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# Initiation of alternative pathway of complement, and development of novel liposomal coatings

ANNA ADLER



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### Abstract

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The complement system is a central part of the innate immune system, and is an essential part in recognizing and clearing non/altered-self surfaces in the body. This thesis comprises of projects in which the initiation of the alternative pathway (AP) of complement in the fluid phase as well on various artificial and lipid surfaces has been studied. We have also synthesized and evaluated polymer-lipids as liposome coatings to suppress innate immune activation with focus on complement regulation.

In paper I we investigated how “C3b-like” C3(H<sub>2</sub>O) is in regards to form an initial fluid phase AP C3 convertase. Even though C3(H<sub>2</sub>O) could form a C3 convertase, it was much slower in comparison to the convertase generated by C3b.

In paper II the contact activation of C3 on various artificial and lipid surfaces as a potential targeted AP activation pathway was explored. C3 bound selectively to lipid surfaces with negatively charged phospholipids and cholesterol, activated platelets and apoptotic cells. Thus, AP was initiated without prior proteolytic cleavage of C3 nor by preformed C3(H<sub>2</sub>O) on specific surfaces in a selective manner.

In paper III and IV, synthetic phosphatidylcholine inspired polymer-lipids consisting of poly(2-methacryloyloxyethyl phosphorylcholine)-conjugated lipids (PMPC-lipids) with different degrees of MPC polymerization were synthesized. The protein adsorption, with focus on complement proteins onto the PMPC-lipids were evaluated, indicating that PMPC-lipids with a longer polymer chain are better to suppress protein adsorption.

In paper V fragmented heparin-conjugated (fHep) lipids were investigated for their potential ability to recruit complement regulators to a lipid bilayer surface for complement regulation. This study indicated that fHep-liposomes could recruit the main fluid phase regulator of the AP, factor H, as well as the coagulation regulator antithrombin from human plasma.

To conclude, the results from this thesis indicates that C3(H<sub>2</sub>O) in the fluid phase is a poor initiator of the AP, however contact activated C3 could be targeting activation pathway for the AP. We could also successfully synthesize PMPC-lipids and fHep-lipids for protein suppression and potential complement regulation on coated liposomes.

**Keywords:** immunology, complement system, C3, liposomes, polymer-lipids

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*To my family*

*“We look up at the same stars and see such different things”*

*George R.R. Martin*



# List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

- I. Fromell, K., **Adler, A.**, Åman A., Manivel, V.A., Huang, S., Dührkop, C., Sandholm, K., Ekdahl, K.N., and Nilsson, B. (2020) Assessment of the role of C3(H<sub>2</sub>O) in the alternative pathway. *Frontiers in Immunology*, 11:530
- II. Manivel, V.A., **Adler, A.**, Hamad, O., Duehrkop, C., Ebert, R., Teramura, Y., Ekdahl, K.N., Fromell, K.<sup>§</sup> and Nilsson, B.<sup>§</sup>. A targeted binding and activation of native C3 without proteolytic cleavage induced by contact with biosurfaces. Manuscript
- III. **Adler, A.**<sup>§</sup>, Inoue, Y.<sup>§</sup>, Sato, Y., Ishihara, K., Ekdahl, K.N., Nilsson, B., and Teramura, Y. (2021) Synthesis of poly(2-methacryloyloxyethyl phosphorylcholine)-conjugated lipids and their characterization and surface properties of modified liposomes for protein interactions. *Biomaterials Science*, 9(17):5854-5867
- IV. **Adler, A.**, Inoue, Y., Ekdahl, K.N., Baba, T., Ishihara, K., Nilsson, B., and Teramura, Y. (2021) Effect of liposome surface modification with water-soluble phospholipid polymer chain-conjugated lipids on interaction with human plasma proteins. *Journal of Materials Chemistry B*, 10(14):2512-2522
- V. **Adler, A.**, Fritsch, M., Fromell, K., Leneweit, G., Ekdahl, K.N., Nilsson, B., and Teramura, Y. Regulation of innate immune system by fragmented heparin-conjugated lipids on lipid bilayer membranes. Manuscript

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## Contribution to the included publications

- I. Planned, carried out, analyzed and assisted in the interpretation of the results together with the first and last author for the experiments related to C3(H<sub>2</sub>O) generation, characterization, convertase formation in fluid phase as well as complement activation on surfaces. Contributed to writing the manuscript.
- II. Contributed to study conception and design, as well as major contribution to analysis, interpretation and compilation of the data, and writing the manuscript.
- III. Planned and performed the experiments related to protein interaction with the polymer-lipids, as well as imaging of liposomes together with the last author. Major contributions to liposome fabrication and characterization, as well as assisting in analysis and interpretation of all the results included in the manuscript. Took lead in writing the manuscript together with the last author.
- IV. Designed the project together with the last author. Major contribution to data collection for experiments involving liposome production, characterization and protein analysis. Took lead in data interpretation and compilation, and writing the manuscript together with the last author.
- V. Designed the project together with the last author. Major contribution to data collection, interpretation and compilation for all experiments, as well as writing the manuscript with assistance from the last author.

## Additional work outside the scope of the thesis

1. Ekdahl, K.N., Mohlin, C., **Adler, A.**, Åman, A., Manivel, V.A., K, Sandholm, K., Huber-Lang, M., Fromell, K., Nilsson, B. (2019) Is generation of C3(H<sub>2</sub>O) necessary for activation of the alternative pathway in real life? *Molecular Immunology*, 114(353-361)
2. Messerer, D.A.C., Vidoni, L., Erber, M., Stratmann, A.E.P., Bauer, J.M., Braun, C.K., Hug, S., **Adler, A.**, Ekdahl, K.N., Nilsson, B., Barth, E., Radermacher, P., Huber-Lang, M. (2020) Animal-free whole blood sepsis model to study changes in innate immunity. *Frontiers in Immunology*, 11:571992
3. **Adler, A.**, Manivel, V.A., Fromell, K., Teramura, Y., Ekdahl, K.N., Nilsson, B. (2022) A robust method to store complement C3 with superior ability to maintain the native structure and function of the protein. *Frontiers in Immunology*, 13:891994
4. Suzuki, H., **Adler, A.**, Huang, T., Kuramochi, A., Ohba, Y., Sato, Y., Nakamura, N., Manivel, V.A., Ekdahl, K.N., Nilsson, B., Ishihara, K., Teramura, Y. (2022) Impact of spontaneous liposome modification with phospholipid polymer-lipid conjugates on protein interactions. *Science and Technology of Advanced Materials*, 23(1):845-857



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# Abbreviations

$\alpha_2M$	$\alpha_2$ -macroglobulin
ABC	Accelerated blood clearance
ANA	Anaphylatoxin domain in C3
AP	Alternative pathway
ARGET-ARTP	Activator regenerated by electron transfer-atom transfer radical polymerization
AT	Antithrombin
BCA	Bicinchonic acid
BSA	Bovine serum albumin
C1-9	Complement protein 1-9
C3*	Contact activated C3
C3(H <sub>2</sub> O)	C3 in which the thioester has been broken by water
C3(met)	C3 treated with methylamine
C3(KSCN)	C3 treated with potassium thiocyanate
C3(H <sub>2</sub> O)Bb	AP C3 convertase
C3(x)	C3 where the x is the reactive nucleophile or chaotropic agent
iC3b	C3b cleaved by factor I
C3bBb	AP C3 convertase
C3bBbP	AP C3 convertase stabilized by properdin
C3BbC3bP	AP C5 convertase
C4b2b	CP and LP C3 convertase
C4bC2bC3b	CP and LP C5 convertase
C4BP	C4b-binding protein
C5b-9	Membrane attack complex
C <sub>12</sub> -SH	1-dodecanethiol
C <sub>12</sub> -SAM	C <sub>12</sub> -self assembled monolayer
C14	1,2-Dimyristoyl-sn-glycerol
C16	1,2-Dipalmitoyl-sn-glycerol
C18	1,2-Distearoyl-sn-glycerol
Ca <sup>2+</sup>	Calcium ion
CARPA	Complement activation-related pseudoallergy
CP	Classical pathway
CR1-3	Complement receptor 1-3
Cu <sup>2+</sup>	Copper ion
CUB	Uegf,B domain in C3

$\Delta D$	Change in dissipation
$\Delta f$	Change in frequency
DAF	Decay acceleration factor
DAMPs	Damage-associated molecular patterns
DLS	Dynamic light scattering
DPPC	1,2-Dipalmitoyl-sn-glycero-3-phosphocholine
DPPE	1,2-Dipalmitoyl-sn-glycero-3-phosphoethanolamine
DPPG	1,2-Dipalmitoyl-sn-glycero-3-phosphoglycerol
DTT	Dithiothreitol
EDTA	Ethyledinitrilotetraacetic acid
EGTA	Ethylene glycol-bis( $\beta$ -aminoethyl ether)-N,N',N'-tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EPR	Enhanced permeability and retention
Fc-region	Fragment crystallizable region
fHep	Fragmented heparin
FITC	Fluorescein isothiocyanate
F/T	Freeze/thaw
HRP	Horseradish peroxidase
kDa	Kilo Dalton
KnC	Lysine (K), numbers (n), and cysteine (C)
KSCN	Potassium thiocyanate
LC-MS/MS	Liquid chromatography with tandem mass spectrometry
LNK	Linker region in C3
LP	Lectin pathway
IEX	Ion exchange chromatography
Ig	Immunoglobulin
mAb	Monoclonal antibody
MAC	Membrane attack complex
MASP	Mannose-binding lectin-associated serine protease
MBL	Mannose binding lectin
MCP	Membrane cofactor protein
MeO-PEG	Methoxy-polyethylene glycol
Mg <sup>2+</sup>	Magnesium ion
MPC	2-methacryloyloxyethyl phosphorylcholine
MG	Macroglobulin-like domain in C3
NaCl	Sodium chloride
NH <sub>2</sub>	Amine
OH	Hydroxyl
PAMPs	Pathogen-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PDI	Polydispersity index
PEG	Polyethylene glycol
PMNs	Polymorphonuclear leukocytes

PMPC	Poly(2-methacryloyloxyethyl phosphorylcholine)
PRRs	Pattern recognition receptors
QCM-D	Quartz crystal microbalance with dissipation monitoring
RES	Reticuloendothelial system
RT	Room temperature
sC5b-9	Soluble membrane attack complex
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography
SiO <sub>2</sub>	Silicon dioxide
TED	Thioester domain in C3
TRAP6	Thrombin activating peptide-6
VBS <sup>++</sup>	Veronal-buffered saline with Ca <sup>2+</sup> and Mg <sup>2+</sup>



# Thesis outline

This thesis is based on five papers, where we in detail have studied how the alternative pathway of complement is initiated, as well as developed and evaluated novel liposomal coatings, in regards to immune suppression and regulation. To put the present investigations into a context, the thesis is structured to give a general description of the immune system followed by a more in-depth theoretical background to the field of complement, with focus on up-to-date plausible mechanisms for initiation of the alternative pathway. Thereafter, the concept of liposomes is introduced, and this section also highlights the challenges with current liposomal coatings, and why new liposomal coating strategies are needed. The present investigations are then described one by one, where the aims, main methods used, and the obtained results are discussed in separate sections for each individual paper. Lastly, all the work included in this thesis is summarized together with concluding remarks and future perspectives.



