membrane bound and soluble regulatory proteins of complement system. Adapted with the permission from [47]. Copyright 1998 Pharmacological Reviews

Regulatory Proteins	Ligand	Concentrations (µg/mL)	Point of Action	Functional Activity
Membrane Bound Regulators				
DAF (CD55)	C3b, C4b	N.A	CP,AP	Accelerates decay of C3 convertases, dissociates bound components from ligands
MCP (CD46)	C3b, C4b	N.A	CP, AP	Inhibits the formation of convertases, cofactor for factor I
CR1(CD35)	C3b, C4b	N.A	CP, AP	Accelerates decay of C3 and C5 convertases, dissociates bound components from ligands, cofactor for factor I
CD59 (Protectin)	C8,C9	N.A	TP	Inhibits pore formation on the host cells
Soluble Regulators				
C1INH	C1s, C1r, MASP2	200	CP,LP	Dissociates C1 complex and inactivates MASP2
C4BP	C4b	250	CP	Accelerates decay of CP convertase, dissociates bound components from C4b, cofactor for factor I
Factor H	C3b	500	AP	Accelerates decay of AP convertase, dissociates components from C3b, cofactor for factor I
Factor I	C3b, C4b	35	AP, CP	Cleaves and inactivates the ligands with any of cofactors
Clusterin (SP-40,40)	C5b-7	100	TP	Inhibits the insertion of MAC components into the host cells
Vitronectin (S-protein)	C5b-7	300	TP	Inhibits the insertion of MAC components into the host cells
Carboxy-peptidase N	C3a,and C5a	30	CP, LP,AP	Inactivation of anaphylatoxins

2.1.5. Complement Associated Activities

All three complement activation pathways lead to the generation of distinct effector molecules or complexes either in solution or on an activator surface, which establishes three main immune responses or biological functions (**Figure 2.1**). The first is opsonization that refers to marking the surface with certain complement proteins-called

opsonins, -such as C3b, C4b, or MBL in order to facilitate an immune clearance of target by the reticuloendothelial system (RES) [48]. The second is inflammation that denotes the recruitments of the phagocytes and other immune inflammatory cells after the release of the anaphylatoxins (small inflammatory peptides) such as C3a and C5a [49-51]. The third is cytolysis that occurs due to the assembly of the MAC components onto lipid bilayer membrane [52, 53]. To date, these three main immune responses have been used as indicator metrics to assess the complement activation of a foreign entity. Moreover, those proteins associated with each immune response have been utilized for measurement of complement activation.

2.2. Monitoring Complement Activation

2.2.1. Traditional Approaches

As described in the previous section, once the complement activation is triggered by the presence of foreign entities, three main complement mediated-immune responses occur and they are associated by the complement proteins, fragments or complexes that are either deposited on the activator surface or released from the activator surface. Therefore, they have been used as markers for assessing the complement activation. To date, versatile strategies and methods have been established in order to examine the reaction mechanisms and level of the complement activation caused by foreign entities as summarized in **Table 2.3**.

For most immune studies, hemolytic assays have been extensively used to assess the total complement activation [54, 55]. This assay measures the bystander hemolysis ability of the MAC components on erythrocytes (red blood cells) in the presence of foreign entities. Moreover, this measurement can be also specified against the activation pathways in order to identify the type of complement activation pathways that is triggered. Therefore, erythrocytes have been either sensitized with antibodies and mannan or they have been used from different sources (e.g. sheep, or rabbit) for identifying different roles of complement activation. In particular, the classical and lectin pathway-specific hemolytic assays use the antibody sensitized sheep erythrocytes and the mannan

coated erythrocytes, respectively while the alternative pathway uses guinea pig, rabbit, or chicken erythrocytes as target cells [56-58]. These assays also help to display deficiencies of a specific complement protein, which can be measured by the absence of the MAC-mediated cell lysis. The main benefits of using a hemolytic assay are (i) the straightforward determination of the total complement activation in most mammalian species' serum (ii), commercially available and (iii) inexpensive.

Table 2.3 List of methods have been conducted to monitor complement activation

Methods	Principles	Advantages	Disadvantages
Hemolytic Assay	Measures the release of hemoglobin due to lysis of red blood cells	Assessment for the entire complement system Final read out	No standard protocol Short lifetime of red blood cells Time dependent results
ELISA	Measures the colorimetric change when the analyte concentration increases at a analyte specific antibody coated plate	Sensitive Highly protein specific Commercially available kits Available standardized protocols Multiple set of measurement	Kinetic information not available Relatively expensive
Crossed Immuno-electrophoresis	Measures altered electrophoretic mobility when a protein is cleaved into its different fragments	Detectable conformation alteration within the protein detectable	Limited by lability of the protein and denaturation and solubility of gels used Obtained protein amount in arbitrary unit Not applied to large scale quantitative assessments Kinetic studies not feasible due to long experimental hours
Radial Immunodiffusion	Measures the diameter of precipitin ring formed by the interaction of the analyte and antibody at the optimal concentration	Simple to conduct Minimal equipment Inexpensive Quantitative measurement possible for analyte or precipitating antibody	Reproducibility False positive reactions due to salt or detergents Need large amount of protein Not feasible for large number of assays Long experiment time
Turbidimetry	Measures the intensity of transmitted light through immune complex in the solution	Automatic experimental setup High volume testing capability Relatively rapid Relatively cheap	More accurately quantitates large immune complex Destructively scattered light Background scattering interfere the measurement Requires high concentration of immune complexes
Nephelometry	Measures the intensity of scattered light caused by immune complexes in the solution	Shorter analysis time Steady state measurements Highly automated, Simple to operate, More sensitive compared to turbidimetry	Scatter angle limits the sensitivity Expensive Restricted from the measurement ranges, requirements for controls
Western Blot	Measures the electrophoretic transfer of proteins to an immunoblotting matrix depending on protein's molecular mass	Direct measurements Cleavage of proteins into its fragments detectable Commercially available reagents	Complex Requires highly trained people Difficult Quantification

Immune complexes, conversion products of complement proteins, as well as the reductions of native complement proteins in serum upon activation have been detected by radial immunodiffusion (RID), turbidimetry and nephelometry[59-62]. These measurements are more specifically done by monitoring the decrease in the concentration of complement proteins, C3 and C4 in serum, yielding an indication of complement consumption by the pathway initiating proteins. Such information can also depict the efficiency of complement activation [63]. More specifically, RID measurements are

based on precipitation reactions of antibody-antigen that occurs in the agarose gel. Generally, the antibody of interest is incorporated into an agarose gel and then the serum samples containing different concentrations of protein are poured in wells that are covered by antibodies. Later, the proteins of interest diffuse in the gel as a function of time and react with the incorporated antibody. Once the protein-antibody reaction reaches to equilibrium, a ring of precipitation occurs and the size of the obtained ring increases in diameter as the concentration of the protein increases. Nephelometry and turbidimetry utilize the intensity of the scattered and transmitted light through the immune complexes in the solution respectively [60].

Similar to RID measurements, crossed immunoelectrophoresis has also been used to determine the cleavage of C3 to its fragments, C3b or C3d, in serum when exposed to an activating surface. This measurement is based on the migration of an antigen into the agarose gel containing an antibody under the direction of an electric field. For complement activation, crossed immunoelectrophoresis utilizes an agarose gel containing a C3c antibody that helps analysis of C3 cleavage. Although this assay can give information about cleaved products of C3 proteins, it is not practical for commercial use due to protein lability outside of the body, the lack of control measurements, its minimal measurement capabilities, and a long experimental protocol. Another popular form of quantifying complement activation is the enzyme linked immunosorbent assay (ELISA), which has been mainly used to assess in vitro complement activation. Typically concentrations of the sC5b-9 complex-also known as soluble MAC- are measured for investigation of terminal complement activation. The sC5b-9 complex is created when it fails to be incorporated into a lipid bilayer membrane and alternatively interacts with the regulatory serum protein, vitronectin (also known as the S-protein) [64].

Taken together, sC5b-9 targeted-ELISA measurements indicate the generation of sC5b-9 in serum upon complement activation by foreign particulates in serum but both sC5b-9 targeted- ELISA and hemolytic measurements are not sufficient to evaluate all of the complement associated-biological activities. These two measurements only point out the termination of the complement system, giving little or no information about which pathway is initiated and what kinds of anaphylatoxins are generated. Therefore, the pathway specific ELISA kits have been developed for further classification of immune

responses, such as C4d and Bb. For example, C4d is a scission derivative of the C4b fragment and primarily indicates the activation of the classical or lectin pathways whereas Bb is released from the dissociation of the C3 convertase and specifies the activation of the alternative pathway [65].

ELISA measurements have been also tuned for measuring released anaphylatoxins including C3a, C4a, and C5a upon exposure of a foreign surface in plasma or serum. From these measurements, C5a was determined to be the most potent inflammatory mediator, indicating 20 folds and 2500 folds stronger activity than to C3a and C4a, respectively [60], yet C5a was later considered to be a poor indicator of complement mediated inflammation due to the fact that C5a was immediately recognized by the receptor, C5aR. Thus, the concentration of C3a has used as a gauge for the activity of total complement mediated inflammation [60]. The use of different ELISA kits has provided insights into mechanistic factors of the complement cascade associated with cytolysis and inflammation with high accuracy and sensitivity. Moreover, **Table 2.4** summarizes commercially available ELISA kits used for assessment of complement activation.

Table 2.4. Complement activation products measurable by ELISA Complement activation

Marker	Originated by	Pathways Involved	Indications
C4d	Cleavage of C4b by factor I	CP/LP	Initiation of complement activation through CP and LP
Bb	Dissociation of C3 and C5 convertases	AP	Initiation of complement activation through AP and amplification loop
iC3b	Cleavage of C3b by factor I	AP	Surface recognition and amplification loop
C3a	Cleavage of C3 by C3 convertases	CP, LP,AP	Surface recognition and amplification loop
C5a	Cleavage of C5 by C5 convertases	CP, LP,AP	Convertase Stability
sC5b-9	Inactivation by solubilizing MAC	CP, LP,AP	Convertase Stability, C5b-C9 stability, CD59 action

ELISA is the key method to evaluate the hypersensitivity reactions specifically named as complement activation-related pseudoallergy (CARPA) because anaphylatoxins, mainly C3a and C5a, are generated upon complement activation by nanosized pharmaceutical carriers (*e.g.* liposomes, micelles, emulsions, nano- and micro-capsules).

Then, they trigger both mast cells and basophiles to release the secondary mediators (*e.g.* histamine, thromboxane A₂, and platelet activating factor) [66-68]. These mediators subsequently amplify the effective immune responses, leading to anaphylaxis in humans and animals [69-72]. As a complementary to ELISA, the versatile animal models have been also used to determine the hypersensitivity reactions in human against the nanosized pharmaceutical carriers. For examples, the reactions in pigs have been also reproduced in dogs, rats, and monkeys because the results vary from each other in terms of level of sensitivity and spectrum of symptoms. However, practical obstacles of animal models are the slow, labor intensive (*e.g.* required to conduct surgery) and requirement of special equipments. Therefore, the evaluation of CARPA model is conducted mostly with ELISA kits today. Nevertheless, ELISA based quantification is still not sufficient for complement activation along with CARPA studies because it is relatively slow and requires expensive labeled antibodies as well as it is restricted to measuring a single output of the serum or plasma exposed to an activating surface. Moreover, the ELISA itself can lead to production of inconclusive results for the lack of real-time data or reposition of released C3a and C5a to the activator surface. Therefore, new approaches have been required to monitor complement activation in addition to traditional methods used for measurement of the soluble activation products.

2.2.2. Surface Science Approaches

Surface sensitive analytical tools have brought to different perspective to the complement activation studies since they make possible to determine the complement proteins that are bound to the activator surface in real time and label free format. In this context, activator surface becomes a sensor where the changes are detected upon binding of complement proteins whereas the sensor bound proteins can be opsonins, components of either convertases or membrane attack complex. To date, versatile surface sensitive analytical tools such as ellipsometry, surface plasmon resonance (SPR), quartz crystal microbalance with dissipation monitoring (QCM-D) have been employed in order assess the interactions of the complement proteins with the sensor surfaces. Particularly, SPR is optical measurement technique that has been mainly used for investigation of the

biomolecular reactions such as binding kinetics and affinity constants among complement proteins [73, 74] because a detailed knowledge of individual interactions among the complement proteins are necessary for understanding of both complement activation and regulation [75]. Fundamentally, the SPR measurement technique records the changes of electron density around the sensor surface (*e.g.* gold) upon addition of the samples. This measurement principle is also connected to changes in the refractive index of the medium close (within 300 nm) to a sensor. The SPR measurement responses are presented as sensorgrams, a plot of resonance units (RU) as a function of time. As such, a 1 RU corresponds to 1 pg/mm² for most of the protein samples. Moreover, the SPR sensor surface has been also decorated with 100-200 nm thick carboxymethylated dextran-matrixes which are selectively and easily functionalized with distinct chemical moieties, allowing the immobilization of the protein to the surface through the covalent coupling strategies [76]. For the general investigations of the protein-protein interactions, one of the interaction partners is immobilized onto the SPR sensor surface while the other one remains in fluid phase. With this experimental methodology, the both binding and kinetic constants of other complement proteins (*e.g.* factor B, factor H, complement receptor 1 or properdin) to bound C3b have been determined [76-78].

Likewise, the ellipsometry is an optical measurement that specifically measures the changes in ellipsometric angles of polarized light upon reflection from a planar surface. Ellipsometry has been also used to detect the proteins of both coagulation and complement system at the model interfaces [79-81] since it determines the amount of bound proteins on reflecting sensor surfaces (*e.g.* silicon or gold coated silicon) both in air and solution with a mass sensitive in the range of 5–10 ng/cm². Both SPR and ellipsometry measurements provide similar results although optical unit of ellipsometry is more complicated than that of SPR. Recent complement activation studies has been also started to utilize acoustic based measurement techniques such as quartz crystal microbalance since it generally simpler and less expensive than the optical based measurements [82, 83]. The physical principle of QCM technique relies on the changes in frequency of the oscillating quartz sensor upon adsorption of mass. The important aspect on the QCM technique is to have limited demands for optical quality of the surface and the biological specimens. The QCM technology has been recently improved by the

introduction of the dissipation monitoring technology, named as quartz crystal microbalance with dissipation (QCM-D) [83]. With the new QCM-D technique it is also possible to do simultaneous measurements of adsorbed material mass and its viscoelastic properties [82] with the fact that estimated mass sensitivity of QCM-D reaches to 17.7 ng/cm^2 . Overall, the surface sensitive analytical tools that have been used for complement activation studies have their own strengths and weaknesses which are briefly summarized in **Table 2.5**

Table 2.5. List of surface science approaches with advantages and disadvantages

Technique	Principle	Advantages	Disadvantages
Ellipsometry	Change in the state of polarized light upon reflection in a surface	Accurate measurement of ultra-thin films of thickness <10nm	Expensive optical unit High throughput screening
SPR	Change in refractive index at the sensor surface	Relatively small amount of protein needed. Faster response time	Expensive optical unit High throughput screening Sensitive to temperature
QCM-D	Change in oscillating frequency of piezoelectric devices upon mass loading	Less expensive than optical methods High throughput screening	Longer response time Mass deposition in QCM-D measurements is higher than the optical measurements due to bound water molecules

2.3. Determination of Complement Activation at Model Interfaces

Surface sensitive analytical tools that have been used in complement activation studies are briefly summarized in previous section. Four different model interfaces have been combined with surface sensitive analytical tools in order to assess the complement activation. They are inorganic surfaces, protein coatings, self-assembled monolayers and polymers as schematically shown in **Figure2.2**.

Figure 2.2 Model Interfaces used for determination of complement activation

2.3.1. Inorganic surfaces

When the complement system is activated on inorganic surfaces exposed to blood, plasma, or serum, specific complement proteins accumulate on the surface, which can either act as an opsonin to recruit phagocytes or as an initiator to promote the complement cascade to form enzymatic complexes that results in inflammation as well as formation of membrane attack complex [84]. As a means of investigating the pathway specific complement activation, a wide range of surface sensitive tools have been employed [85-91]. Out of all surface sensitive analytical tools, ellipsometry was the most commonly used one for monitoring the complement activation at inorganic interfaces (**Table 2.6**), where each surface is incubated with blood, plasma, or serum and further analyzed with the addition of specific complement associated antibodies [85, 92-97]. This has led to observations on the role of incubation time for surfaces like silicon, aluminum and titanium that were incubated in heparinized plasma for different periods of time in order to identify the specific pathway of activation [93]. The obtained results gave a strong indication of activation through the classical pathway on titanium with a minimal contact time due to low levels of IgG antibodies and no detectable C3c antibodies. As the

contact time increased, the results showed signs of alternative pathway activation from the increase in C3c antibodies on the titanium surface [93, 95]. Silicon and aluminum also demonstrated a time dependence for incubation where smaller time scales yielded minimal antibody adsorption on silicon and significant amounts on aluminum. For the prolonged exposure time, C3c antibody deposition increased on both surfaces, suggesting that the alternative pathway activation is initiated.

Table 2.6. List of inorganic surfaces used in complement activation studies

Inorganic surfaces	Method	Findings	Ref.
Silicon	Ellipsometry	Complement activation occurs via surface induced conformation changes in adsorbed proteins Conformational changes of proteins are larger extent on hydrophobic (methylated) silicon than hydrophilic (hydroxylated) silicon	[93]
Glass	Ellipsometry	Negligible level of complement activation was observed	[98]
Platinum	Ellipsometry	The amount of deposited anti-C3 was higher	[86]
Titanium	Ellipsometry	The amount of deposited anti-C3 was lower due to high degree of surface displacement phenomena of protein adsorption Initially trigger the classical pathway- Anti C1q and IgG deposition- and after certain time serum incubation alternative pathway takes over because anti-C3c deposition increases while anti-C1q and anti-IgG decreases	[86, 93, 95, 96]
Chromium	Ellipsometry	Large deposition of anti-C1q induced to conclude the classical pathway activation	[94]
Gold	Ellipsometry	Bare gold caused low level of complement activation	
Aluminium	Ellipsometry	Anti-C3c deposition was time and C1q dependent. There was no anti-C3c deposition when C1q was not present in serum that exposed to surface	[93]
Zirconium	Ellipsometry	No Anti-properdin and higher amount anti-factor H detected, suggesting that the complement proteins bind to zirconium but it immediately downregulated by factor H.	[92]

Similarly, zirconium surfaces were also incubated in heparinized-plasma and serum and further analyzed with more specific antibodies (IgG, C3c, factor H, properdin, and C3d) to confirm the presence of C3 convertases and the initiating factors of complement activation [92]. These surfaces showed adsorption for each antibody correspondingly except for properdin. The failure to detect properdin and the presence of

bound factor H indicated that complement activation is initiated by zirconium but it is also simultaneously down-regulated by factor H. In other words, factor H interferes at an early stage in the complement cascade, and prevents the assembly of a convertase because it mediates the rapid degradation of the C3 convertase. The findings invoke suppositions that implanted zirconium is opsonized through the deposition of C3b and is not capable of inducing other cascade reactions through the complement system, shedding a new light on zirconium interactions with the innate immune system and how understanding of specific complement interactions could lead to more biocompatible zirconium implants through methods like surface functionalization.

Surface modification of inorganic materials has also demonstrated different responses from the innate immune system [94, 95]. Notably studies of complement activation on chromium surfaces cleaned with ethanol or peroxide prior to incubation in serum [94]. These treatments significantly change the surface chemistry on chromium, reducing carbon contaminants and increasing the oxidative species. So by using ellipsometry and the addition of antibodies reacting with IgG, C1q, and C3c, the amount of deposited C3c antibodies increased as a function of time and were found to be higher on ethanol treated surfaces. Furthermore, ethanol-treated chromium surfaces were found to initiate complement activation through the classical pathway by the detection of C1s antibodies at short incubation times whereas the obtained data for peroxide treated surfaces was not sufficient to define an activation pathway. This study demonstrates the importance of surface chemistry and how this solid interface is the deterministic factor in inorganic substrate complement activation. As such, tailoring the chemistry of these materials through different coatings or chemical techniques could yield desired properties for biosensing and other biomedical applications.

2.3.2. Protein Coatings

It is known that free circulating proteins in the blood do not trigger complement activation until they are deposited onto foreign surfaces with different protein conformations. So far, many studies have primarily focused on the most abundant proteins found in blood such as the immunoglobulins A, G and M (IgA, IgG and IgM,

respectively) (**Table 2.7**). Elwing et al. was one of the first researchers to compare the adsorption profile of IgG on both hydrophobic and hydrophilic silicon surfaces using ellipsometry [81]. The results showed that IgG adsorption plateaued after 10 min resulting with little or no desorption from the surface during buffer rinsing, suggesting that there is the irreversible IgG adsorption on hydrophobic surfaces. However, on hydrophilic surfaces IgG adsorption reached a maximum after a few min which was followed by decay despite the continual addition of IgG. Interestingly, buffer rinsing IgG coatings on hydrophilic silicon surfaces resulted in an appreciable amount of desorbed IgG, suggesting that there is a reversible IgG adsorption on hydrophilic surfaces. The IgG coated surfaces were subsequently immersed into human serum and along with C3 specific antibodies, yielding a substantial increase in antibody deposition. These findings established a new methodology for analyzing protein coated materials with surface sensitive techniques, revealing the presence of IgG coatings on both hydrophilic and hydrophobic silicon surfaces enhances the deposition of abundant serum proteins such as C3.

Table 2.7. List of protein coatings used in complement activation studies

Protein Coatings	Substrate	Method	Findings	Ref.
IgG	Glass (Hydrophilic and hydrophobic)	Ellipsometry	Reversibly bound IgG onto hydrophilic glass did not enhance the C3 deposition. Irreversible bound IgG onto hydrophobic glass enhanced the C3b deposition	[99]
IgG	Methylated silicon	Ellipsometry	Preadsorbed IgG initiated the classical pathway and after 30 min alternative pathway took over the complement activation due to an unknown mechanism.	[96, 100-103]
IgA	Methylated silicon	Ellipsometry	Preadsorbed IgA activated alternative pathway since there was a lag phase in deposition of increased amount of serum proteins, anti-C3c and anti-properdin.	[101]
IgM	Methylated and aminated silicon	Ellipsometry	Preadsorbed IgM initiated complement activation via calcium-independent C1q-bypassed like classical pathway	[103]
C3	Polystyrene	QCM-D	Preadsorbed C3 was able to generate C3 convertase because its conformation has changed to C3 (H ₂ O)	[104]
C3b	Polystyrene	SPR, QCM-D	Preadsorbed C3b on polystyrene participate in alternative pathway C3 convertase formation.	[104, 105]

Factor H	Polystyrene	QCM-D	Immobilized factor H on the surface reduced the deposition of C3 fragments, suggesting the down regulation of complement activation	[106]
Fibrinogen	Polystyrene	QCM-D	Preadsorbed fibrinogen did not have binding site for C3b, suggesting that no convertase formation was observed	[104]
HSA	Polystyrene	QCM-D	Preadsorbed HSA has a binding site for C3b, allowing alternative pathway convertase formation	[104]

Liu and Tengvall et al. further explored the complement activation behavior of both IgG coated hydrophilic and hydrophobic surfaces [99, 100]. The first noteworthy finding from this study was that neither the bare hydrophobic or hydrophilic surfaces trigger the complement activation. However, when both surfaces were coated with IgG, there was an appreciable increase in the level of the complement activation. As a way to correlate the deposition of complement proteins to the previous studies, ellipsometry was used in conjunction with the addition of C3 antibodies. The result showed C3 deposition on IgG coated hydrophobic surfaces but not on hydrophilic surfaces. This was believed to be due to the high degree of desorption of IgG from hydrophilic surfaces. Additionally, when the thickness of the IgG layer increased, C3 deposition increased appreciably. Together, these results elucidate how a sufficiently uniform protein coating can completely change the surface properties of a material, taking on the identity of the protein corona itself. Moreover, Tengvall et al. also investigated the complement activation pathways on IgA and IgM coatings on the same silicon surfaces and compared it with the previous findings for IgG coatings [101, 103]. IgA coated surfaces triggered the complement activation via the alternative pathway illustrating a general lag in the adsorption of serum proteins C3 and properdin antibodies [101]. As for IgM coated surfaces, ellipsometry demonstrated no surface bound C1q and C3c antibodies but a noticeable amount of C3d antibodies, which advocates the down regulation of complement activation by factor H and cofactor I [103]. In order to determine the specific mechanism, IgM coated surfaces that are used to activate the complement system was also examined at the end of incubations in C1q, factor B, and C2 depleted human serums, explicating that they trigger complement activation without C1q and factor B. However, C2 was pivotal for immediate activation. These findings sanctioned the use of

surface science techniques for the real time analysis of protein interactions with materials leading to observations of IgM coated surfaces activates the classical pathway in a unique fashion bypassing C1q. As a whole, these results demonstrate how pathway dependent analyses can determine specific interactions of protein coatings and their interactions with the innate immune system.

A more recent study by Sellborn et al. incubated polystyrene (PS) and silicon dioxide coated with IgG in serum to further explore their complement activating properties on different substrates [102]. Using QCM-D, the adsorption of C3 fragments were monitored in real time and were correlated to the level of generated soluble C3a and C5b-9 by ELISA kits as a means of quantifying the total complement activation. As a result, complement activation was much higher on IgG coated PS surfaces whereas the silicon dioxide coated surfaces had significantly lower activation due to the poor surface coverage of IgG as well as the conformation of bound IgG. The true novelty of this study was the first time to employ QCM-D instead of ellipsometry for analyzing the adsorption of complement cascade activators on protein coated surfaces and direct evidence of protein coated substrates that are more activating than others. Utilizing the QCM-D technique, Andersson et al. went on to investigate whether complement activation occurs on the top of an adsorbed plasma protein layer [104]. This was done through the stepwise assembly (factor B to factor D to C3) of the alternative pathway convertase on PS surfaces coated with IgG, fibrinogen and human serum albumin (HSA). The PS surfaces coated with IgG and HSA both showed convertase formation but not the fibrinogen coated surfaces, suggesting that fibrinogen does not have a binding site for C3b. Exposing potential mechanistic factors of pre-adsorbed IgG with C3b that lead to complement activation through the alternative pathway. Although previous studies have shown that bound IgG initiates the complement activation via the classical pathway, this study was the first to demonstrate how IgG can also activate the alternative pathway. An even more novel finding was that surface bound HSA promoted the covalent binding of C3b, resulting in its participation in convertase assembly.

By combining the nanoscale precision of surface sensitive techniques and the selective adsorption of serum proteins, different immune responses can be also studied in vitro. Highlighting the importance of understanding these systems through specific

protein-protein interactions, the findings further suggest that interactions of materials with the innate immune system can be tailored by pre-coating a material with a known complement activator or regulator, essentially giving the material a new identity. As such, these findings could lead to new forms of stealth pharmaceuticals or medical implants that can either modulate or inhibit complement activation depending on the protein coating.

2.3.3. Self-Assembled Monolayers

As a widely utilized covalent surface functionalization scheme, self-assembled monolayers (SAMs) enable precise control over interfacial properties of a material surface, including hydrophilicity/hydrophobicity as well as the type and density of functional groups[107]. Likewise, tailoring the composition of SAMs influences numerous physicochemical properties; such variations also have a significant influence on corresponding biological activities, including protein adsorption and cellular adhesion [108-114]. It is within this purview that there has been significant attention placed on understanding how SAM features influence on complement activation (**Table 2.8**).

Table 2.8. List of SAMs used in complement activation studies

SAMs	Functional Group	Method	Findings	Ref.
Mercaptopropionic acid(MPA)	-COOH	Ellipso-metry	On an inactivating surface, a MPA layer induced minimal complement activation	[115]
Mercaptoglycerol (MG)	-OH		On an inactivating surface, a MG layer caused pronounced productions of iC3b and C5b-9 in serum as well as increased C3 deposition on the surface. MG layers showed high affinity for IgG. At lower serum concentrations, the classical pathway activation seemed to dominate complement activation	[115]
Oligo ethylene oxide (OEO)	-OH	SPR	On OEO monolayers, minimal h-IgG adsorption occurred and approximately three orders of magnitude less than methyl-terminated monolayers.	[108, 116]
Mercapto-dodecane	-CH ₃	SPR	A monolayer on gold showed minimal alternative pathway activation	
Mercapto-undecanol	-OH	SPR	A monolayer on gold adsorbed C3b resulting in complement activation through the alternative pathway.	[117]

Combined SAMs: 11-mercapto-1-undecanol (C11-OH) N-nonanethiol N-dodecanethiol N-hexadecanethiol [CH₃]	-OH and CH ₃	SPR	Mixed monolayer surfaces on gold resulted in activating the complement system. Increasing the C11-OH content increased C3b adsorption.	[118]
11-amino-1-undecanethiol (C11-NH₂)	-NH ₂	SPR	Amine terminated SAM surfaces adsorbed less C3b compared to hydroxyl terminated SAMs and concentrations were comparable to methyl terminated SAMs.	[119]
Tri(ethyleneglycol)-terminated alkanethiol (HS-TEGOH)	-OH	SPR	HS-TEGOH monolayers showed strong complement activation as compared to HS-mPEG surfaces.	[120]
Methoxy-terminated polyethylene glycol thiol (HS-mPEG)	-OCH ₃	SPR	The surfaces did not induce complement activation until long periods of storage and became stronger activators with increasing time. Degradation of the HS-mPEG monolayer with UV irradiation yielded an increase in C3b adsorption.	[120]
Combined SAMs: C11-NH₂ [NH₂] 11-mercaptoundecanoic acid [COOH] Dodecanethiol [CH₃]	-NH ₂ , -COOH, -CH ₃	SPR	Monolayers of combined methyl and amine end groups were not potent activators. Monolayers of combined amine and carboxylic acid end groups demonstrated complement activation through C3b adsorption.	[121]

Liu et al. first investigated complement activation on bare and thiol-modified gold substrates [115]. A key significance of the work was that it sets as a precedent for applying surface-sensitive measurement techniques to study complement activation of SAM-coated surfaces. Bare and mercaptoglycerol (MG)-coated gold surfaces were incubated within whole serum, and then a C3-antibody was added to detect bound C3 in the active form. The antibody binding was detected by ellipsometric measurement and indicated C3-antibody only adsorbed onto the serum-treated MG-coated gold surfaces, not the bare gold substrates, indicating that the active form of C3 protein deposits only on MG-coated gold surfaces. Supplementary, ELISA measurements showed significantly enhanced production of fluid-phase iC3b and C5b-9 on MG-coated surfaces compared to bare gold substrates and 3-mercaptopropionic acid (MPA)-coated gold surfaces. Taken together, this work established a correlation between the extent of active C3 deposition on

a SAM-coated surface and corresponding MAC production and also demonstrated the different SAM chemistries induce different degrees of complement activation [115].