# Introduction

## The human immune system

The human immune system is a highly complex biological system consisting of a network of different organs, cells and proteins that work together to defend the body from foreign invaders and altered host cells by self-nonself discrimination. The self-nonself discrimination is based on the ability of the immune system to recognize distinctive molecular patterns on non-/altered-self surfaces and distinguish them from self-surfaces. This is a key aspect of the immune system, as it allows for elimination of the threat and maintaining host homeostasis while avoiding damaging healthy host tissue (1). The human immune system can be divided into two main parts, the innate and the adaptive immune system (2).

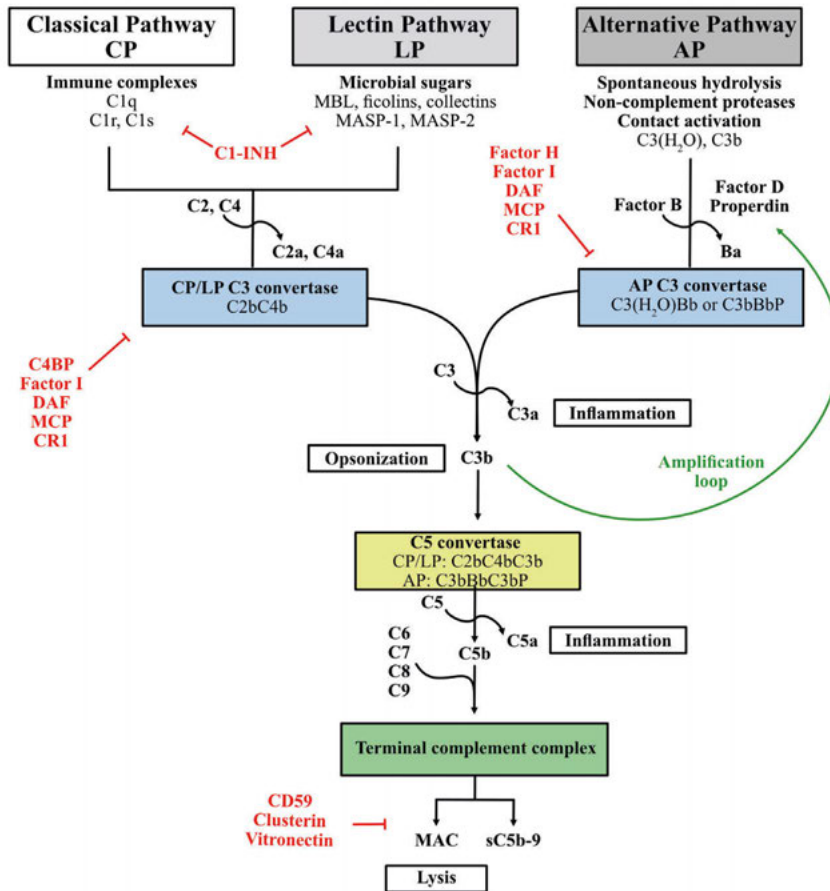
Humans are born with the innate immune system, which by many is referred to as non-specific immunity, and it is the first line of defense against foreign invaders. It consists of barriers, e.g., the skin and mucosal layers, soluble mediators such as the complement system, membrane bound receptors, and innate immune cells including; natural killer cells, innate lymphoid cells, dendritic cells and phagocytes. The innate immunity is basically “pre-programmed” from birth, and is mainly based on pattern recognition receptors (PRRs), that recognizes common pathogenic structures, so called pathogen-associated molecular patterns (PAMPs). The PRRs also recognizes damage-associated molecular patterns (DAMPs), which are endogenous molecules expressed and/or released by damaged or dying cells (3). It reacts fast to non-/altered-self, and in a similar fashion every time, even upon repeated exposures to the same foreign invader.

The adaptive immune system, also known as acquired immunity, involves specialized immune cells, T- and B-cells, which helps protecting the body from specific invaders. It is based on antigen-specific receptors which are “acquired” over the lifetime as the body is exposed to foreign antigens. For example, B-cells produces specific antibodies against the antigens expressed by the foreign invader. The adaptive immunity is trained to become better at recognizing specific pathogenic structures upon repeated exposures. When the adaptive immunity encounters a foreign invader for the first time, it is quite slow in mobilizing an immune response. However, upon repeated exposures

to that specific foreign invader, it generates a successively more specific, faster and powerful immune response against that specific antigen, due to the generation of memory T- and B-cells (1). The innate and adaptive immune system have distinctive functions and roles, however they are linked together, for example via the complement system, to collectively protect the body from foreign invaders.

## The complement system

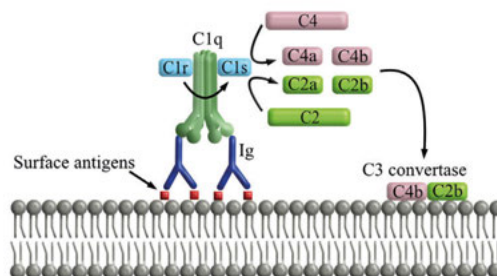
The complement system is a central part of the innate immunity, operating in tissues, plasma, and intracellularly (4). It was discovered in the 1890s when it was demonstrated that a heat-labile substance in human plasma “complemented”, i.e., aided, antibodies in lysing bacteria (5). And as of today, over 130 years later since its discovery, we know that the complement system is not just one substance, but a very potent, complex and tightly regulated cascade system consisting of over 50 different plasma and cell surface proteins (6, 7). The complement system can be initiated via three distinct pathways: the classical pathway (CP), the lectin pathway (LP) and the alternative pathway (AP), where complement protein 3 (C3) plays a central role as it supports the activation of all the pathways (8, 9). After the initial activation, often triggered by non/altered-self surfaces, the pathways merge in the downstream steps of the terminal pathway. As the cascade proceeds the signal is amplified, and generates the same effector molecules, which are involved in elimination of foreign invaders via opsonization, inflammation, and lysis (Figure 1) (2).



**Figure 1.** Simplified overview of the complement system and the main complement regulators. The complement system can be initiated via three distinct pathways: CP, LP and AP. CP is generally initiated by C1q binding to antigen-antibody complexes. LP is initiated by MBL/ficolin/collectin binding to microbial sugars such as mannose. AP can be initiated by spontaneous hydrolysis of C3 to C3(H<sub>2</sub>O), without proteolytic cleavage, or by non-complement protease cleavage of C3 to C3b. CP and LP generate the C3 convertase C4bC2b, while AP generates C3(H<sub>2</sub>O)Bb and the amplification C3 convertase C3bBbP. The C3 convertases can cleave C3 into the anaphylatoxin C3a and opsonin C3b, and subsequently generate the CP/LP C5 convertases C4bC2bC3b and AP C5 convertase C3bBbC3bP. The C5 convertases can cleave C5 into the anaphylatoxin C5a and C5b. C5b together with C6, C7, C8 and multiple copies of C9 can generate the MAC complex or the fluid phase counterpart sC5b-9. The complement system is tightly regulated by different fluid phase and membrane bound regulators. The main complement regulators are illustrated in red. C1-INH; C1-inhibitor, C4BP; C4b-binding protein, CR1; complement receptor 1, DAF; decay acceleration factor, MBL; mannose binding lectin, MASP1-2; mannose-binding lectin-associated serine protease 1-2, MCP; membrane cofactor protein, and P; properdin.

## Classical pathway

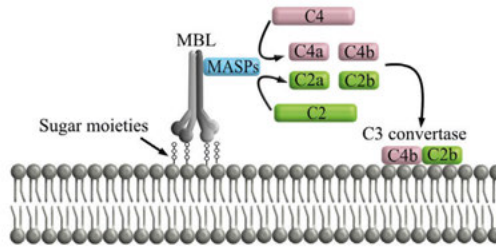
The CP was the first pathway to be discovered, and is mainly activated by the binding of C1 to antigen-antibody complexes, thereby providing a link between the innate and adaptive immunity (10). It can also be initiated in an antibody independent way, by e.g., pentraxins such as C-reactive protein and serum amyloid P component bound to pathogenic surfaces or damaged cell membranes, which then are recognized by C1. C1 is a multiprotein complex and consists of the pattern recognition molecule C1q, and two copies of each of the serine proteases C1r and C1s (7). C1q binds to the fragment crystallizable (Fc)-regions of immunoglobulin G (IgG) and IgM. When C1q binds to clusters of antibody Fc-regions it undergoes conformational changes promoting autocatalytic enzymatic activity in C1r. C1r can then cleave and activate C1s. Once C1s is activated it proteolytically cleaves C4 into C4a and C4b, and C2 into C2a and C2b. C4b and C2b forms the C3 convertase C4bC2b, previously known as C4bC2a (11), on the surface of the pathogen (Figure 2). The C3 convertase cleaves C3 into C3a, an inflammatory mediator, and C3b, an opsonin, and initiates amplification and downstream effector functions of the complement system including inflammation, opsonization, phagocytosis, B-cell stimulation and lysis of the target cell (2, 12).



**Figure 2.** Overview of the initiation of classical pathway of complement. C1q binds to Ig on pathogenic surfaces and undergo conformational changes which activates C1r and C1s. C1s cleaves C4 into C4a and C4b and C2 into C2a and C2b. C4b and C2b binds to the surface and forms the C3 convertase C4bC2b. Illustration adapted from Afshar-Kharghan (13). Ig; immunoglobulin.

## Lectin pathway

The LP is initiated via the binding of pattern recognition molecules like mannose-binding lectin (MBL), ficolins and collectins to microbial sugars, such as mannose, N-acetylglucosamine or fucose. The binding activates mannose-binding lectin-associated serine proteases (MASPs), which then cleaves C4 and C2 to initiate the downstream proteolytic steps identical to the CP (Figure 3) (14).



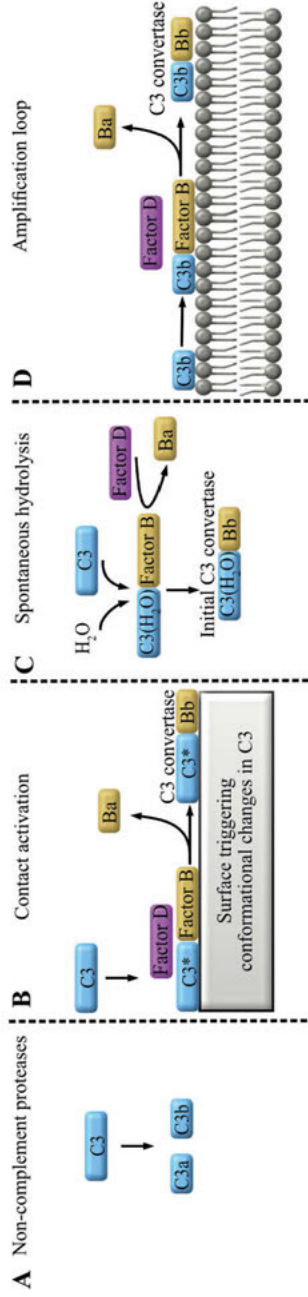
**Figure 3.** Overview of the initiation of lectin pathway of complement. The binding of MBL to microbial sugars activates MASPs. MASPs cleave C4 into C4a and C4b and C2 into C2a and C2b. C4b and C2b binds to the surface and form the C3 convertase C4bC2b, same C3 convertase as formed by the classical pathway but through a different initiation pathway. Illustration adapted from Afshar-Kharghan (13). MASPs; mannose-binding lectin-associated serine proteases, and MBL; mannan binding lectin.

## Alternative pathway

While CP activation is specific for antibodies and LP is activated by specific sugar moieties, Pillemer *et al.*, suggested in the 1950s that properdin, the only known positive regulator of complement (15, 16), was the specific initiator of the AP (17). Later in the 1970s Lachmann *et al.*, put forward the tickover theory, in which the AP is theorized to be spontaneously activated. Lachmann with collaborators suggested that this spontaneous activation of AP could be triggered by non-complement proteases and/or interactions with surfaces (18, 19, 20). In the 1980s Pangburn with coworkers discovered that native C3 can undergo slow spontaneous thioester hydrolysis in solution and generate a “C3b-like” molecule, and named it C3(H<sub>2</sub>O). C3(H<sub>2</sub>O) is generated in solution due to nucleophilic attack by water molecules to the internal thioester in native C3, which leads to conformational changes within the molecule without proteolytic cleavage and release of the C3a-peptide (21, 22). C3(H<sub>2</sub>O) in solution, in the presence of magnesium ions (Mg<sup>2+</sup>), can bind factor B, which then can be cleaved by factor D to Ba and Bb. This results in the formation of an initial fluid phase C3 convertase, C3(H<sub>2</sub>O)Bb, which can initiate AP by cleaving C3 to C3a and C3b (2, 22, 23). However, the initiating mechanism of the AP is still under debate. Today the main theories of AP activation are; (a) cleavage

of C3 into C3a and C3b by non-complement proteases (Figure 4A) (24, 25). (b) By specific or non-specific contact-activation of C3 at different surface interfaces, which leads to conformational changes within C3 to a “C3b-like” surface bound molecule which can form an initial surface bound C3 convertase (Figure 4B) (26, 27). (c) Spontaneous thioester hydrolysis of native C3 to C3(H<sub>2</sub>O), which then can form an initial fluid phase C3 convertase. This C3 tickover mechanism is the main AP activation described in most textbooks (Figure 4C) (2, 12). The potential role of properdin to act as an initiator of AP activation has been hard to resolve, and it has been a controversial topic since its discovery in the 1950s by Pillemer *et al.* (28).

C3b from all the three activation pathways can bind covalently and opsonize foreign surfaces, thus creating a positive feedback loop for the AP by generating an amplifying C3 convertase (C3bBb), which in its turn can cleave C3 to C3a and C3b thereby further amplifying the signal (Figure 4D) (2). The half-life of C3bBb is around 90 s (29). While properdin is not essential for convertase AP activation, the association with properdin stabilizes the C3 convertase, and can lead to up to a 10-fold increase in its half-life (16). This is important to generate an efficient complement response. The CP and LP are very efficient activators of complement. However, the AP is a major contributor in the amplification of the complement cascade, and it has been proven to generate most of the C3b and around 80-90 % of the terminal pathway complex C5b-9 (30, 31).

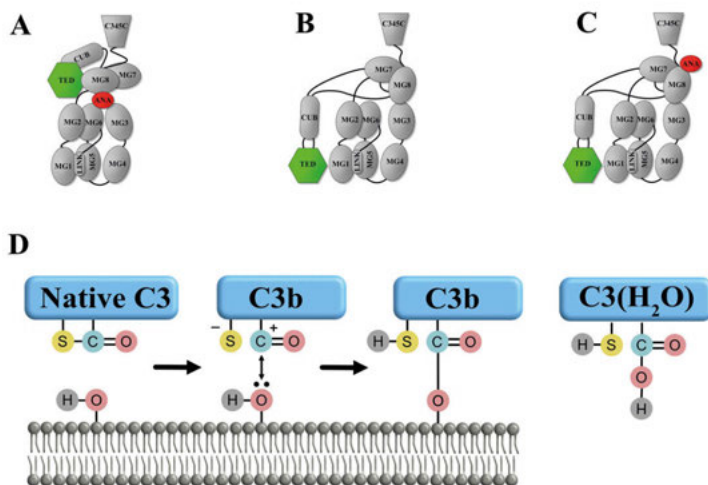


**Figure 4.** Overview of the current suggested mechanisms of initiation and amplification of AP. **(A)** Tickover of AP by cleavage of C3 to C3a and C3b by non-complement proteases. **(B)** Contact activation of C3 (C3\*) on various leading to conformational changes of C3 which allows it to form a surface bound C3 convertase. **(C)** Classical textbook illustration of the tickover of AP. C3 in solution is spontaneously hydrolyzed and forms C3(H<sub>2</sub>O), which binds factor B. Factor B is then cleaved by factor D to Ba and Bb. The C3(H<sub>2</sub>O)Bb forms an initial fluid phase C3 convertase which can further cleave C3 into C3a and C3b. **(D)** The AP also works as an amplification loop where generated C3b molecules by all C3 convertases formed by the CP, LP and AP have the potential to bind factor B and D to generate more C3b, thus amplifying the complement activation. Figure C and D are adapted from Afshar-Kharghan (13). AP; alternative pathway, C3\*, contact activated C3, CP; classical pathway, and LP; lectin pathway.

## C3 and C3(H<sub>2</sub>O)

C3 is a central complement protein, and was first isolated by Müller-Eberhard with coworkers in the 1960s (32). It is the most abundant complement protein in humans, with a plasma concentration between 0.7-1.2 mg/mL in healthy adults (8). It belongs to the  $\alpha_2$ -macroglobulin family ( $\alpha_2$ M), which besides C3 and  $\alpha_2$ M also includes C4 and C5 (33). C3 is a 185 kilo Dalton (kDa) large protein consisting of two chains, an  $\alpha$ -chain (110 kDa) and a  $\beta$ -chain (75 kDa) linked together by a disulfide bridge that together forms 13 distinct domains; eight macroglobulin-like (MG) domains, a linker region (LNK), an anaphylatoxin domain (ANA), a C345C domain, Uegf,B (CUB) domain, and a thioester domain (TED). The C3  $\alpha$ -chain consists of MG6 $_{\alpha}$ , MG7-8, ANA (which contains C3a) CUB, TED, and C345c domains, while MG1-MG5, MG6 $_{\beta}$  and LNK domains makes up the C3  $\beta$ -chain (33, 34, 35). The structures of native C3, C3b and C3(H<sub>2</sub>O) are illustrated in (Figure 5A-C).

Upon proteolytic activation of C3, a C3 convertase generated by any of the activation pathways, cleaves off the C3a (9 kDa) peptide from the  $\alpha$ -chain, thus generating C3b (176 kDa) (Figure 5B). This leads to conformational changes within the C3 molecule and subsequent exposure of an intramolecular thioester bond in C3b, which is hidden in a hydrophobic pocket in native C3 (35, 36). The exposed thioester bond enables for nascent C3b to bind covalently to hydroxyl (-OH) or amino (-NH<sub>2</sub>) groups on target surfaces in its immediate vicinity and contributes to the local amplification of the complement cascade (37, 38, 39) (Figure 5D). If the nascent C3b does not bind covalently, the activated acyl group (R-C=O) in the exposed thioester is rapidly inactivated (half-life  $\approx$  60  $\mu$ s) by hydrolyzation (28, 40).

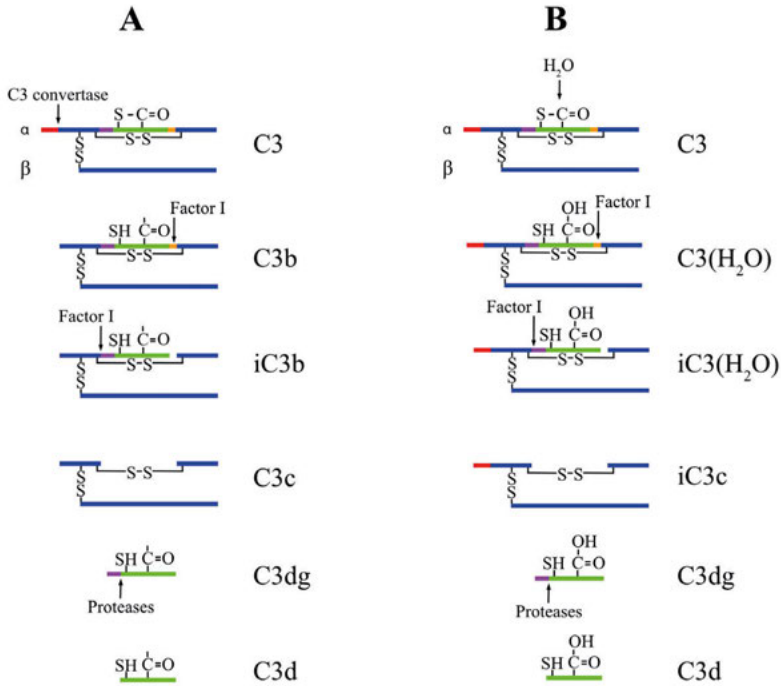


**Figure 5.** Conformation of; (A) native C3, (B) C3b, (C) C3(H<sub>2</sub>O). The C3 structures are adapted from Gros *et al.* (35). Macroglobulin-like (MG) domains, linker region (LNK), anaphylatoxin domain (ANA) (red) which contains C3a, C345C, Uegf, B (CUB) domain, and a thioester domain (TED) (green) which contains the thioester. (D) Simplified illustration of the thioester bond in C3. When C3 is cleaved into C3a and C3b, the thioester bond in nascent C3b becomes exposed and the activated acyl group is susceptible for covalent binding to hydroxyl groups (or amino groups, not shown) on foreign surfaces. If the acyl group is hydrolyzed by water, as it is in C3(H<sub>2</sub>O), it cannot bind covalently to a surface.

To further regulate the complement activation, the  $\alpha'$ -chain of C3b is inactivated by proteolytic degradation by factor I in association with co-factors in three proceeding steps, which prevents further convertase formation as well as altering the function of the protein. In two steps, the C3f (2 kDa) fragment is cleaved off from C3b thereby generating iC3b (175 kDa). iC3b cannot bind factor B, thereby blocking its ability to generate a functional C3 convertase (18, 41, 42). iC3b is further cleaved which release C3c (135 kDa) from the target bound C3dg (40 kDa) fragment. Proteases cleave C3dg into C3d (32 kDa) and C3g (8 kDa), while the C3  $\beta$ -chain (75 kDa) stays intact. The generated C3 fragments have different biological activities, and can evoke different cellular and molecular effects by interacting with various complement receptors (Figure 6A) (8).

As previously described, native C3 can undergo conformational changes without proteolytic cleavage due to slow spontaneous thioester hydrolysis in solution, thus generating C3(H<sub>2</sub>O). C3(H<sub>2</sub>O) has a “C3b-like” conformation, but with an intact  $\alpha$ -chain. The rate of hydrolysis of C3 has been estimated to 0.2-0.4 % per hour in plasma (22, 43, 44). Since the activated acyl group in the exposed thioester is hydrolyzed by water, C3(H<sub>2</sub>O) cannot bind covalently to

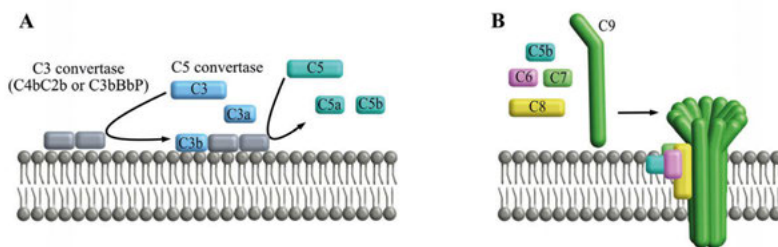
surfaces (28). C3(H<sub>2</sub>O) is also susceptible by degradation by factor I in the presence of co-factors such as factor H (Figure 6B) (23). In contrast to C3b, C3(H<sub>2</sub>O) is a hemolytically inactive molecule, thus it cannot alone activate the complement cascade all the way to the terminal pathway (21, 22).