Silin et al. primarily studied the adsorption kinetics of BSA and human IgG (hIgG) on different SAM chemistries due to their net surface charges. SAM coated gold surfaces consisting of 16-hexadecanethiol (C16), 16-mercaptohexadecanoic acid, 16-mercaptohexadecanol, p-(12-mercapto-1-oxododecanyl) phenol (CPheOH), 2-aminoethanethiol, oligo ethylene oxide (11-mercaptodecyl hexa (ethylene oxide)) (OEO) were used to determine the protein adsorption kinetics on different SAM chemistries by using the SPR measurement technique. Each protein was added after establishing a steady baseline in intensity where the fluctuations in the relative intensity as a function of time yielded protein adsorption to the surface. This study resulted into minimal IgG adsorption onto OEO coated surfaces, whereas, C16 and CPheOH coated surfaces were two orders of magnitude higher in IgG adsorption, suggesting that IgG has a selectivity against different SAM chemistries [108]. Disley et al. then applied these specific interactions of hIgG to interfacial SAM layers by fabricating an SPR based immunosensor on a SAM coated-silver substrate [116].

Following this line of SPR analysis, Hirata et al. explored the covalent immobilization of C3b on SAM coated gold surfaces with different SAM chemistries. Specifically, monolayers of 11-mercapto-1-undecanol (C11-OH) and 1-dodecanethiol (C12) on sensor surfaces contained hydroxyl and methyl groups, respectively. The interactions of these surfaces with C3b were measured by incubating the SAM-gold substrates within 10% diluted human serum and subsequent applying a C3-antibody. The amount of antibody immobilization was measured by the shift in the reflected intensity as a function of the incident angle. C3b was found to have a high affinity to C11-OH surfaces but not C12. This was believed to be due to the internal thioester bond of the C3b protein interacting with the nucleophilic hydroxyl groups on the C11-OH surfaces. Furthermore, the novelty of this work was that the adsorption of C3b on C11-OH surfaces was also dependent on the salt in the medium, where saline solutions containing Mg^{2+} showed less C3b adsorption. Furthermore, different SAM chemistries can have also specific effects on complement activation, and thus great care must be taken when analyzing complement protein interactions with different saline solutions [117].

In order to validate the hypothesis that hydroxyl covered surfaces were the driving factor in C3b adsorption, Hirata et al. conducted a similar experiment using mixed-SAMs on gold surfaces. The same SPR techniques and antibodies were used, but the C11-OH content was varied with different molar ratios of C12, n-nonanethiol, and n-hexadecanethiol. A direct correlation was found in the immobilization of the C3b-antibody and increasing C11-OH content. These results show that selectively mixing SAM chemistries with different functional groups on surfaces directly affects complement protein adsorption. Ultimately it also leads to the further analyses of SAM chemistries with different nucleophilic groups on gold surfaces [118].

Continuing the studies using SAM-coated gold surfaces and SPR measurements, Toda et al. hypothesized that all nucleophilic groups should be potential complement activators. In order to confirm this claim the surfaces were initially coated with 11-amino-1-undecanethiol (C11-NH₂) and interactions between C3b and amino groups on the surface was carefully studied. Additionally, gold surfaces coated with C11-OH and C12 were also used as control measurements, respectively. Upon C3b antibody immobilization, C11-NH₂ coated surfaces demonstrated significantly less C3b adsorption than C11-OH surfaces, which agreed well with ELISA measurements of C3a-desArg (a fluid phase constituent of the C3 protein that is released after C3 cleavage). In order to clarify the minimal complement activation of amino covered surfaces, antibodies reacting to C1q, IgG, and HSA were also applied to determine a potential pathway of activation as well as the effect of other serum proteins. Notably, C11-NH₂ and C12 surfaces showed a high affinity towards HSA. These findings suggested that interactions of serum proteins with amino covered surfaces potentially disrupt C3b immobilization, indicating that amino-covered surfaces may not be effective activators of the alternative pathway due to the masking of other serum proteins [119].

Regardless of this finding, other studies began to report nucleophilic amino groups being potent activators of the complement system. To elucidate these contradictions, Toda et al. continued to analyze the interactions of C3b with mixed-SAM coated gold surfaces using SPR measurement technique. Each surface consisted of different molar ratios of C11-NH₂ and C12, as well as C11-NH₂ and 11-mercaptopundecanoic acid (C11-COOH). Moreover, each individual SAM surface was

also used as a control surface, including the surface coated with C11-OH. The content of C11-NH₂ was varied with each mixed SAM, where the maximum C3b-antibody immobilization occurred when the surface density was NH₂: COOH (%55: %45). Interestingly, the concentration of HSA-antibodies showed a minimum at this surface density but correspondingly increased with C11-NH₂ content. For the mixed SAM coatings consisting of C11-NH₂ and C12, minimal adsorptions of C3b and C1q antibodies were measured suggesting that there is no complement activation. These results correlated well with ELISA measurements measuring the resultant MAC production. These measurements also showed that C11-OH and C11-NH₂:C11-COOH surfaces yielded the largest sC5b-9 release in serum where their respective concentrations were statistically the same. Thus, there is a strong proof that the binding of HSA to nucleophilic amino groups mitigates C3b adsorption while electrostatic interactions at the SAM-protein interface play an important role in complement protein adsorption. Subsequently, the formation of the MAC must also be dependent on these interactions and need to be considered when designing biocompatible artificial surfaces [121].

The fabrication of biomimetic surfaces using SAMs has also been explored with different SAM chemistries [122]. Recently, Goda et al. utilized these chemical processes to coat gold surfaces with SAMs containing zwitterionic phosphorylcholine (PC) ligands (PC-SAM). This established a biomimetic coating on a gold surface for examining CRP adsorption dynamics at the lipid-protein interface. According to the bivalent binding model, there are two primary reactions of CRP to PC-SAM coated surfaces. The first is the recognition of CRP to a PC ligand. Second, the CRP-containing the surface forms a second bond with a nearby PC ligand. Since CRP is pentameric protein complex, there can be up to five bonds but more than two is generally ignored due to the extremely low probability of occurrence [123]. The effects of the physicochemical properties such as lateral PC density, surrounding pH, and the interactions of CRP with the PC-SAM coated surfaces were monitored using SPR measurement technique. As a result, CRP binding affinity was independent of ligand density in the first ligand reaction which agreed well with the theoretical adsorption model. However, the second binding reaction yielded unintuitive results where the increase in density decreased CRP-ligand interactions. The adsorption model also shows a correlation with the optimized surface density of PC

ligands to the surface density of phospholipids in natural bilayers. Since the PC ligands are covalently bound to SAM surfaces and have no lateral fluidity, these results suggest that the lateral fluidity of lipid bilayers attributes to the multivalent binding of CRP to PC ligand surfaces. Furthermore, the pH of the medium affects the binding affinity of CRP to PC through conformational changes in CRP. This was measured by the binding of an anti-mCRP aptamer which specifically bonds to the monomeric form of CRP. In this conformation CRP is known to elicit proinflammatory responses in tissue microenvironments where the pH is more acidic [124-128]. As a whole, these results demonstrate that surface sensitive techniques can translate to understanding the behavior of proteins in different microenvironments that lead to complement activation. Setting a precedence for the future study of SAM-protein interactions and designing artificial surfaces [129].

Surprisingly, there has only been two surface measurement techniques applied to the study of complement protein adsorption on SAM surfaces: ellipsometry and SPR [75]. From these studies, two main characteristics arise for understanding complement protein-SAM interfaces. First of all, the SAM chemistry at the interface directly affects protein adsorption. Secondly, the medium of the protein-SAM interaction is critical for protein conformation and subsequent protein adsorption. Thus, SAMs as a means to study surface coatings with other techniques such as QCM-D could allow the development of methodologies to manipulate specific protein interactions based on these parameters. There is also a plethora of different SAM and SAM-ligand chemistries with various properties that have still yet to be tested [107]. The selective functionalization of each SAM and a comprehensive study of complement protein interactions with these surfaces should lead to a better understanding of the mechanisms behind complement protein adsorption and the initial binding factors that result in the subsequent assembly of protein complexes.

2.3.4. Polymers

Synthetic and natural biocompatible polymer coatings have been a quintessential part of biomedical research [130, 131], leading to polymer-based applications such as

biosynthetic materials [132, 133], and drug delivery platforms [134, 135] that have translated to new forms of medical and pharmaceutical treatments [136, 137]. The utility of these materials stem from their conformational properties, giving a wide range of chemistries that can interact with biological systems [138]. However, these properties can result in undesired immune responses, specifically arisen from complement activation [139-142]. Given the scope of polymeric materials, understanding the properties that elicit adverse immune responses is key to their current application [20, 143]. Thus, the aim of this section is to review the interfacial interactions of complement proteins with polymer coated surfaces that result in activating or inhibiting the innate immune system (Table 2.9)

Table 2.9. List of polymers used in complement activation studies

Polymers	Method	Findings	Ref.
Polystyrene	Ellipsometry, TIRF, QCM	On activating surfaces, C3 adsorption induces a conformational change that results in factor B binding and formation of a functional convertase. Additions of HSA to polystyrene surfaces resulted in fractional C3 desorption. Activating surfaces coated with vinyl pyrrolidone showed higher C3 and IgG adsorption but a decrease in complement activation	[82, 144]
Poly(methyl methacrylate) (PMMA)	Ellipsometry TIRF,	Factor B passively adsorbs to PMMA surfaces. Additions of HSA to PMMA surfaces resulted in fractional C3 desorption	[144]
Poly(ethylene terephthalate) (PET)	QCM	Comparing spin coated surfaces of PET, polydimethylsiloxane, polyethylene, and PMMA; PET demonstrated the highest C3 surface adsorption.	[104]
Poly(ethylene glycol) (PEG)	Ellipsometry, SPR	PEG coatings on an activating surface demonstrated low protein adsorption. PEG modified activating surfaces inhibited complement activation. Ultraviolet Degradation of a PEG-monolayer on an activating surface results in strong activation.	[145]
Chitin/Chitosan	Ellipsometry	Chitosan coated surfaces weakly activated complement due to the binding of anti-fibrinogen. Acetylation of non-activated surfaces resulted in strong activation due to the absence of anti-fibrinogen.	[146]
Poly(alkyl methacrylate) (PAM)	QCM	On activating surfaces, increasing the alkyl ester side chain length of PAM decreases complement activation	[147]
Dextran	SPR	Immobilized dextran on activating surfaces increased complement activation, but the soluble polymers could not induce activation even in high concentrations	[120]

Poly(vinyl alcohol) (PVA)	SPR	Immobilized PVA on activating surfaces increased complement activation, but the soluble polymers could not induce activation even in high concentrations. Complement activation by hydrated PVA coated surfaces showed a dependence on water content.	[120, 148]
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Malmsten et al. was one of the first researchers to test complement activation on polymer coated surfaces with poly (methyl-methacrylate) (PMMA) and polystyrene (PS) using surface sensitive techniques [144]. These polymers were spin coated onto silicon substrates and they were subsequently incubated with C3, HSA, and factor B. Utilizing in-situ ellipsometry and total internal reflection fluorescence spectroscopy (TIRF); the adsorption profile of C3, HSA, and Factor B was investigated. Upon the addition of C3 to each surface, it was found that PMMA adsorbed a slightly higher amount of C3 than PS. For analyzing the competitive adsorption between different serum proteins, the polymer surfaces were pre-coated with C3 then fluorescently labeled HSA and Factor B were both added in a sequential manner. This resulted in the slight desorption of C3 by HSA on both of the polymer coated surfaces. Subsequently, the addition of factor B after HSA caused C3 desorption yielded higher amount of factor B binding to C3 on polystyrene surfaces while factor B passively adsorbed to PMMA coatings. Since factor B induces the convertase formation of C3b to C3bBb in the complement cascade, it selectively binds to the C3b, subunit of C3, suggesting that C3 takes a more active conformation when immobilized on polystyrene surfaces [144]. This supports previous observations of alternative pathway activation on polystyrene surfaces.

Jokiranta et al. further explored the binding mechanisms of the alternative pathway proteins on polymer surfaces using SPR measurement technique, specifically, C3b which was enzymatically or amine coupled to polystyrene and carboxylated dextran (CD) coated gold surfaces. The protein adsorption to these surfaces was quantified as fluctuations in the relative intensity over time with the addition of four other complement proteins: factor B, factor H, C5, and sCR1. It was determined that factor H has three different binding interactions with the C3b fragments, C3c and C3d but factor B selectively bound to C3d. In addition, C5 and sCR1 also showed preferential binding to C3c but not C3d. As a result, this sets the base for the mapping of specific protein interactions on polymer coated substrates with surface sensitive techniques. Explicating

the analytical capabilities of interfacial studies needed to determine the sequential protein interactions that lead to complement activation [105].

Taking this lead, Andersson et al. utilized polystyrene coated QCM-D crystals to analyze the adsorption of the alternative pathway proteins and the formation of the C3bBb convertase [82]. Using the QCM-D measurement technique, C3 and C3b were incubated with the QCM crystals followed by the sequential addition of factor B, factor D, and C3. The protein adsorption was measured as a dip in frequency over time, revealing that C3 and C3b both generated the C3 convertase via the binding of C3 fragments to the PS surface. This finding agreed well with immunoassay measurements showing a significant production of the soluble C3 fragment, C3a. Upon the addition of properdin to the system, the convertase reaction showed a substantial amplification, demonstrating that C3 convertases were formed directly on the PS surface. Furthermore, to determine the effect of factors I and H on the convertase formation, these regulatory proteins were also incubated with the PS-C3 and PS-C3b surfaces in the presence of properdin. As expected, C3b was shown to be susceptible to cleavage by factors I and H but adsorbed C3 did not, suggesting a conformational change of C3 on PS [82]. Knowing that PS is a potent activator of the alternative pathway, Andersen et al. also used the QCM-D technique to demonstrate that coating PS and silicon surfaces with a plasma polymerized vinyl pyrrolidone (PVP) coating can reduce complement activation [149]. This PVP coating resulted in the strong adsorption of serum proteins suggesting that the coating of a protein corona mitigated complement activating factors. These works establish a correlation between the extent of complement protein adsorption to polymer surfaces and the regulatory factors that result in convertase formation, further demonstrating the utility of analyzing polymer surfaces with surface sensitive techniques.

In a similar fashion, Berglin et al. used QCM-D to investigate the complement protein adsorption properties of poly (alkyl methacrylate) coated gold surfaces with varying alkyl chain lengths. Polymer coatings consisting of: poly (octadecyl methacrylate) (POMA), poly (isobutyl methacrylate) (PIBMA), poly (hexyl methacrylate) (PHMA), poly (lauryl methacrylate), and poly (butyl methacrylate) (PBMA) were spin coated on to gold QCM-D crystal surfaces and incubated with human serum. The amount of C3 deposition was quantified by the immobilization of a C3c-antibody resulting in a

correlation between the chain length of the polymer and the C3 adsorption, where larger chains had more amounts of C3 proteins. These results suggest that polymers with greater chain mobility or smaller alkyl chain lengths are less likely to adsorb C3 to the surface. Despite the authors claim for measuring complement activation, there was no subsequent study of the MAC formation from these surfaces. Thus, these studies do not truly validate the polymer surface as a complement activator but indicate the deposition of precursor proteins as a result of polymer conformation [147].

Correspondingly, other analyses of complement activating polymer surfaces continued to use QCM-D measurement technique to quantify the initial protein interactions that lead to complement activation. Andersson et al. went on to measure C3 deposition on bare and polymer coated titanium surfaces consisting of polyethylene (PE), poly (ethylene terephthalate) (PET), PMMA, and poly (dimethylsiloxane) (PDMS). Each surface was incubated with human serum followed by the addition of C3c-antibodies which were used to measure adsorbed C3. As a result, PET showed the highest C3c-antibody immobilization which was contradictory to the immunoassay results for the sC5b-9 production, suggesting that PET surfaces activate the complement system the least. Notably, all of the other surfaces showed clear correlations between C3c-immobilization and concentrations of sC5b-9. Therefore, the inconsistency in the PET coated surface is assumed to be due to either a conformational change in C3 as it binds to the polymer surface, or there is a substantial blockage of C3 fragments by other serum proteins. Immunoassays of C3a concentrations also showed no statistical significance between all of the polymers and the bare titanium surface. This further demonstrates that protein conformation and other serum proteins are key factors in complement activation on immobilized polymer surfaces while multiple assays must be used to reveal polymer-protein interfacial reactions [104].

A typical polymer coating poly (ethylene glycol) (PEG) was also investigated by Arima et al. using SPR measurement technique. Utilizing their expertise in SAMs, gold surfaces were coated with monolayers of tri-(ethylene glycol)-terminated alkanethiol (HS-TEGOH) and methoxy-terminated PEG thiol (HS-mPEG) respectively, and incubated within serum. The addition of various antibodies reacting with C1q, C3b, HSA, IgG, and IgM were subsequently used to measure the surface concentration of

complement proteins. It was found that HS-mPEG showed minimal protein adsorption, consisting mostly of C3b. However, surfaces coated with HS-TEGOH showed a four-fold increase in the amount of surface bound C3b. Surprisingly, despite their extensive experimental design for SAMs, interactions of complement proteins were not confirmed with immunoassays and therefore little information was obtained about their activations of the complement system. In addition, HS-mPEG can oxidize as a function of time, resulting in an increase of C3b adsorption to the surface. This correlated well with accelerating the oxidation process of the HS-mPEG monolayer using ultraviolet (UV) irradiation which increased C3b adsorption to the surface [145]. A follow up study was conducted since the validation of the MAC formation was neglected. This time only HS-mPEG was revisited and the ELISA results correlated well with the protein adsorption, as such that the surface is a more potent activator of the MAC as it oxidizes [150].

Continuing this form of analysis, Arima et al. analyzed the effects of polymers carrying surface hydroxyl groups when soluble or immobilized. Gold surfaces were used as platforms to immobilize dextran and PVA to aldehyde terminated SAMs for SPR analysis. These coated surfaces were incubated with serum and anti-C3b antibodies were added to detect bound C3b. It was found that both PVA and dextran immobilized surfaces were effective activators of the complement system from the C3b-antibody concentrations and ELISA measurements but did not produce the MAC when solubilized with serum. This suggests that the physicochemical properties of the polymer can greatly modulate complement activation [120]. Arima et al. then went on to determine the effects of water content in PVA and how it influences on complement activation using SPR measurement technique. PVA layers were prepared on gold surfaces modified with aldehyde groups by spin coating. These surfaces were then annealed, resulting in the crystallization of the PVA layer, and were subsequently incubated with serum. Thus, with the addition of a C3b-antibody to detect bound C3b it was determined that more hydrated PVA coated surfaces weakly adsorb C3b. This correlated well with ELISA measurements showing minimal MAC production. However, less hydrated layers yielded opposite results and were shown to be a strong complement activator. Altogether, these findings suggest that the functionalization and hydration of polymers are pivotal factors in activating the complement cascade [148].

Naturally occurring polymers, such as chitosan, were also explored as potential inhibitors against complement activation. Using ellipsometry, Benesch et al. have used aminopropyltriethoxysilane (APTES) coated silicon surfaces to form a uniform chitosan layer. These surfaces were incubated with serum and compared with two known activating surfaces, hydrophilic silicon and IgG coated silicon and one weak-activating surface, hydrophobic silicon. After incubation, antibody immobilization reacting to complement proteins was used as indicators of complement activation. It was found that the chitosan coated surfaces adsorbed a large amount of serum proteins but low amounts of C1q, C3c, and IgG proteins. Modifying the chitosan layer through acetylation lead to a dramatic increase in C3c-antibody immobilization, suggesting that acetylated chitosan is a strong activator of the alternative pathway. Although these results are indicative to the initial steps of complement activation, no investigations of MAC production were made. These results demonstrate the importance of different polymer chemistries and how modification leads to different protein reactions [146].

Although there have been extensive efforts in understanding the interactions of complement proteins with polymeric materials, there have been relatively few studies involving surface sensitive techniques. Given the breadth of these materials, there are still superfluous numbers of surfaces that have not yet to be tested [151, 152]. From the current works, protein-polymer interactions are dependent on the hydration, conformation, physical state (immobilized or solubilized), and surface chemistry of the material. Explicitly, the functional groups at the surface lead to changes in protein conformation and the competitive binding of other serum proteins. In addition, there has not yet to be any surface sensitive studies on changing the physicochemical properties of the environment and the binding of complement proteins to immobilized polymer surfaces. Therefore, there is still a lot of potential for future studies of polymers with the complement system that could yield new insights to the design of new biocompatible surfaces.

2.4. Interaction of Complement Proteins with the Lipid Membranes

Complement activation by lipid membranes of pathogenic entities is critical for the host defense. The liposomes have been mainly used as a model system to mimic the lipid membranes (or lipid bilayers) of biological systems and past studies confirmed that the liposomes interact with the complement proteins in the same ways on which erythrocytes membranes and human lymphoblastic cell lines do [142-147]. Additionally, it is important to note that the liposomes have been also utilized as drug carrying vehicles (*e.g.* Doxil) in the most of the recent pharmaceutical applications. This demands additional efforts to understand the complement activation by lipid membranes. To date, complement activation by the liposomes have been mainly assessed as degree of complement activation by using the traditional techniques indicated in previous sections [153-160]. Once the liposomes are incubated with the body fluid (*e.g.* serum), the versatile complement proteins are deposited on the liposome surface. These deposited proteins determine the fate of the liposomes and the duration that stays in the blood circulation before they are cleared by the reticuloendothelial system (RES) [161, 162]. Therefore, many studies have been conducted to determine the factors that play role in degree of complement activation by liposomes (*e.g.* strong or weak) (**Table 2.10**) [163-168].

Table 2.10. The factors that play role in degree of complement activation by liposomes

Adapted from [160].

Factors	Strong Complement Activation	Weak Complement Activation
Charge	Negative/Positive	Neutral
Cholesterol	Presence	Absence
Saturation of acyl chains	Unsaturated	Saturated
Fatty acid chain length	Shorter	Longer
Size	Large	Small

More specifically, liposome surface charge has attracted the most attention as a key determinant of the complement activation. Thereafter, Chonn et al. has primarily shown how the surface charge of liposomes can separate two distinct groups based on which complement pathway can be activated [163]. This study showed that neutral charged liposomes with compositions of PC: Chol (55:45) or PC: Chol: DMPE (35: 45: 20) failed to trigger complement activation, and negatively charged liposomes containing phosphatidylglycerol (PG), phosphatidic acid (PA), cardiolipin, phosphatidylinositol (PI) or phosphatidylserine (PS) were capable of activating the classical pathway of the complement system in human serum in a Ca^{2+} dependent manner while positively charged liposomes containing stearylamine or 1,2-bis(oleoxyloxy)-3-(trimethylammonio)propane triggered the alternative pathway. Yet, these findings have come with contradictions where negatively charged multilamellar liposomes made of 10% dicetyl phosphate (DCP) predominantly activated the alternative pathway instead of the classical pathway in rat serum [169]. Additionally, the liposomes containing positively charged lipid, stearylamine, did not activate the alternative pathway unless glycolipids were present [170]. Taken together, it is generally claimed that a positive surface charge is required for complement activation through the alternative pathway while a negative charge is required for the classical pathway activation irrespective of presence of contradictory results. Nevertheless, how liposomes activate the complement system at molecular level in the context of surface charge remains unknown.

In order to investigate the interaction of the proteins with the lipid molecules which are incorporated into liposomal systems, versatile solution based assays have been used in general manner as presented in **Table 2.11** [171].

Table 2.11. Solution based methods- adapted with the permission from the reference [171].

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Methods	Advantages	Disadvantages
Liposome sedimentation assay	In vitro and in vivo proteomic analysis Possible to do measurements with and without labeling	Challenging to quantitate equilibrium constant
Photoactivated cross linking assay	In vitro and in vivo proteomic analysis	Low sensitivity Low yield of protein binding

Isothermal titration calorimetry (ITC)	Assess the thermodynamic parameters in label free format	Low sensitivity Require higher amount of protein consumption
Electron spin resonance (ESR) assay	Possible stoichiometry calculation , selective	Requires post-hoc membrane modification
Fluorescence based assays	Higher sensitivity Measuring the affinity constant	Labelling required Problems associated with light scattering and auto-fluorescence

More specifically, the liposomes which are already prepared with the lipids of interest are mixed with the target protein and the lipid-protein complex is then separated from the unbound proteins by employing centrifugation at the high speed in the case of sedimentation assays [171]. After this process, the protein-liposome complex is contained in pellet while the unbound proteins stay in the supernatant. Then, the protein fractions are quantified by the SDS-PAGE. Moreover, the isothermal titration calorimetry (ITC) is the most common technique that is utilized for the assessment of protein-lipid interactions in label free format. ITC typically measures the change in heat generation (exothermic reaction) or absorption (endothermic reaction) during the interaction of the proteins with the lipid based systems in the solution. Within this assay, thermodynamic parameters including Gibbs free energy, association and dissociation constant can be measured [172]. For solution based assays, the following requirements must be there for the correct assessment of the protein bindings to lipid based systems in terms of liposomes;

- After immobilization of the proteins onto liposomes, protein should retain the affinities against the other proteins or their functions
- The proteins should bind to the liposomes firmly
- Upon protein binding, the liposomes should preserve the integrity
- The efficiency of protein binding should be high

As a summary, solution based liposomes assays are not suitable to do real time measurements in a quantitative manner. Therefore, how complement proteins bind to

liposomes at molecular level in order to trigger the complement activation still remains as a challenging question, leading to the use of supported lipid bilayers combined with surface sensitive techniques to develop a fundamental understanding about interactions between complement proteins and lipid bilayers. Such knowledge can be used to ideally design optimal lipid formulations for liposomal drug carriers.

Given the scope of lipid membrane interactions that trigger complement activation and the need of a model membrane platform that is compatible with the surface sensitive measurements, supported lipid bilayer (SLB)s have become a ideal model platform for analyzing the binding of complement proteins to lipid membranes. SLBs are two dimensional biomimetic membranes that self-assemble on solid supports. These bilayers have been extensively used to investigate the fundamental characteristics of cellular membranes and other membrane-associated processes that have led to the development of biosensors for drug delivery and various industries. SLBs are readily fabricated on a variety of substrates including: silicon oxide, aluminum oxide, titanium oxide, and gold using either vesicle fusion method or a solvent assisted self-assembly (SALB) process. Using versatile sensor surfaces makes SLB to be compatible with the whole range of surface-sensitive analytical techniques. The fabrication of SLBs also allows the tuning the chemical composition in terms of surface charge, membrane fluidity, and cholesterol content. The stability and interfacial properties of SLBs make them an ideal platform for a critical analysis on the complement activation by various lipid configurations.

With this scope, Klapper et al. was the first to demonstrate the nonproteolytic activation of the complement component C3 on a neutral charged SLBs prepared by vesicle fusion method on silicon dioxide[173] The binding of C3 to the bilayers was monitored using QCM-D, indicating that C3 binds to the SLB. Correlating the amount of bound C3 to its lateral dimensions, it was estimated that each C3 protein covers approximately 30% of the surface area on the membrane. Factor H and factor I were sequentially added to confirm whether the C3 protein took the form of conformationally changed and hydrolyzed C3 (C3 (H₂O)) when bound to the membrane. With the addition of factor H, a decrease in frequency shift was measured corresponding to the binding of factor H to C3. Then, with the addition of factor I an increase in frequency was observed indicating C3 cleavage by factor I forming iC3 (H₂O) -inactivated C3 (H₂O). As a

complementary to the QCM-D, two different experimental approaches were also used to further clarify the role of C3 proteins on complement activation. Firstly, ELISA measurements were conducted to examine the generation of activation products, C3a and sC5b-9, in the fluid phase when the liposomes which has same lipid composition with the SLBs were incubated with lepirudin plasma, indicating that complement activation by liposomes is time and dose dependent and can be also inhibited by compstatin or chelators (*e.g.* EGTA and EDTA). Secondly, SDS PAGE along with Western blot analyses was used to investigate the binding of C3 proteins to liposomes using a strategy where liposomes were initially incubated with C3 and subsequently Factor H and Factor I were added. The findings indicated that C3 initially binds to liposomes in the form of C3 (H₂O) and then it turns to iC3 (H₂O). More specifically, iC3 (H₂O) functions as an opsonin and is recognized by CD11b receptors on polymorphonuclear monocytes (PMN) to stimulate the phagocytosis of opsonized liposomes. Taken together, this study provided insights into liposomes with the implications for fate of liposomes.

Looking forward, learning from the past studies can be applied to design experimental studies seeking to extend the fundamental understanding of the interactions between complement proteins and lipid membranes in the context of initiation of complement activation, convertase assembly along with regulation, and membrane attack complex assembly along with regulation.

2.5. Immobilization schemes of complement proteins at the target interfaces

All complement activation pathways converge at the step of C3 activation into C3b by convertases and deposition of C3b to nearby activator surfaces. General knowledge about C3 activation and C3b deposition is very critical for fundamental understanding of complement activation and regulation at C3 level since the C3 protein is a main player for amplification of complements mediated responses. Principally, once the C3b proteins are generated, it is known that they bind to biologically relevant activator surfaces through four different mechanisms as shown in **Figure 2.3**. At first mechanism, the C3b protein is spontaneously hydrolyzed upon generation and then physically adsorbs to activator surface via non covalent linkage. At the second and third mechanisms, the C3b

is covalently bound to activator surface which contains amine groups and hydroxyl through an amide linkage or an ester linkage, respectively. At last mechanism, the ester linkages, unlike amide linkage, are not firm or stable against the spontaneous cleavage. Depending on time, temperature, solution pH or nucleophile ester linkages can be broken and accordingly make C3b to leave the surface (e.g. it can bind to nucleophile moiety). Concomitantly all mechanisms rely on the carbonyl group (C=O) of C3b [21]. However, as seen in **Figure 2.3**, the C3b also contains a sulfhydryl (-SH) moiety which is not found in native C3 and might associate to the activator surface through sulfhydryl moiety since both carbonyl and sulfhydryl moieties are located at same chain of C3b which is considered as the surface association sites [174, 175].

To date, in order to clarify the nature of the C3 binding to activator surface (e.g. zymosan) the radiolabeled C3b proteins were incubated with zymosan surfaces in the different buffer solutions with variable pH values [161]. After incubation, the treated surfaces were washed and the remaining C3b proteins on the surface were determined by the changes in particle-associated-radioactivity. The findings indicated that the chemical bond is stable at acidic solution where it can be breakable upon incubation under alkaline solutions with higher pH values. Moreover, Bhakdi et al. has also proven the covalent attachment of C3b by adding of I-labeled C3 on the antibody-covered sheep erythrocytes [176]. The findings indicated that C3b binds to membrane bound proteins and on erythrocytes via NH_2OH though an ester bond [177-179]. Later, it has been found that the highest amount dissociation of C3b from macromolecules present on erythrocytes while zymosan requires detergents in addition to NH_2OH . Furthermore, the binding studies at fluid phase where C3 proteins are triggered by trypsin in the presence of small molecules carrying -OH or free NH_2 groups have been also employed in order to clarify the covalent bonds by C3 [180, 181]. The results specifically showed that the C3b binding creates an ester bond at neutral pH whereas it turns to an amide bond at the pH values greater than pH 9. On the other hand, the ester bond occurs in the form of R-O-CO-C3b instead of R-CO-O-C3b where R corresponds to molecules on a target surface or a molecule in the fluid phase. Collectively, although the covalent bond is a crucial element of the association of the C3b onto the target surface, it may not be the only determinant.