**Figure 6.** Schematic illustration of the activation and degradation of (A) C3 and (B) C3(H<sub>2</sub>O), 185 kDa. The fragments represent: red = C3a, 9 kDa, blue = C3c, 135 kDa, purple = C3g, 8 kDa, green = C3d with thioester group, 32 kDa, and orange = C3f, 2 kDa. Adapted from Huber-Lang *et al.* (24).

## Terminal pathway

The continuous deposition of C3b by the C3 convertases generates a high local surface density of C3b on the target surface. The C3b associates with the C3 convertase, which favors generation of a C5 convertase (C4bC2bC3b/C3bBbC3bP) (Figure 7A). Thus, the convertase shifts specificity from C3 to C5 (45, 46). The C5 convertase cleaves C5 into the potent anaphylatoxin C5a, and C5b which initiates the assembly of the membrane attack complex (MAC, C5b-9) or, its fluid phase counterpart, sC5b-9. The MAC and sC5b-9 consist of C5b, C5, C6, C8 and multiple copies of C9, and are responsible for the downstream complement effects in the terminal pathway (Figure 7B). The incorporation of MAC into a cell membrane often leads to lysis of the target cell (2).



**Figure 7.** Overview of the terminal pathway of complement with (A) the generation of a C5 convertase which (B) proceeds to initiate the assembly of the membrane attack complex. Illustration adapted from Afshar-Kharghan (13).

## Complement regulators

The complement system is a very potent system and is tightly regulated by both surface bound and soluble regulators to avoid responses that produce excessive complement-mediated damage of self-tissues. Dysregulation or over-activation in the complement cascade, caused by e.g., inherited complement protein deficiencies or defect complement regulators, is associated with a number of different diseases. For example, defects in factor H have been linked to age-related macular degeneration and atypical hemolytic uremic syndrome (47). There are regulators operating at all levels in the complement cascade, from blocking complement activation to preventing MAC formation (48). For example, C1-inhibitor (C1-INH) blocks CP and LP activation, and factor I, factor H, decay acceleration factor (DAF), membrane cofactor protein (MCP), C4b-binding protein (C4BP), and complement receptor 1 (CR1) interfere with the C3 convertases, while CD59, clusterin and vitronectin regulate the terminal pathway (48, 49) (Figure 1). The work in this thesis is mainly focused on the complement regulators factor I, factor H and C4BP.

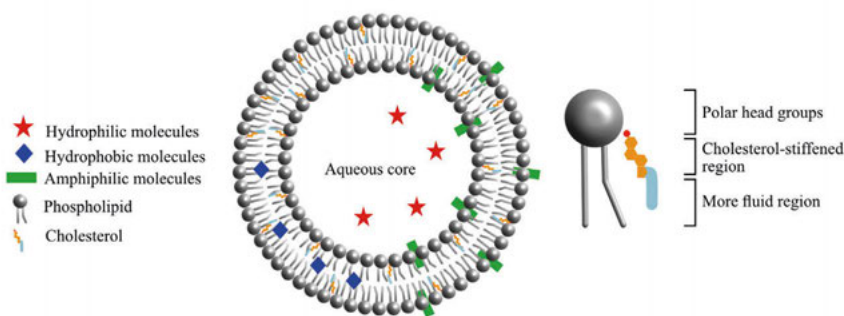
Factor I is a serine protease which proteolytically cleaves C3b, C3(H<sub>2</sub>O) and C4b by using cofactors such as C4BP and factor H. Factor I interferes with the generation of both the CP/LP and AP C3 convertases. C4BP is the main fluid phase regulator of the CP and LP. It binds to C4b and can displace C2 from the C3 convertase (2, 50, 51). Factor H is not only a cofactor for factor I-mediated degradation of C3b. It is also an important fluid phase regulator of the AP as it competes with factor B for C3b binding, as well as it can accelerate the decay of the C3 convertases by dissociate factor Bb from the AP C3 convertase (52). Factor H is an important regulator to restrict C3b degradation to host cells. It can distinguish between self and non-self surfaces by specific binding to self-structures as glycosaminoglycans, like heparan sulfate (53) and sialic acid glycans (54, 55), which are abundant on healthy host cell surfaces. However, some pathogens have developed strategies to mimic the host self-

markers and “hijack” the hosts complement regulators by recruiting them to their own surface to evade recognition by the complement system (56, 57).

As previously mentioned, complement can clear foreign invaders, however the complement system is also very important in the clearance of immune complexes, apoptotic-, necrotic-, and modified self-cells (e.g., cancer cells), in a non-inflammatory manner (7). The lipid bilayer membrane of a cell is asymmetric, meaning that the lipids in the inner and outer layers are different. The outer layer mainly contains glycolipids and derivatives of phosphatidylcholine, whereas the inner layer, facing the cytosol, consists of derivatives of negatively charged (anionic) phospholipids like phosphatidylethanolamine and phosphatidylserine (58). Upon e.g., platelet activation or cell apoptosis, the phospholipids “flip-flops”, thus the negatively charged phospholipids are translocated to the outer layer of the membrane, resulting in a very different cell morphology. Thus, the surface is recognized as an altered-self surface, on which complement activation can take place (48).

## Liposomes

Bangham with colleagues first described liposomes, which are artificial lipid vesicles, in the 1960s when they observed that phospholipids in an aqueous solution can form spherical bilayer membranes (Figure 8) (59). In general, liposomes are made up of lipids, e.g., phospholipids, and cholesterol, which are amphiphilic molecules, having one hydrophobic and one hydrophilic end. In an aqueous solution the phospholipids will spontaneously orient themselves in the most energetically favorable structure, a lipid bilayer, which is the main structure of cell membranes (58). The lipids can be unsaturated or saturated, meaning that in unsaturated lipids one of the hydrocarbon tails have at least one *cis*-double bond, which creates a kink in the tail. The length and saturation state of the lipids hydrocarbon tails, as well as cholesterol content affects membrane fluidity as it influences how the lipids in the membrane are packed against each other (58). Due to the structural similarities between liposomes and biological membranes, liposomes are a useful tool in research to mimic a simple cell membrane. Although liposomes lack the complexity of biological membranes, they can be used to study e.g., lipid-protein interactions (60).



**Figure 8.** Schematic model of a liposome. Illustration adapted from Guimaraes *et al.* (61).

## Applications of liposomes

Liposomes have an internal aqueous compartment entrapped by either a single or multiple concentric lipid bilayer(s), and range in size from around 30 nm to several  $\mu\text{m}$  in diameter (60). During the 1970s the use of liposomes as drug carriers was suggested as it was demonstrated that liposomes could entrap hydrophilic drugs within their aqueous core (62, 63). The amphiphilic nature of the phospholipid and cholesterol molecules also gives liposomes the ability to carry other amphiphilic molecules, as well as hydrophobic compounds in the lipid bilayer (61). And as of today, thanks to the biodegradable and biocompatible properties of liposomes, and their ability to encapsulate a broad range of different molecules, they are not only used as a drug delivery system (64) but also by e.g., the food-, agricultural-, and the cosmetic industry (65).

Liposomal drug encapsulation provides a physical barrier between the encapsulated drugs and the biological environment, which is especially desired in administration of cytotoxic drugs. Liposomal encapsulation protects drugs from premature degradation, as well as it reduces off target toxicity. It can also improve tumor targeting via enhanced permeability and retention (EPR) effect or by attaching functional ligands to the liposome surface. The EPR effect of liposomes is mainly ascribed to highly permeable vessels (“enhanced permeability”) and defective lymphatic drainage (“enhanced retention”) which exist in some but not all tumors (66), thus enabling accumulation of liposomes and other macromolecules in a tumor-selective manner (67, 68). A size of 100-200 nm is considered the optimal size range for liposomes to exploit the EPR effect (69). Long-circulating liposomes in the bloodstream is also necessary to obtain efficient liposome-based drug delivery systems. However, since the immune system protects the body from non-self surfaces, liposomes included, unprotected liposomal drug delivery systems are recognized, opsonized as well as degraded immediately after injection by the innate immune system,

where the complement system plays a major role (70, 71, 72, 73). Therefore, since the discovery of liposomes several coating strategies have been developed, and avoiding complement activation is one of the keys to obtain long-circulating liposomes.

## Polymers

### Polyethylene glycol

A popular strategy to make various biomaterials, such as liposomes, biocompatible is to coat them with polymers (74). Biomaterials are materials intended to interact with a biological system, and the term biocompatibility describes the property of a material being compatible with living tissue. Thus, when a biocompatible biomaterial is exposed *in vivo* to the body or bodily fluids it should ideally not generate a toxic or immunological response (75). The gold standard surface modification of liposomes as of today is using polyethylene glycol (PEG), which is a polymer with repeating units of ethylene glycol. PEG is a flexible, uncharged and water-soluble synthetic polymer, which e.g., can be conjugated to the head-group of phospholipids. PEGylation of liposomes have been shown to increase their circulation time, reduce protein adsorption and immune activation, and the subsequential removal by the reticuloendothelial system (RES) (76, 77). PEGylation increase liposome stability by preventing aggregation, as well as reducing protein adsorption through steric hindrance. PEG also binds to water molecules, thus a hydration layer is formed around the liposomes, which also reduces protein binding to the surface (78, 79). In 1995 Doxil® got regulatory approval in the US to be used to treat ovarian cancer and AIDS-related Kaposi's sarcoma, thus it was the first approved PEGylated liposomal drug delivery system (80, 81). Today, there are a number of approved liposome-based formulations both with and without PEGylation, and several formulations are currently being evaluated in clinical trials (64). However, Dams *et al.*, were first to report that PEGylated liposomes are rapidly cleared from the circulation upon repeated injections in rats (82). This phenomenon is called accelerated blood clearance (ABC) effect, which is attributed to the generation of anti-PEG IgM (83, 84). Its relevance for human liposomal drug delivery system is still under debate. Nevertheless, developing new alternative liposomal coatings as well as cell surface coatings which can suppress immune activation is of interest.

### Poly(2-methacryloyloxyethyl phosphorylcholine)

Taking inspiration from nature, biomimetic materials such as biocompatible polymers which mimic cell membranes have been designed (85). This concept of molecular mimicking of materials with membrane inspired properties, more

specifically the phospholipids of the outer cell membrane layer, led Nakabayashi and Ishihara to design the synthetic phosphatidylcholine inspired polymer 2-methacryloyloxyethyl phosphorylcholine (PMPC) (86). MPC polymers are strongly hydrophilic and retain water, which suppresses protein adsorption and also inhibits cell adhesion to MPC polymer-coated materials in contact with plasma or whole blood (87). MPC is a methacrylate bearing a phosphorylcholine group in the side chain and this structure is similar to the phospholipid polar groups in biological membranes. MPC has a neutral charge under physiological conditions (88). EVAHEART® a left ventricular assist device, Endeavor® a cardiovascular stent, and Proclear® soft contact lenses are all examples of medical devices which have all been successfully coated with MPC-based polymers (89). In this thesis we investigate if PMPC-lipid modified liposomes could be an alternative liposome coating strategy to PEGylation (90).