Contrary to what is believed, the hydrophobic interactions may also involve into the C3b linkage to the target surfaces through two different scenarios. First, hydrophobic interactions present as an internal element of interaction among the labile binding site of the C3b and the target surface [160]. Second, hydrophobic interactions among C3b and target surface may create a condition for covalent attachment through an ester bond.

Figure 2.3 Mechanism of C3b interaction with activator surfaces Redrawn from the permission from [182]. Copyright 1988, Annual Reviews. Native C3 protein contains an internal thioester domain which is exposed when it is split by C3 convertases (C3bBb or C4b2a) into C3a

and C3b. The internal thioester may be hydrolyzed by water and then interact with activator surface through non covalent linkage **(i)** or may create a covalent amide bond between C3b and exposed amino groups on the activator surface **(ii)** or may create a covalent ester bond between C3b and exposed hydroxyl group on activator surface **(iii)**. Ester linkages are broken by nucleophile (e.g. methylamine) which liberates the activator surface from the covalently bound C3b **(iv)**.

Taken together, the way of C3b binding to the activator surface determines the subsequent interaction of C3b with factor H or factor B. It is known that if the surface bound- C3b interacts with factor H along with factor I, the C3b is inactivated and iC3b is generated on the surface, which is no longer capable of pursuing the sequential complement cascade [183]. On the other hand, if factor B binds to the surface bound-C3b, the complement activation ensues with amplification of C3b deposition, generation of both C3 and C5 convertases as well as assembly of membrane attack complex [184, 185]. Moreover, C3b on different activator surfaces (e.g. bacteria, viruses, parasites, altered cells and immune complexes) has different affinity against factor H and factor B depending on the way of C3b binding to surface [186]. For example, Fearon et al. revealed that after the removal of sialic acid from the surface of erythrocytes and other cell surfaces, the amount of factor H binding to C3b bound-surface reduced in compared to C3b bound surface that still contains sialic acid [187-190]. As a summary, the previous studies have utilized the complex lipid membrane systems (*e.g.* erythrocytes and zymosan) to discriminate the impact of the covalently and noncovalently bound C3b on the association of other complement proteins since C3b is the key for the complement activation and immune responses. However, they do not reveal the exact surface features allowing C3b binding and their mode of action due to the complexity of the model platforms. Therefore, the major factors such as membrane surface charge and functional groups remain to be elucidated.

2.6. PhD in Context of Literature

Overviews of the complement system, used-methods and applied-model interfaces to investigate in vitro complement activation are introduced in detail at this chapter. However, general information regarding interactions of the complement proteins with the lipid bilayer interfaces has been lagged although the lipids are one of the most abundant matrix ingredients of membranes of bacteria, cells and the liposomal drug delivery vehicles. Additionally, it is also well known fact that functional properties of the proteins significantly are differed by the interactions among a protein and a lipid bilayer interface. To date, the liposomal systems have been mainly employed as a model platform to investigate the interactions between complement protein and lipid bilayer interface using the various techniques summarized in **Table 2.11**. Even if some useful information is extracted by those techniques, it has been still challenging to gain impactful insights about the interaction of complement proteins with the lipid bilayer interfaces and further protein-protein interactions at those interfaces. Therefore, in this dissertation supported lipid bilayers (SLBs) are proposed a promising model platform because the SLBs can be integrated with a wide range of surface sensitive analytical tools and they can provide insights into interaction kinetics, including determination of protein uptake and resultant conformational changes—all of which are purported to be closely related to triggering complement activation to varying extents. In this thesis, four main studies were conducted as follows;

First of all (**Chapter 4**), it is now known that positively and negatively charged liposomes strongly trigger the complement activation while neutral liposomes weakly trigger complement action. Additionally, positively charged liposomes activate the alternative pathway whereas negatively charged liposomes initiate the classical pathway. In other words, surface charge of liposome surface determines the complement activation pathways. It has been reported that C1q protein is responsible for the recognition of negatively charged liposomes through classical pathway. However, there is still no solid information about the interactions of the complement proteins-C3, C3b and properdin—that are known to be initiators of alternative pathway with the lipid bilayer interfaces. Therefore, I investigated the membrane recognition ability of complement proteins-C3,

C3b and properdin- on supported lipid bilayers depending on the membrane surface charge. Conducting the quartz crystal microbalance-dissipation (QCM-D) technique and ELISA measurements, I compared recognition abilities of complement proteins against the lipid bilayer surface depending on the membrane surface charge.

Secondly (**Chapter 5**), the alternative pathway is the most important complement activation route among others because it contributes to an amplification loop- in other words positive feedback loop- in order to enhance the complement mediated response against the target or foreign surface, even after the complement activation is triggered by lectin or classical pathways. This unique characteristic of alternative pathway comes from the continuous cycle of C3b generation by C3 convertases because the C3b proteins reinitiate the alternative pathway and produce newly formed convertases at target surfaces. However, it also known that the C3b protein generates an alternative pathway C3 convertase when it is covalently bound to the target surface that contains hydroxyl and amine group. In order to generate in vitro alternative pathway convertases, different model surfaces have been used. However, those surfaces generally lack from-non-fouling surface characteristics, which makes very difficult to eliminate the nonspecific protein interactions and to understand the stepwise alternative pathway convertase assembly. Towards this goal, I have established two different C3b tethering schemes-covalent and noncovalent tethering- to create functional complement convertase assemblies at lipid bilayer interfaces. Then, I compared the enzymatic properties of both covalent and noncovalently created C3 convertases and subsequently tested the compstatin, complement inhibitor, at enzymatically activate convertases.

Thirdly (**Chapter 6**), after the complement activation is triggered, it is terminated by the assembly of the membrane attack complex (MAC), also known as terminal attack complex (TCC). The assembly of MAC on lipid bilayer interfaces causes the lysis, which makes the target to be cleared by the immune system through rupture of targets. In order to understand the factors that support the MAC assembly on lipid bilayer interfaces, different liposomal systems have been previously utilized. However, the stepwise assemblies of MAC components at lipid bilayer interfaces were not fully understood. Therefore, in this chapter I monitored the real-time assembly of MAC components on the supported lipid bilayers while altering the surface charge of the membrane.

Lastly (**Chapter 7**), when the MAC components are assembled at the lipid bilayer of target, a pore forms and target is cleared from the circulation independent of the nature of target, whether or not it is a host cell. Therefore, soluble MAC inhibitors-vitronectin and clusterin- are present in our blood to prevent the possible damages that might occur on host cells by MAC assembly. To date, the inhibitor activities of vitronectin and clusterin on MAC assemblies have been investigated by different methods such as hemolysis and ELISA based measurements. Although these measurements depict the overall inhibition activity of MAC assembly, the mechanisms of the actions of MAC inhibitors at real time format remain challenging. Therefore, utilizing the knowledge I obtained from the previous work, I examined the mechanisms of actions of two well known complement inhibitors-clusterin and vitronectin- to prevent the MAC assembly using supported lipid bilayer platform combined QCM-D measurement.

By combining all these studies conducted at this thesis, the findings can contribute to a broader understanding of the factors that influence interactions of complement protein with lipid bilayer interfaces and using surface sensitive techniques along with the SLB platform leaves a multitude of pathway specific studies left to be tested which can guide the design of synthetic lipid bilayers with immunocompetent features.

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Chapter 3

Experimental Methodology

As mentioned in the previous chapter, supported lipid bilayer platforms have proposed to use as a model interface in order to investigate lipid-protein and protein-protein interactions that play key roles in complement activation, amplification, regulation, and termination. Therefore, this chapter introduces the materials and preparations that are utilized experimentally including the working principles, instrumentation, and experimental protocols.

3.1. Rationale for the selection of materials and experimental techniques

In this chapter, the experimental methodologies used throughout this thesis will be described in detail and presented in three main sections; (i) materials (ii) materials preparation (iii) experimental techniques. First, the materials used and their sources are listed, specifically the solution compositions consisting of lipids, buffers, proteins, and serum reagents. Lipid reagents were used as a basis for the model membrane platforms, supported lipid bilayers and liposomes. For the design of different membrane surface properties on a specified platform, the lipid reagents consisted of a predetermined lipid head groups to differentiate nucleophilic groups or electrostatic charge (*e.g.* positive, neutral, negative charge, or maleimide functionalities). The buffer reagents were specific to each material and protocol and were utilized for self-assembly processes of SLBs and liposomes, or for diluting protein reagents in physiological mediums. The protein reagents used were purified complement proteins and regulators that are primary constituents in complement activation. Explicitly the proteins that are involved in the assembly and inhibition of the amplification loop of the alternative pathway and the membrane attack complex. The serum reagents were used to incubate liposomes for the quantification of total complement activation.

Secondly, the materials preparations are described. This section introduces the use of lipid molecules to produce model lipid membrane surfaces. Lastly, the final section introduces the relevant experimental techniques used. These techniques required the use of instrumentation such as a zetasizer or a dynamic light scattering device to determine the size and surface charge of prepared liposomes. A quartz crystal microbalance with dissipation measurement techniques was also utilized to monitor the formation of supported lipid bilayers and the interaction of complement proteins onto the self-assembled membranes. Since it is capable of real-time analysis, this allowed experiments to be done *in situ* with label free measurements reducing contamination and other variables that could add to the uncertainty. Techniques using photobleaching for fluorescence microscopy and fluorescence recovery were utilized to further visualize the interactions between obtained bilayers and proteins. To determine the immune response, enzyme linked immunosorbent assays specific to measuring the concentration of the C5b-

9 terminal complement complex were conducted. This was used as a metric to determine the total complement activation of liposomes incubated with serum reagents.

3.2. Materials

3.2.1. Lipid Reagents

1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-Dioleoyl-*sn*-glycero-3ethyl-phosphocholine (chloride salt) (DOEPC), 1,2-dioleoyl-3-trimethylammonium-propane (chloride salt) (DOTAP), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1'- *rac*glycerol) (sodium salt) (POPG), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-[4-(*p*-maleimidophenyl) butyramide] (Sodium salt) (MPB-PE), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (Ammonium salt) (Liss Rhod-PE fluorophore) were purchased either in chloroform or as a powder from Avanti Polar Lipids (Alabaster, AL, USA). All of the lipids were stored at -20 °C until conducting the experiments. The chemical structures of the phospholipid molecules utilized in this thesis are presented in **Figure 3.1**.

Figure 3.1 Chemical structures of the phospholipids used in this thesis.

3.2.2. Buffer Reagents

For the aqueous buffer solutions, deionized water was obtained from a Millipore purification system ($R > 18\text{M}\Omega$, Millipore, Molsheim, France). An aqueous buffer containing 10 mM Tris, 150 mM NaCl [pH 7.5], was used as the running buffer for forming SLB platforms for all of the experiments unless otherwise stated. This buffer was also used to dilute the proteins that were mentioned in chapters 4, 6, and 7 to the appropriate test concentration. Another aqueous buffer containing a 10 mM Tris [pH 7.2] solution with a combination of 150 mM NaCl and 5 mM MgCl_2 was used to dilute other proteins mentioned in Chapter 5 to the appropriate test concentration. For chapter 7, a representative protein free precursor solution was prepared by diluting a 50% glycerol solution with a Tris buffer. The protein concentration was then varied as follows: 1, 5, 10, 20, and 50 nM, prior to the injection of clusterin onto the supported lipid bilayer platforms. Normally a glycerol solution combined with a stock concentration of protein can induce dramatic changes in the QCM-D measurement signals, frequency, and dissipation shifts. This is due to the difference in viscosity and density of the buffer that is being flown over a surface. Therefore, the flowing Tris solution must be exchanged after the bilayer formation with a representative protein free buffer-glycerol solution in order to prevent dramatic shifts in frequency and dissipation from these viscosity and density variations. After establishing a stable QCM-D response, clusterin solution was injected and then rinsed with a Tris buffer to remove the residual glycerol solution. Thus, this blocks the effect of glycerol from associating with the MAC components.

3.2.3. Protein Reagents

Purified human C3 (Catalog no. A113), C3b (Catalog no. A114), properdin or Factor P (Catalog no. A139) proteins, iC3b (Catalog no. A115), C3a (Catalog no. A118), factor B (Catalog no. A135), factor D (Catalog no. A136), C5b-6 (Catalog no. A122), C7 (Catalog no. A124), C8 (Catalog no. A125), and C9 (Catalog no. A126) were purchased from Complement Technology (Tyler, TX) and stored at -80°C . The regulatory proteins, human recombinant vitronectin (Catalog no. MBS691940), and clusterin (Catalog no.

MBS1265506), were obtained from Mybiosource (San Diego, California, USA) and stored at -20°C . Recombinant vitronectin was received as a lyophilized powder and hydrated to a stock concentration of 1 mg/mL in deionized water containing 0.1 wt% bovine serum albumin (BSA). Recombinant clusterin was received in a phosphate-buffered saline (PBS) solution [pH 7.4] containing 50% glycerol. Compstatin (Catalog No. 2585) were purchased from Tocris Bioscience (Bristol, UK). The peptides were received in powder forms and stored at -20°C . Fluorescein isothiocyanate (FITC)-labeled polyclonal C3c antibodies (Catalog no. F0201) were purchased from Dako (Denmark). **Table 3.1** presents the list of proteins and peptides used in this thesis.

Table 3.1. List of proteins and peptides used in this thesis

Protein	Molecular Weight (kDa)	Serum Concentration ($\mu\text{g/mL}$)	Isoelectric point	Storage Buffer
C3	185	1200 to 1500	5.9	10 mM sodium phosphate, 145 mM NaCl, pH 7.2
C3b	176	N.A.	5.7	10 mM sodium phosphate, 145 mM NaCl, pH 7.3
iC3b	176	N.A.	5.7	10 mM sodium phosphate, 145 mM NaCl, pH 7.2
Factor B	93	200 to 210	5.6 -6.1	10 mM Sodium phosphate, 145 mM NaCl, pH 7.2
Factor D	24	1 to 2	7.4	10 mM sodium phosphate, 145 mM NaCl, pH 7.3
Properdin	53	20	>9.5	10 mM Sodium phosphate, 145 mM NaCl, pH 7.3
C5b-6	285	N.A.	6-6.5	10 mM HEPES, 120 mM NaCl, pH 7.2
C7	92.40	49 to 70	6-6.5	10 mM Sodium phosphate, 145 mM NaCl, pH 7.3
C8	151	50 to 80	6.2-7.5	10 mM sodium phosphate, 145 mM NaCl, pH 7.3
C9	71	47 to 70	4.7	10 mM sodium phosphate, 145 mM NaCl, pH 7.3
Vitronectin	52	300	N.A	Water containing 0.1 wt % bovine serum albumin
Clusterin	27	100	N.A.	Phosphate-buffered saline [pH 7.4] containing 50% glycerol
C3a	9.089	N.A	>9.5	Phosphate buffered saline, pH 7.3
Compstatin	1.55	N.A	N.A	10 mM TRIS, 150mM NaCl 5mM MgCl_2 pH 7.5

N.A, not applicable.

3.2.4. Serum Reagents

Normal human serum and C2 depleted normal human serum where it has been depleted of the complement C2 protein (C2-Dpl) was purchased from Complement Technology (Tyler, TX). Along with a Mg-EGTA buffer (100 mM MgCl₂, 100 mM EGTA [pH 7.3]). All serum samples were stored at -80 °C until doing experiments. Immediately before an experiment, the serum samples were diluted with a PBS [pH 7.2] buffer solution that was supplemented with 0.15 mM CaCl₂ and 0.5 mM MgCl₂ at 1:200 volumetric ratios. A 10 mM EDTA aqueous solution was also added at the end of serum incubation cycles to terminate further complement activation.

3.3. Lipid Preparations

3.3.1. Vesicle preparation

Vesicles, termed as liposomes, are small spherical lipid bilayer entities that encapsulate an aqueous solution within[1]. There is a wide range of versatile methods to prepare vesicles and each method is responsible for the production of different types of vesicles which are classified depending on the size and number of lipid bilayers within the vesicle [2]. There are 4 main type of vesicles; multilamellar vesicles (MLV), small unilamellar vesicles (SUV), large unilamellar vesicles (LUV) [3] and giant unilamellar vesicle (GUV) [4] as shown in **Figure 3.2**. The vesicles are generally prepared by either a sonication or extrusion method except for the GUVs. The use of sonication does not create a uniform size distribution and can potentially lead to metal contamination, lipid degradation, and the generation of heat and aerosols. Therefore the extrusion method is the most commonly used and requires the mechanical extrusion of multilamellar vesicles through a filter membrane with defined pore size, resulting in unilamellar vesicles with a narrow size distribution.

Figure 3.2 Schematic representation of vesicle classification. Multilamellar vesicles (MLV) are made of over one closed lipid bilayers while small, large, and giant unilamellar vesicles (SUV, LUV and GUV, respectively) are made of only one closed lipid bilayers.

Extrusion method is used to prepare the unilamellar vesicles (LUVs) as shown in **Figure 3.3**. Initially lipid powders of interest are suspended in chloroform and then the appropriate amount of lipids are mixed with the desired molar ratio into a glass flask. Next the chloroform is evaporated under a stream of N₂, leaving behind a film of dried lipids in the vessel forming thin lipid films. Subsequently the dried lipid sample is placed in the vacuum desiccators overnight to remove the residual chloroform through vacuum assisted evaporation. A physiologically relevant buffer solution is then added to the thin lipid films and swelling occurs. Large multilamellar vesicles (LMV) are created with shaking and/or vortexing. The as-produced large multilamellar vesicles are then downsized using a mini-extruder apparatus through polycarbonate membranes.

Figure 3.3 Schematic representation of the mechanism of unilamellar vesicle formation via extrusion method

3.3.2. Supported Lipid Bilayer Formation

Supported lipid bilayers (SLB) are a two-dimensional membrane-mimicking surface that is compatible with a wide range of surface-sensitive measurement techniques, including acoustic and optical biosensors of various kinds [5-8]. SLBs can be readily formed on silica-based materials through vesicle fusion (14) or solvent-assisted lipid bilayer (SALB) [9-13] formation methods, and the chemical composition of the bilayer, including electrical surface charge, membrane fluidity, and cholesterol content, can be adjusted. More specifically, vesicles fusion method involves a complex sequence of steps including vesicle adsorption, fusion, spreading, rupture and reassembly of lipid bilayers, which yield to complete SLB formation (**Figure 3.4**). To date, a variety of experimental conditions, including vesicle size [14, 15] lipid composition [16], solution

pH [17], osmotic pressure [18], and freeze-thaw pretreatment [19] have been identified for reproducible bilayer fabrication via vesicle fusion method.

Figure 3.4 . SLB formation via vesicle fusion method. Adapted with the permission from reference [20]. Copyright 2015 Royal Society of Chemistry

On the other hand, the SALB process is a simpler and more versatile approach that involves lipid deposition in alcohol (e.g. isopropanol), followed by solvent-exchange with an aqueous buffer (**Figure 3.5**). Its optimization is straightforward and supported lipid bilayers can be formed on a wide range of substrates, including gold, aluminum oxide, titanium oxide, and silicon oxide [9, 12, 21]. Altogether the straightforward operation and high reproducibility lends strong potential for SALB fabrication approach to serve as the basis for the scale-up production of supported lipid bilayer platforms for research that translates to clinical applications.

Figure 3.5 Solvent assisted SLB formation. Lipids dissolved in isopropanol are not adsorbed onto hydrophilic substrates (e.g. glass or silicon oxide). As water content increases in the lipid-isopropanol mixture, micelles start to form, which can also adsorb to the substrate. When the water content reaches to 100%, the lipid bilayers are spontaneously formed on both the substrate and solution in the form of liposomes. Adapted with the permission from [22]. Copyright 2010 AIP Publishing LLC

3.4. Experimental Technique

In this section, the principles and analyses behind the experimental techniques used are described.

3.4.1. Dynamic Light Scattering- Principle and Analysis

Dynamic light scattering (DLS) is a noninvasive technique that measures the size distribution profile of particles in solution [23]. Its utility is based on two main characteristics of the particles in solution: Tyndall scattering and Brownian motion [24]. The Tyndall scattering is described as the isotropic scattering of incident light from the particles in solution while Brownian motion is defined as a random diffusion of particles in solution. By combining these two behaviors, the scattered light intensity from the particle undergoing Brownian motion in the solution fluctuates in time. Therefore, the

DLS measurement technique analyzes the time dependent fluctuations of the scattering light intensity arising from the particles undergoing Brownian motion by interpreting these fluctuations into autocorrelation functions which are described as follows:

$$g^2(q; \tau) = \frac{\langle I(t)I(t+\tau) \rangle}{\langle I(t) \rangle^2}, \quad (\text{Eq. 3.1})$$

Where $g^2(q; \tau)$ is defined as a second order autocorrelation function for a particular wave vector (q) and delay time (τ) and I is the intensity of scattered light.

The correlation becomes high at very short time decays due to particles not having enough time to change their initial position, whereas at longer time delays, the correlation decays exponentially. This means that there is no correlation among the scattered intensities from the initial and final position of the particles. Therefore, the correlation is strongly dependent on the probability density function of the particles that move. The probability density function is defined as:

$$P(r, t|0,0) = (4\pi Dt)^{(-\frac{3}{2})} \exp\left(-\frac{r^2}{4Dt}\right), \quad (\text{Eq. 3.2})$$

D is the diffusion coefficient of the particles and is defined as how fast the particles move with diameters, r , of particles that are assumed as spheres. Moreover, according to the Stokes-Einstein equation, D alters depending on the particle size (R), temperature (T), and viscosity of liquid (η) that is used as a dispersant [23].

$$D = \frac{k_B T}{6\pi\eta R}, \quad (\text{Eq. 3.3})$$

Where k_B is Boltzmann's constant. In order to calculate the diffusion coefficient of particles, two different approaches have been employed to solve autocorrelation functions: the Cumulant method [25] and the CONTIN algorithm [26].

Experimental protocol: A 90Plus particle size analyzer with a 658 nm monochromatic laser (Brookhaven Instrument, Holtsville, NY) was used to determine the size distribution and polydispersity of the prepared liposome samples. The light scattering was monitored at a fixed angle of 90° and the intensity-weighted size distribution was obtained from the intensity autocorrelation by the Cumulant method. Before the measurement plastic, UV-Vis transparent, disposable, single sealed cuvettes (Sigma Aldrich, St. Louis) were cleaned and rinsed with a filtered-diluent solution. The liposome samples were diluted in a 10:1 volumetric ratio with 10mM Tris [pH 7.5] buffer solution

containing 150 mM NaCl ensuring sufficient scattering intensity was observed. All measurements were conducted at 24 °C.

3.4.2. Zeta Potential

Zeta potential is a measurement used to determine the surface charge on a particle dispersed in a liquid. Besides the particle size distribution, it is one of the next most common parameters that is used for understanding a particle's behavior. The net surface charge on a particle attracts the counter ions with the opposite charge in the dispersant liquid and thereafter an electrical double layer is generated around the particles as shown in **Figure 3.6**. The electrical double layer is formed by two distinct regions: Stern and diffuse layers. The Stern layer is the inner region where the ions that carry the opposite surface charge with the particle and ions are firmly bound to particle surface while the diffuse layer is the outer region where the ions that have positive and negative charges are distributed over a region depending on the balance among attractive electric forces and the random thermal motion. Particularly, there is a boundary at the inside of the diffuse layer where the ions are more firmly bound to the particles than the ions at outside of the boundary. This boundary is known as the hydrodynamic shear plane which is the key determinant for the zeta potential calculation. More specifically, the zeta potential of a particle suspension is experimentally investigated by an electrophoresis technique which is based on the movement of different charged particles towards electrodes with opposite charges that are driven by an applied electric field. Therefore, the zeta potential measurement technique utilizes the changes in frequency shifts, also known as Doppler shifts, between the light scattered from the moving particle under an incident beam. By measuring the Doppler shift, the velocity of a charged particle (electrophoretic mobility of the particle) under an electric field is estimated and then obtained electrophoretic mobility is used to calculate the zeta potential via Henry's equation as follows:

$$\mu_E = \frac{2\varepsilon\zeta f(ka)}{3\eta}, \text{ (Eq. 3.4)}$$

Where μ_E is the electrophoretic mobility, ϵ is the dielectric constant of the dispersant liquid, ζ is the zeta potential, $f(ka)$ is Henry's function, k is the reciprocal length of the electrical double layer with a thickness of $1/k$, a is the radius of the particle, and η is the viscosity of the dispersant liquid. The $f(ka)$ has been defined according to the Huckel approach and Smoluckowsky approach [27]. If the particle size is much smaller than the double layer then $f(ka)$ equals 1 (Huckel approach) while if the particle size is much larger than the double layer which is the case when the ionic strength is high, $f(ka)$ equals to 1.5 (Smoluckowsky approach).

Figure 3.6 Schematic representation of zeta potential of a negatively charged particle in a dispersant solution

Experimental Protocol: A Malvern Zetasizer Nano ZS ZEN 3600 (Malvern Instruments Inc, UK) with a 633 nm laser wavelength was used to determine the zeta potential of prepared liposomes. Before the experiment, disposable DTS1070 Folded capillary cells (Malvern Instruments) were rinsed and cleaned with a filtered dispersant solution and then liposome samples were diluted to a 10:1 volumetric ratio with 10mM Tris [pH 7.5] buffer solution containing 10 mM NaCl ensuring a sufficient scattering intensity. The temperature of all experiments was set at 24 °C.

3.4.3. Quartz Crystal Microbalance with Dissipation Monitoring

The quartz crystal microbalance (QCM) is a nanogram sensitive weighing balance that utilizes the piezoelectric properties of an AT cut-quartz crystal as the working principle (**Figure 3.7**). Typically, A piezoelectric material, such as a quartz crystal, is mechanically deformed (expansion or contraction) when exposed to an external electric field. When the electrical field is removed, the quartz crystal turns back to the original shape. These variations in electromechanical response of a quartz crystal generate an acoustic wave in the thickness shear mode of the crystal.

Figure 3.7 Schematic Representation of Piezoelectric Properties of Quartz Crystal. (i) a quartz crystal, (ii) the induction of the piezoelectric effect by the application of an external force, (iii) reverse piezoelectric effect to induce oscillations for QCM measurements; adapted from ref. [28, 29]

By applying an alternating potential over a quartz crystal which is sandwiched between two metal electrodes, the quartz crystal starts to deform periodically, making the crystal oscillate with the same frequency as the applied potential. At the resonance frequency, the oscillation displays a total harmonic behavior with a maximized amplitude and minimal energy loss. The typical resonance frequency of a quartz crystal is on the order of MHz and alters depending on the thickness of the crystals. A higher resonance frequency is generally associated with a higher sensitivity which is only possible when the quartz crystal is very thin. The common resonance frequency of a quartz crystal is 5MHz which is obtained from a $\sim 300 \mu\text{m}$ thick quartz crystal. Since the resonance frequency is related to the crystal thickness, any changes that occur on the surface of the quartz crystal induce a shift in the resonance frequency. Therefore, when a uniform mass

is adsorbed onto the quartz crystal the resonance frequency shifts. This relationship between the mass and resonance frequency is stated by Sauerbrey in 1959 as follows [30]:

$$\Delta m = \frac{C}{n} \times \Delta f, \text{ (Eq. 3.5)}$$

Where Δm is the adsorbed mass, Δf is the frequency change, n is the harmonic number and, C is the sensitivity constant and it is:

$$C = \frac{t_q \times \rho_q}{f_o}, \text{ (Eq. 3.6)}$$

Where t_q is the thickness of the quartz, ρ_q is the density of quartz, and f_o is the resonance frequency of quartz. The sensitivity constant (C) has been estimated as -17.7 ng/Hz.cm^2 for 5MHz quartz crystals [31]. There are three main assumptions for the Sauerbrey relationship to remain valid. The first, the adsorbed mass must be relatively smaller than the mass of the quartz crystal. The second, the adsorbed mass must be maintain rigidity on the quartz crystal. The third, the adsorbed mass must be homogenously distributed over the surface of the quartz crystal. This assumption was introduced at earlier times of using a QCM to monitor the deposition of metal thin films under a vacuum and at standard pressures.

In addition to measuring the changes in the resonance frequency upon mass adsorption, the QCM with dissipation (QCM-D) has developed to detect the changes in the viscoelastic properties of an adsorbed mass which makes the QCM attractive for biosensing applications. Initially, conducting a QCM measurement in a liquid environment was not possible due to the variable contributions from liquid viscosity and elasticity that would be measured in frequency change. Yet new developments with QCM-D has allowed simultaneous measurements through monitoring the changes in resonance frequency and the energy dissipation when exciting the quartz crystal at its resonance frequency while recording the decay in the free oscillations after removing the driving voltage (**Figure 3.8**).

Figure 3.8 Schematic representation of QCM-D monitoring. (a) A piece of quartz crystal that is sandwiched between electrodes. (b) The driving voltage of quartz crystal is turn on. When the driving voltage is turn off, the quartz crystal is covered with (c) a rigid film and (d) a viscous film.

Moreover, the dissipation factor (D) is described as [32]:

$$D = \frac{1}{\pi f \tau}, \text{ (Eq. 3.7)}$$

Where f is frequency, τ is the decay time constant. Since it is dimensionless [33], it is also defined as:

$$D = \frac{1}{Q} = \frac{E_{\text{dissipated}}}{2\pi E_{\text{stored}}}, \text{ (Eq. 3.8)}$$

Where Q is the quality factor, $E_{\text{dissipated}}$ is the energy loss per oscillation and E_{stored} is the total energy stored while the piezo is oscillating. The dissipation factor is typically on the order of 10^{-6} . Since the Sauerbrey relation is based on how rigid the film is on the crystal, the changes in frequency and energy dissipation is again derived for the viscous liquid environments, described as [34]:

$$\Delta f = -\frac{1}{2\pi t_q \rho_q} \sqrt{\pi f \rho_l \eta_l}, \text{ (Eq. 3.9)}$$

$$\Delta D = -\frac{1}{t_q \rho_q} \sqrt{\frac{\rho_l \eta_l}{\pi f}}, \text{ (Eq. 3.10)}$$

Where ρ_l and η_l are the density and viscosity of the liquid, respectively.

In this thesis, the QCM-D was utilized in order to monitor lipid and protein adsorption processes by tracking the changes in resonance frequency and energy dissipation of a silicon oxide coated quartz crystal as a function of time.

Experimental Protocol: A Q-Sense E4 instrument (Q-sense AB, Gothenburg, Sweden) was used and the data was collected for the 3rd, 5th, 7th, 9th, and 11th overtones, and the reported QCM-D data was the 5th overtone and normalized accordingly ($\Delta f_{n=5}/5$).

Substrate Preparation: A quartz crystal (model no. QSX303) which has a diameter of 14 mm, a fundamental frequency of 5MHz, and a sputter-coated, 50nm thick layer of silicon dioxide were purchased from Q-sense AB (Gothenburg, Sweden). Immediately before the experiments, silicon oxide coated quartz crystals were sequentially rinsed with 2% SDS, water, and ethanol followed by drying with nitrogen gas. The crystals were then treated with an oxygen plasma with an expanded plasma cleaner (model no; PDC-002- Harrick Plasma, Ithaca, NY) at the maximum radiofrequency (RF) power for 2 min.

Measurement Conditions: QCM-D measurements were conducted under continuous flow conditions, with the flow rate defined as 100 μ L/min for all steps in the bilayer formation and washing processes and 41.8 μ L/min for the protein addition as controlled by a Reglo Digital peristaltic pump (Ismatec, Glattbrugg, Switzerland). The temperature of the flow cell was fixed at 24.0 $\pm$ 0.5 $^{\circ}$ C.

3.4.4. Fluorescence Microscope

Fluorescence is used to characterize specific substances such as molecules that absorb light at particular energies for a certain time and simultaneously emit the light of a lower energy. This is governed by an electronic transition within a molecule that is mechanistically driven by three different steps as seen **Figure 3.9**. At the first step, electrons are triggered from the ground state to an excited state or higher energetic state

upon absorption of light. Next, some of the gained energy is dissipated through an internal conversion or vibrational relaxation of the excited electrons. Lastly, the electrons return to the ground state by emitting a light that has a longer wavelength and therefore a lower energy. The final difference between the absorption (excitation) and emission of the energy is termed as a Stoke shift.

Figure 3.9 Jablonski diagram displaying stepwise excitation and emission processes

Fluorescence microscopy is a type of optical microscope that utilizes the fluorescence of a fluorescently labelled substance to generate a visible image for the visualization or tagging of specific molecules. In this thesis, fluorescence microscopy was used to visualize supported lipid bilayers containing 0.5 wt % fluorescently labelled lipid (rhodamine) that were conjugated to C3b proteins and visualized using an inverted epifluorescence Eclipse TE 2000 microscope (Nikon) equipped with a 60 $\times$ oil immersion objective (NA 1.49) and an Andor iXon + EMCCD camera (Andor Technology, Belfast, Northern Ireland). The acquired images consisted of 512 $\times$ 512 pixels with a pixel size of 0.267 $\mu\text{m} \times$ 0.267 μm . A fiber-coupled mercury lamp (Intensilight C-HGFIE, Nikon) was used as a light source to illuminate the fluorescently labelled substances, and excite the fluorophores within the bilayer.

Experimental Protocols: Glass substrates were obtained from Menzel Gläser (Braunschweig, Germany) with dimensions of 25 mm $\times$ 75 mm $\times$ 170 μm . Prior the experiment, the glass slides were rinsed with ethanol and water, followed by nitrogen gas drying. The glass slides were subject to oxygen plasma for 5 to 10 min and mounted in a

custom-made microfluidic flow cell (stick-Slide I0.1 Luer, Ibidi, and Munich, Germany). The flow cell had a chamber volume of 350µL and fitted on the microscopy XY stage. All measurements were conducted under continuous flow conditions with a flow rate of 50µL/min for all steps in the bilayer formation. The washing processes as well as protein and antibody additions were controlled by a Reglo Digital peristaltic pump (Ismatec, Glattbrugg, Switzerland).

3.4.5. Fluorescence Microscopy after photobleaching

Fluorescence recovery after photobleaching (FRAP) is a fluorescence based-technique to determine the two dimensional lateral mobility of fluorescently labelled molecules known as fluorophores [35]. A defined area on the sample is photobleached with maximum laser intensity and then the bleached molecules diffuse and exchange with the molecules that are present in unbleached areas, resulting in a time dependent recovery of the fluorescence within the bleached area. **Figure 3.10** shows the mechanism of the FRAP measurement on the supported lipid bilayer.

The obtained FRAP data was evaluated according to the following equation:

$$f(t) = \frac{[F(t)-F(0)]}{[F(i)-F(0)]}, \text{ (Eq. 3.11)}$$

Where $F(i)$ is the initial fluorescence intensity prior to bleaching, $F(0)$ is the fluorescence intensity inside the bleached area when $t=0$ and $F(t)$ is the fluorescence intensity of the recovering area at the time t . The $f(t)$ is fitted with the following equation [36];

$$f(t)=F(t) - \langle F(i) - F(0) \rangle \cdot \left\{ 1 - \exp \left(-2 \frac{\tau_D}{t} \right) \left[I_0 \left(2 \frac{\tau_D}{t} \right) - I_1 \left(2 \frac{\tau_D}{t} \right) \right] \right\}, \text{ (Eq. 3.12)}$$

Where τ_D is the characteristic diffusion time, I_0 and I_1 are single fitting parameters which are modified by a Bessel function. Furthermore, the diffusion coefficient is estimated by the equation as follows:

$$\tau_D = \frac{\omega^2}{4D}, \text{ (Eq. 3.13)}$$

Where D is the diffusion coefficient and ω is the radius of the bleached area at $t=0$.

Figure 3.10 A schematic representation of FRAP in supported lipid bilayers.

(i) Fluorescently labelled lipid molecules within the bilayer are uniformly distributed. (ii) A circular area is bleached by high pulse laser. (iii) Bleached lipid molecules start to diffuse, where some move away from the bleached area and the other emitting light molecules move inside. (iv) A Uniform distribution of bright and bleached molecules is formed and the dark spot from the bleaching disappears.

Experimental protocol: To conduct fluorescence recovery after photobleaching (FRAP) measurements, a circular spot of 20 μm diameter was photobleached for 5 s using a 532 nm, 100 mW laser (Klasech Laser Technologies, Dortmund). Fluorescence micrographs were imaged for 90 s at 1 s intervals, and the corresponding lateral diffusion coefficients were obtained based on the Hankel transform method.

3.4.6. Enzyme linked Immunosorbent Assays or Complement Activation Assays

An Enzyme linked immunosorbent assay (ELISA) is a plate based- immunological assay that utilizes the recognition concept that is similar to a lock and key. The key is represented by an antigen which is a substance that stimulates antibodies when introduced to the body. The lock is the antibody which identifies and passivates the newly recognized foreign substances (antigens). Therefore, by using this lock-key (antibody-antigen) recognition mechanism, the ELISA detects and quantifies an analyte

of interest (antigen or antibody) in bodily fluids such as human serum or plasma. Three different ELISA measurement formats have been introduced. They are direct, indirect and sandwich assays. A sandwich ELISA is the most commonly used due to its superior properties in terms of sensitivity and flexibility compared to the others. A double antibody sandwich TCC C5b-9 ELISA was used here. This ELISA follows the seven-step procedures as shown in **Figure 3.11**. The first step shows a polystyrene microplate that is coated with a known quantity of the capture antibody of interest. Second, the unbound antibodies on the plate are removed and the uncoated regions on the plate are passivated or sealed with irrelevant proteins such as BSA in order to eliminate the false positive signals. Third, the antigen containing sample is added to the plate that has already bound antibodies and after incubation, the unbound antigen is removed through rinsing. Fourth, biotinylated- antigen specific antibodies are added and incubated for a defined time and the unbound antibodies are removed through rinsing. Fifth, the enzyme conjugated antibodies are added and incubated. Sixth, the substrate solutions are added and converted by an enzyme into a colored product. At the seventh and final step, a microplate reader is employed to measure the optical density (OD) values of the plate wells to detect the presence and quantity of the antigen.

Figure 3.11 Schematic diagram of a double sandwich ELISA protocol

Experimental Protocol: An enzyme-linked immunosorbent assay (ELISA) was conducted in order to measure the amount of generated sC5b-9 complex induced by liposome samples in human serum. A human terminal complement complex (TCC) C5b-9 specific ELISA kits were purchased from Mybiosource (San Diego, California) and all

kits and reagents were stored at 4 °C before experiments. Liposomes samples were incubated in serum for 60 min at 37 °C in a shaking bath. In typical experiments, 10 µL liposome samples are mixed with 40 µL of serum in Eppendorf tubes. The concentration of TCC C5b-9 products was determined by optical density measurements at a 450 nm wavelength.

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Chapter 4

Recognition of Membrane Surface by Complement Proteins*

The complement system is an important part of the innate immune response and promotes the clearance of foreign particulates such as pathogens, biomaterials, and nanomedicines. There is great interest in understanding how complement proteins interact with lipid membrane interfaces, especially in the context of recognizing foreign particulates (e.g., liposomal nanomedicines). In this chapter, we established a supported lipid bilayer platform in order to investigate the effect of membrane surface charge (positive, negative, or neutral) on the adsorption of three complement proteins– C3, C3b and properdin – involved in the initiation of alternative pathway complement activation. The role of membrane surface charge is found to be key determinant in modulating complement protein adsorption and the maximum protein uptake is occurred under conditions favoring electrostatic attraction or repulsion depending on the tested protein.

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4.1. Introduction

The complement system is the first line of host defense that responds to pathogenic invaders [1-3] (*e.g.*, bacteria, viruses) and damaged host cells [4-6] (*e.g.*, apoptotic or necrotic cells) by promoting their clearance. A complex and highly orchestrated sequence of complement proteins, typically soluble and cell surface proteins found in extracellular fluids, act in coordinated fashion to mediate a rapid and vital immune response. The initiation of complement activation is a critical first step of recognition and occurs through one of three distinct pathways [7, 8]; antibody-antigen interaction on the target surface (*classical pathway*) [9], binding of a mannan-binding lectin (MBL) to terminal sugar molecules on the surface of microorganisms (*lectin pathway*) [10], or nonspecific deposition of complement proteins on the surface of foreign particulates (*alternative pathway*) [11, 12].

In contrast to the highly specific initiatory steps involved in the classical and lectin pathways, the alternative pathway of the complement system is continuously and spontaneously activated at a low and regulated level [13]. When a foreign particulate is detected, the level of activation is amplified through the deposition of pattern recognition proteins from the alternative pathway onto the target surface [14, 15]. The deposited proteins have multiple functions, including acting as opsonins which facilitate clearance by the reticuloendothelial system (RES) as well as initiators which initiate a proteolytic cascade to generate potent effector molecules that stimulate (*recruit*) immune cells or induce complement-mediated lysis [16-18]. For these reasons, there is great interest in understanding the factors which influence the adsorption of complement proteins onto target surfaces. While conventional assays to study the complement system focus on measuring hemolysis [19, 20] or generation of soluble complement products [21-25], there is growing attention to investigate the deposition of complement proteins on target surfaces due to their distinct functional properties over generated proteins. Towards this goal, flow cytometry combined with confocal microscopy have been initially employed as a reliable method to identify which complement proteins are bound to the target surfaces (*e.g.* liposomes and nanoparticles) as well as to monitor the uptake of complement-bound target surfaces by the individual cells (*e.g.* polymorphonuclear

leukocytes, PMNs) [26-28]. Furthermore, the ELISA measurements have been also utilized to quantitate the bound C5b-9 complexes at eukaryotic cells[29]. However, these assays require tedious sample preparations and specific antibodies that identify the surface bound proteins. Therefore, recently, surface-sensitive analytical tools such as ellipsometry, surface plasmon resonance (SPR) and quartz crystal microbalance-dissipation (QCM-D) have been introduced to measure the adsorption of complement proteins in a label-free and real-time measurement format [30, 31]. Such measurements are typically performed in one of two ways. The most common option is to incubate a model surface with serum, followed by exchange with an aqueous buffer solution. Then, adsorbed proteins at a model surface are identified by addition of a specific antibody that recognizes a complement protein [31-33]. Antibody binding to the serum treated-surface infers the presence of the deposited complement protein which reveals surface-mediated complement activation [33-35]. To date, this approach has enabled the study of the adsorption of certain complement proteins onto many medically relevant model surfaces such as inorganic substrates [31, 36-38], protein-coated substrates [31], polymeric surfaces [39-42] and self-assembled monolayers (SAMs) [43-45]. Another option is to add purified complement proteins and directly monitor their binding to the surface; however, there are few studies in this direction despite the utility of this measurement format for analyzing protein adsorption [46-49]. Indeed, direct measurement of protein adsorption onto target surfaces would be advantageous because, in many previous studies, the C3b pattern recognition protein was the main focus and it was covalently attached to the target surface beforehand in order to monitor its interaction with other complement proteins [50, 51]. The covalent attachment of complement proteins to the substrate was generally chosen because it permits efficient use of protein reagents [52-54]. However, this approach prevents monitoring of real-time target recognition through deposition of the complement proteins. Therefore, understanding the factors which influence the noncovalent adsorption of complement proteins onto target surfaces is still an outstanding subject due to its important role in target recognition [44]. Moreover, there has been limited exploration of the deposition of complement proteins in general on more biologically relevant interfaces such as lipid membranes, with only one investigation relating to the deposition of C3 protein on zwitterionic lipid bilayers [26].

Given the scope of lipid membranes involved in triggering complement activation, there is significant potential for investigating how lipid membrane properties influence complement protein deposition. To date, lipid membrane investigations have been principally limited to solution-based liposome systems, as studied in many *in vitro* and *in vivo* studies that focus on measuring the generation of complement products [55-62]. In the context of opsonization, these studies have shown that negatively charged liposomes are cleared more rapidly from the circulation through RES uptake due to binding of serum proteins (opsonins) [60, 63, 64]. Moreover, it is known that both negatively and positively charged liposomes activate the complement system significantly more than neutral liposomes [65, 66]. While the functional consequences of membrane surface charge are relatively well understood, the influence of membrane surface charge on the deposition of complement proteins remains to be studied. Indeed, there is growing evidence that the role of electrostatic forces in governing protein adsorption can be complex and the electric dipole of the protein itself should also be considered in terms of its binding affinity and orientation to a target surface [67-72].

The goal of this study is to investigate how membrane surface charge influences the adsorption of three different pattern recognition proteins – C3b, C3 and properdin – onto lipid bilayer membranes. Historically, C3 and C3b proteins are the most widely studied pattern recognition proteins and properdin (Factor P) is a newly identified one [15, 73]. In order to achieve this goal, we sought to extend the capabilities of supported lipid bilayers which have been recently employed to study other aspects of the innate immune response, including membrane association of membrane attack complex proteins and inhibition thereof [74, 75]. Supported lipid bilayers with positive, neutral or negative surface charges were fabricated by the solvent-assisted lipid bilayer (SALB) method, and the quartz crystal microbalance-dissipation (QCM-D) measurement technique was utilized in order to track protein adsorption in a real-time, label-free format. The membrane surface charge of the supported lipid bilayer was varied based on tuning the lipid composition. Taken together, the findings in this work help to establish a platform to study the adsorption of pattern recognition proteins onto lipid bilayer membranes.

4.2. Materials and Methods

4.2.1. Protein Reagents

Purified human C3, C3b, C3a and properdin (Factor P) proteins were purchased from Complement Technology (Tyler, TX). As presented in **Table 4.1**, C3 is the most abundant complement protein in plasma and has a molar mass of 185 kDa. C3b protein is a 176 kDa fragment of the C3 protein that is obtained through proteolytic conversion. Both C3 and C3b proteins have isoelectric points below 6 and possess negative net charges under neutral pH conditions [76, 77]. By contrast, properdin is a much smaller protein with a molar mass of 53 kDa and has an isoelectric point around 9.5 [78-80]; hence, it has a positive net charge under neutral pH conditions. Also, properdin monomers are known to self-associate forming dimers, trimers, and tetramers in the relative portion of 26:54:20 [81-83]. C3a is an inflammatory peptide which forms upon cleavage of C3 proteins. The stock aliquots of complement proteins were prepared in a 10 mM sodium phosphate [pH 7.2] buffer solution with 145 mM NaCl, and were stored at -80 °C until the experiment. Immediately before experiment, the proteins were diluted in a 10 mM Tris [pH 7.5] buffer solution with 150 mM NaCl to the appropriate test concentration. Additionally, it is important to note that the maximum protein concentration set as 500 nM for the experiments because properdin concentration in normal human serum is the lowest (approximately 500 nM) compared to the other proteins, C3 (7-8 μ M) and C3b (not known how much C3 proteins are converted into C3b and C3b). However, in the case of C3a, since it has the smallest size among other proteins, the peptide concentration set as 3 μ M in order to obtain a clear QCM-D measurement signal response.

Table 4.1. Characteristics and function of pattern recognition proteins involved in the alternative pathway of complement activation

Protein	Molecular Weight (kDa)	Serum Concentration (µg/mL)	Isoelectric point	Function
C3	185	1200-1500	5.9	Responsible for the amplification of complement activation through generation of active C3b
C3b	176	N.A*	5.7	An opsonin as well as a structural component of enzymatic convertases
C3a	9	N.A*	>9.5	Anaphylatoxin
Properdin	53	20	>9.5	Positive regulator of alternative pathway of complement system

*N.A., not applicable.

4.2.2. Lipid Reagents

1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-ethylphosphocholine (chloride salt) (DOEPC), 1,2-dioleoyl-3-trimethylammonium-propane (chloride salt) (DOTAP) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (sodium salt) (POPG) were obtained in lyophilized powder form from Avanti Polar Lipids (Alabaster, AL). The lipid powders were stored at -20 °C until experiment and were then diluted in the appropriate organic solvent. The chemical structures of the phospholipid molecules are presented in **Figure 4.1**. The DOEPC and DOTAP lipids are positively charged lipids while POPG is a negatively charged lipid and DOPC is a zwitterionic lipid.

Figure 4.1 Chemical structure of phospholipid molecules used in this chapter. The net charges correspond to the state under neutral pH conditions.

4.2.3. Serum Reagents

Normal human serum and normal human serum depleted of complement C2 protein (C2-Dpl) were purchased from Complement Technology. Mg-EGTA buffer (100 mM MgCl₂, 100 mM EGTA [pH 7.3]) was also supplied from Complement Technology. All serum samples were stored at -80 °C until experiment. Immediately before experiment, the serum samples were diluted with PBS [pH 7.2] buffer solution supplemented with 0.15 mM CaCl₂ and 0.5 mM MgCl₂ at a 1:200 volumetric ratio. A 10 mM EDTA aqueous solution was added at the end of incubation cycles in order to terminate complement activation.

4.2.4. Quartz Crystal Microbalance with Dissipation (QCM-D) Monitoring

A Q-Sense E4 instrument (Q-sense AB, Gothenburg, Sweden) was utilized in order to monitor lipid and protein adsorption processes by tracking changes in resonance frequency and energy dissipation of a silicon oxide-coated quartz crystal (model no. QSX303) as a function of time. The QCM-D measurement data were collected at the 3rd, 5th, 7th, 9th, and 11th odd overtones, and the reported QCM-D data were all obtained at the 5th overtone and normalized accordingly ($\Delta f_{n=5}/5$). Immediately before the experiment, the QCM-D sensor substrates were sequentially rinsed with 2% SDS, water, and ethanol followed by drying with nitrogen gas. The substrates were then subjected to oxygen plasma treatment (Harrick Plasma, Ithaca, NY) at the maximum radiofrequency (RF) power. All QCM-D measurements were conducted under continuous flow conditions, with the flow rate defined as 100 $\mu\text{L}/\text{min}$ for all of the steps in the bilayer formation and washing processes and 41.8 $\mu\text{L}/\text{min}$ for the protein addition as controlled by a Reglo Digital peristaltic pump (Ismatec, Glattbrugg, Switzerland). The temperature of the flow cell was fixed at 24.0 ± 0.5 °C. In order to estimate the molecular ratio of adsorbed proteins, the Sauerbrey model was applied in order to convert frequency shifts into mass density values as follows:

$$\Delta m = -C \frac{\Delta f_n}{n},$$

where Δm is the adsorbate mass, C is the proportionality constant ($17.7 \text{ ng}/\text{cm}^2$), Δf is the frequency shift, and n is the odd overtone number of the resonance frequency.

4.2.5. Supported Lipid Bilayer Formation

The solvent-assisted lipid bilayer (SALB) formation method [84, 85] was employed in order to prepare supported lipid bilayers, as previously demonstrated for charged lipid compositions [86]. For SALB experiments, the DOPC, DOEPC and DOTAP lipid powders were dissolved in isopropanol at a stock concentration of 10 mg/mL. The POPG lipid powder was dissolved in ethanol at a concentration of 5 mg/mL and incubated around 40 °C in order to promote solubilization. Before the experiment, the lipid solutions were mixed to the desired molar ratio and diluted in isopropanol to a

final concentration of 0.5 mg/mL. As part of the SALB experimental protocol, a measurement baseline was first recorded in aqueous buffer solution (10 mM Tris [pH 7.5] with 150 mM NaCl) (Step 1), followed by exchange with isopropanol solution (Step 2). Then, 0.5 mg/mL lipid in isopropanol was deposited onto the substrate (Step 3), and incubated for approximately 10 min before a solvent-exchange step was performed as aqueous buffer solution was reintroduced leading to bilayer formation (Step 4). After bilayer formation, 0.1 mg/mL BSA was added (Step 5) in order to verify the completeness of bilayer formation.

4.2.6. Vesicle Preparation

Lipid powders were suspended in chloroform and then mixed to the desired molar ratio. A dried lipid film was formed by evaporating the chloroform upon exposure to a gentle stream of nitrogen gas. The dried lipid films were then placed in a vacuum desiccator overnight in order to remove residual chloroform. After storage, the dried lipid films were hydrated in 10 mM Tris [pH 7.5] buffer solution with 150 mM NaCl and subjected to vortexing. The resulting vesicle suspension was extruded a minimum of 13 times through a track-etched polycarbonate membrane with 200 nm diameter pores by using a Mini Extruder apparatus (Avanti Polar Lipids).

4.2.7. Dynamic Light Scattering

The size distribution of vesicle samples was characterized using a 90Plus particle size analyzer (Brookhaven Instruments, Holtsville, NY) with a 658 nm monochromatic laser. The scattering was monitored at a fixed angle of 90°. The intensity-weighted size distribution was obtained from the intensity autocorrelation by the cumulant method. The vesicles sizes are reported by the average effective hydrodynamic diameter and the sample polydispersity. All measurements were conducted at 24 °C.

4.2.8. Zeta Potential Measurements

The electrophoretic mobility of vesicles in solution was determined by laser Doppler velocimetry and phase analysis light scattering measurements. A Malvern Zetasizer Nano ZS ZEN 3600 (Malvern Instruments Inc, UK) with a 633 nm wavelength laser was used for all measurements. More specifically, the vesicle samples were diluted in a 10:1 volumetric ratio with 10 mM Tris [pH 7.5] buffer solution with 10 mM NaCl in order to eliminate the charge shielding effects that occur in a buffer solution with high salt content (150mM NaCl). Although the exact zeta potential values of liposomes obtained in a buffer solution with 10mM NaCl was different than the one with 150mM NaCl, the trends of zeta potential values of liposomes remain same. The temperature of all experiments was set at 24 °C.

4.2.9. Complement Activation Assay

An enzyme-linked immunosorbent assay (ELISA) was conducted in order to measure the amount of generated sC5b-9 complex induced by liposome samples in human serum. Human terminal complement complex (TCC) C5b-9 specific ELISA kits were purchased from Mybiosource (San Diego, California) and all kits and reagents were stored at 4 °C before experiment. Liposome suspensions in PBS solution were incubated in normal human serum (NHS), 10 mM Mg-EGTA treated NHS, C2-depleted NHS and properdin-depleted NHS with a 1:1 (v:v) diluted serum: liposome ratio. The liposome-serum mixture was incubated for 60 min at 37 °C in a shaking bath with a speed of 200 rpm. The reactions were then stopped by addition of a 10 mM EDTA solution. The concentration of TCC C5b-9 products was determined by optical density measurements at 450 nm wavelength. Zymosan (Z4250, Sigma Aldrich; was the positive control and NHS in the absence of liposomes (diluted instead with blank PBS) was the negative control. Before experiment, the zymosan sample was activated through treatment in a boiling water bath for one hour, followed by centrifugation at 4000 rpm for 30 minutes, removal of the supernatant, and resuspension of the pellet in PBS solution.

4.2.10 Statistical Analysis

Measurements were conducted in triplicate and the results are expressed as mean $\pm$ standard deviation (SD) of the mean. Statistical analysis was performed using the OriginPro 9 software program (OriginLab Corporation, Northampton, MA). One way analysis of variance (ANOVA), followed by the Bonferroni test was conducted and a P value of less than 0.05 was considered to be statistically significant.

4.3. Results and Discussion

4.3.1. Supported Lipid Bilayer Fabrication

In order to determine how membrane surface charge affects the adsorption behavior of C3, C3b and properdin proteins onto lipid membrane surfaces, we first established a supported lipid bilayer (SLB) platform on a silicon dioxide substrate by employing the solvent-assisted lipid bilayer (SALB) formation protocol as outlined above. The SLB fabrication process was followed in real-time by QCM-D measurement analysis in order to characterize the mass and viscoelastic properties of the adlayer on the basis of resonance frequency (Δf) and energy dissipation (ΔD) shifts, respectively. Representative QCM-D sensorgrams are presented in **Figures 4.2b** and SLB formation occurred for all lipid compositions, as indicated by Δf and ΔD shifts of -25.6 ± 2.0 Hz and $0.6 \pm 0.3 \times 10^{-6}$, respectively, which agree with previously reported values [74, 84]. The lipid composition was varied in order to create a negatively or positively charged or neutral membrane surface. The chosen zwitterionic lipid was DOPC; for charged lipid compositions, 70 mol% DOPC was mixed with 30 mol% DOEPC or DOTAP for positively charged SLBs or 30 mol% POPG for negatively charged SLBs.

Figure 4.2 Design of supported lipid bilayer platform to investigate membrane association of pattern recognition proteins (a) Schematic of supported lipid bilayer platform and protein binding to charged lipid bilayers. (b) Quartz crystal microbalance-dissipation (QCM-D) measurements provide a label-free measurement technique in order to measure changes in resonance frequency and energy dissipation upon formation of supported lipid bilayer via the SALB fabrication method. The numbers denote the injection of (1) isopropanol, (2) lipid in isopropanol, and (3) buffer, respectively.

The surface charge of the resultant SLBs was estimated by preparing lipid vesicles of equivalent lipid membrane composition for zeta potential measurements (**Figure 4.3**). The zeta potential of the negatively charged 70/30 DOPC/POPG vesicles was -50.4 ± 2.9 mV while the zwitterionic DOPC vesicles had a zeta potential of -3.9 ± 1.3 mV. On the other hand, the zeta potential values of the positively charged 70/30 DOPC/DOEPC and 70/30 DOPC/DOTAP vesicles were 59.5 ± 2.6 mV and 25.2 ± 3 mV, respectively. This is because the DOEPC contains a cationic ethylated phosphate group while the DOTAP lacks from it, suggesting that molecular features of lipid head group play an important role in the net charge of lipid vesicles. Hence, selected lipid compositions are suitable for preparing different SLBs with variable surface charges and molecular features.

Figure 4.3 Zeta potential measurements for different lipid vesicle samples

After SLB formation, 0.1 mg/mL bovine serum albumin (BSA) was added to bare and SLB-coated silicon dioxide substrates. The BSA adsorption was quite significant on silicon dioxide (ΔF approximately -17Hz) while it was significantly reduced (ΔF approximately -2 Hz) when the silicon dioxide surface was covered with zwitterionic SLBs, suggesting that the frequency shifts upon BSA adsorption on zwitterionic SLBs are acquired due to the area that was not covered by lipid bilayers. Considering that BSA adsorption is negligible at zwitterionic SLBs, the bilayer completion is estimated by the subtracting the frequency shift of BSA adsorption on lipid bilayer from that on bare silicon dioxide surface. Thus, the degree of SLB coverage on the silicon dioxide substrate was estimated to be >90% complete [87]. Moreover, the BSA blocking step also passivated any exposed areas on the silicon dioxide substrate, ensuring that subsequent detection of complement protein adsorption occurred only on the lipid bilayer surface. Following the blocking step, the QCM-D measurement signals were normalized and the baseline signals were reestablished in order to focus on membrane association of the pattern recognition proteins. After stable baseline signals were recorded for 10 min, 500

nM of the chosen protein – purified human C3, C3b or properdin – was injected under continuous flow conditions.

4.3.2. Membrane Association of Complement Proteins

4.3.2.1. C3 Protein

The QCM-D sensorgrams of the Δf and ΔD shifts for 500 nM C3 protein adsorption onto different-charge SLB platforms are presented in **Figures 4.4.a** and **4.4.b**, respectively. Significantly higher protein adsorption was observed to negatively charged SLBs, with final Δf shifts of -16.2 ± 1.1 Hz. By contrast, the final Δf shifts were -3.5 ± 0.8 Hz for protein adsorption onto positively charged 70/30 DOPC/DOEPC SLBs and there were only minor shifts around -1 Hz on neutral and positively charged 70/30 DOPC/DOTAP SLBs. In all cases, protein adsorption led to only minor ΔD shifts, indicating that membrane-associated C3 protein possesses a rigid conformation. Based on repeated experiments, it was determined that C3 adsorption onto negatively charged SLBs is statistically significant compared to the cases on other SLBs (**Figure 4.4.c**). Considering the negative net charge of C3 protein under the tested conditions [77], this finding suggests that there is a positively charged domain within the C3 protein which is involved in membrane association onto negatively charged SLBs [22, 48]. Beyond net charge, the dipole moment of the protein is another important factor in the context of how electrostatic forces can mediate C3 adsorption onto both negatively and positively charged SLBs [68, 70, 71]. Indeed, while C3 adsorption was greatest onto negatively charged SLBs, moderate C3 binding was also observed onto positively charged SLBs. Of note, C3 adsorption onto 70/30 DOPC/DOEPC SLBs was greater than onto 70/30 DOPC/DOTAP SLBs, which is consistent with attractive electrostatic forces modulating protein adsorption because 70/30 DOPC/DOEPC SLBs have more appreciable positive surface charge than 70/30 DOPC/DOTAP SLBs.

Following our observation that C3 protein adsorption is most appreciable on negatively charged SLBs, we further investigated the effect of bulk protein concentration on the adsorption uptake on negatively charged SLBs. As proteins are relatively large

macromolecules, the rate of protein uptake is limited by their diffusion in bulk solution, which in turn depends on the bulk protein concentration [88]. With increasing protein concentration, the adsorption kinetics had a shorter time scale and a larger final Δf shift was observed across the test range (63 to 500 nM C3 protein), as shown in **Figures 4.4.d** and **4.4.e**. The final Δf shifts ranged from -2.5 ± 0.6 Hz at 63 nM protein to -16.2 ± 1.1 Hz at 500 nM protein. There were negligible ΔD shifts always below 0.3×10^{-6} in all cases. There was a clear dependence of the absolute frequency shift due to bound C3 protein as a function of the bulk protein concentration (**Figure 4.4.f**). This trend supports that the rate of protein adsorption is diffusion-limited up to a relatively large uptake. Taken together, the results indicate that C3 protein moderately prefers adsorption onto negatively charged SLBs which occurs in a protein concentration-dependent manner.

Figure 4.4 Influence of membrane surface charge on C3 protein adsorption. After SLB formation, the QCM-D Δf and ΔD shifts were normalized to zero and then 500 nM C3 protein was added after 10 min baseline. Changes in (a) normalized frequency and (b) energy dissipation are presented as a function of time. Arrow indicates protein injection. (c) Changes in absolute frequency shifts on different SLB platforms. Changes in (d) normalized frequency and (e) energy dissipation as function of time for C3 protein adsorption onto 70/30 DOPC/POPG supported lipid bilayer at various bulk protein concentrations. (f) Changes in absolute frequency shifts as a

function of bulk protein concentration. Error bars indicate the average + standard deviations of three independent ($n=3$) measurements.