## Surface recruitment of complement regulators

PEG-lipids suppress immune activation on phospholipid surfaces. However, they cannot regulate the complement system. There are a couple of studies published where the goal has been to recruit complement inhibitors to the surface of e.g., cells and liposomes to mimic the complement evasion strategy of some bacteria. Peptides have been used to coat biomaterials to regulate complement activation on surfaces, for example the 5C6-peptide, which can recruit factor H to the surface (52, 91), and streptococcal M-protein derived peptides to recruit C4BP (92).

Heparin is another well-established coating strategy to improve the hemocompatibility of e.g., medical devices (93), cells (94, 95) and liposomes (96). Heparin is a linear polysaccharide, mainly made up of repeating units of highly sulfated disaccharides (93, 97). Structurally heparin is related to the cell surface proteoglycan heparan sulfate which is expressed on the vascular endothelium surface as a glycocalyx (98). Heparin is naturally found intracellularly in mast cell granules. Cell surface modification with heparin has been used to improve graft survival during cell transplantation (99). The idea is that the heparin coating protects the cell surface by mimicking the glycocalyx of the cells since transplanted cells are damaged or destroyed due to hemoincompatibility when therapeutic cells are infused into patients' blood stream (100, 101). Heparin can bind and enhance the activity of the serine protease inhibitor antithrombin (AT). AT is one of the most important regulators of coagulation as it can inhibit several of the coagulation cascade enzymes, such as thrombin and factor Xa (102).

Our group have previously synthesized fragmented heparin-lipids (fHep-lipids) with the aim to mimic the glycocalyx of cells. The heparin-lipids consists of different numbers of fragmented heparin chains (one to nine) which are conjugated to a PEG-lipid (103). Besides the ability of heparin to bind AT, complement regulators factor H and C4BP have heparin binding sites, thus in theory allowing for surface recruitment of these regulators by the heparin (53, 104, 105, 106). This could be a potential strategy to regulate complement activation on the surface of modified liposomes and cells.

# Aims

The overall objective of this thesis was to further investigate mechanisms of AP activation, as well as to develop new biocompatible liposome coatings which can evade the complement system.

The specific aims of each paper were:

- I                Investigate how effective C3(H<sub>2</sub>O) is to form an initial fluid phase AP C3 convertase.
- II                Investigate contact activation of C3 on various artificial and lipid surfaces as a potential targeted AP activation pathway.
- III                Synthesize synthetic phosphatidylcholine inspired polymer-lipids consisting of poly(2-methacryloyloxyethyl phosphorylcholine)-conjugated lipids (PMPC-lipids) with different degrees of MPC polymerization to use as a liposomal coating.
- IV                Fabricate liposomes with different PMPC-lipids and study the interaction between human plasma proteins and the modified liposomes.
- V                Investigate if fHep-lipids can recruit complement regulators factor H and C4BP to modified lipid bilayer surfaces, using liposomes, for complement regulation.

# Materials and methods

In this section the main experimental concepts included in the thesis are discussed. In the materials and methods section of each paper the methodological descriptions and reagents used are more comprehensively described.

## Generation of C3(H<sub>2</sub>O)

C3(H<sub>2</sub>O) is generated in the body at a slow rate through spontaneous hydrolysis of the intramolecular thioester bond. The generation of C3(H<sub>2</sub>O) from native C3 can be facilitated by chaotropic agents (e.g., potassium thiocyanate, (KSCN), and guanidine), nucleophiles (e.g., methylamine and ammonia) (21, 107), at the interface of gas (44) or by slow freezing and thawing (F/T), from -20 °C to room temperature (RT) (108). In paper I and II C3(H<sub>2</sub>O) was generated by either treating native C3, purified in-house according to Hammer et al., (109), with 0.33 M KSCN, 0.2 M methylamine and/or by slow F/T.

## Size exclusion chromatography (I)

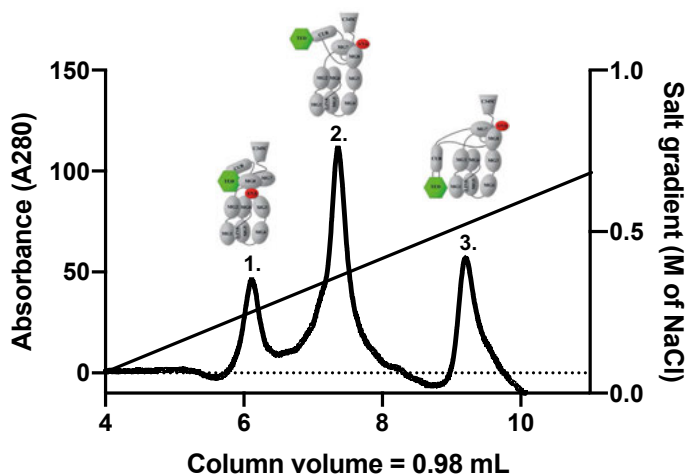
Size exclusion chromatography (SEC) can be used to separate molecules of different size. The SEC column is packed with porous resin beads. When a sample is added to the SEC column, large molecules are unable to enter the beads and will elute first. Whereas small molecules can diffuse into the pores of the beads thus eluting after the large molecules due to the increased residence time in the column (110). In paper I SEC (Enrich™ SEC 650 10 × 300 Column coupled to an NGC™ Chromatography System (Bio-Rad, USA)), was used to investigate if specific antibodies formed complexes with methylamine treated C3 (C3(met)). Briefly, 0.5 μM F/T C3(met) (185 kDa) was pre-incubated with 0.25 μM in-house generated monoclonal antibody (mAb) anti-C3a 4SD17.1 (150 kDa) (111, 112) in Veronal-buffered saline (VBS<sup>++</sup>, 5 mM sodium barbiturate, pH 7.4; 145mM sodium chloride (NaCl); 0.15 mM calcium ions (Ca<sup>2+</sup>); 0.5 mM Mg<sup>2+</sup>) for 60 min at 37 °C. mAb-C3 complex formation was confirmed by elution of the proteins in an earlier fraction due to its larger size (300+ kDa). C3b (0.5 μM) which lacks the C3a domain was

used as a negative control, and human fibrinogen (340 kDa) was used as a positive control for mAb-C3 complex formation.

## Ion exchange chromatography (I)

To ensure that the activity of C3(H<sub>2</sub>O) was not influenced by non-converted native C3 (with intact thioester), cation exchange chromatography (IEX) was used to separate native C3 from C3(H<sub>2</sub>O). Native C3 and C3(H<sub>2</sub>O) have the same molecular weight and cannot be separated using SEC (113, 114, 115). However, IEX can be used since their charge differ. C3(H<sub>2</sub>O) is more positively charged partially due to the rearrangement and exposure of the positively charged C3a-domain. The elution profiles of treated C3 to generate C3(H<sub>2</sub>O) have previously been described (22, 108, 113), and when separated using IEX native C3 (peak 1) elute first followed by an intermediate form of C3(H<sub>2</sub>O) (peak 2), and lastly C3(H<sub>2</sub>O) (peak 3) (Figure 9).

In paper I, a Mono S 5/50 GL column (GE Healthcare, Bio-Sciences AB, Uppsala, Sweden) connected to the NGC™ Chromatography System was used. The Mono S column was packed with negatively charged beads to capture the positively charged molecules. The flow-rate was set to 0.5 mL/min at RT and a gradient was established from 0 to 0.85 M NaCl in 20 mM phosphate buffer pH 6.8. After application of the sample, a gradient of the high salt phosphate buffer was added to the column to elute the proteins. This to elute the less positively charged molecules first at a low salt concentration, followed by the elution of positively charged molecules of increasing strength, which elutes at higher salt concentrations (116).



**Figure 9.** Schematic representation of the elution profiles of the different forms of C3 generated when treating native C3 with 0.2 M methylamine at pH 8.0 for 30 min, on a mono S column using a high salt gradient elution buffer (NaCl in 20 mM phosphate buffer pH 6.8). **Peak 1**; native C3, **peak 2**; C3(H<sub>2</sub>O) intermediate, and **peak 3**; C3(H<sub>2</sub>O) The green domain on the C3 represent the domain containing the thioester and the red domain represents C3a. NaCl; sodium chloride.

## Blood collection (I, II, IV, V)

Blood was drawn by peripheral venipuncture from healthy human volunteers who had not received any medication for a minimum of 10 days prior to donation. Ethical approval for the use of human blood was obtained from the regional ethics board of Uppsala (diary no 2008/264). The blood was collected in Vacutainers® containing either ethylenedinitrilotetraacetic acid (EDTA) or lepirudin. EDTA is a chelating agent which binds Ca<sup>2+</sup> and Mg<sup>2+</sup>. Thus, all Ca<sup>2+</sup> and Mg<sup>2+</sup>-dependent reactions will be blocked in EDTA plasma, including all the complement pathways (49). Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) can be added together with Mg<sup>2+</sup> to specifically block Ca<sup>2+</sup>-dependent reactions, such as the CP and LP, while the AP remains active (117). The anticoagulant lepirudin is a thrombin inhibitor, and does not affect the complement system like other anticoagulants. Therefore, the complement system remains active in lepirudin plasma (118). The collected blood samples were subjected to different centrifugation steps to obtain plasma or to isolate specific cells. EDTA (4 mM) and lepirudin (50 µg/mL) plasma were prepared by centrifugation of collected human whole blood at 2000-2500 × g, for 15 min at RT. The EDTA plasma was stored at -80 °C until use, while lepirudin plasma was used fresh.

## Cell isolation

Platelet rich plasma (PRP) was prepared by centrifugation of EDTA (4 mM) or lepirudin (50 µg/mL) treated human whole blood, at  $150 \times g$  for 15 minutes at RT. To further isolate the platelets, they were pelleted and washed in Tyrode's buffer (119). Platelets in EDTA or lepirudin treated PRP, or isolated platelets were activated using thrombin activating peptide-6 (33.5 µM, TRAP6). P-selectin (CD62p) was used as a marker to confirm platelet activation.

Polymorphonuclear leukocytes (PMNs) and peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque Plus density gradients (GE, Healthcare, Uppsala, Sweden) according to the instruction from the manufacturer. To induce apoptosis the PMNs and PBMCs were incubated with camptothecin (5 µM) for 6 h at 37 °C, or tunicamycin (15 µg/mL) for 2 h at 37 °C. Induction of apoptosis, i.e., flip-flop of the cell membrane thus exposing phosphatidyl serine (120) was measured using fluorescein isothiocyanate (FITC)-Annexin V staining apoptosis detection kit (BD, Pharmingen) according to the instructions of the manufacturer. The PMNs and PBMCs were then incubated with C3 (250 µg/mL) for 30 min at RT. Flow cytometric analysis was used to assess the binding of C3, and if the bound C3 were conformationally changed by using the polyclonal antibody (pAb) FITC-anti-human-C3c (DakoCytomation) and AlexaFlour 488-labelled anti-human C3a mAb 4SD17.3, respectively. Competitive binding of Annexin V (Sigma Aldrich) and native C3 was also tested by sequential addition of the two proteins at a concentration of 100 µg/mL to non-apoptotic and apoptotic PMNs.