4.3.2.2. C3b Protein

The QCM-D sensorgrams of the Δf and ΔD shifts for 500 nM C3b protein adsorption onto different-charge SLB platforms are presented in **Figures 4.5a** and **4.5.b**, respectively. While C3b still exhibited greatest adsorption onto negatively charged SLBs, its binding preference for negatively charged SLBs over other SLBs was markedly lower than that of C3 protein. The final Δf shift on the negatively charged SLB was -4.5 ± 1.4 Hz, versus final Δf shifts around -1 to -3 Hz on the neutral and positively charged SLBs. Again, the final ΔD shifts were nearly negligible in all cases, suggesting a rigid conformation of membrane-associated C3b protein. Comparatively, the extent of C3b protein adsorption mirrored the results obtained for C3 protein adsorption onto neutral and positively charged SLBs, with the same binding preference for DOPC/DOEPC 70/30 SLBs over DOPC/DOTAP 70/30 SLBs arising from stronger electrostatic attraction (**Figure 4.5.c**). Furthermore, while tempered, the binding preference for negatively charged SLBs is consistent with the presence of a cationic patch near the purported membrane association domain of C3b protein [76, 89]. Further experiments on negatively charged SLBs showed that the extent of C3b protein uptake and corresponding adsorption kinetics varied with the bulk protein concentration, as indicated by the Δf and ΔD shifts (**Figures 4.5.d** and **4.5.e**). Likewise, the absolute frequency shifts upon association of C3b varied with the bulk protein concentration and support that C3b protein adsorption is diffusion-limited (**Figure 4.5.f**). Overall, the results indicate that C3b protein preferentially binds to negatively charged SLBs, albeit the preference is weaker than that of C3 protein and the total protein uptake is relatively low under all tested conditions.

Figure 4.5 Influence of membrane surface charge on C3b protein adsorption. After SLB formation, the QCM-D Δf and ΔD shifts were normalized to zero and then 500 nM C3b protein was added after 10 min baseline. Changes in (a) normalized frequency and (b) energy dissipation are presented as a function of time. Arrow indicates protein injection. (c) Changes in absolute frequency shifts upon association of the C3b protein on different SLB platforms. Changes in (d) normalized frequency and (e) energy dissipation as function of time for C3b protein adsorption onto 70/30 DOPC/POPG supported lipid bilayer at various bulk protein concentrations. (f) Changes in the absolute frequency shifts on negatively charged SLBs as a function of bulk C3b protein concentration. Error bars indicate the average + standard deviations of three independent ($n=3$) measurements.

4.3.2.3. Properdin

The QCM-D sensorgrams of the Δf and ΔD shifts for 500 nM properdin adsorption onto different-charge SLB platforms are presented in **Figures 4.6.a** and **4.6.b**, respectively. Compared to C3 and C3b proteins, properdin exhibited prodigious adsorption onto negatively charged SLBs with a final Δf shift of -43.4 ± 2.9 Hz. Interestingly, a significant final ΔD shift of $4.4 \pm 0.6 \times 10^{-6}$ was also recorded. Taking into account that there is a positive increase in slope of the ΔD vs. Δf plot upon properdin adsorption, membrane-associated properdin has viscoelastic character. Such behavior is

likely due to the attachment of multiple properdin as properdin is known to form low-order aggregates (2-4 monomers) in solution [82, 83].

On the other hand, properdin adsorption onto the other tested SLBs was tempered yet still appreciable. There was negligible protein adsorption onto 70/30 DOPC/DOEPC SLBs whereas adsorption onto 70/30 DOPC/DOTAP SLBs led to Δf and ΔD shifts of -6.4 ± 1.1 Hz and $1.2 \pm 0.2 \times 10^{-6}$, respectively. Also, properdin binding to neutral DOPC SLBs led to Δf and ΔD shifts of -4.9 ± 0.6 Hz and $0.6 \pm 0.2 \times 10^{-6}$, respectively. Hence, there was moderate binding to both DOPC and 70/30 DOPC/DOTAP SLBs, whereas there was significantly less binding to 70/30 DOPC/DOEPC SLBs, as indicated in **Figure 4.6.c**. Taking into account that properdin is highly positively charged, this finding is consistent with the fact that 70/30 DOPC/DOEPC SLBs have greater positive surface charge than 70/30 DOPC/DOTAP SLBs and hence properdin does not bind to 70/30 DOPC/DOEPC SLBs due to repulsive electrostatic forces. Furthermore, the absolute frequency shift onto negatively charged SLBs due to adsorption of properdin was seven-fold higher than to the other SLBs indicating high selectivity on the basis of membrane surface charge. Therefore, membrane association of properdin to negatively charged SLBs was probed as a function of bulk protein concentration. The final Δf and ΔD shifts are presented in **Figures 4.6.d** and **4.6.e**, respectively. Protein binding was detectable down to 8 nM concentration and both measurement signals indicated concentration-dependent behavior. The absolute frequency shifts varied by more than 40-fold across the tested concentration range (**Figure 4.6.f**). The data indicates that properdin demonstrates strongly preferential adsorption onto negatively charged SLBs with a bound state that exhibits viscoelastic character, supporting that the protein binds in an oligomeric state [90].

Figure 4.6 Influence of membrane surface charge on properdin adsorption. After SLB formation, the QCM-D Δf and ΔD shifts were normalized to zero and then 500 nM properdin was added after 10 min baseline. Changes in (a) normalized frequency and (b) energy dissipation are presented as a function of time. Arrow indicates protein injection. (c) Changes in absolute frequency shifts due to adsorption of properdin on different SLB platforms. Changes in (d) normalized frequency and (e) energy dissipation as function of time for properdin adsorption onto 70/30 DOPC/POPG supported lipid bilayer at various bulk protein concentrations. (f) Changes in absolute frequency as a function of bulk properdin protein concentration. Error bars indicate the average + standard deviations of three independent ($n=3$) measurements.

4.3.3. Comparison of Membrane Association of C3, C3b, and Properdin

In order to compare noncovalent membrane association of the different proteins, **Table 4.2** presents the final frequency and dissipation shifts which were obtained under identical experimental conditions in each experimental set. The QCM-D measurement results indicate that membrane association depends on membrane surface charge and the greatest adsorption was observed on negatively charged SLBs for all tested proteins. Interestingly, C3 and its conformationally distinct C3b subunit exhibit striking differences in total protein uptake considering the nearly equivalent molar masses of the two proteins. C3 protein adsorption to negatively charged SLBs was almost four-times

greater than that of C3b protein. The main difference between C3 and C3b is the missing C3a subunit which is released through C3 cleavage. Of note, C3a is highly cationic peptide with an isoelectric point of 9.5 [79, 80].

Table 4.2. QCM-D measurement response at the equilibrium state of protein adsorption on different SLBs

Proteins	DOPC		DOPC/DOTAP (70/30)		DOPC/DOEPC (70/30)		DOPC/POPG (70/30)	
	ΔF	ΔD	ΔF	ΔD	ΔF	ΔD	ΔF	ΔD
	(Hz)	(10^{-6})	(Hz)	(10^{-6})	(Hz)	(10^{-6})	(Hz)	(10^{-6})
C3	1.42 $\pm$ 1.2	0.85 $\pm$ 0.1	0.85 $\pm$ 0.6	0.2 $\pm$ 0.3	3.48 $\pm$ 0.8	0.49 $\pm$ 0.3	16.2 $\pm$ 1.1	0.24 $\pm$ 0.2
C3b	1.67 $\pm$ 1.6	0.13 $\pm$ 0.1	0.78 $\pm$ 0.7	0.24 $\pm$ 0.2	2.89 $\pm$ 0.6	0.33 $\pm$ 0.1	4.54 $\pm$ 1.35	0.21 $\pm$ 0.1
Properdin	4.86 $\pm$ 0.6	0.57 $\pm$ 0.2	6.4 $\pm$ 1.06	1.28 $\pm$ 0.2	0.70 $\pm$ 0.1	0.08 $\pm$ 0.03	43.4 $\pm$ 2.9	4.43 $\pm$ 0.6

Figure 4.7 Comparison of C3a peptide adsorption onto supported lipid bilayers with varying membrane surface charge. Changes in QCM-D (a) resonance frequency and (b) energy dissipation as a function of time. 3 μ M C3a peptide was added to pre-formed supported lipid bilayers at $t = 10$ min

To further understand the basis for the different binding preferences of C3 and C3b proteins, we conducted additional membrane binding experiments with C3a peptide (**Figure 4.7**). Following SLB fabrication as described above, 3 μ M C3a peptide was added to the SLB platforms in order to probe membrane association. The QCM-D

measurement results indicated that the C3a peptide preferentially adsorbed onto negatively charged SLBs with final Δf shifts around -4 Hz. By contrast, markedly less C3a peptide adsorption was observed onto neutral SLBs with final Δf shifts around -1.5 Hz and there was negligible adsorption onto positively charged SLBs. This finding supports that the positively charged C3a domain is important for promoting C3 membrane association on negatively charged SLBs, *i.e.*, likely as a consequence of attractive electrostatic interactions.

Hence, our findings support that the C3a domain plays an important role in modulating membrane association of C3 protein to negatively charged SLBs, and this difference helps to explain why C3 display high specificity for negatively charged membrane compositions while C3b has weaker discrimination for membrane surface charge. On the other hand, properdin is much smaller yet exhibited more appreciable adsorption with high preference for negatively charged membrane compositions, likely due to its highly positive surface charge and tendency to oligomerize. The corresponding high energy dissipation shifts for properdin adsorption support that it maintains a membrane-associated oligomeric state which may enable its reported multifunctional capabilities.

4.3.4. Role of Membrane Surface Charge in Triggering Complement Activation

In order to evaluate the degree of complement activation, the charged liposomes which carried same charge with SLBs were prepared and then incubated in normal human serum for 60 min and then the amount of generated TCC sC5b-9 complex was measured (**Figure 4.8.a**). Comparably high levels of sC5b-9 complex ($\sim 1.5 \mu\text{g/mL}$) were induced in the presence of negatively charged 70/30 DOPC/POPG and positively charged 70/30 DOPC/DOTAP liposome samples. A lower amount of TCC sC5b-9 complex ($\sim 0.5 \mu\text{g/mL}$) was generated in the presence of the 70/30 DOPC/DOECP liposome sample whereas the DOPC liposome samples induced negligible production of TCC sC5b-9 complex comparable to baseline levels. The observation that both negatively and positively charged liposomes, but not zwitterionic liposomes, are strong complement activators in normal human serum is consistent with literature reports [89, 90].

Interestingly, a key finding in our experiments is that the 70/30DOPC/DOEPC liposomes have a greater positive zeta potential than 70/30 DOPC/DOTAP liposomes, yet the 70/30 DOPC/DOTAP liposomes induce appreciably greater TCC sC5b-9 complex production. While other nanoparticulate systems have shown a correlation between the positive zeta potential and the degree of complement activation – determined on the basis of C3a, C5a, or TCC C5b-9 complex generation – our evidence points to the chemical structure of the molecular species involved in complement activation playing a governing role over the absolute surface charge of the nanoparticulate.

Figure 4.8 TCC C5b-9 measurements for assessment of liposome-mediated complement activation. (a) Measured concentration of TCC sC5b-9 product in normal human serum (NHS). (b) Measured concentration of TCC sC5b-9 product 10 mM Mg-EGTA treated normal human serum (MgEGTA-NHS). (c) Measured concentration of TCC sC5b-9 product in C2-depleted normal human serum (C2 dpl-NHS). All values are normalized by subtracting the baseline value for TCC sC5b-9 production in PBS solution. Error bars indicate the average + standard deviations of three independent ($n=3$) measurements.

In order to verify the pathway by which the different liposome samples undergo complement activation, we also incubated liposomes in 10mM Mg-EGTA treated NHS and C2-depleted NHS in which only the alternative pathway is functional and performed similar ELISA measurements (**Figures 4.8.b** and **4.8.c**). Similar levels of complement activation were observed for both positively charged liposome samples as compared to the respective NHS cases, whereas there was essentially no released sC5b-9 product in the negatively charged 70/30 DOPC/POPG liposome sample over the baseline or zwitterionic DOPC liposome cases. These data support that the alternative pathway is activated by the positively charged liposomes, both those containing DOTAP or DOEPC

phospholipid, whereas the classical pathway is mainly activated by the negatively charged liposomes [56, 60].

In the context of the protein adsorption studies, there are two major implications of the *in vitro* data. First, the uptake of pattern recognition proteins does not appear to correlate with the extent of complement activation. Second, within the subgroup of cationic liposomes, the molecular features of the cationic phospholipid appear to be more important than the absolute surface charge in determining the extent of complement activation. In light of these findings and the previously reported preferential uptake of negatively charged liposomes by the RES, it appears that the binding of pattern recognition proteins to lipid membranes has greater direct impact for opsonization rather than for the generation of the terminal complement complex.

Following this line, then one may ask why membrane association of pattern recognition proteins is insufficient for triggering complement activation via the alternative pathway, i.e., the negatively charged membrane case. Based on the findings reported here, such questions could be further investigated in future studies in order to understand how the conformation and surface density of membrane-associated proteins influence their activity in downstream protein-protein interactions. Indeed, with the surface science capabilities utilized in this study, it becomes possible to address these questions in a systematic and quantitative manner, in turn revealing deeper insights into the biophysical regulation of the complement system.

4.4. Conclusion

In this chapter, we have investigated how membrane surface charge regulates the adsorption properties of pattern recognition proteins implicated in the alternative pathway of complement activation. Using the QCM-D measurement technique, the adsorption of three different proteins – C3, C3b and properdin – to charged supported lipid bilayers was investigated. It was identified that all three proteins prefer adsorption onto negatively charged supported lipid bilayers, with variable charge selectivity over the other tested membrane compositions. There were significant differences in the specific details of protein adsorption for each tested protein, including total uptake and membrane charge

selectivity. The observed trends in protein adsorption also correlate well with the known trends in RES clearance arising through opsonization. Moreover, the experimental results of this study introduce a biomimetic approach to explore the interactions between complement proteins and lipid membrane interfaces at the molecular level, providing insights into pattern recognition proteins that are responsible for the initiation of complement activation at lipid bilayer surface. Such information can be important to control the complement activation by lipid bilayer surfaces at the initial stage as well as be helpful to design biomimetic lipid membrane coatings with immunocompetent properties. Collectively, the SLB platforms combined with QCM-D measurement technique can be a valuable tool to study complement activation at lipid bilayer systems in order to enlarge the current knowledge and understanding.

References

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Chapter 5

Convertase Assembly on Membrane Surfaces*

The self-assembly formation of complement convertases – essential biomacromolecular complexes which amplify innate immune responses – is triggered by protein adsorption. In this chapter, a supported lipid bilayer platform was utilized in order to investigate the effects of covalent and noncovalent tethering strategies on the self-assembly of alternative pathway C3 convertase components, starting with C3b protein adsorption followed by factors B and D. Quartz crystal microbalance-dissipation (QCM-D) experiments measured the real-time kinetics of convertase assembly onto supported lipid bilayers. The results indicate that the nature of C3b immobilization onto supported lipid bilayers is a critical factor governing convertase assembly.

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5.1. Introduction

The complement system is a key part of the innate immune response which defends against pathogenic entities such as microbes, apoptotic cells and immune complexes [1-4]. A combination of soluble and membrane-bound complement proteins acts synergistically in order to eliminate invading entities by either labeling them for phagocytosis or producing anaphylatoxins (chemoattractants) to recruit immune cells [1]. Furthermore, complement proteins are able to directly kill invading entities, particularly lipid membrane-enclosed particulates (*e.g.*, bacteria), through the assembly of a pore-forming membrane attack complex (MAC) [1]. Collectively, all these effector responses are known as complement activation, and there is a delicate balance of regulatory factors which manage basal complement levels because effector molecules such as anaphylatoxins and MAC complexes can act indiscriminately[5, 6]. Indeed, if complement activation is not tightly controlled, generation of excessive effector molecules may lead to an immune imbalance, resulting in complement-mediated disorders (autoimmune diseases) or serious tissue damage associated with self-complement attack [7-10].

Complement proteins are normally inactive in blood circulation, and become active only once they interact with pathogenic entities. Indeed, protein adsorption on target surfaces initiates complement activation, and the specific complement proteins involved in the adsorption process dictate the complement response through either the classical, lectin or alternative pathway [11-13]. The classical pathway is triggered by C1q protein adsorption while the lectin pathway is activated by attachment of mannan-binding lectin. On the other hand, the alternative pathway is initiated by the spontaneous hydrolysis of C3 in the solution phase, or adsorption of one or more recognition molecules, namely C3, C3b, and properdin. After initiation, all three pathways converge leading to the formation of a surface-bound enzymatic complex, most prominently the C3 convertase which catalyzes the cleavage of complement C3 protein in blood. This proteolytic event generates two different protein fragments, C3a and C3b. The smaller fragment, C3a, is a biomarker peptide that functions as an anaphylatoxin in order to induce inflammation. In contrast, the larger fragment, C3b, is a biologically active

fragment that covalently attaches to the free hydroxyl or amino groups that present on the proteins and carbohydrates bound target surfaces, forming covalent ester or amide bonds, respectively [14], thereby stimulating phagocytosis or MAC-induced cytolysis. Moreover, surface-adsorbed C3b provides binding sites for factor B and other complement proteins such as the regulatory complement factor H [15, 16]. The binding of factor B leads to the formation of a C3bB proconvertase and this macromolecular complex is subsequently cleaved by factor D in order to yield an alternative pathway C3 convertase, C3bBb, which in turn promotes generation of more C3b proteins [17, 18]. As a result, the close relationship between C3 convertase assembly and C3b generation instigates a positive feedback loop which amplifies total complement activation independent of which pathway was initially triggered [4, 11, 19, 20]. Moreover, the newly generated C3b can be subsequently associated with the surface bound C3bBb protein complex to create a C5 convertase which cleaves C5 into C5b that participates into termination of complement cascade by formation of membrane attack complex (MAC). However, if the surface bound C3b is not at its right conformation, it is no longer capable of generating C3 and C5 convertases. Furthermore, when attached C3b is associated with factor H instead of factor B, the alternative pathway convertase is no longer assembled [21-23]. Hence, the self-assembly of functional convertases is regulated and involves a highly orchestrated sequence of protein-surface and protein-protein interactions.

To probe the stepwise assembly of alternative pathway convertases on model surfaces, surface-sensitive measurement approaches have proven useful. Nilsson *et al.* first conducted surface plasmon resonance (SPR) experiments in order to monitor convertase assembly as well as drug-mediated inhibition (*i.e.*, compstatin peptide) of complement activation [24]. Of note, in that study, the convertase initiator, C3b, was covalently attached to the carboxymethyl dextran-coated sensor surface through amine coupling. Using similar covalent attachment schemes, the effects of other complement regulatory proteins such as the decay accelerating factor (DAF, CD55) and properdin (factor P) on convertase assembly and complement activation have also been investigated [25, 26]. In later studies, different C3b immobilization schemes – covalent amine coupling or activated hydroxyl coupling – were compared, revealing that the type of immobilization affects C3b conformation although both conformations facilitated

convertase assembly [27, 28]. While oriented C3b immobilization schemes support *in situ* convertase assembly, there are technical challenges such as high degrees of nonspecific protein adsorption when using conventional carboxymethyl dextran surfaces [29]. In an alternative approach, Sarrias *et al.* demonstrated the attachment of biotinylated C3b onto streptavidin-coated surfaces in an oriented manner [30], enabling investigation of alternative pathway C5 convertases [31]. Furthermore, on polystyrene surfaces, quartz crystal microbalance-dissipation (QCM-D) measurements have detected convertase assembly arising from noncovalent C3b adsorption [32]. Accordingly, there is debate about the importance of the attachment scheme – covalent versus noncovalent coupling – between C3b protein and the target surface [33].

Such questions become particularly important when considering that existing convertase assembly studies have focused on interactions occurring on synthetic biomaterial surfaces with fouling properties – characteristics which contrast starkly to those of biological interfaces such as non-fouling lipid membranes. Towards this goal, artificial membrane platforms such as supported lipid bilayers (SLBs) platforms are widely utilized for immunological studies and are an ideal choice for studying convertase assembly in the context of biological membranes [34, 35]. SLBs can be investigated using a wide range of surface-sensitive measurement techniques and their lipid composition is tunable in order to control parameters such as membrane surface charge and functional group presentation, the latter of which facilitates biomolecule conjugation [36]. The SLB platform has facilitated mechanistic investigations into how complement inhibitors such as clusterin and vitronectin interfere with MAC assembly [37, 38]. Covalent immobilization of complement proteins to SLBs remains to be investigated, and identifying an optimal scheme to form convertase assemblies on lipid membranes is an elusive goal.

The aim of the present chapter is to develop an experimental platform that permits the assembly and evaluation of functional convertases at lipid membrane interfaces. Noncovalent and covalent immobilization schemes were utilized in order to attach C3b protein to SLBs, followed by addition of complement factors B and D to trigger convertase assembly. The individual steps of convertase assembly were monitored in real-time by QCM-D measurements, with fluorescence microscopy employed in order to

confirm selective, covalent attachment of C3b to functionalized SLBs. The results obtained provide guidance on useful strategies for establishing complement convertases at lipid membrane interfaces towards functional evaluation and inhibitor studies.

5.2. Materials and Methods

5.2.1. Protein Reagents

Purified human C3, C3b, iC3b, factor B, and factor D proteins were obtained from Complement Technology (Tyler, TX). FITC-labeled polyclonal C3c antibodies were obtained from Dako (Glostrup, Denmark). Compstatin was received in a powder form from Tocris (Bristol, UK) and reconstituted within an appropriate buffer solution. As presented in **Table 5.1**, C3 is the most abundant complement protein in plasma and has a molar mass of 185 kDa. C3b protein is a 176 kDa fragment of the C3 protein that is obtained through proteolytic conversion. Both C3 and C3b proteins have isoelectric points below 6 and possess negative net charges under neutral pH conditions [16, 39]. Factor B is a serine protease that interacts with surface-bound C3b and solution-phase C3(H₂O) in order to form membrane bound-C3bB and soluble C3(H₂O)B proconvertases, respectively [40]. Factor D is another serine protease that cleaves factor B when factor B is bound to C3b or C3 (H₂O) [41, 42]. iC3b is the inactivated form of C3b, and consequently lacks binding sites for complement factors and C5. Since iC3b does not interact with factor B, membrane association of iC3b cannot trigger convertase assembly. Compstatin is a cyclic C3-binding peptide with a molecular weight of 1550.77Da and a sequence of ICVVQDWGHRCT-NH₂ [43]. It is the first non-host derived complement inhibitor which is a promising candidate for being complement targeted-therapeutic agent because it inhibits the cleavage of native C3 proteins by alternative and classical pathways C3 convertases [44]. Stock aliquots of complement proteins were prepared in a 10 mM sodium phosphate [pH 7.2] buffer solution with 145 mM NaCl, and were stored at -80 °C. FITC-labeled antibody and compstatin were stored at -20°C. Immediately before experiment, the proteins were diluted to the appropriate test concentration in a 10 mM Tris [pH 7.5] buffer solution with 150 mM NaCl and 5 mM MgCl₂.

Table 5.1. Characteristics and function of the complement proteins that involved in the formation of alternative pathway convertases

Protein	Molecular Weight (kDa)	Serum Concentration (μg/mL)	Isoelectric Point	Key Function
C3	185	1200-1500	5.9	Responsible for the amplification of complement activation through generation of active C3b.
C3b	176	N/A	5.7	An opsonin as well as a structural component of enzymatic convertases or mediates phagocytosis and cytolysis and structural component of both C5 alternative and classical pathway convertase.
iC3b	176	N/A	5.7	Inactivated form of C3b
Factor B	93	200-210	5.6-6.1	Catalytic subunit of C3 alternative pathway convertase
Factor D	24	1-2	7.4	Cleaves factor B that is then bound to C3b.

5.2.2. Lipid Reagents

1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-[4-(p-maleimidophenyl)butyramide] (sodium salt) (MPB-PE), and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (sodium salt) (POPG) were obtained in lyophilized powder form and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (Ammonium salt) (Rh-PE fluorophore) was received in chloroform from Avanti Polar Lipids (Alabaster, AL). The lipid powders were stored at -20 °C until experiment and were then diluted in isopropanol.

5.2.3. Quartz Crystal Microbalance-Dissipation (QCM-D) Monitoring

A Q-Sense E4 instrument (Biolin Scientific, Gothenburg, Sweden) was used to monitor *in situ* adsorption processes by monitoring real-time changes in resonance frequency and energy dissipation of a silicon oxide coated-quartz crystal (model no. QSX303) at multiple odd overtones ($n = 3, 5, 7$ overtones). All reported data herein was recorded at 25 MHz ($n = 5$ overtone) and normalized accordingly ($\Delta f_{n=5}/5$). Before experiment, the QCM-D sensor surfaces were washed sequentially with 1 wt% SDS,

water and ethanol, followed by drying with a gentle stream of nitrogen gas. The sensor surfaces were then exposed to oxygen plasma treatment (Harrick Plasma, Ithaca, NY) at the maximum radiofrequency (RF) power in order to remove any residual organic contaminants. After establishing baseline signals in aqueous buffer solution, the QCM-D measurements were conducted under continuous flow conditions, with the flow rate defined as 100 $\mu\text{L}/\text{min}$ for all steps of bilayer formation and washing processes and 41.8 $\mu\text{L}/\text{min}$ for protein addition, as controlled by a Reglo Digital peristaltic pump (Ismatec, Glattbrugg, Switzerland). The temperature of the flow cell was fixed at 24.0 ± 0.5 $^{\circ}\text{C}$.

5.2.4. Epifluorescence Microscopy

Fluorescence microscopy experiments were conducted on SLBs containing 0.5 mol% Rhodamine-DHPE lipid by using an inverted epifluorescence Eclipse TI microscope (Nikon) equipped with a 60 $\times$ oil immersion objective (NA 1.49), and an Andor iXon+ EMCCD camera (Andor Technology, Belfast, Northern Ireland). The SLBs were formed in a microfluidic channel configuration (sticky-Slide IV0.4 Luer, Ibidi, Munich, Germany), and 500 nM C3b was injected onto SLB-coated surfaces at a flow rate of ~ 50 $\mu\text{L}/\text{min}$ for 15 min, followed by injection of 100 $\mu\text{g}/\text{mL}$ FITC-labeled C3c at the same flow rate for 15 min. The acquired images consisted of 512×512 pixels resulting in an image size of 136 $\mu\text{m} \times 136$ μm . The samples were visualized using TRITC (rhodamine–DHPE lipid) and FITC (C3c antibody) filter sets by a mercury lamp (Intensilight C-HGFIE; Nikon Corporation).

5.2.5. Fluorescence Microscopy after Photobleaching (FRAP)

A 20 μm -wide circular spot was photobleached with a 532 nm, 100 mW laser beam, followed by time-lapsed recording for total of 2 min. Diffusion coefficients were determined based on the Hankel transform method [45]. For all fluorescence microscopy imaging experiments, thin glass coverslips (Menzel Gläser, Braunschweig, Germany) were assembled with commercially available microfluidic chambers as described above.

5.2.6. Supported Lipid Bilayer (SLB) Formation

The solvent-assisted lipid bilayer (SALB) formation method was employed in order to prepare SLBs. For SALB experiments, the DOPC and MPB-PE lipid powders were dissolved in isopropanol at a stock concentration of 10 mg/mL and 1 mg/mL, respectively. The POPG lipid powder was dissolved in ethanol at a concentration of 5 mg/mL and incubated at around 40 °C in order to promote complete solubilization. Before experiment, the lipid solutions were mixed to the desired molar ratio and diluted in isopropanol to a final concentration of 0.5 mg/mL. As part of the SALB experimental protocol, a measurement baseline was first recorded in aqueous buffer solution (10 mM Tris [pH 7.5] with 150 mM NaCl) (Step 1), followed by exchange with isopropanol solution (Step 2). Then, 0.5 mg/mL lipid in isopropanol was deposited onto the substrate (Step 3), and incubated for approximately 10 min before a solvent-exchange step was performed as aqueous buffer solution was reintroduced leading to bilayer formation (Step 4). After bilayer formation, 0.1 mg/mL BSA was added (Step 5) in order to verify the completeness of bilayer formation and passivate any exposed surfaces.

5.2.7. Statistical Analysis

Measurements were conducted in triplicate and the results are expressed as mean $\pm$ standard deviation (SD) of the mean. Statistical analysis was performed using the OriginPro 9 software program (OriginLab Corporation, Northampton, MA). One way analysis of variance (ANOVA), followed by the Bonferroni test was conducted and a P value of less than 0.05 was considered to be statistically significant.

5.3. Results and Discussion

5.3.1. Tethering Strategies for Protein Attachment at Lipid Membrane Interfaces

Convertase assembly on the SLB platform was initiated by identifying an optimal scheme for C3b protein attachment. Two tethering strategies were employed based on

covalent and noncovalent conjugation, respectively (**Figure 5.1**). Both approaches began with SLB formation on silicon oxide by employing the solvent-assisted lipid bilayer (SALB) fabrication method [46]. A key advantage of the SALB method is that it enables formation of SLBs from a wider range of lipid compositions than the conventional vesicle fusion method. For noncovalent tethering, the chosen lipid composition was 70 mol% DOPC and 30% POPG which provides a negatively charged SLB that supports C3b protein adsorption, which is mediated by a positively charged domain within the C3b protein. On the other hand, for covalent tethering, a mixture of DOPC and maleimide-functionalized MPB-PE lipids was utilized for SLB preparation, with the molar fraction of MPB-PE lipid ranging between 5 to 30 mol%. The maleimide functional group enables the formation of covalent thioether bonds between the MPB-PE lipid headgroup and sulfhydryl groups of the C3b protein [47].

Figure 5.1 Schematic representation of biomimetic C3b conjugation based on covalent and noncovalent interaction. (i) The maleimide functionalized SLBs are created. The MPB-PE lipids specifically react with the cysteine or SH terminated C3b protein, resulting in a covalent

conjugation of C3b protein to the bilayers. (ii) The negatively charged SLBs are created for the noncovalent conjugation of C3b to the bilayer.

The SLB formation process was tracked by the QCM-D measurement technique (**Figure 5.2**). Simultaneous changes in the resonance frequency (Δf) and energy dissipation (ΔD) were recorded as a function of time and reflect the mass and viscoelastic properties of the adlayer, respectively [48].

Figure 5.2 QCM-D Characterization of SLB Formation via SALB Procedure (a) Schematic representation of the quartz crystal microbalance-dissipation (QCM-D) measurement technique, which was utilized in order to monitor in real-time the formation of supported lipid bilayers and subsequent protein adsorption onto the supported lipid bilayer platform. The changes in resonance frequency and energy dissipation reflect the mass and viscoelastic properties of the adsorbate, respectively. For a representative case, changes in (b) frequency and (c) energy dissipation were recorded as a function of time during execution of the SALB procedure. Arrows indicate (i) buffer injection, (ii) isopropanol injection, (iii) injection of lipids dissolved in isopropanol, and (iv) buffer exchange. (d) Changes in frequency shifts as a function of time upon

0.1 mg/mL bovine serum albumin (BSA) adsorption onto bilayer-coated and bare silicon oxide surfaces, respectively. The arrow indicates the time point of BSA addition.

For all lipid compositions, the final QCM-D measurement responses corresponded to Δf and ΔD values of -26 ± 3 Hz and $< 1 \times 10^{-6}$, respectively, which agree well with literature values for SLB systems [46, 49, 50]. The detailed values for each lipid composition are reported in **Table 5.2**.

Table 5.2. Frequency and Dissipation shifts for SLBs made of different lipid composition

SLB Lipid composition	Δ Frequency (Hz)	Δ Dissipation (10^{-6})
DOPC	-25.4 $\pm$ 2.2	0.45 $\pm$ 0.3
DOPC:MPB-PE (95:5)	-28.3 $\pm$ 1.2	0.69 $\pm$ 0.2
DOPC:MPB-PE (90:10)	-28.5 $\pm$ 1.9	0.59 $\pm$ 0.1
DOPC:MPB-PE (85:15)	-27.2 $\pm$ 1.4	0.66 $\pm$ 0.3
DOPC:MPB-PE (80:20)	-26.2 $\pm$ 0.9	0.69 $\pm$ 0.2
DOPC:MPB-PE (75:25)	-25.5 $\pm$ 1.0	0.68 $\pm$ 0.2
DOPC:MPB-PE (70:30)	-26.1 $\pm$ 1.2	0.76 $\pm$ 0.3
DOPC:POPG (70:30)	-25.6 $\pm$ 1.9	0.57 $\pm$ 0.3

Of note, attempts to form SLBs containing 30 mol% MPB-PE lipid were also conducted using the vesicle fusion method, but did not succeed. This observation is in line with previous literature reports of SLB formation with up to 20 mol% maleimide-functionalized lipid, and demonstrates the utility of the SALB method for this application [49-51]. Fluorescence recovery after photobleaching (FRAP) experiments confirmed the formation of homogenous SLBs and it was determined that the diffusion coefficient decreased with increasing MPB-PE lipid fraction [51] (**Figure 5.3**). Collectively, these characterization experiments validate SLB formation, thereby enabling subsequent investigation of C3b protein attachment through the different tethering strategies.

Figure 5.3. Fluorescence Recovery After Photobleaching (FRAP) Measurements of Maleimide-Functionalized SLBs. The FRAP measurements were conducted before and after C3b protein immobilization on 90/10 mol% DOPC/MBP-PE SLBs. Post-bleaching images were recorded at 2 sec intervals for 2 min. The -1 sec images refer to the time point immediately before photobleaching commenced. All scale bars are 20 μm . Similar experiments were also conducted on 80/20 mol% DOPC/MBP-PE SLBs. In the absence of C3b protein, the lipid diffusion coefficient on 90/10 and 80/20 mol% DOPC/MBP-PE SLBs was $2.30 \pm 0.12 \mu\text{m}^2/\text{s}$ and $1.76 \pm 0.03 \mu\text{m}^2/\text{s}$, respectively. The effect of C3b protein attachment on the lipid diffusion coefficient was negligible.

5.3.2. Comparison of Covalent and Noncovalent Attachment Schemes

Figure 5.4 presents the comparison of C3b protein attachment to SLBs. After completion of SLB formation, the QCM-D measurement signals were normalized and the baseline signals were reestablished. After 10 min stabilization, 500 nM C3b protein was added to a negatively charged 70/30 mol% DOPC/POPG SLB and a maleimide-functionalized 70/30 mol% DOPC/MPB-PE SLB (**Fig. 5.4 a,b**). C3b protein attachment to the negatively charged SLB led to Δf shifts of $-7.2 \pm 1.8 \text{ Hz}$ and a negligible ΔD shift

of $0.1 \pm 0.06 \times 10^{-6}$. By contrast, appreciably more protein adsorption occurred on the maleimide-functionalized SLB with Δf and ΔD shifts of -16.2 ± 2.0 Hz and $0.83 \pm 0.15 \times 10^{-6}$, respectively (**Fig. 5.4.c**). Importantly, the $\Delta f/\Delta D$ ratio provides insight into the structural properties of the attached protein, and the differences in the $\Delta f/\Delta D$ ratio for the noncovalent versus covalent tethering strategies suggest that membrane-associated C3b proteins have a different conformation in each case.

Figure 5.4 . Comparison of Covalent and Noncovalent Tethering Strategies for C3b Protein Attachment. Changes in QCM-D (a) frequency and (b) energy dissipation as a function of time upon addition of C3b to SLBs (indicated by arrow) via covalent (red circle) and noncovalent (blue square) immobilization schemes. The covalent tethering approach utilizes a 70/30 mol% DOPC/MPB-PE SLB whereas the noncovalent tethering is based on a 70/30 DOPC/POPG SLB. (c) Summary of absolute frequency shifts for C3b protein attachment to SLBs by the two different immobilization strategies. (d) Summary of absolute frequency shifts for iC3b protein

attachment to SLBs by the two immobilization strategies. Each data is the average + standard deviation of three independent ($n = 3$) measurements

The attachment of inactive C3b (iC3b) to the two SLB platforms was also investigated (**Figure 5.4 d**). iC3b has the same molecular weight and surface charge density as C3b protein, albeit with a different protein conformation that lacks both proteolytic function and binding sites for other complement proteins as well as no active sulfhydryl functional groups. Upon addition of 500nM iC3b protein to the negatively charged and maleimide-functionalized SLB platforms, protein adsorption was observed, as indicated by Δf shifts (**Figure 5.5.a,b**). In both cases, the final Δf shift was around -6 Hz. Importantly, there was significantly more C3b attachment to maleimide-functionalized SLBs, as compared to iC3b attachment. By contrast, C3b and iC3b attachment to negatively charged SLBs was equivalent. Collectively, these findings support that covalent attachment of C3b is driven by formation of the covalent thioether linkages, whereas electrostatic interactions results in appreciably less bound protein for the noncovalent tethering approach.

Figure 5.5 Comparison of Covalent and Noncovalent Tethering Strategies for iC3b Protein Attachment. Changes in QCM-D (a) frequency and (b) energy dissipation as a function of time upon addition of iC3b to SLBs (indicated by arrow) via covalent (red circle) and noncovalent (black square) immobilization schemes.

5.3.3. C3b Protein Conjugation to Maleimide-Functionalized SLBs

The covalent conjugation of C3b protein to maleimide-functionalized SLBs was further characterized by varying the MPB-PE molar fraction, as presented in **Figure 5.6**. 500 nM C3b protein was added to maleimide-functionalized SLBs, and the Δf and ΔD shifts were monitored as a function of time (**Figure 5.6 a, b**). Upon C3b protein adsorption, the Δf shift decreased while ΔD shifts slightly increased. With increasing MPB-PE molar fraction in the lipid composition, the Δf shift decreased until reaching saturation of attached protein. The ΔD shifts showed a proportional increase but always remained below 1×10^{-6} . As presented in **Table 5.3**, the Δf shift ranged from -5.3 ± 0.42 Hz at 5 mol% MPB-PE up to -16.18 ± 2.01 Hz at 30 mol% MPE-PE. Buffer washing By contrast, there were negligible Δf and ΔD shifts upon C3b protein addition to 100 mol% DOPC SLBs. Due to the negligible ΔD shifts in all cases, the molar surface density of attached C3b protein was estimated by the Sauerbrey relationship. The surface densities varied from 0.53 ± 0.04 pmol/cm² at 5 mol% MPB-PE until reaching saturation around 1.70 ± 0.39 pmol/cm² at 25 mol% MPB-PE (**Figure 5.6 c**). Hence, the amount of covalently bound C3b protein was tunable based on MPB-PE molar fraction.

Figure 5.6 QCM-D kinetics for the conjugation of C3b on maleimide functionalized bilayers. Changes in (a) frequency and (b) dissipation for functional lipid bilayers of varied MPB-PE concentration after addition of C3b at the point that is indicated by arrow. (c) Summary of the C3b conjugation is displayed as a function of MPB-PE content.

Table 5.3. Final values of the frequency and dissipation shifts after C3b conjugation to SLBs depending on the MPB-PE content

MPB-PE Content (%)	Δ Frequency (Hz)	Δ Energy Dissipation (10^{-6})
0	-1.67 ± 1.56	0.12 ± 0.06
5	-5.36 ± 0.42	0.11 ± 0.01
10	-8.46 ± 1.01	0.30 ± 0.24
15	-12.08 ± 2.75	0.23 ± 0.24
20	-12.76 ± 3.86	0.35 ± 0.21
25	-16.91 ± 3.92	0.78 ± 0.31
30	-16.18 ± 2.01	0.83 ± 0.15

To determine if membrane-associated C3b protein is recognizable by conformation-specific antibodies, additional QCM-D experiments were conducted on 90/10 mol% DOPC/MPB-PE SLBs, as shown in **Figure 5.7**. In these experiments, C3b protein was added to SLBs as described above, followed by addition of 100 $\mu\text{g/mL}$ FITC-labeled C3 antibody which recognizes a fragment within the C3b protein. C3b conjugation and subsequent antibody binding steps led to Δf shifts around -8 and -43 Hz, respectively (**Figure 5.7 a**). The corresponding ΔD shifts were around 0.8×10^{-6} and 5×10^{-6} , respectively. A control experiment was also performed whereby C3c antibody was directly added to the SLB in the absence of C3b protein (**Figure 5.7 b**). In this case, there was no antibody binding, indicating selective binding of C3 antibody to membrane-associated C3b. Fluorescence microscopy experiments confirmed the selective attachment of FITC-labeled C3 antibody were added to SLBs without or with attached C3b protein (**Figure 5.7 c**). While no antibody binding was observed to the SLB without C3b, a marked increased in fluorescence intensity was observed when antibody was added to the C3b-coated SLB.

Figure 5.7 Antibody Recognition of Membrane-Associated C3b Protein on a Maleimide-Functionalized SLB. Changes in QCM-D (a) frequency and (b) energy dissipation as a function of time upon sequential addition of C3b and C3c antibody (indicated by arrows) onto a maleimide-functionalized SLB composed of 90/10 mol% DOPC/MPB-PE lipid composition. (c) Fluorescence micrographs of FITC-labeled C3c antibody binding to equivalent SLBs without (left) and with (middle) covalently tethered C3b protein. The TRITC filter set in the left panel reflects the status of SLBs labeled with 0.5 mol% rhodamine-DHPE lipid. The scale bar is 20 μm .

Similar experiments were performed for noncovalently attached C3b protein (Figure 5.8 a,b) and there were similar amount of antibody binding onto negatively charged SLBs. These results support that C3b proteins are present on the negatively charged and maleimide functionalized SLBs that creates platforms to study convertase assembly depending on the C3b tethering strategies.

Figure 5.8 Antibody Recognition of Membrane-Associated C3b Protein on a Negatively Charged SLB. Changes in QCM-D (a) frequency and (b) energy dissipation as a function of time upon sequential addition of C3b and C3c antibody (indicated by arrows) onto a negatively charged SLB composed of a 70/30 mol% DOPC/POPG lipid composition.

5.3.4. Convertase Assembly

To test this hypothesis, we explored stepwise assembly of the alternative pathway C3 convertase which was achieved by sequential addition of C3b, a mixture of factor B and factor D, and C3 protein as presented in **Figure 5.9 a**. Both covalent and noncovalent immobilization of C3b protein on the SLB was tested. The lipid compositions for all of the following experiments were either 70/30 mol% DOPC/MPB-PE (covalent scheme) or 70/30 mol% DOPC/POPG (noncovalent scheme). The QCM-D Δf and ΔD shifts as a function of time are shown in **Figures 5.9 b** and **5.9 c**, respectively.

After C3b attachment which was performed exactly as described above, a mixture of 200 nM factor B and 200 nM factor D solutions was added in order to form the C3bBb convertase. Upon addition of factors B and D, there was a general decrease in frequency and increase in the dissipation shifts for both SLB platforms. For covalently conjugated C3b on the maleimide-functionalized SLB, the Δf and ΔD shifts were -2.49 ± 0.44 Hz and $0.23 \pm 0.1 \times 10^{-6}$, respectively. For noncovalently conjugated C3b on the negatively charged SLB, the Δf shift was lower around -1.4 ± 0.5 Hz and the ΔD shift was negligible.

Figure 5.9 Assembly of alternative pathway C3 convertase components onto SLB platforms (a) Schematic illustration of convertase assembly at lipid membrane interface. (i) C3b protein attachment to SLB. (ii) Addition of complement factors B and D to membrane-associated C3b. In case of functional C3b, this leads to formation of C3bBb convertase. (iii) C3 protein addition in order to detect functional activity of membrane-associated convertase. QCM-D measurements tracked the corresponding changes in (b) frequency and (c) energy dissipation as a function of time.

Afterwards, 500 nM C3 protein was added in order to determine if the assembled C3bBb convertase is enzymatically active at the lipid membrane interface. On the maleimide-functionalized SLB, C3 addition induced a significant decrease in resonance frequency and increase in dissipation with final values of -16.59 ± 4.85 Hz and $0.89 \pm 0.16 \times 10^{-6}$, respectively. These measurement responses are attributed to the convertase cleaving the native C3 protein into C3a and C3b fragments, and the generated C3b protein can either be recruited to the C3 convertase (C3bBb) in order to form the C5 convertase or it becomes covalently conjugated to free maleimide groups on the SLB surface. In marked contrast, there was no change in the measurement signals when C3 was added to the negatively charged SLB, indicating a non-functional or incomplete

convertase assembly. Collectively, these results support that factor B in the presence of factor D is associated with covalently bound C3, thus creating a functional alternative pathway convertase at the lipid membrane interface. In summary, the results indicate that covalent immobilization of C3b enables functional convertase assembly, whereas the noncovalent scheme did not result in a functional assembly despite a significant amount of bound C3b protein.

Table 5.4. QCM-D measurement responses obtained from addition of individual convertase components on the SLB platforms

Assembly Stage	Covalent Tethering		Noncovalent Tethering	
	Δ Frequency (Hz)	Δ Dissipation (10^{-6})	Δ Frequency (Hz)	Δ Dissipation (10^{-6})
(i) C3b	-16.59 ± 4.85	0.89 ± 0.16	-6.36 ± 2.08	0.12 ± 0.09
(ii) Factors B & Factor D	-2.99 ± 0.76	0.39 ± 0.16	-1.44 ± 0.50	-0.21 ± 0.07
(iii) C3	-26.50 ± 7.91	0.97 ± 0.22	-1.15 ± 0.21	0.06 ± 0.01

5.3.5. Roles of Factors B and D in Convertase Assembly

We also differentiated the interaction of individual factor B, factor D, and the mixture of factor B and factor D with maleimide functionalized SLBs in the presence and absence of C3b proteins on the membrane surfaces. QCM-D measurement responses are presented in Figure 6 upon protein addition. When a mixture of 200nM factor B and 200nM factor D added to C3b bound maleimide functionalized SLBs (**Figure 5.10 a**), the frequency and dissipation values reached to 2.49 ± 0.44 Hz and $0.25 \pm 0.08 \times 10^{-6}$, respectively as displayed in **Figure 5.10 b and c**. When the factor B (200nM), the factor D (200nM), the mixture of the factor B (200nM) and factor D (200nM), were added to maleimide functionalized SLBs (**Figure 5.10 d**) in the absence of C3b, the all protein additions induced a drop in frequency shifts and there were no changes in dissipation shifts (**Figure 5.10 e and f**). Moreover, when the protein adsorbed surfaces were rinsed with the buffer solution, a small increase in the frequency shifts was detected as a result of a loss in adsorbed protein mass, suggesting that the factor B and factor D proteins reversibly bound to the membrane surface due to weak interaction between the membrane

surface and the proteins. However, when the mixture of the factor B and factor D was added to the membrane surface, the final frequency shifts were comparable with the one on the C3b conjugated membrane surface and there was no protein mass loss upon rising with the buffer solution, indicating that the mixture of the factor B and factor D forms a more stable protein assembly.

Figure 5.10 Role of Factor B and factor D in the convertase assembly. After binding of C3b to bound to maleimide functionalized (DOPC/MPB-PE 70/30), the mixture of factor B and factor D is added at $t=10$ min (a). Then, the changes in frequency (b) and dissipation (c) were recorded as a function of time. As a control, after the fabrication of maleimide functionalized SLBs (d), individual factor B (i), factor D (ii) and the mixture of factor B and factor D (iii) were also added at $t=10$ min. The changes in frequency (e) and dissipation (f) were tracked upon addition of proteins.

5.3.6. Inhibition of C3 Cleavage

To further demonstrate the potential of the SLB platform for evaluating candidate pharmacological inhibitors of convertase function, we investigated the effect of the known complement inhibitor, compstatin (Fig. 5.11 a). After establishing the covalently immobilized C3bBb convertase on the SLB platform, the QCM-D measurement

responses were normalized to the baseline values, followed by addition of C3 protein in either the presence or absence of compstatin. When 500 nM C3 protein was added alone to the membrane-associated convertase assembly, convertase-mediated C3 cleavage led to normalized Δf and ΔD shifts of -26.50 ± 7.91 Hz and $0.97 \pm 0.22 \times 10^{-6}$, respectively (**Figs. 5.11.b** and **11. c**). In contrast, the simultaneous addition of premixed 500 nM C3 and 50 μ M compstatin in a 1:100 molar ratio led to normalized Δf and ΔD shifts of -2.80 Hz and -0.06×10^{-6} , respectively—a striking reduction of almost 90% in the measurement responses.

As a control, 50 μ M compstatin was individually added to the membrane-associated convertase assembly, and the normalized Δf and ΔD shifts were 2.21 Hz and -0.53×10^{-6} , respectively. Taking into account the measurement responses obtained when C3 and compstatin were added together *versus* when compstatin was added alone, the results support that compstatin caused total inhibition of convertase function, with the small Δf shift attributed to peptide binding to membrane-associated convertase assemblies. Collectively, the results indicate that compstatin is able to inhibit C3 cleavage by the membrane-associated C3bBb convertase, thereby demonstrating the utility of the SLB platform for evaluating the performance of candidate inhibitors in a biologically relevant membrane environment.

Figure 5.11 Compstatin-mediated inhibition of C3 cleavage from C3bBb bound membrane surface. (a) After the C3bBb convertases were created on maleimide functionalized SLBs, QCM-D measurement responses were normalized and after 10 min measurement baselines (i) C3, (ii) compstatin and (iii) the mixture of compstatin and C3 were added as presented. The changes in (b) frequency and (c) dissipation shifts were recorded as a function of time upon individual sample additions.

5.4. Conclusion

In this chapter, we have investigated two different tethering strategies in order to form functional complement convertase assemblies at lipid membrane interfaces. While both covalent and noncovalent immobilization schemes promoted C3b protein attachment to the SLB platform, it was discovered that only covalently attached C3b protein facilitated downstream protein-protein interactions leading to functional convertase assembly. The QCM-D measurement approach enabled real-time measurement of the protein-protein

interactions resulting from convertase assembly as well as detection of C3 cleavage which is indicative of functional C3bBb convertases. Importantly, the label-free measurement readout was capable of measuring compstatin-mediated inhibition of convertase function, thus demonstrating the utility of this measurement platform for candidate inhibitor evaluation. Taken together, the results suggest that SLBs combined with QCM-D measurement technique can be as a complementary test platform to validate the binding of the soluble inhibitors to the membrane bound complement complexes (e.g. alternative pathway convertase). Other than complementary test platform, the SLBs also can be used as an unique platform because they have a great potential to host cell surface bound complement inhibitors such as C receptor type I (CR1), decay accelerating factor (DAF), membrane cofactor protein (MCP) in their natural state due to the fact that the SLBs maintain lateral mobility of lipid molecules even after the protein conjugation as in living cells. This characteristic makes possible to investigate the interactions of cell surface bound complement proteins with targets that are bound to the SLB surface such as enzymatic convertases, which is beyond the scope of conventional complement inhibitor platforms.

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Chapter 6

MAC Assembly on Membrane Surface*

Complement activation results in formation of membrane attack complex (MAC), a lytic immune pore that disrupts the lipid bilayer and lyse the pathogens (e.g. bacteria and viruses) therapeutic agents (e.g. liposomal drugs) and the host cells (e.g. red blood cells). The MAC is assembled from the sequential association of the five individual fluid phase or soluble proteins, C5b, C6, C7, C8 and C9 on the target surfaces. In this chapter, we fabricated the supported lipid bilayer platform to monitor the real time assembly of the MAC components, C5b-6, C7, C8 and C9 depending on the membrane surface charge.

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6.1. Introduction

As presented in Chapter 4 and Chapter 5, we have focused on the initiation and activation of the complement system through the alternative pathway since it is responsible for 80 to 90% total complement response through the positive feedback loop [1-3]. Once the complement system is activated irrespective of the initiation routes, it results (yields) in the formation of a membrane attack complex (MAC or C5b-9), a transmembrane pore, on the target surface from the sequential and irreversible association of five fluid phase complement proteins, C5b, C6, C7, C8 and C9, that induces membrane lysis of target entities [4]. The stepwise assembly of the MAC is initiated by the generation of unstable C5b protein through the proteolytic cleavage of C5 protein by enzymatically active C5 convertases [5-8]. The C5b protein is rapidly associated with C6 protein to form a stable C5b-6 complex [9]. Subsequent association of C7 creates a hydrophobic C5b-7 complex that can partially attach or adhere to the lipid bilayer surface [10, 11]. Thereafter, C8 is bound the surface attached C5b-7 complex resulting in the formation of C5b-8 complex. This binding event is considered as the first step for the insertion of the multimeric protein complex into the lipid bilayer and yields a small leaky pore with a diameter of 0.9nm [12, 13] as time passes the pore gets bigger [14]. Finally, multiple copies (12-18) of C9 proteins binds to surface bound C5b-8 complex and then they polymerize to assemble a transmembrane pore with a ring structure that has a diameter of 10 nm [15-17].

To date, many biochemical and cellular assays has been employed in order to investigate lytic nature of MAC assembly by using model platforms including erythrocytes and artificial lipid bilayers (*e.g.* liposomes and black lipid membranes (BLM)). The antibody coated-erythrocytes have been incubated in the serum samples since erythrocytes do not have endogenous trigger to initiate the complement activation. The findings indicated the erythrocytes are lysed by the MAC assembly which has been later standardized as hemolysis assay for the final readout of complement activation. On the other hand, the lytic nature of MAC assembly has been also determined by the measuring either changes in electrical properties of planar lipid bilayers (BLM) or release profile of fluorescein dye that is encapsulated within the liposomes upon sequential

addition of the purified MAC components [18-21]. Results indicated that the C5b-8 complexes are inserted into the bilayers that make the bilayers leaky or permeable due to the existence of small pores while after C9 protein addition the C5b-9 complexes are assembled onto the bilayers with more than one C9 protein, resulting a transmembrane pore.

Moreover, the effect of protein stoichiometry on the MAC assembly has been also investigated by employing electron microscopy [16, 22, 23]. The number of C9 proteins per C5b-8 complex determines the diameter of the MAC forming pore. The minimal pore size corresponded to 3 nm and 4nm for three and four C9 proteins per C5b-8 complex, respectively [24]. The number of C9 protein differs the obtained final MAC structure (*e.g.* pore size) [25]. The size variations of the MAC pores have been also estimated for the different C9:C5b-8 molar ratios (0 to 12) by conducting the liposome swelling assays [14]. When C9 protein is added to the C5b-8 bound complexes on the liposomes, the size of the channel increased as indicated by the relative rate of swelling. Step wise addition of increasing amount of C9 from the molar ratio of 0 to 12 induced to a corresponding increase in the size of the MAC forming channels. By combining with the recent structural analyses the stoichiometry of the MAC composition with a complete pore structure and a network of interaction interfaces that determine its multi hit MAC assembly mechanism have been identified [26, 27]. Although the previous structural analyses for the pore formation have been inferred from structures of bacterial cytolysins, the visualization of the MAC assembly was only recently accessible [27-29].

Collectively, the existing approaches have been relied the direct assesment of the MAC assembly and functional activity on free standing-lipid bilayer membranes which makes difficult to use surface sensitive characterization tools. Therefore, the supported lipid bilayer (SLB) combined with quartz crystal microbalance with dissipation (QCM-D) measurement technique has provided a great platform to investigate the step by step MAC assembly depending on the membrane surface charge in a real time, label free format. In order to achieve this goal, we established supported lipid bilayers with positive, neutral or negative surface charges by employing the SALB method, and the quartz crystal microbalance-dissipation measurement technique was utilized in order to track sequential protein adsorption upon addition of MAC components.

6.2. Materials and Methods

6.2.1. Reagents

Human complement proteins C5b-6, C7, C8, and C9 were purchased from Complement Tech (Texas, USA). **Table 1** provides the concentrations used in this chapter.

Table 6.1. Protein Concentrations used for MAC Assembly

Protein	Concentration ($\mu\text{g/mL}$)	Molarity (nM)
C5b-6	5	17.54
C7	5	54.11
C8	5	33.11
C9	60	846.07

An aqueous buffer containing 10 mM Tris, 150 mM NaCl [pH 7.5] was used in all experiments. 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-ethylphosphocholine (chloride salt) (DOEPC), and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (sodium salt) (POPG) were purchased either in chloroform or powder form from Avanti Polar Lipids (Alabaster, AL). Immediately before the experiment, zwitterionic DOPC lipid powder was dissolved in isopropanol at a final concentration of 10 mg/ml. POPG and DOEPC lipid molecules were received in chloroform. First, the chloroform was evaporated with a stream of nitrogen air in order to create dried lipid films. Then, the dried lipid films were dissolved in an appropriate organic solvent, *e.g.*, ethanol or isopropanol. The negatively charged POPG lipids were dissolved to 5 mg/mL in ethanol at an elevated temperature (at or above 40 °C) in order to improve solubility. Prior to experiment, the desired lipid compositions were diluted to the experimental lipid concentration of 0.5 mg/mL.

6.2.2. Quartz Crystal Microbalance with Dissipation Monitoring

Quartz Crystal Microbalance with Dissipation (QCM-D) measurement technique was employed via a Q-Sense E4 instrument equipped with flow modules (Q-Sense AB, Gothenburg, Sweden) in order to track the changes in resonance frequency (ΔF) and energy dissipation (ΔD) of an oscillating silicon oxide-coated quartz crystal. The crystal was excited at its fundamental resonance frequency of 5 MHz and odd overtones ($n = 3, 5, 7$, and 11) in order to generate the thickness shear mode. The reported data were recorded at the third overtone and all the experiments were performed under continuous flow conditions at a flow rate of $100\mu\text{L}/\text{min}$ for bilayer formation and $41.8\mu\text{L}/\text{min}$ for injection of MAC components, as controlled by a peristaltic pump.

6.3. Results and Discussion

6.3.1. Charged lipid bilayer formation by solvent-assisted lipid bilayer (SALB) formation method

To study the effect of membrane electrostatic surface charge on MAC formation, we created planar bilayer membrane platforms with different surface charges (positive, negative and neutral) by way of tuning the lipid composition as also introduced in Chapter 4. The synthetic lipid molecules used in the experiments included zwitterionic DOPC, cationic DOEPC, and anionic POPG. More specifically, 30% molar fraction of POPG or DOEPC lipids were mixed with the remaining fraction of DOPC lipids in order to make negatively or positively charged lipid bilayer membranes, respectively. Experiments were also conducted with 100% DOPC lipid composition.

Figure 6.1 Design of charged supported lipid bilayer platforms by the SALB formation method. QCM-D monitoring of the supported lipid bilayer formed on SiO₂ by the SALB formation method. **(a)** The schematic display charged lipids used to form a planar bilayer as follows: Left) DOPC:POPG (70:30), Middle) DOPC Right) DOPC:DOEPC (70:30). Color key: yellow: DOPC; red: DOEPC; blue: POPG. **(b)** Changes in frequency (ΔF , blue) and energy dissipation (ΔD , red) were recorded as a function of time at the third overtone, $n=3$. QCM-D signatures of planar bilayer formation by the SALB formation method were obtained by the following experimental sequence: (Step 1) Add Tris buffer solution; (Step 2) Inject isopropanol solution; (Step 3) Add 0.5 mg/mL lipid molecules in isopropanol; (Step 4) Exchange isopropanol to Tris buffer solution; (Step 5) add BSA protein (0.1 mg/mL) to check the completeness of the bilayers. **(c)** Adsorbed lipid mass surface density was calculated by the Sauerbrey equation. **(d)** Percentage of bilayer completion was calculated based on BSA adsorption to bare SiO₂ substrate (control) and the bilayer after completion of the SALB procedure.

Figure 6.1.b shows the representative QCM-D kinetic measurements for supported lipid bilayer formation on SiO₂ by the SALB formation method. Initially, the aqueous buffer solution (10 mM Tris, 150 mM NaCl, pH 7.5) was injected into the QCM-D measurement cell (Step 1) in order to establish stable baseline signals for the frequency and energy dissipation responses. After establishing the baselines, isopropanol solution was added into the measurement cell (Step 2) which led to drastic changes in the frequency and energy dissipation signals during mixing isopropanol with buffer. During the mixing stage, the obtained dramatic changes mainly arose from the differences in

density and viscosity of the buffer and isopropanol solutions, respectively. Afterwards, a freshly prepared 0.5 mg/ml lipid solution composed of DOPC or DOEPC:DOPC mixture (30:70) dissolved in only isopropanol, or POPG:DOPC (30:70) dissolved in a mixture of ethanol and isopropanol was injected into the measurement cell (Step 3). There were small changes in frequency of around -6 Hz and no changes in energy dissipation during DOPC and DOEPC: DOPC (30:70) lipid deposition on SiO₂. On the other hand, deposition of the POPG:DOPC (30:70) lipid mixture on SiO₂ corresponded to a - 4.7 Hz frequency shift and a 1.9×10^{-6} dissipation shift. In the final step, the isopropanol solution was gradually exchanged with buffer solution (Step 4) in order to form a complete planar lipid bilayer. Additionally, in order to check the completeness of the formed bilayer, 0.1 mg/ml BSA protein was finally added to detect nonspecific adhesion (Step 5). There was no significant change due to BSA protein adsorption in all cases, suggesting that high-quality lipid bilayers were formed in order to investigate MAC assembly.

As quantitative measures, the changes in final frequency and dissipation values after bilayer formation and BSA adsorption are summarized in **Table 6.2**. The obtained final frequency and dissipation values are consistent with the literature [30-32]. For rigid films such as a planar lipid bilayer, Sauerbrey equation was used to calculate the adsorbed mass on the substrate based on frequency shifts [33]. The attached mass on SiO₂ was approximately 450ng/cm² after bilayer formation and was independent of lipid composition as shown in **Figure 6.1.c**. In order to quantify the bilayer completion depending on lipid composition, the frequency shifts obtained by BSA adsorption on bare SiO₂ are normalized according the frequency shifts obtained by BSA adsorption on a planar lipid bilayer. The calculation indicated that bilayer completion was more than ~ 97% complete irrespective of the lipid composition used to form the bilayer as shown in **Figure 6.1.d**.

Table 6.2. QCM-D measurement responses obtained for various SALB platforms and their nonspecific interactions with BSA protein

SLB Platform	SALB		BSA	
	ΔF (Hz)	ΔD (10^{-6})	$I\Delta F$ (Hz)I	$I\Delta D$ (10^{-6})I
DOPC	24.79 $\pm$ 2.92	0.04 $\pm$ 0.031	0.51 $\pm$ 0.21	0.26 $\pm$ 0.2
DOPC:DOEPC (70:30)	26.28 $\pm$ 0.69	0.49 $\pm$ 0.002	0.64 $\pm$ 0.25	0.9 $\pm$ 0.1
DOPC:POPG (70:30)	26.16 $\pm$ 1.20	0.55 $\pm$ 0.18	0.71 $\pm$ 0.48	0.06 $\pm$ 0.1

The average and standard deviation of each measurement value are reported for three independent experiments.