## SDS-PAGE (I, II, IV)

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is a commonly used method to separate proteins by size in a polyacrylamide gel under the influence of an electrical field. The proteins are first denatured and labelled with sodium dodecyl sulfate (SDS), a negatively charged detergent, to obtain proteins with uniform charge-to-mass ratio. The samples can be run under non-reduced or reduced conditions. Under reduced conditions the samples are treated with e.g., dithiothreitol (DTT), which is a reagent which reduces disulfide bonds. The samples are then added to a polyacrylamide gel and an electrical field is applied. The negatively charged SDS-labelled proteins will start to migrate towards the positive end of the gel. Small proteins will move fast through the polyacrylamide gel, whereas large proteins will get tangled up in the gel mesh, thus proteins of different molecular weights are separated. A dye, usually Coomassie brilliant blue, is then added to visualize the proteins (121).

SDS-PAGE was used to investigate if  $\alpha$ -chain of the different forms of C3(H<sub>2</sub>O) in paper I could be cleaved in the presence of factor I and H. The binding of proteins to liposomes incubated in human plasma was also studied using SDS-PAGE in paper IV. The samples were analyzed on a 4–20 % gradient gel (Mini-PROTEAN® TXG™ Precast Gels, Hercules, CA, USA, BioRad) with Laemmli sample buffer (BioRad) under both non-reduced and reduced conditions, using DTT (25 mM). The electrophoretic separation was performed at 200 V for 30 min. The proteins were visualized on the gel using Coomassie brilliant blue staining and destaining solution (BioRad) according to the manufacturer's instructions.

## Hemolytic activity by the AP (I)

Determination of hemolytic activity of the AP of the different C3(H<sub>2</sub>O) preparations were performed as described in Nilsson and Nilsson (122). When normal human serum, with a fully functional complement system is added to rabbit erythrocytes, the complement system is activated which ultimately leads to lysis of the rabbit erythrocytes through the insertion of the lytic MAC complex. The release of hemoglobin is measured by absorbance; thus, the hemolysis level is related to the complement activity in a sample. In short, to investigate the hemolytic activity of the prepared C3(met) in paper I, C3 depleted serum (Complement Technology, TX, USA) was carefully mixed with native C3 (positive control), C3b (negative control), C3(met) and F/T C3. These C3 spiked serum samples were then added to the rabbit erythrocytes and the release of hemoglobin was measured after incubation.

## ELISA (I, II)

Sandwich enzyme-linked immunosorbent assay (ELISA) is based on immobilization of a coating antibody, with epitope binding sites to the antigen of interest, to a solid phase, e.g., high binding microtiter plate. After, the sample is added and the antigen of interest is captured by the coating antibody. This is followed by the addition of a detection antibody, which is usually labeled with an enzyme. When the enzyme substrate, usually a chromogenic substrate, is added to the plate a color change occurs due to a reaction with the immobilized enzyme/antibody/antigen complex. The generated color signal corresponds to the antigen concentration (123).

In paper I and II in-house sandwich ELISAs for C3c, C3(H<sub>2</sub>O), and C3a were used to analyze total C3, the generation of C3(H<sub>2</sub>O), as well as complement activation.

## Capillary immune electrophoresis (I, II, IV, V)

Wes is an automated capillary immune electrophoresis method used to quantify and characterize proteins and protein modifications (124). Briefly, to prepare a protein sample for Wes it is first heat denatured and labelled with SDS to reach a uniform charge-to-mass ratio. The samples are loaded into capillaries containing with stacking and separation matrices. Proteins are separated based on their molecular weight by applying an electrical field to the capillaries, i.e., electrophoretic separation. Ultraviolet light is then used to cross-link the proteins to the capillary wall. Detection of specific proteins is achieved by flowing a primary antibody against the target protein through the capillary. This step is then followed by detection using a horseradish peroxidase (HRP)-tagged secondary antibody. Luminol and peroxide are then flowed through the capillaries to visualize the bound antibodies by generating a chemiluminescent signal when reacting with HRP. The immunodetected signal, at specific molecular weight is presented in electropherograms as the peak area, i.e., the area under the curve is proportional to the amount of protein in each capillary (125). The electropherograms are analyzed in the Compass software, which also converts the electropherogram into a virtual blot.

In paper I, Wes was used to compare the efficiency of AP C3 convertase formation in the fluid phase between native C3, C3b, C3(met), and C3(KSCN). The different C3 preparations (70 µg/mL) were mixed with factor B (100 µg/mL) and factor D (0.5 µg/mL) in VBS<sup>++</sup> at 37 °C. The cleavage of factor B to Bb was measured over time (0-120 min) in Wes using the mAb anti-human factor Bb (10 µg/mL, BioRad) as the primary antibody, which binds to both intact factor B and the cleaved product Bb.

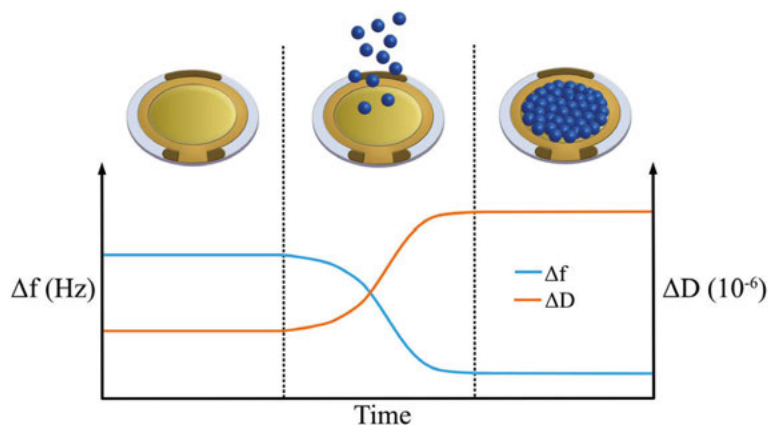
Wes was also used together with the primary antibodies mAb 7D84.1 (20 µg/mL) and mAb 169.7 (70 µg/mL) to analyze C3 binding to platelets (paper II) and liposomes (paper IV), respectively. Lastly, the recruitment of factor H and AT, and the cleavage of C3b to iC3b on fHep-lipid modified liposomes were analyzed using the primary antibodies goat anti-human factor H (4 µg/mL, RnD), rabbit anti-human AT III (diluted 1/1000, Sigma Chemical Company), and pAb rabbit anti-human C3c (diluted 1/100, Dako), respectively.

## QCM-D (II, III, V)

Quartz crystal microbalance with dissipation monitoring (QCM-D) analyses were performed using the QCM-D Pro (Biolin Scientific AB, Gothenburg, Sweden). QCM-D is a technique used to analyze the interaction between a sensor surface and molecules in real-time without labelling (Figure 10). An

altering voltage is applied to the sensor which excite the sensor to resonance. The resonance frequency,  $f$ , is monitored as a function of time. The resonance frequency of the sensor depends on its mass (thickness). The more mass on the sensor the more negative the  $f$  will be. Therefore, changes in frequency ( $\Delta f$ ) are used to analyze the adsorption and desorption of molecules to the sensor surface. The Sauerbrey equation be used to calculate the mass changes on the sensor based on the frequency (126). The QCM-D also measures the dissipation ( $\Delta D$ ), which is the energy loss. The dissipation gives insight to the mechanical properties of the surface-adhering layer. The D-value can in combination with the  $\Delta f$  be used to quantify mass, thickness and viscoelastic properties of soft layers (127, 128, 129).

QCM-D was used in paper II to analyze the C3 binding and AP C3 convertase formation on various artificial surfaces. In paper III and V gold sensors (Bio-lin) were incubated with 1-dodecanethiol (2 mM,  $C_{12}$ -SH) to prepare hydrophobic  $C_{12}$ -self assembled monolayer ( $C_{12}$ -SAM) surfaces. The  $C_{12}$ -SAM sensors was then used for the incorporation of a monolayer of PMPC-lipids and fHep-lipids to analyze their interaction with various purified plasma proteins.

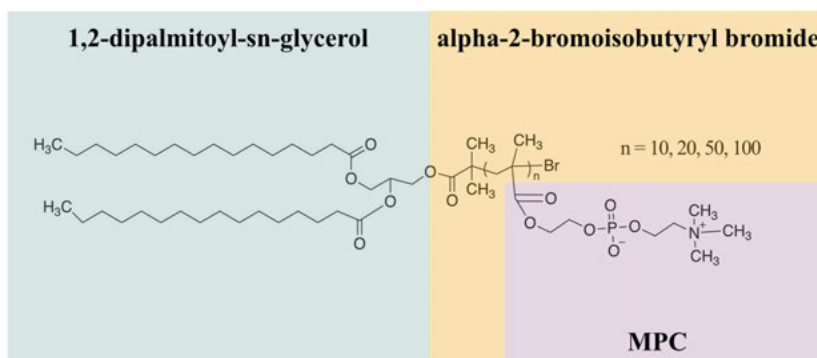


**Figure 10.** Illustration of the principle of QCM-D. When molecules over time adsorb to the sensor surface the mass change will cause a decrease in frequency ( $\Delta f$ , blue line) and the dissipation ( $\Delta D$ , red line) will generally increase depending on the properties of the adsorbed molecule layer. Illustration adapted from Biolin Scientific (130). QCM-D; Quartz crystal microbalance with dissipation monitoring.

## Synthesis of PMPC-lipids (II, III)

Activator regenerated by electron transfer-atom transfer radical polymerization (ARGET-ARTP) (131) was used to synthesize PMPC-lipids (Figure 11). In brief, an ATRP-initiator, in our case a bromide-lipid, was synthesized by

adding either 1,2-dimyristoyl-sn-glycerol (acyl chain with 14 carbons (C14)), 1,2-dipalmitoyl-sn-glycerol (C16), or 1,2-distearoyl-sn-glycerol (C18) to  $\alpha$ -2-bromoisobutyryl bromide. The bromide-lipid initiator was mixed with MPC-monomers to prepare PMPC-lipids with a degree of MPC polymerization ranging from 10 to 100. The polymerization degree was controlled by adjusting the initial molar ratio of MPC to the lipid initiator.

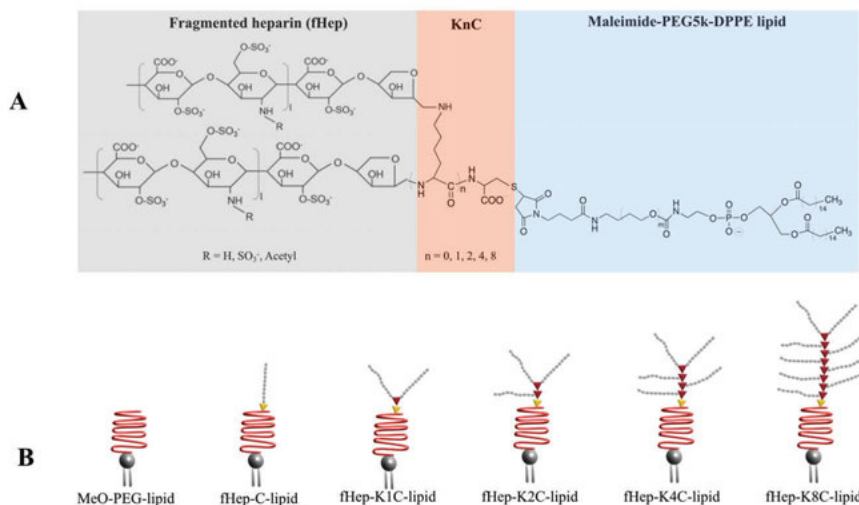


**Figure 11.** Structural formula of a PMPC-lipid with 1,2-distearoyl-sn-glycerol. 1,2-dimyristoyl-sn-glycerol and 1,2-dipalmitoyl-sn-glycerol were also synthesized which have shorter acyl chains. PMPC-lipid with different degrees of polymerization were synthesized and used.

## Synthesis of fHep-lipids (V)

The synthesis of fHep-lipids was performed according to Asawa et al. (103) (Figure 12A and B). Briefly, fHep was obtained by mixing unfractionated heparin in a solution with sulfuric acid and sodium nitrite for 15 min at RT. After, the pH was adjusted to 7.0, and the heparin solution was dialyzed against water, and lyophilized to obtain fHep powder. Next, maleimide-PEG-lipids were prepared by mixing N-hydroxysuccinimide-PEG-maleimide, triethylamine and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE) in dichloromethane for 48 h at RT. Maleimide-PEG-lipids were obtained after precipitation with diethyl ether (132). Next, a linker consisting of just a cysteine, or lysine-cysteine (K1C), lysine-lysine-cysteine (K2C), lysine-lysine-lysine-lysine-cysteine (K4C), or lysine-lysine-lysine-lysine-lysine-lysine-lysine-lysine-cysteine (K8C), was conjugated to the mal-PEG-lipids via thiol-maleimide Michael addition click reaction. This introduced 1, 2, 3, 5 or 9 positively charged amine groups at the end of the C-lipid, K1C-lipid, K2C-lipid, K4C-lipid and K8C-lipid, respectively. The available amine groups on the KnC-lipids allowed for conjugation to an aldehyde group on fHep via Schiff chemistry, followed by reduction with sodium cyanoborohydride (133). To assure no amine groups were left unreacted, succinic anhydride was added

converting the remaining amine groups into carboxylic groups. The fHep-lipid solution was then purified with spin columns and lyophilized, and a powder of fHep-C-lipid, fHep-K1C-lipid, fHep-K2C-lipid, fHep-K4C-lipid and fHep-K8C-lipid were obtained.



**Figure 12.** (A) fHep-lipid. The fHeps are conjugated to a linker with different numbers ( $n = 0, 1, 2, 4, 8$ ) of lysine (K) and one cysteine (C), KnC. The KnC linker is in its turn is conjugated to a maleimide-PEG5k-lipid. (B) Illustration of the different fHep-KnC-lipids used in paper V, as well as the MeO-PEG-lipid used as control. DPPE; 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine, fHep; fragmented heparin, and MeO-PEG-lipid; methoxy-polyethylene glycol-lipid.

## Liposome preparation (II, III, IV, V)

In this thesis liposomes were fabricated using the thin-film hydration method followed by hand-extrusion to prepare small (<200 nm) unilamellar liposomes. The lipids and cholesterol were dissolved in ethanol and mixed in the desired molar ratio. For the liposomes produced in paper II, a gentle nitrogen stream was used to evaporate the ethanol and generate a thin lipid film. Whereas in paper III, IV and V a rotary evaporator was used to remove the ethanol and to generate a thin and uniform lipid film. The lipid film was dried for 24 h *in vacuo* to ensure complete removal of the ethanol. Phosphate buffered saline (PBS) or MQ-H<sub>2</sub>O was used to hydrate the lipid film under constant stirring at RT for 2 h. The liposomes were then extruded through polycarbonate membranes with different pore sizes to reduce the size and to generate a homogeneous liposome suspension, with an acceptable polydispersity index (PDI) ( $\leq 0.1$ ). The liposomes were then characterized using dynamic light scattering (DLS).

In paper V fHep-K8C-lipids, which are not soluble in any organic solvent, could not be added directly to the lipid film. Therefore, the method of spontaneous incorporation of fHep-lipids to pre-made liposomes were used (103, 134). Briefly, liposomes containing 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC)/cholesterol 1/1 by molar ratio, were prepared with the thin-film hydration method. The liposomes were extruded through 1000, 400 and 200 nm polycarbonate membranes. The liposomes were then incubated with fHep-K8C-lipids overnight. Since liposomes are very small, and hard to pellet, ultracentrifugation ( $100,000 \times g$ , 60 min at 4 °C) was used to remove non-incorporated fHep-K8C-lipids.

## DLS (II-V)

DLS was used to characterize the liposome size, PDI, which gives a value on the width of the particle size distribution, and zeta potential, which gives information about the particle surface charge. DLS uses a laser to illuminate macromolecules and nanoparticles in a fluid, and depending on the Brownian motion of the particles, which occurs due to the bombardment of solvent molecules, the light will be scattered at different intensities. Small particles scatter little light but diffuse fast, whereas large particles scatter more light and diffuse slowly. This particle motion, can then be used to determine size and PDI of the particles (135, 136).

A charged particle in a conducting liquid, e.g., a negatively charged liposome in a low salt buffer, is surrounded by a double layer of ions. The ions closest to the surface is known as the Stern layer, and is composed of tightly bound ions of the opposite charge to the surface charge. In the example of a negatively charged liposome, the Stern layer will consist of strongly associated positively charged ions. And the Stern layer is surrounded by a layer called the slipping plane, which consist of loosely associated ions. The ions in the slipping plane of a negatively charged liposome will have a negative charge. The zeta potential is defined as the potential measured at the boundary between the ions in solution and the associated ions that move with the liposome as a single unit, when an electrical field is applied (137, 138).

The zeta potential of a particle can be determined with DLS by measuring how fast a particle moves towards its corresponding electrode under the influence of an applied electrical field. The velocity is proportional to the zeta potential, and depending on how fast a particle move the light will be scattered differently, which can then be used to calculate the zeta potential of a particle (136).

In paper II, III, IV and V DLS was used to characterize the prepared liposomes size, PDI and zeta potential. In paper III and V DLS was used to assess the long-term stability of PMPC- and fHep-liposomes.

## Liposome isolation

Ultracentrifugation (Beckman, Optima™ LE-80K Ultracentrifuge with the Type 70.1 Ti rotor, Beckman Coulter, Bromma, Sweden) at  $100,000 \times g$  for 60 min at 4 °C was used to isolate and pellet the small (<200 nm) prepared liposomes.

## Protein quantification on liposomes (III)

In paper III the Micro BCA kit™ (Thermo Scientific), according to the manufacturer's instructions, was used to quantify protein adsorption to liposomes incubated in human plasma. Briefly, bicinchonic acid (BCA) analysis is a colorimetric assay which can be used to determine the total protein concentration in a sample. It is based on the reduction of copper ions ( $\text{Cu}^{2+}$ ) to  $\text{Cu}^+$  under alkaline conditions by peptide bonds as well as by cysteine, cystine, tryptophan, tyrosine (139).  $\text{Cu}^+$  and BCA then form a purple complex, and the absorbance can be measured at 562 nm. Thus, the generated color signal corresponds to the protein concentration in the sample (140).

## Statistics (I-V)

GraphPad Prism was used for statistical analyses and to generate graphs.  $P < 0.05$  was considered significant, and the calculated p-values were defined as follows: \* $p < 0.05$ , \*\* $p < 0.001$ , \*\*\* $p < 0.001$  and \*\*\*\* $p < 0.0001$ . Statistical tests have been chosen accordingly depending on the experimental design, for details please see individual papers.

# Results and Discussion

## PAPER I

The spontaneous hydrolysis of the internal thioester in C3(H<sub>2</sub>O) has been the main mechanism to explain AP activation. In previous studies C3(H<sub>2</sub>O) has been described to have a “C3b-like” conformation and properties (21, 22). However, it has been under debate of how “C3b-like” C3(H<sub>2</sub>O) actually is with regard to its ability to initiate the AP. Therefore, the aim of paper I was to investigate how effective C3(H<sub>2</sub>O) is to form an initial fluid phase C3 convertase.

To generate C3(H<sub>2</sub>O), native C3 was subjected to methylamine, F/T or KSCN. In this paper these forms of C3(H<sub>2</sub>O) were referred to as C3(x), where the x represents the reactive nucleophile or chaotropic agent used. To confirm that C3(H<sub>2</sub>O) was generated, the exposure of the C3a region due to the disruption of the thioester bond and the subsequential conformational change within the C3 molecule after methylamine treatment was analyzed using SEC. The SEC analysis indicated that the anti-C3a antibody (150 kDa) formed a complex with C3(met) (185 kDa) as the complex eluted earlier in the same fraction as fibrinogen (340 kDa). No complex formation was observed between the negative control C3b, which lacks the C3a domain, and the antibody.

When analyzing the different C3(x) preparations with IEX it was observed that the elution profile of C3(met) generated three distinct peaks. Native C3 eluted in peak 1, peak 2 contained an intermediate form of C3(H<sub>2</sub>O) referred to as C3(x)<sub>1</sub>, and peak 3 the fully conformationally changed C3(H<sub>2</sub>O) referred to as C3(x)<sub>2</sub>. The C3a domain was fully exposed in C3(x)<sub>2</sub> and likely just partially exposed in the C3(x)<sub>1</sub>, this giving rise to the different elution profiles (35). Since C3b lacks the C3a region it could not be used as a control in this experimental set-up to determine the conformation of the molecules in the different C3(x) peaks. By F/T the C3(met) the majority of the treated C3 was then eluted in peak 3. Similar elution profiles were observed for C3(KSCN) and F/T C3. Besides F/T the C3(met) to completely convert C3(met)<sub>1</sub> into C3(met)<sub>2</sub>, in theory the C3 could have also been treated for a longer time and/or with a higher concentration of methylamine (108).

Native C3, C3(met)<sub>1</sub> and C3(met)<sub>2</sub> isolated from the IEX were then subjected to cleavage by factor I and H. The cleavage of C3 was analyzed using SDS-PAGE. Native C3 was, as expected, not cleaved by factor I and H, whereas the positive control C3b was (22). The C3(met)<sub>1</sub> was partially cleaved and C3(met)<sub>2</sub> was almost completely cleaved by factor I and H, further indicating that these two forms of C3 had a different conformation. The hemolytic activity of C3(met) and C3 F/T preparations were also measured. C3 depleted human sera spiked with the different C3 preparations, indicated that the C3(met) and F/T C3 were deprived of their hemolytic activity. This indicated further that the thioester was disrupted, which was in line with previous findings that C3(H<sub>2</sub>O) lacks hemolytic activity (21, 22).

Next, Wes immunoassay was used to study how effective these different forms of C3 were to generate a C3 convertase of the AP in a pure system. C3 was incubated together with a mixture of the purified complement proteins factor B, factor D in VBS<sup>++</sup> over time (0-120 min) (Figure 13) (141). The generation of factor Bb was an indication that an initial C3 convertase of the AP had been formed. The results clearly showed that native C3 and C3b were efficient at initiating the AP, whereas neither of the IEX isolated C3(met)<sub>1,2</sub> were very efficient activators of the AP in the fluid phase.