6.3.2. Initial strategy to form MAC assembly on SLBs

Figure 6.2 Assembly of MAC components on supported lipid bilayer platforms MAC formation is initiated by the incubation of hydrophilic C5b-6 and C7 precursors (1). During the incubation of these two protein precursors, C7 interacts with C5b-6 and causes a hydrophilic-amphiphilic transition to result in precomplex C5b-7 formation and its membrane association. Addition of C8 proteins (2) to membrane-bound C5b-7 precomplex leads both formation of the

C5b-8 intermediate complex and its insertion into the hydrophobic core of the membrane. Subsequent introduction of C9 (**3**) results in an interaction with the membrane-inserted C5b-8 intermediate complex causing significant changes in C9 protein conformation and this makes 12-18 copies of C9 polymerize circularly to generate the final MAC complex(**3**). Using QCM-D tracking, changes in frequency (**a**) and dissipation (**b**) were monitored in order to validate MAC formation as a function of time. Three different kinds of SLB platforms composed of DOPC: POPG (70:30)(black square); DOPC:DOEPC(70:30)(red circle) and DOPC(blue triangle) were used to determine the effects of membrane surface charge on MAC formation. After completion of the SALB procedure and validation of bilayer completeness, the previously incubated C5b-6 and C7 proteins were added in the form of a precomplex C5b-7 to the SLB platform. Then, C8 and C9 were added to the membrane-associated C5b-7 precomplex, respectively. (**c**) Schematic representation of MAC formation on the negatively charged SALB platform.

We first investigated the effect of membrane surface charge on MAC self-assembly on the planar lipid bilayer. The QCM-D signatures of the self-assembly of MAC components from the sequential addition of C5b-7, C8 and C9 proteins on three different types of lipid bilayer membranes are presented in **Figure 6.2.a and 6.2.b**. The initial measurement baseline was recorded during the validation of lipid bilayer quality via BSA adsorption and then normalized. Immediately before the injection of C5b-7 precomplex, 5 $\mu\text{g/ml}$ of C5b-6 and 5 $\mu\text{g/ml}$ of C7 protein precursor were incubated for 30 min in order to generate the C5b-7 precomplex adduct at room temperature. Once the obtained precomplex was added onto the zwitterionic (DOPC), positively (DOEPC: DOPC, 30:70) and negatively charged (POPG: DOPC, 30:70) bilayers (**Figure 6.2, arrow 1**), changes in frequency and dissipation values were recorded as a function of time. The QCM-D signatures varied depending on the lipid compositions. Adsorption of the C5b-7 precomplex on the negatively charged SALB resulted in frequency and dissipation values of -16.2 ± 2.6 Hz and $2.3 \pm 0.5 \times 10^{-6}$, respectively. However, in the case of the positive charged lipid bilayer, the changes in frequency and dissipation values were -3.4 ± 2.2 Hz and $0.2 \pm 0.2 \times 10^{-6}$, respectively, whereas addition of the C5b-7 precomplex onto zwitterionic lipid bilayer membranes resulted in almost no binding based on the frequency and energy dissipation values. It has been previously reported that

C5b-7 interacts with only negatively charged liposomes through a hydrophobic interaction [34]. Therefore, our findings are consistent with the scientific literature.

After association of the C5b-7 precomplex into the lipid bilayers, 5 $\mu\text{g/ml}$ of C8 protein was added onto the C5b-7 precomplex containing lipid bilayers (**Figure 6.2, arrow 2**). The variations in frequency and dissipation responses depended on the lipid composition, reflecting C5b-7 precomplex association. On negatively charged lipid bilayer membranes, the frequency and dissipation shifts due to the presence of C8 proteins were -6.1 ± 1.0 Hz and $0.05 \pm 0.20 \times 10^{-6}$, respectively. Likewise, the frequency and dissipation shifts were negligible for both the positively charged and zwitterionic lipid bilayers (< 2 Hz and $\sim 0.5 \times 10^{-6}$). The interaction between the C5b-7 precomplex and C8 leads to formation of a C5b-8 intermediate complex on negatively charged lipid bilayers [35]. This interaction is crucial for further association of C9 proteins in order to make the MAC self-assembly. Finally, 60 $\mu\text{g/ml}$ C9 protein—the final MAC component—was injected to the membrane bound C5b-8 intermediate complex (**Figure 2, arrow 3**). The absolute frequency and dissipation shifts on the negatively charged bilayer were -11.0 ± 2.3 Hz and $1.6 \pm 0.4 \times 10^{-6}$, respectively. However, there were no significant changes in frequency and dissipation responses obtained from the assembly of C9 proteins on both zwitterionic and positively charged lipid bilayers which is similar to the changes obtained from the association of the C5b-7 precomplex and C8 proteins. These small changes in frequency and dissipation of both positively charged and zwitterionic lipid bilayer membranes were caused by non-specific interactions between protein and the bilayer. The detailed schematic of MAC formation is presented in **Figure 2C**. The changes in absolute frequency, energy dissipation, and mass binding responses during the self-assembly of MAC components are presented in **Table 6.3**. As a result, the obtained frequency and dissipation shifts were quite appreciable on negatively charged lipid bilayers. Therefore, negatively charged lipid bilayers were utilized for further studies in order to investigate the molecular assembly of MAC components.

Table 6.3. QCM-D measurement responses obtained for the addition of individual MAC components on the SLB platform

SLB Platforms	C5b-7		C8		C9	
	ΔF (Hz)	ΔD (10^{-6})	ΔF (Hz)	ΔD (10^{-6})	ΔF (Hz)	ΔD (10^{-6})
DOPC	1.52	0.077	0.39	0.00023	1.35	0.0972
DOPC:DOEPC (70:30)	3.44 $\pm$ 2.16	0.20 $\pm$ 0.21	1.62 $\pm$ 0.60	0.33 $\pm$ 0.188	3.11 $\pm$ 0.46	0.11 $\pm$ 0.04
DOPC:POPG (70:30)	16.21 $\pm$ 2.59	2.34 $\pm$ 0.49	6.17 $\pm$ 0.96	0.49 $\pm$ 0.202	10.95 $\pm$ 2.27	1.55 $\pm$ 0.37

The changes in final frequency and dissipation values were reported after the addition of individual MAC components in the order of C5b-7, C8 and C9, respectively. The C5b-7 precomplex was formed during a 30 minute incubation of MAC initial precursors of C5b-6 and C7. The average and standard deviation of each measurement value are reported for three independent experiments.

6.3.3. C5b-6 and C7 in MAC assembly

Figure 6.3 Complement components C5b-6 and C7 create a C5b-7 precomplex which associates on the negatively charged lipid bilayer in order to initiate MAC formation. Changes in normalized frequency (a) and dissipation (b) are presented as a function of time. The initial MAC precursors including C5b-6 (red circle), C7 (black square), and C5b-7 (blue up triangle) were individually injected onto the negatively charged bilayers after a stable baseline

obtained from BSA addition. C5b-7 was formed at the end of a 30 minute incubation period of C5b-6 and C7. (c) Schematic diagram depicts the interaction of initial MAC precursors with the negatively charged bilayer.

Individual C5b-6 and C7 protein precursors and the C5b-7 precomplex were utilized in order to further investigate the preferential association of these proteins on negatively charged lipid bilayers in order to initiate MAC self-assembly formation as shown in Figure 3. First of all, after formation of the bilayer, 5 $\mu\text{g/ml}$ of C5b-6 protein was added to the negatively charged lipid bilayer as shown in **Figures 6.3a** and **6.3b**. Adsorption of C5b-6 onto the bilayer did not induce remarkable variations in both frequency and dissipation values. The association of C5b-6 to the cell surface is still unclear to date [36, 37]. It has been reported that C5b-6 loosely interacts with the cell membrane but the specific membrane molecules modulating this interaction are still unknown [36]. However, Silversmith et al. has reported that the composition of lipid vesicles determines the interaction of C5b-6 and C5b-7 complexes with liposomes based on light scattering intensity measurements [37, 38]. It was observed that liposomes formed by 100% phosphatidic acid (PA) or phosphatidylglycerol (PG) interacted with C5b-6, unlike liposomes composed phosphatidylcholine (PC), phosphatidylserine (PS), or phosphatidylinositol (PI) [37]. Although the C5b-6 complex interacts with the liposomes made of PA or PG lipids, it is not capable of interacting with further C7 protein to prolong the MAC assembly. Therefore, the C5b-7 complex which is formed by incubation of C5b-6 and C7 in solution is considered as an initiator of MAC assembly.

When C7 protein was added onto the negatively charged lipid bilayer, there were only minor changes, with frequency and energy dissipation shifts of -2.5 Hz and less than 0.5×10^{-6} , respectively. However, when C5b-6 interacts with C7, the C5b-7 precomplex spontaneously transferred from the solution to the targeted negatively charged SALB where it attached to the lipid bilayer. Therefore, the frequency values increased almost 10-fold while changes in energy dissipation increased almost 6-fold compared to the individual association of C5b-6 and C7 proteins. The reported mechanism for interaction of the C5b-7 precomplex with the negatively charged lipid bilayer is the presence of an irreversible hydrophilic-amphiphilic transition during the interaction between C5b-6 and

C7 protein precursors [35]. In other words, a conformational alteration occurs due to binding of C7 to C5b-6 which causes the exposure of an already existing internal hydrophobic domain with the surrounding environment. This alteration creates a bilayer targeting site in the C5b-7 precomplex which appears to involve the C7 protein even though the binding of individual C7 protein to negatively charged lipid bilayers is negligible [10]. In terms of affinity to the negatively charged lipid bilayer surface, it has been shown that the deposition level of the C5b-7 precomplex can also be enhanced by the presence of two sialylated molecules of erythrocyte membranes such as gangliosides and glycoporin through ionic interactions [39].

6.3.4. C8 in MAC assembly

Figure 6.4 Electromagnetic interaction is essential for the activity of C8 proteins regardless of the presence of the precomplex C5b-7. The changes in frequency (a) and energy dissipation (b) are tracked as a function of time. C8 protein was added right after stabilization of BSA (wine hexagon) and association of the C5b-7 precomplex (green tetragon). The binding of C8 to membrane-associated C5b-7 precomplex creates a C5b-8 complex. (c) Schematic diagram presents the interaction of C8 proteins with a bilayer in the presence or absence of the C5b-7 precomplex.

The preferential association of C8 protein in the presence and absence of the C5b-7 complex on the lipid bilayer membrane surface was examined in order to demonstrate

the formation of the C5b-8 intermediate complex as shown in **Figure 6.4**. Once 5 μg of C8 protein was added to the bare lipid bilayer and the C5b-7 precomplex-containing lipid bilayer, the changes in frequency and dissipation responses were quite comparable (**Figure 6.4. a, b**). The interaction of C8 with the bare lipid bilayer led to small changes in final frequency and dissipation responses (-5.1 Hz and 0.09×10^{-6} , respectively). In the case of the presence of the C5b-7 precomplex on the lipid bilayer, the variations in frequency and dissipation responses were slightly different than in the absence of the C5b-7 precomplex. They were -6.8 Hz and 0.5×10^{-6} , respectively. It can be inferred that the changes in QCM-D responses were independent of the presence of the C5b-7 precomplex on the lipid bilayer. It has been reported that the positively charged C8 protein interacts with negatively charged C5b-7 precomplex through electrostatic interactions [40]. Therefore, it is not possible to confirm the formation of the C5b-8 intermediate complex based on obtained results because C8 would interact with the C5b-7 precomplex or the lipid bilayer itself as depicted in **Figure 6.4 c**. In order to prove the existence of C5b-8 precomplex, further investigation is necessary using C9 proteins.

6.3.5. Transformation of C9 monomer to MAC self-assembly complex

Figure 6.5 Polymerization of C9 proteins depend on the existence of the membrane-inserted C5b-8 complex in order to form MAC. The changes in frequency (a) and energy dissipation (b)

are presented as a function of time. C9 proteins were injected after the stabilization of BSA (purple star) and association of C5b-8 complex (pink left triangle). (c) Schematic representation demonstrates the interaction of C9 with a bilayer and membrane inserted C5b-8.

As a final test, 60 $\mu\text{g/ml}$ of C9 protein was added to the negatively charged lipid bilayer in order to investigate the association of C9 in the presence and absence of C5b-8 intermediate complex. The changes in frequency and dissipation shifts are presented in **Figure 6.5 a, b**. In the absence of all MAC precursor proteins, C9 proteins interacted non-specifically with the lipid bilayers so the changes in frequency and energy shifts were negligible and the obtained frequency and dissipation shifts were -3.1 Hz and 0.1×10^{-6} , respectively. These small changes indicated that the nonspecific interaction of C9 did not affect the viscoelastic properties and integrity of the lipid bilayers. However, when the same concentration of C9 proteins was injected into the lipid bilayer which contains the C5b-8 intermediate complex, the variations in frequency and dissipation responses were about -14.0 Hz and 1.7×10^{-6} , respectively. These appreciable changes designate the importance of the C5b-8 intermediate complex for association of C9 proteins. For example, the light scattering measurements were previously performed and the obtained light scattering intensity data showed that C9 did not interact with lipid vesicles unless the C5b-8 intermediate complex was present on the lipid vesicles [37]. In addition, C9 primarily interacts with C8 within the C5b-8 intermediate complex and this creates a C9 polymerization site which is essential for further MAC formation. 12-18 copies of C9 are necessary to polymerize circularly to create the MAC [41, 42]. If the concentration of C9 is lower, then C5b-9 forms due to the fact that the C9 polymerization stops before ring closure occurs [17]. The final MAC formation schematic is presented in **Figure 6.5c**. The presence of the C5b-8 intermediate complex on the lipid bilayer decreases the time required for C9 polymerization because it lowers the activation energy required for spontaneous polymerization of C9 [43, 44]. During this process, the hydrophilic-amphiphilic transition occurs from the monomeric C9 to polymeric C9 complex which is able to insert the whole polymeric C9 complex itself into the hydrophobic core of the lipid bilayer. Based on our results, the initial association of C9

proteins to C5b-8 bound membrane occurred within 10 min, which might be preliminary evidence of C9 polymerization.

6.4. Discussion

Supported lipid bilayers constitute a two-dimensional membrane-mimicking surface that is compatible with a wide range of surface-sensitive measurement techniques, including acoustic and optical biosensors of various kinds[45, 46]. Supported lipid bilayers can be readily formed on silica-based materials by vesicle fusion[47] or solvent-assisted lipid bilayer (SALB) formation methods[30], and the chemical composition of the bilayer can be adjusted, including electrical surface charge, membrane fluidity, and cholesterol content. For vesicle fusion, a variety of experimental conditions, including vesicle size[48], lipid composition[49], solution pH[50], osmotic pressure[51], and freeze-thaw pretreatment[52] need to be optimized for reproducible bilayer fabrication which is difficult for non-expert users. On the other hand, the SALB process is a simpler and more versatile approach that involves lipid deposition in alcohol, followed by solvent-exchange with aqueous buffer. Its optimization is straightforward and supported lipid bilayers can be formed on a wide range of substrates, including gold, aluminum oxide, and titanium oxide in addition to silicon oxide [30, 53-56]. Straightforward operation and high reproducibility lends strong potential for SALB fabrication approaches to serve as the basis for scale-up production of supported lipid bilayer platforms for clinical applications.

Importantly, in conjunction with label-free biosensing measurements, supported lipid bilayers enable quantitative kinetic tracking of molecular processes occurring on membrane interfaces. The quartz crystal microbalance with dissipation (QCM-D) monitoring technique has demonstrated significant promise for bioanalytical measurements by simultaneously recording changes in resonance frequency and energy dissipation of an oscillating quartz crystal due to a mass adsorbate[57]. The concept has been extended to molecular diagnostics in biological fluids, motivating the exploration of this measurement technique towards clinical applications, including high-sensitivity predictive detection of complement activation.

6.5. Conclusion

In this chapter I monitored the real-time assembly of the membrane attack complex (MAC) on SLBs whilst altering the surface charge of the membrane. Using the SALB method, the lipid composition of the membrane was tailored to form neutral, negative, and positive charged membrane surface respectively. MAC components were then added in sequence, starting with the C5b-7 pre-complex, C8, and C9. The binding of each MAC component were tracked as a function of time by QCM-D and the results showed the C5b-7 pre-complex preferentially bound onto negatively charged membranes forming the MAC after adding C8 and C9. Interestingly, the binding of C5b-7 to both neutrally and positively charged SLBs did not have any measurable change and were unable to form the terminating complex. Moreover, the previous works have also indicated that the MAC generation can be observed in the presence of positively and negatively charged liposomes. Such knowledge has been mainly gained by the ELISA measurements which detect the generation of non-lytic MAC assembly, sC5b-9 complex, in the solution. Therefore, the previous reports not necessarily focus on the MAC assembly on the liposome surface. Moreover, since ELISA measurements utilize the serum as a complement source, the protein corona presents on the liposome surface and alters in terms of protein composition depending on the liposome surface charge. Principally, the protein corona can interrupt the interaction of MAC components with the liposome surface and themselves. Nevertheless, the influence of protein corona on MAC assembly is still not clearly known due to the fact that the protein corona is complex and its composition can alter as a function of time. Therefore, this work clearly distinguish the MAC assembly at the lipid bilayer interface from the one formed in solution.

To date, this is the first direct evidence of the MAC generation being an electrostatically driven event, explicitly to negatively charged membranes. Collectively, such knowledge is quite valuable due to the fact that the MAC assembly creates a transmembrane pore at the lipid bilayer membrane and a pore can be formed on the host cell surface, which causes adverse immune responses. Therefore, it is important to know which factors trigger the MAC assembly at the lipid bilayer membrane in order to control the undesired responses. Additionally, the MAC assembly can also occur on the

liposomes- based drug delivery vehicles and it can lyse the liposomes which make the liposomes to release their contents. In other words, it leads to liposome clearance from the circulation before they are delivered to the diseased area in the body. Therefore, the MAC dependent release of liposome contents appears to be one of the most important factors in determining the biological fate of liposomes. As a result, the presented work in this chapter can open a new door for designing new lipid formulations of liposomes that can resist against the MAC assembly.

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Chapter 7

Inhibition of MAC Assembly on Membrane Surface*

Complement activation plays an important role in innate immune defense by triggering formation of the membrane attack complex (MAC), which is a biomacromolecular assembly that exhibits membrane-lytic activity against foreign invaders, including various pathogens and biomaterials. Understanding the details of MAC structure and function has been the subject of extensive work involving bulk liposome and erythrocyte assays. However, it is difficult to characterize the mechanism of action of MAC inhibitor drug candidates using the conventional assays. To address this issue, a biomimetic supported lipid bilayer platform was employed in this chapter to investigate how two MAC inhibitors, vitronectin and clusterin, interfere with MAC assembly in a sequential addition format, as monitored by the quartz crystal microbalance-dissipation (QCM-D) technique.

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7.1. Introduction

The complement system is a key part of the innate immune system, which is involved in eliminating foreign invaders such as microbes and particulates [1-4]. Activation of the complement system, termed complement activation, consists of a proteolytic cascade involving more than 30 proteins that form macromolecular complexes in plasma and on membrane surfaces, leading to opsonization, anaphylatoxin production, and cytotoxicity via formation of the membrane attack complex (MAC) [2, 5]. Fundamentally, the mechanisms of complement activation and downstream events involve molecular recognition [6], with surface features of various pathogens and biomaterials inducing activation through either the classical, lectin, or alternative pathway [7, 8]. Recently, a number of studies [9, 10] have demonstrated that *in vitro* and *in vivo* complement activation caused by biomaterials is related to surface characteristics such as the degree of hydrophobicity, surface charge density, and the presence or absence of specific functional groups [11-16]. Thus far, attention has been placed on the relationship between complement activation and biomaterial properties, with less emphasis on understanding how downstream processes such as MAC formation are affected.

The multi-protein MAC induces membrane lysis of target surfaces upon sequential association of MAC components that include C5b, C6, C7, C8 and C9 proteins [17-19]. Initially, C5b interacts with C6 and C7 in solution, leading to the formation of a stable C5b-7 complex which can associate with target membranes via a hydrophobic domain in C7. Then, C8 and C9 proteins interact with the membrane-associated C5b-7 complex in a sequential manner in order to assemble the MAC, which leads to formation of a transmembrane pore that causes membrane lysis [20-22]. Due to the potent nature of the functional MAC structure, human cells possess natural inhibitors in the form of soluble and membrane-bound proteins which prevent self-destruction [23, 24]. Complement inhibitors such as soluble vitronectin (S-protein), clusterin, and membrane-bound CD59 selectively block cytotoxic MAC assembly [25-29]. Specifically, vitronectin and clusterin are proposed to bind to the C5b-7 precomplex, preventing its association on the cell membrane which is a necessary step for MAC assembly [30, 31]. The details of how

complement inhibitors work are still being unraveled and have been the subject of significant experimental efforts.

To date, investigation of MAC assembly and its inhibition has focused on human erythrocyte and synthetic liposome studies in bulk solution. Hemolysis assays are commonly employed and provide a direct readout that is based on membrane lysis. On the other hand, in order to characterize formation of the MAC assembly itself, electron microscopy has been employed to examine the morphology of MAC assemblies formed by either sequential *in situ* addition of individual MAC components to lipid vesicle solutions or *in vitro* incubation of erythrocytes in human serum [20, 32]. In addition, light scattering intensity measurements have followed real-time MAC assembly by tracking the interaction between MAC components in the presence or absence of lipid vesicles [33, 34]. Furthermore, human, rabbit, or sheep erythrocytes have been incubated in human serum followed by detection of membrane-bound and soluble C5b-9 complexes by immunoblotting [27, 35]. Collectively, the existing measurement approaches primarily support direct assessment of MAC assembly structure and functional activity. However, the existing measurement platforms are difficult to utilize in order to determine the mechanism of action of complement inhibitors, lending motivation to the development of new measurement platforms that address this issue, particularly in the context of blocking membrane association of MAC components [36-38].

Towards this goal, we recently introduced a supported lipid bilayer (SLB) platform that offers label-free investigation of MAC assembly through sequential addition of purified components including the C5b-7, C8 and C9 complement proteins. Taking advantage of the compositionally flexible solvent-assisted lipid bilayer (SALB) fabrication method, the lipid composition of the SLB platform is tunable and it was identified that the MAC assembly preferentially forms on negatively charged lipid bilayers, in part due to the electrostatic-dependent membrane association of the C5b-7 complex [39]. A key advantage of the SLB platform is its compatibility with label-free, surface-sensitive measurement techniques such as the quartz crystal microbalance-dissipation (QCM-D) technique which was utilized in the aforementioned study [39] and other biomacromolecular studies [40-42]. QCM-D measurements can monitor interfacial processes including complement protein adsorption and corresponding structural

transformations involved in MAC assembly by tracking changes in the resonance frequency and energy dissipation of a lipid bilayer-coated, oscillating quartz crystal, which are in turn proportional to the mass and viscoelastic properties of the adsorbate, respectively [43, 44].

Expanding on the development of an SLB platform to reconstitute the MAC assembly, the goal of the present chapter is to investigate the quantitative stoichiometry of the MAC assembly and to determine how two known complement inhibitors, vitronectin and clusterin, function as MAC antagonists in the context of lipid membranes. Collectively, our findings support that the SLB platform in conjunction with QCM-D monitoring offers a promising approach to characterize the mechanism of action of MAC inhibitors, thereby opening the door to the classification of existing and investigational drug candidates in this class.

7.2. Materials and Methods

7.2.1. Protein Reagents

Purified human C5b-6, C7, C8, and C9 native proteins were purchased from Complement Tech (Tyler, Texas, USA). The C proteins were stored at -80°C. Human recombinant clusterin (27 kDa) and vitronectin (52 kDa) proteins were obtained from Mybiosource (San Diego, California, USA), and stored at -20°C. Recombinant vitronectin protein was received in lyophilized powder form and reconstituted at a stock concentration of 1 mg/mL in water containing 0.1 wt% bovine serum albumin (BSA). Recombinant clusterin protein was received in phosphate-buffered saline (PBS) [pH 7.4] containing 50 % glycerol. Immediately before experiment, all proteins were diluted in 10 mM Tris buffer [pH 7.5] with 150 mM NaCl to the desired protein concentration.

7.2.2. Quartz Crystal Microbalance-Dissipation

A Q-Sense E4 instrument (Q-sense AB, Gothenburg, Sweden) was utilized in order to monitor lipid and protein adsorption processes by tracking the changes in

resonance frequency and energy dissipation of a silicon oxide-coated quartz crystal (model no. QSX303) as functions of time. The QCM-D measurement data were collected at the 3rd, 5th, 7th, 9th, and 11th odd overtones, and the reported QCM-D data were all obtained at the 5th overtone and normalized accordingly ($\Delta f_{n=5}/5$). Immediately before experiment, the QCM-D sensor substrates were sequentially rinsed with 2% SDS, water, and ethanol followed by drying with nitrogen gas. The substrates were then subjected to oxygen plasma treatment (Harrick Plasma, Ithaca, NY) at the maximum radiofrequency (RF) power. All QCM-D measurements were conducted under continuous flow conditions, with the flow rate defined as 100 μ L/min for all steps in the bilayer formation process and 41.8 μ L/min for protein addition as controlled by a Reglo Digital peristaltic pump (Ismatec, Glattbrugg, Switzerland). The temperature of the flow cell was fixed at 24.00 ± 0.5 °C. In order to estimate the molecular ratio of adsorbed proteins, the Sauerbrey model was applied in order to convert frequency shifts into mass density values as follows:

$$\Delta m = -C \frac{\Delta f_n}{n},$$

where Δm is the adsorbate mass, C is the proportionality constant (17.7 ng/cm²), Δf is the frequency shift, and n is the odd overtone number of the resonance frequency [45].

7.2.3. Supported Lipid Bilayer Formation

The solvent-assisted lipid bilayer (SALB) formation method was used in order to prepare supported lipid bilayers. 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (sodium salt) (POPG) were purchased in lyophilized powder form from Avanti Polar Lipids (Alabaster, AL). Before experiment, DOPC lipid powder was dissolved in isopropanol at a stock concentration of 10 mg/mL. POPG lipid powder was dissolved in ethanol at a stock concentration of 5 mg/mL and heated to around 40°C in order to promote solubilization. Then, a 70%/30% molar fraction of DOPC and POPG lipids was prepared by mixing lipids and diluted in isopropanol to a final concentration of 0.5 mg/mL. As part of the SALB experimental protocol, a measurement baseline was first recorded in aqueous buffer solution (10 mM

Tris [pH 7.5] with 150 mM NaCl) (Step 1), followed by exchange with isopropanol solution (Step 2). Then, 0.5 mg/mL lipid in isopropanol was deposited onto the substrate (Step 3), and incubated for approximately 10 min before a solvent-exchange step was performed as aqueous buffer solution was reintroduced leading to bilayer formation (Step 4). After bilayer formation, 0.1 mg/mL BSA was added (Step 5) in order to verify the completeness of bilayer formation.

7.2.4. Membrane Attack Complex Assay

The C5b-6, C7, C8 and C9 proteins were used in a sequential addition assay format in order to establish the membrane attack complex on the supported lipid bilayer platform, as monitored by the QCM-D technique. Before experiment, protein aliquots were diluted with aqueous buffer solution (10 mM Tris, 150 mM NaCl [pH 7.5]). The C5b-5 and C6 proteins were pre-complexed in order to form a conjugate for membrane association. The C5b-7 complex was formed by mixing 20 nM C5b-6 and 50 nM C7 for a 30 min period of incubation at room temperature. Then, the C5b-7 precomplex was added to the supported lipid bilayer, followed by sequential addition of 30 nM C8 protein and then 200 nM C9 protein.

7.2.5. MAC Inhibitor Studies

In order to investigate the inhibitory effects and mechanism of actions of recombinant clusterin and vitronectin protein on MAC assembly two different strategies were applied based on the previous studies. , The inhibitors are mixed with C5b-7 precomplex or added beforehand to the supported lipid bilayer platform (**Figure 7.1**). In the first experimental format, varying concentrations of clusterin or vitronectin between 1 and 200 nM were mixed with 20 nM C5b-7 complex and incubated at room temperature for 30 min before experiment. Then, the solution containing C5b-7 complex and inhibitor was added to the bilayer before sequential addition of C8 and C9 proteins. Alternatively, varying concentrations of clusterin or vitronectin between 1 and 200 nM were added to the bilayer followed by sequential addition of C5b-7, C8, and C9 proteins.

Figure 7.1 Experimental Strategies to Test Inhibitors of MAC Assembly. Two strategies were employed. In the first strategy, (i) the inhibitor and C5b-7 complex were mixed in solution and then (ii) the mixture was added to the SLB platform, followed by sequential addition of (iii) C8 and (iv) C9 proteins. In the second case, (i) the inhibitor was first added to the SLB platform followed by sequential addition of (ii) C5b-7, (iii) C8 and (iv) C9 proteins to the SLB platform.

As mentioned above, stock clusterin was supplied in a 50% glycerol solution. It is important to take into account the specific glycerol fraction because glycerol affects the solution viscosity and QCM-D measurements are sensitive to changes in the viscosity of the bulk solution. For this reason, we carefully performed measurements in order to verify that glycerol has negligible effects on the supported lipid bilayer (**Figure 7.2**). In the clusterin experiments, the supported lipid bilayer was initially formed in pure aqueous buffer solution and then the solution was exchanged with aqueous buffer solution which contained a glycerol fraction equivalent to that of the clusterin solution prepared for the given experiment. Upon exchange with the glycerol-water mixture, minor frequency and dissipation shifts were observed, as expected due to the change in solution viscosity. After clusterin and C5b-7 addition was performed via either strategy, exchange with aqueous buffer solution was completed and additional steps in the MAC formation process (C8 and C9 protein addition) were conducted in pure aqueous buffer solution.

Figure 7.2 QCM-D monitoring to identify the effect of glycerol on the SLB. Changes in the frequency (a) and dissipation (b) were recorded as a function of time at the fifth overtone, $n=5$ in order to identify the effect of glycerol. Initial measurement baselines were obtained after the bilayer quality was validated via BSA attachment and then the experimental sequences were followed: (Step 1) Injection of representative glycerol solution; (Step 2) Inject 50nM Clusterin (Black Square); (Step 3) buffer rinsing.

7.3. Results and Discussion

7.3.1. Design of MAC Assay Platform

In order to test the effect of inhibitors on MAC assembly, we first established a supported lipid bilayer platform on silicon oxide. The bilayer was fabricated by using the SALB method [39, 46, 47], which involves the deposition of lipids dissolved in alcohol onto the solid support followed by solvent-exchange with aqueous buffer solution to promote bilayer formation. The selected lipid composition was 70 mol% DOPC and 30 mol% POPG in order to prepare negatively charged supported lipid bilayers which represent the surface of the most biologically relevant membranes of cell and bacteria because the MAC assembly also specifically occurs at negatively charged lipid bilayer membrane [39]. The QCM-D technique allows label-free measurement of the mass and viscoelastic properties of the adsorbed material, enabling detailed characterization of the fabricated lipid bilayer and each step of protein addition. After forming the lipid bilayer, the final frequency and dissipation shifts are -25.0 ± 1.4 Hz and $0.6 \pm 0.3 \times 10^{-6}$, respectively, and the values are in agreement with past reported values [39, 46, 47]. An additional step of BSA protein addition to bare and bilayer-coated substrates indicated that the bilayers are >95% complete and passivated remaining parts of the substrate in order to specifically detect membrane association of MAC proteins to the lipid bilayer. Thereafter, as part of the MAC assay described in **Figure 7.3 a**, the change in frequency shift was recorded as a function of time upon addition of protein components of the membrane attack complex, with C5b-7, C8 and C9 proteins added in sequential format (**Figure 7.3 b**).

Each stage of protein addition (indicated by the respective arrows) led to a negative frequency shift. Because the frequency shift is negatively proportional to the adsorbed mass, the frequency shifts indicate that the proteins attach to the lipid bilayer and the adsorbed mass of each protein can be quantified (**Figure 7.3. c**). By taking into account the molecular weights of each protein, the molar ratio of bound proteins can be determined. Based on this approach, it was determined that approximately 1.25 C8 and 3.60 C9 proteins were attached to the lipid bilayer per 1 C5b-7 protein. The calculated

C5b-7:C8 molar ratio is in good agreement with literature reports that indicate a 1:1 stoichiometry [33]. From these calculations, we also determined that the measured stoichiometry of the C5b-8 intermediate complex to C9 protein is 3.4, which agrees well with the literature range between 3 and 16 [27, 33, 48, 49]. Taken together, the results indicate that the platform offers a quantifiable, label-free assessment of bound protein mass, leading us to investigate how the two inhibitors each affect MAC assembly.

Moreover, it is also important to note that this study specifically focuses on the MAC assembly on the negatively charged lipid bilayers because the purified MAC components significantly interact with the negatively charged bilayers rather than other lipid bilayers that carry positive and neutral surface charge (see **Chapter 6**). However, there is a still possibility that MAC assembly can occur on other lipid bilayers in the presence of serum proteins. In other words, protein corona layer that forms on the lipid bilayers can induce the MAC assembly on those bilayers.

Figure 7.3 QCM-D Monitoring of MAC Assembly on the SLB Platform (a) Schematic illustration of MAC formation via sequential addition format. Changes in (b) frequency and (c) Sauerbrey mass were monitored as functions of time after sequential addition of C5b-7 complex, C8 protein, and C9 protein onto the 70:30 mol% DOPC:POPG SLB platform. The SLB on silicon oxide was initially formed by the SALB method, and then the measurement time was normalized.

At $t = 0$ min, the MAC assay was initiated and the C5b-7 complex was added at $t = 20$ min. Then, C8 protein was added at $t = 80$ min and C9 protein at $t = 150$ min. The adsorbed mass was calculated by the Sauerbrey relationship and the mass sensitivity constant for a 1Hz frequency shift is 17.7 ng/cm^2 .

7.3.2. Effect of Vitronectin on MAC Assembly

Using the supported lipid bilayer platform, we first investigated the effect of vitronectin on membrane association of the C5b-7 complex. The C5b-7 complex and vitronectin were mixed before experiment, and then added to the lipid bilayer platform (**Figure 7.4 a**). With increasing vitronectin concentration, there was a general decrease in the frequency shift associated with protein adsorption (**Figure 7.4. b**). In order to investigate details of the vitronectin concentration- dependent effects, the maximum frequency shifts are presented as a function of vitronectin concentration in **Figure 7.4. c**. Interestingly, at lower vitronectin concentrations (5 nM and below), the frequency shifts were lower than the value obtained from the association of C5b-7 complex in the absence of inhibitor. These results support that some of the C5b-7 complex interacted with vitronectin in the solution and loses the ability to associate on the bilayer at lower vitronectin concentration. That is, when vitronectin directly interacts with C5b-7 in solution, the resulting vitronectin-C5b-7 complex is prevented from subsequently binding to the lipid bilayer. By contrast, at higher vitronectin concentrations, there is a clear increase in the total adsorbed protein mass. In particular, at 100 nM and higher vitronectin concentrations, there are large frequency shifts which suggest that vitronectin was the dominant adsorbed species because the C5b-7 protein concentration was fixed at 20 nM.

Figure 7.4 Influence of Vitronectin on Membrane Association of the C5b-7 Complex (a) Schematic illustration shows addition of the C5b-7 complex which was preincubated with vitronectin. Panel (b) presents the QCM-D frequency shift associated with C5b-7 + vitronectin addition to the SLB platform as a function of vitronectin concentration. The C5b-7 complex concentration was fixed at 20 nM. The proteins were added at $t = 20$ min. Panel (c) shows absolute value of the maximum QCM-D frequency shift due to the amount of total adsorbed protein as a function of vitronectin concentration based on the data presented in Panel (b). Each data point is the average + standard deviation of three independent ($n = 3$) measurements. (d-e) Corresponding schematic illustration and QCM-D measurement data for the addition of vitronectin to the SLB platform, followed by addition of C5b-7 complex to the same platform. Panel (f) shows absolute values of the maximum QCM-D frequency shifts due to the amounts of adsorbed vitronectin and C5b-7 complex as a function of vitronectin concentration based on the data presented in Panel (e). Each data point is the average + standard deviation of three independent ($n = 3$) measurements.

In order to clarify the effect of vitronectin on C5b-7 adsorption, a sequential addition protocol was followed in parallel experiments across the same range of inhibitor concentrations (**Figure 7.4.d**). **Figure 7.4.e** presents the individual frequency shifts associated with membrane association of vitronectin followed by C5b-7 protein. Again, with increasing vitronectin concentration, there was an increase in vitronectin uptake. On

the other hand, increasing concentrations of vitronectin inhibited subsequent membrane association of C5b-7. As presented in **Figure 7.4.f**, the frequency shifts were proportional to the vitronectin concentration ranging up to -19 Hz at 200 nM concentration. Likewise, prior membrane association of vitronectin suppressed membrane association of C5b-7, with negligible C5b-7 addition at high vitronectin concentrations. Particularly strong inhibition was observed at vitronectin concentrations above 50nM. Taken together, the data obtained from both experimental strategies support that vitronectin inhibits membrane association of C5b-7 via direct interaction with C5b-7 which prevents binding to the lipid bilayer and via competitive membrane association onto the supported lipid bilayer which restricts the number of available adsorption sites.

Following addition of the i) premixed C5b-7 and vitronectin or ii) vitronectin followed by C5b-7, there was next sequential addition of C8 and C9 proteins in order to investigate the effect of surface-bound vitronectin. In **Figure 7.5**, the frequency shifts associated with C8 and C9 protein addition are presented as a function of vitronectin concentration. Independent of the route by which the supported lipid bilayer was treated with vitronectin, subsequent membrane association of C8 and C9 proteins decreased with increasing vitronectin concentration. There is a nearly linear trend in inhibition between 5 and 100 nM vitronectin, with minimal inhibition below this concentration range. Collectively, the results are consistent with vitronectin blocking membrane association of C5b-7, in turn preventing membrane association of C8 and C9 proteins as well.

Figure 7.5 Effect of Vitronectin on the Sequential Addition of C8 and C9 Proteins to the SLB Platform (a) Schematic illustration of sequential addition of C8 protein to the SLB platform after vitronectin and C5b-7 addition. (b) QCM-D maximum frequency shift associated with C8 protein addition as a function of vitronectin concentration for each strategy. Each data point is the average + standard deviation of three independent ($n = 3$) measurements. (c-d) Corresponding schematic illustration and QCM-D measurement data for C9 protein addition as a function of vitronectin concentration.

7.3.3. Effect of Clusterin on MAC Assembly

In addition to vitronectin, we also studied the inhibitory effect of clusterin on membrane association of the C5b-7 complex. Initially, clusterin and C5b-7 complex were

premixed and incubated for 30 min at room temperature (**Figure 7.6.a**). **Figure 7.6.b** presents the QCM-D frequency shifts for the addition of premixed C5b-7 and clusterin to the SLB platform. With increasing clusterin concentration, there was a trend towards increased frequency shifts as reflected in **Figure 7.6. c**. Low clusterin concentrations had negligible effect on protein uptake, while there was a linear correspondence between clusterin concentration and protein uptake at 10 nM clusterin and above. The data indicate that clusterin attached to the lipid bilayer together with the C5b-7 complex, clarifying a previous report [50] which suggested that clusterin prevents attachment of C5b-7 complex to the membrane. In parallel experiments, clusterin was directly added to the supported lipid bilayer in the absence of the C5b-7 complex (**Figure 7.6.d**). Even at high clusterin concentrations, there was only minimal attachment to the lipid bilayer (less than 5 Hz in all cases) (**Figure 7.6.e**). In turn, the amount of attached C5b-7 on the lipid bilayer surface did not depend on the bulk clusterin concentration (**Figure 7.6.f**).

In order to further investigate the effect of clusterin on membrane association of C8 and C9 proteins, C8 and C9 proteins were sequentially added after initial addition of premixed clusterin-C5b-7 or after sequential addition of clusterin and then the C5b-7 complex. Changes in frequency shifts associated with C8 protein addition are presented as a concentration of clusterin (**Figures 7.7.a, b**). For both experimental strategies, the changes in frequency shift were largely independent of clusterin concentration at low and moderate inhibitor concentrations whereas there was some inhibition of C8 protein attachment at high concentrations (20 nM clusterin and above). This moderate inhibitory effect of clusterin on C8 protein attachment is consistent with previous dye release experiments [28]. After C8 addition, C9 protein was next added and the changes in frequency shifts are presented as a function of clusterin concentration (**Figure 7.7.c, d**). Interestingly, the amount of bound C9 increased with increasing clusterin concentration, which is also consistent with the previous finding [25] that clusterin is known to bind to multiple sites on C9.

Figure 7.6 Influence of Clusterin on Membrane Association of the C5b-7 Complex (a) Schematic illustration shows addition of the C5b-7 complex which was preincubated with clusterin. Panel (b) presents the QCM-D frequency shift associated with C5b-7 + clusterin addition to the SLB platform as a function of vitronectin concentration. The C5b-7 complex concentration was fixed at 20 nM. The proteins were added at $t = 20$ min. Panel (c) shows absolute value of the maximum QCM-D frequency shift due to the amount of total adsorbed protein as a function of clusterin concentration based on the data presented in Panel (n). Each data point is the average + standard deviation of three independent ($n = 3$) measurements. (d-e) Corresponding schematic illustration and QCM-D measurement data for the addition of clusterin to the SLB platform, followed by addition of C5b-7 complex to the same platform. Panel (f) shows absolute values of the maximum QCM-D frequency shifts due to the amounts of adsorbed clusterin and C5b-7 complex as a function of clusterin concentration based on the data presented in Panel (e). Each data point is the average + standard deviation of three independent ($n = 3$) measurements.

Figure 7.7 Effect of clusterin on the sequential addition of C8 and C9 proteins to the SLB platform (a) Schematic illustration of sequential addition of C8 protein to the SLB platform after clusterin and C5b-7 addition. (b) QCM-D maximum frequency shift associated with C8 protein addition as a function of clusterin concentration for each strategy. Each data point is the average + standard deviation of three independent ($n = 3$) measurements. (c-d) Corresponding schematic illustration and QCM-D measurement data for C9 protein addition as a function of clusterin concentration.

7.3.4. Comparison of Inhibition by Clusterin versus Vitronectin

It is known that vitronectin and clusterin inhibit the MAC assembly through two different mechanisms. First, they interact with the domain of C5b-7 that is responsible of

insertion of C5b-7 complex into the membrane and thus prevents the C5b-7 insertion, or in other words solubilise the C5b-7 complex (so called sC5b-7) to make unable of C5b-7 insertion to the membrane. Second, they interact with the C9 proteins and block the interaction of C9 with the membrane bound C5b-8 complex and its polymerization with other C9 proteins to create a pore in the membrane. In this chapter, the inhibitory mechanisms of vitronectin and clusterin on MAC assembly were investigated at the light of previous findings.

The interaction of vitronectin and clusterin with supported lipid bilayers was determined by using the QCM-D technique. **Figure 7.8.a** presents the changes in attached protein mass density upon addition of vitronectin and clusterin as a function of protein concentrations. The adsorbed mass of vitronectin proteins increased when the concentration of vitronectin increased ranging from 12 to 344 ng/cm², and occurs due to electrostatic attraction because vitronectin has a positively charged domain [31] that prefers membrane association onto negatively charged lipid bilayers. However, when the C5b-7 complex was incubated with vitronectin, vitronectin blocked subsequent membrane association of the C5b-7 complex at lower concentrations which results in less adsorbed protein, as reflected in **Figure 7.8.b**. The higher concentration of vitronectin induced an increase in total protein attachment due to adsorption of both C5b-7 complex and vitronectin. These findings are consistent with past reports [31, 48] that vitronectin inhibits MAC formation through occupation of the membrane binding site of the C5b-7 complex and extends this knowledge to directly show that vitronectin has a high propensity to attach to negatively charged lipid bilayers.

In the case of clusterin, there was only minor adsorption of the inhibitor to the SLB platform in all cases, with mass shifts always below 50 ng/cm² (**Figure 7.8.a**). Although clusterin did not directly interact with the SLB platform, the preincubation of clusterin with the C5b-7 complex induced an appreciable change in the attached mass of total protein which depended on the clusterin concentration as depicted in **Figure 7.8.b**. Based on these results, we conclude that clusterin attached to the C5b-7 complex but did not block the membrane association site of the C5b-7 complex. Collectively, the QCM-D measurement results obtained on the SLB platform demonstrate that vitronectin and clusterin differentially interfere with the C5b-7 complex as part of their roles as

complement inhibitors. Looking forward, there is excellent potential to utilize the SLB platform with additional sensing modalities such as combined QCM-D and electrical impedance spectroscopy measurements [49] in order to investigate how complement inhibitors interfere with other processes involved in MAC assembly such as pore formation.

Figure 7.8 Comparison of Inhibition by Vitronectin and Clusterin on Membrane Association of the C5b-7 Complex. (a) Changes in adsorbed mass of vitronectin and clusterin upon addition to the SLB platform as a function of inhibitor concentration. (b) Changes in adsorbed mass due to inhibitor + C5b-7 complex upon joint addition to the SLB platform as a function of inhibitor concentration. Each data point is the average + standard deviation of three independent ($n = 3$) measurements.

7.4. Conclusion

In this chapter, we have investigated the association of MAC components onto the SLB platform in the presence and absence of known MAC inhibitors. First, we measured the quantitative stoichiometry of adsorbed MAC components on negatively charged SLBs. Compared to existing attempts to determine stoichiometry from light scattering and electron microscopy, our QCM-D measurement approach yields high precision data based on label-free mass-sensitive detection. Extending these capabilities, the specific inhibitory mechanisms of vitronectin and clusterin towards the MAC assembly were identified using a combination of two experimental strategies which focused on the direct protein-protein interaction with the C5b-7 complex and competitive membrane association of the inhibitor to the SLB. The findings support that vitronectin blocked the membrane association of the C5b-7 complex to the SLB platform at low inhibitor concentrations through a direct interaction whereas vitronectin at higher concentrations principally acted via competitive attachment to the SLB platform. By contrast, clusterin directly interacted with the C5b-7 complex and the two proteins adsorbed together onto the SLB. Moreover, the SLB platform combined with QCM-D measurement technique are useful for the assessment of binding of the inhibitor to the target molecule that is already associated at the lipid bilayer surface. However, such information itself is not sufficient to evaluate the overall inhibitory activity. Therefore, complementary assays (e.g. hemolysis assay or ELISA) are necessary to assess the inhibition activities in addition to binding of inhibitors to target molecule. Nevertheless, the SLBs can be promising platforms to validate the inhibitory actions of cell surface proteins since the SLB platform still retain lateral mobility of lipid molecules and the cell surface proteins can be incorporated into SLBs without losing their functions, which has not been done in literature. Taken together, in this chapter the capabilities of the SLB platform has been highlighted to test inhibitors of the MAC assembly, generating critical mechanistic information to guide drug discovery and development with broad applicability to additional classes of inhibitors including small molecules, antibodies and peptides.

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Chapter 8

Conclusion and Future Outlook

In this chapter, conclusions of the findings in this thesis are summarized and two different studies are proposed as future outlook.

8.1. Conclusion

In this dissertation, the interaction of complement proteins with supported lipid bilayers has been investigated with a variety of experimental methodologies. The specific objective was to explore how the membrane surface characteristics influence the initiation, amplification, and termination of complement activation on supported lipid bilayer platforms at the molecular level. Underlying this objective, the overall hypothesis was that the protein-lipid and protein-protein interactions of complement activation occurring at lipid membrane interfaces can be profiled using supported lipid bilayers as a model system. Supported lipid bilayers share many commonalities with colloidal lipid bilayer systems specifically liposomes, in terms of membrane surface charge and functional groups that contribute to the activation of the complement system. In this setting, corresponding studies were conducted as follows:

In chapter 4, charged supported lipid bilayers on silicon oxide were prepared using the SALB method by tuning the precursor lipid composition. The influence of the membrane surface charge (*e.g.* positive, negative, and neutral) on the adsorption of three different alternative pathway initiating proteins (*e.g.* C3, C3b and properdin) were first time studied at the lipid bilayer interfaces. The quartz crystal microbalance-dissipation (QCM-D) technique was utilized to quantify the total protein adsorption onto a supported lipid bilayer in real time. Upon observation, all three proteins preferentially and irreversibly associate onto the negatively charged membranes while the total protein uptake differed based on the tested protein and membrane surface charge. Additionally, the total protein uptake on the negatively charged membranes was also found to be dependent on the bulk protein concentration. Furthermore, ELISA measurements were conducted to monitor the liposomes mediated complement activation pathways. The results showed that positively charged liposomes triggers the alternative pathway while negatively charge liposomes trigger the classical pathway. These findings indicated that although the pattern recognition proteins of alternative pathway preferentially interacted with the negatively charged lipid bilayers, the negatively charged liposomes were not capable of prolonging the complement cascade to generate the terminal complex, suggesting that adsorption preference of pattern recognition proteins itself might not be

only determinant for the activation of complement cascade because the protein corona layer covers the liposome surface in the serum and accordingly alters the liposome surface. Moreover, even if surface bound C3 and C3b proteins do not prolong the complement cascade, they function as opsonins which stimulate the phagocytosis of the protein-bound surface for their immune clearance. Therefore, this chapter highlights the necessity of the complementary assays for designing a synthetic lipid bilayer surface with immunocompetent features.

In chapter 5, maleimide-functionalized and negatively charged supported lipid bilayers were created on silicon oxide and then C3b proteins were first time attached to the membrane interfaces without the requirement of protein tags. The C3b was adsorbed onto the negatively charged membranes through electrostatic (noncovalent) interactions while it was covalently attached to maleimide functionalized membranes through free thiol groups on the C3b. The effects of C3b attachment to the target surface on the assembly of alternative pathway of C3 convertase were investigated with the QCM-D measurement technique. The findings indicated the C3b attachment strategy is a key determinant for convertase assembly. If the C3b was attached to the lipid bilayer surface through the covalent attachment strategy, the C3b was able to create a functional alternative pathway C3 convertase. However, if the C3b was attached to the lipid surface through noncovalent attachment strategy, the functional convertase was not formed and the negligible amounts of convertase proteins were nonspecifically adsorbed. The effects of attachment strategy of initial protein, C3b, on the convertase assembly were clearly examined. Moreover, the functional activity of the C3 convertase on the maleimide functionalized membranes was also tested with the clinically relevant regulatory peptide, compstatin. The results showed that in the presence of compstatin the C3 proteins were not cleaved by the functionally active convertase that were created at the lipid bilayer membrane. The findings in this chapter provide insights into the stepwise assembly of convertase while introducing the attachment scheme of initiator protein to the lipid bilayer interfaces. Taken together, the convertase containing lipid bilayers offers a promising platform to evaluate the test convertase targeted inhibitors.

In chapter 6, the real time assembly of the membrane attack complex onto a supported lipid bilayer depending on the membrane surface charge first time was

investigated by QCM-D measurement technique without requirement of labelling. After the fabrication of the SLB platforms with negative, positive and neutral surface charge on silicon oxide, the molecular components of the membrane attack complex, C5b-6, C7, C8, and C9 was sequentially added. There was no appreciable binding of C5b-6 precomplex to the SLBs while the appreciable binding was only observed at negatively charged SLBs when the C5b-6 procomplex and C7 protein were mixed in solution to create C5b-7 complex before addition to the bilayers, suggesting that the association of C5b-7 complex requires negatively charged membrane surface and the C5b-7 complex itself at the bilayer functions as an initiator of MAC assembly. Further addition of C8 and C9 to the C5b-7 complex bound bilayer surfaces created C5b-8 and C5b-9 complexes, respectively. This study clearly showed a direct evidence of the MAC assembly being an electrostatically driven event through the binding of C5b-7 complex to negatively charged membranes. Collectively, SLB platforms combined with QCM-D measurement technique have a great potential to investigate the molecular assembly of biomacromolecular complexes existed in the complement system.

In chapter 7, negatively charged lipid bilayers were used to quantitate the stoichiometry of the membrane attack complex assembly. After the assembly of MAC components to negatively charged SLB platforms, stoichiometry of each protein was estimated from the bound protein mass. The results indicated that approximately 1.25 C8 and 3.60 C9 proteins were bound to 1 C5b-7 protein that attached to the lipid bilayer. Furthermore, this platform was used to investigate how the MAC assembly is inhibited by two well known inhibitors, clusterin and vitronectin. Two experimental strategies were applied based on previous reported molecular assembly of those inhibitors. The first strategy consisted of decorating the bilayers with inhibitors prior to the addition of the C5b-7 complex. The second strategy used a combination of inhibitors and the C5b-7 complex and was incubated together before being added to the bilayer. The findings showed that vitronectin binds to the negatively charged bilayers through an electrostatic interaction and inhibits the association of the C5b-7 complex in a concentration dependent manner. Subsequently, there was also a reduction of the binding of C8 and C9, suggesting that vitronectin at the bilayer surface was able to block the MAC assembly. Unlike vitronectin, clusterin had little to no binding to the membranes. After attempting

to bind clusterin to the membrane, there were no inhibitory effects observed upon introduction of the C5b-7 pre-complex. The inhibitory action of vitronectin and clusterin on the MAC formation in solution showed vitronectin blocking the association of the C5b-7 complex to the membrane surface at low concentrations. Whereas the inhibitory effects of vitronectin was assessable from the adsorption at higher concentrations and the adsorption of C8 and C9 also reduced accordingly. On the other hand, clusterin directly interacted with C5b-7 such that the pre-complex was still able to bind to the lipid bilayer. Clusterin also altered the subsequent association of other complement proteins involved in the MAC assembly (*e.g.* increase in C9 adsorption). In other words, unexpectedly the clusterin failed to inhibit the MAC assembly. Overall, this study establishes a platform to evaluate the mode of actions of inhibitors against MAC assembly and give insights into drug discovery and development.

Taken together, these works presented in this dissertation also significantly contribute to the fundamental understanding of complement activation occurring at the lipid bilayer interfaces and offers a new platform to characterize the mechanisms of action for a wide range of complement inhibitors including small molecules, antibodies, and peptides.

8.2. Future Outlook

There is a strong potential to continue the research in the direction based on findings presented in this dissertation. In this section, two particular opportunities are discussed and highlighted the potential of utilizing supported lipid bilayer platforms for both fundamental studies relating to the mechanism of complement activation as well as applied studies that involve evaluation of candidate pharmacologic inhibitors, including:

- (i) Investigation of the classical pathway activation on supported lipid bilayers.
- (ii) Comparison of the complement regulation activity between factor H and mini factor H

8.2.1. Investigation of classical pathway activation on supported lipid bilayers

This thesis more specifically demonstrates the adsorption preference of the alternative pathway complement proteins onto SLB platforms along with the protein-protein interactions that lead to the assembly of the C3 convertase (see **Chapter 4 and 5**). In the proposed work, the classical pathway activation will be comprehensively investigated at the lipid bilayer interfaces.

From previous studies, complement activation through the classical pathway has been shown to initiate when an antibody binds to an antigen. The antigen-antibody complex interacts with first subcomponent of the classical pathway, C1q, to initiate the complement cascade. Others reported that the classical pathway is also triggered by antibody-independent manners because C1q can bind to a variety of substances including amyloid P [1], DNA [2], prion[3], C-reactive protein [4], β -amyloid fibers [5], lipopolysaccharides [6] and heparin protamine [7]. C1q is a 460 kDa glycoprotein composed of six globular head groups connected by a triple helix-like stalk chains and present in human serum a concentration of about 100 mg/L [8]. C1q is a main contributor to the formation of the C1 complex- which is formed when doublets of C1s and C1r bind to C1q in the presence of calcium [9]. The obtained C1 complex cleaves C2 and C4 proteins to form the classical pathway C3 convertase.

Recently, liposomes have been used as a packaging system for medicines in most of the recent pharmaceutical applications [10, 11] and their efficacy highly depends on serum protein interactions with liposomes. Particularly the complement proteins that bind to the liposomes and trigger complement activation in an antibody-independent manner. These bound proteins lead to either the opsonization of liposomes along with clearance by phagocytic cells [12-18] or the formation of the membrane attack complex onto liposomes which effects the stability of the liposome membranes and causes the subsequent leakage of the encapsulated content[19, 20]. It is also understood that the initiation of complement activation either through the classical or alternative pathway is dependent on the physicochemical properties and the type of phospholipids used [19, 21]. Chonn et al. reported that positively charged liposomes activate the alternative pathway while negatively charged liposomes trigger the classical pathway [22]. This was supported by other studies, therefore classical pathway activation is primarily initiated by anionic-negatively charged-liposomes, which is most likely from the direct binding of

C1q [23-25]. Together these studies propose that the binding of C1q is driven by the electrostatic interactions between the anionic liposomes and the positively charged collagen like stalks (cationic domain within C1q). Follow up studies using radioimmunoassays to test complement activating properties of liposomes prepared from the different anionic phospholipids including phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidic acid (PA), phosphatidylserine (PS), and cardiolipin (CL), suggest that all phospholipids activate the classical pathway and the highest C1q binding occurs on CL-containing liposomes [26].

Taken together, the factors that initiate the classical pathway have been more or less determined. However, unlike the alternative pathway, there are no studies regarding stepwise assembly of the classical pathway convertase onto sensor surfaces. Therefore, the goal of this study will be to expand the fundamental understanding of C1q binding to supported lipid bilayers made of different anionic lipid composition and its contribution onto classical pathway convertase assembly by using QCM-D measurement technique.

8.2.2. Comparison of the complement regulation activity of mini factor H and factor H on supported lipid bilayers

In this thesis, the mechanisms of actions of complement inhibitors that specifically target the alternative pathway convertase (see **chapter 5**) and the membrane attack complex (see **chapter 7**) were investigated on supported lipid bilayers. The regulation of complement activation is extremely important since an exaggerated level of complement activation can be destructive to host cells and can be associated with different pathological diseases. In order to eliminate these adverse effects of complement activation, our body secretes regulatory soluble and cell surface proteins that control complement activation at several check points (*e.g.* initiation, amplification and termination of complement cascade). However, these regulatory proteins are not always produced at sufficient levels (less or more). Therefore, new tailor-made complement regulators are required in order to take control of exaggerated (*or* unchecked) complement activation in addition to naturally existing regulators. To date, for this purpose a number of promising inhibitors have been announced in drug pipelines of

pharmaceutical companies in **Figure 8.1** [27, 28]. Yet only two of the complement inhibitors are available for clinical studies, specifically, the C1 inhibitor (plasma derived Berinert or recombinant derived Ruconest) and the humanized anti-C5 monoclonal antibody (Eculizumab, Soliris).

Figure 8.1 Complement targets and inhibitors for pharmaceutical intervention in the complement system. Adapted with the permission from ref. [27]. Copyright 2016 Elsevier B.V.

Current ideologies consider factor H-based therapeutics to be more favorable than the current inhibitors (*e.g.* Eculizumab) which only regulate complement activation at the terminal phase. Factor H down-regulates both the alternative and classical pathways of complement activation at the initial phase through different mechanisms including:

- (i) Inhibition of the formation of the alternative pathway's C3 and C5 convertases from the competitive binding of factor B on C3b. [29]

- (ii) Acting as a cofactor for the serine protease factor I to inactivate C3b, which is no longer able to stimulate convertase assembly. [30]
- (iii) Facilitating the dissociation of Bb fragments from the alternative pathway C3 convertases, also known as the decay accelerating activity. [31]
- (iv) Competition with C1q to associate onto a target surface, thus acting as direct down regulator of the classical pathway. [26]

Factor H is a major soluble regulator of the complement system in serum with a concentration of approximately 560 mg/mL [32]. It is particularly composed of 20 short consensus repeat domains (**Figure 8.2**). The C-terminal consisting of the 19-20 domains are necessary for host cell recognition by factor H [33, 34] while the N-terminal, domains 1-4, play a critical a role for both the cofactor and decay accelerating activities of factor H [35, 36]. This leads to the dissociation of Bb fragments from the alternative pathway C3 convertase through competition and the repulsive electrostatic interactions [37, 38].

Figure 8.2 Schematic representation of factor H and mini factor H and their ways of interactions with C3b on host surfaces. Redrawn with the permission from [39] Copyright 2013 The Journal of Immunology

A miniaturized version of factor H (mini-factor H) has also been created and consists of only the N-terminal domains 1-4 and the C-terminal domains 19-20 (**Figure 8.2**) [39, 40]. When this mini factor H binds to C3b, it exhibits the complement regulatory functions that are similar to the full length factor H. The main differences

between the binding characteristics of factor H and mini factor H to sensor bound C3b is [39]:

- (i) Factor H binds to two nearby C3b molecules due to the presence of the long stretch of 14 domains (5-18) among two binding sites.
- (ii) Mini factor H binds to only one C3b molecule, yielding to stronger attachment.

Mini factor H and factor H have also been used for the inhibition of liposome mediated complement activation as shown in an sC5b-9 ELISA assay [41]. The results indicate that mini-factor H is a more potent inhibitor of liposomes-AmBisome- mediated complement activation compared to factor H. However, the existing methods such as hemolysis and ELISA relies on direct assessments of inhibition of complement activation by looking at the generated responses such as hemoglobin release from erythrocytes and reduction in complement final product, sC5b-9, respectively. Therefore it is difficult to determine the mode of action of factor H and mini-factor H, lending motivation to employ a new measurement platform that addresses this issue. The supported lipid bilayer platforms can be employed to elucidate how complement activation is regulated by factor H and mini factor H after binding C3b to the membranes at molecular level.

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List of Publications

Journal Papers

1. Jackman JA; **Yorulmaz Avsar, S.**; Li D; Vladimir Z; Bengt K; Cho NJ. Quantitative Profiling of Liposome Deformation by a Localized Surface Plasmon Resonance Sensor. *ACS Analytical Chemistry* 2016.
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Poster Presentation

1. Yorulmaz, Saziye, et al. " Supported Lipid Bilayer Platform to Test Inhibitors of the Membrane Attack Complex: Insights into Biomacromolecular Assembly and Regulation" Tethered Membrane (TethMem) 2015; Singapore