These experiments were essential to show that we had successfully generated C3(H<sub>2</sub>O), which is described as non-proteolytically activated C3 with “C3b-like” properties but with an intact  $\alpha$ -chain (C3a-domain remaining) and without hemolytic activity. Using SEC, it was proven that the C3a-domain was present in the C3(x) preparation. With the IEX different populations of C3; native C3, C3(met)<sub>1</sub> and C3(met)<sub>2</sub> were isolated due to their different conformations (charge) (21, 22, 114). The known susceptibility of C3(H<sub>2</sub>O) to factor I and H cleavage indicated that the isolated C3(met)<sub>1</sub>, the intermediate form, likely had a more native C3 conformation due to partial cleavage, and the C3(met)<sub>2</sub> had a more “C3b-like” confirmation as it was fully cleaved (23). Upon Wes analysis it was shown that C3(x)<sub>1,2</sub> were not as good activators of AP as native C3 and C3b. C3(x)<sub>1</sub> was a bit faster and more efficient in generating Bb over time compared to C3(x)<sub>2</sub>, again supporting the theory that C3(x)<sub>1</sub> is an intermediate form between native C3 and C3(H<sub>2</sub>O).

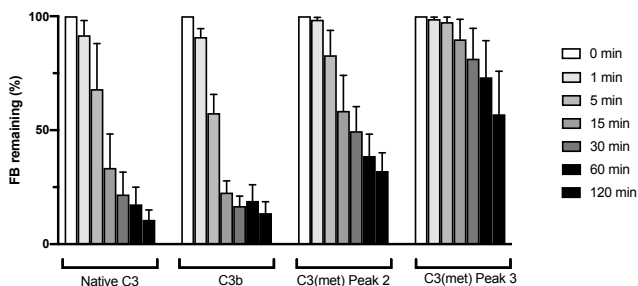
In the early studies of C3(H<sub>2</sub>O) nickel ions was used to study AP convertases, which lead to the convertases being more stable, and likely lead to the overestimation of the C3(H<sub>2</sub>O) activity and “C3b-likeness” (142). As demonstrated in this study the C3(H<sub>2</sub>O) preparation method is important. We speculate that prepared C3(x) batches in early studies of C3(H<sub>2</sub>O) activity may have been “contaminated” with all different populations of C3. And as shown in paper I, if not all native C3 is converted into C3(H<sub>2</sub>O), this will lead to an overestimation of the true C3(H<sub>2</sub>O)Bb convertase efficiency. As previously

investigated by Pangburn the  $C3(x)_1$  can go back to a native C3 structure (114). However, it remains to be investigated which or both of these forms of  $C3(x)_{1,2}$  exists in the body, or if it is an artefact in an artificial system. We can also not exclude the possibility that  $C3(x)$  may behave differently compared to  $C3(H_2O)$  generated in the body.

Theoretically, just one C3b molecule is needed to initiate the AP. However, deposition of a large amount of C3b molecules on the target surface is needed to ensure an immediate and efficient activation of the AP. This, to avoid a long lag phase, thus promoting activation over inactivation (141). The question remains if the low-level, nonspecific generation of  $C3(H_2O)$  via the tickover mechanism is the main activation pathway for AP. Between 0.2-0.4 % of the C3 in the body is estimated to be in a  $C3(H_2O)$  confirmation (22, 43, 44). In paper I we argue that  $C3(H_2O)$  is a poor activator of AP. Firstly, when  $C3(H_2O)$  is generated, it can quickly become inactivated by fluid phase complement regulators. Secondly, if the  $C3(H_2O)$  manage to form an initial  $C3(H_2O)Bb$  fluid phase C3 convertase it is an extremely low probability that the generated C3b molecules will deposit on a target surface (26). But rather, the generated C3b will quickly be hydrolyzed by water, thus not allowing for covalent surface attachment, and can further be inactivated by the fluid phase complement regulators. And thirdly, as shown in this paper, and by Bexborn et al., the  $C3(H_2O)Bb$  C3 convertase is not as efficient as the  $C3bBb$  fluid phase convertase (23). Therefore, only activation of the AP via the  $C3(H_2O)$  tickover mechanism *in vivo* is unlikely the main activation mechanism of the AP. However, the  $C3(H_2O)$  tickover mechanism could have a role in diseases where the AP is dysregulated (143, 144). As well as the generation of  $C3(H_2O)$  could be a mechanism of the body to clear old C3 from the circulation, as in blood the half-life of C3 has been estimated to 72 h (22). In addition, uptake of  $C3(H_2O)$  by cells have been suggested as a source of intracellular C3 (43, 145). It is also important to keep in mind that the APs main function may have evolved into mainly being an amplification pathway, as demonstrated by the potent activation of the CP and LP.

The tickover theory suggested by Lachmann that non-complement proteases, for example release of elastase, thrombin and kallikreins (146, 147), promotes AP by cleaving C3 to C3a and C3b would support activation of AP as large amounts of C3b can be generated quickly, for example by the release of elastase by granulocytes, thereby reducing the lag phase (141, 148). Zhang *et al.*, showed by knocking out factor H and factor D in mice that there was still some AP activation as C3b deposition on sepharose, as well as lysis of rabbit erythrocytes were observed. This may be a result of a convertase bypass mechanism induced by proteases. However, they speculate it can be due to uncontrolled  $C3(H_2O)$  tickover (143). Nevertheless, contact activated C3, which is

conformationally changed C3 into C3(H<sub>2</sub>O)-like molecules deposited on a specific surface might be an explanation of how to achieve selective AP activation.



**Figure 13.** Wes immunoassay monitoring consumption of factor B due to cleavage to Ba and Bb after the addition of factor D to native C3, C3b and C3(met) peak 2 (C3(H<sub>2</sub>O) intermediate) and C3(met) peak 3 (C3(H<sub>2</sub>O)) at different time points from 0-120 min (mean  $\pm$  standard error of the mean,  $n = 4$ ). The more factor B that is remaining the less effective is the generated C3 convertases at initiating AP. From paper I. FB; factor B.

## PAPER II

Paper II was a continuation of paper I. In paper I C3(H<sub>2</sub>O) was demonstrated to form a poor initial fluid phase C3 convertase of the AP. Earlier work has indicated that some surfaces can bind non-proteolytical activated C3 (27, 44, 119, 149, 150, 151). Previously, our group have shown that activated platelets can bind and activate complement on the surface, and C3 deposition was observed despite blocking the activation of the complement system with anti-C1q, EDTA and the selective C3 inhibitor compstatin (119, 152). Further, the platelet-bound C3 was conformationally changed, as demonstrated by the exposure of surface bound “C3b-like” neoepitopes, as well as it could bind CR1 (CD35) and CR3 (CD11b/CD18) again displaying “C3b-like” properties, but with an intact  $\alpha$ -chain. This has been interpreted as recruited preformed C3(H<sub>2</sub>O) (153), alternatively contact activated C3(H<sub>2</sub>O) (119). In paper II we wanted to investigate if the observed C3 deposition and AP activation is due to the binding of preformed C3(H<sub>2</sub>O) or alternatively contact activated native C3. As well as to investigate if this C3 binding and activation without proteolytic cleavage is a targeted activation upon interaction with various artificial and lipid surfaces, rather than a spontaneous and random event.

First, we investigated the binding of native C3, C3b and C3(met) to TRAP6 activated platelets. Non-activated platelets were used as a negative control. To confirm that the platelets were activated the expression of P-selectin was measured as it is translocated to the outer membrane upon activation (154).

The binding of native C3, C3b and C3(met) to platelets was analyzed using flow cytometry and a FITC-labeled polyclonal anti-C3c antibody to detect all C3 bound. The results indicated that native C3 bound in the highest amount to platelets, followed by C3b and lastly C3(met). The C3 binding was enhanced by the presence of properdin (Figure 14A-C). Next, factor B and factor D in the presence of  $Mg^{2+}$  was added to the platelets to build a surface bound AP C3 convertase (141). Following a washing step to remove non-bound complement factors, more C3 as a substrate was added. The functionality of the platelet-bound convertases was assessed using a C3a-peptide ELISA. Unexpectedly, platelets incubated with native C3 generated the most C3a. And for all the C3 convertases the C3a generation was amplified by properdin. A correlation analysis between the measured amount of platelet bound C3, C3b and C3(met) in the flow cytometer and the generation of C3a as determined by C3a-peptide ELISA was also performed. Positive correlations were observed with surface bound native C3 and C3b, while no correlation was found with platelet-bound C3(met), corroborating that native C3 transforms into an C3b-like form of C3 when it binds to the platelet surface. This suggests that contact activated C3 likely has a different functionality compared to surface recruited preformed C3( $H_2O$ ) with regard to AP activation, as C3(met) on the platelet surface was a poor activator of AP. However, in this experiment we have to consider that the C3b used is not nascent C3b, meaning that in the C3b used the thioester is very likely hydrolyzed, due to the purification method, thus it cannot bind covalently to the surface. As observed in these experiments less C3b bound to the platelet surface compared with native C3, but if one considers the C3a generation per mole C3b versus native C3, C3b generated more C3a per molecule. Therefore, it is important to be careful to not interpret these results as contact activated C3 being more effective than C3b. And as discussed in paper I, we cannot exclude that C3(met) may act differently compared to C3( $H_2O$ ) generated in the body.

Next, PMNs and PBMCs were isolated from blood and apoptosis was induced using camptothecin (155). The cells were analyzed by flow cytometry for binding of Annexin V, uptake of PI and binding of C3. Annexin V binds to phospholipids in a  $Ca^{2+}$  dependent way, and have a high affinity for phosphatidylserine, which is exposed on cells where the membrane has flip-flopped. The phosphatidylserine translocation from the inner to the outer membrane occurs for example in activated platelets, apoptotic- and necrotic cells (120). PMNs bound both to Annexin V and C3, while PBMCs which did not bind Annexin V, took up PI into the cytosol demonstrating that this cell type had reach a later stage of apoptosis or necrosis. PBMCs bound only small amounts of C3, corroborating that the flip-flop mechanism exposing phosphatidyl serine on the cell surface may be involved in binding of C3 to the surface. The C3 bound to the PMNs also bound mAb 4SD17.3 indicating that the C3 had undergone conformational changes. In addition, we also investigated if C3 and

Annexin V competed for the same binding site on apoptotic PNM. Annexin V bound to apoptotic PMNs surface reduced the binding of native C3 to the surface, and vice versa (Figure 14D and E). This indicates that the newly exposed negatively charged phospholipid phosphatidyl serine is involved in the activation of native C3. However, the C3 binding was not completely blocked by Annexin V. These experiments do not exclude the possibility that other molecules for example P-selectin and properdin can act as C3/C3(H<sub>2</sub>O) recruiting platforms (151, 153).

Next, liposomes were prepared with the aim to mimic the negatively charged surface of activated platelets and apoptotic cells due to the membrane flip-flop and exposure of phosphatidylserine. The liposomes were prepared with or without cholesterol, and the C3 binding and conformation, as well as the AP convertase formation on the liposome surface were analyzed using QCM-D. The liposomes were flowed onto a silicon dioxide (SiO<sub>2</sub>) sensor surface with the aim to assemble a lipid bilayer. We observed that native C3 bound to negatively charged liposomes containing cholesterol and 20 mol% of the negatively charged lipids phosphatidylcholine or 1,2-dipalmitoyl-sn-glycero-3-phosphoglycerol (DPPG). No C3 binding was observed for liposomes without cholesterol or at lower mol% of phosphatidylcholine and DPPG. Further on, by using a mAb against neoepitopes exposed in surface bound C3b, we could determine that conformationally changed C3 was present on the negatively charged liposome surfaces. We could also build a AP C3 convertase on the DPPC:cholesterol:DPPG 40:40:20 mol% when adding factor B and properdin, followed by factor D and more C3, and the formation of the convertase could be inhibited by factor H.

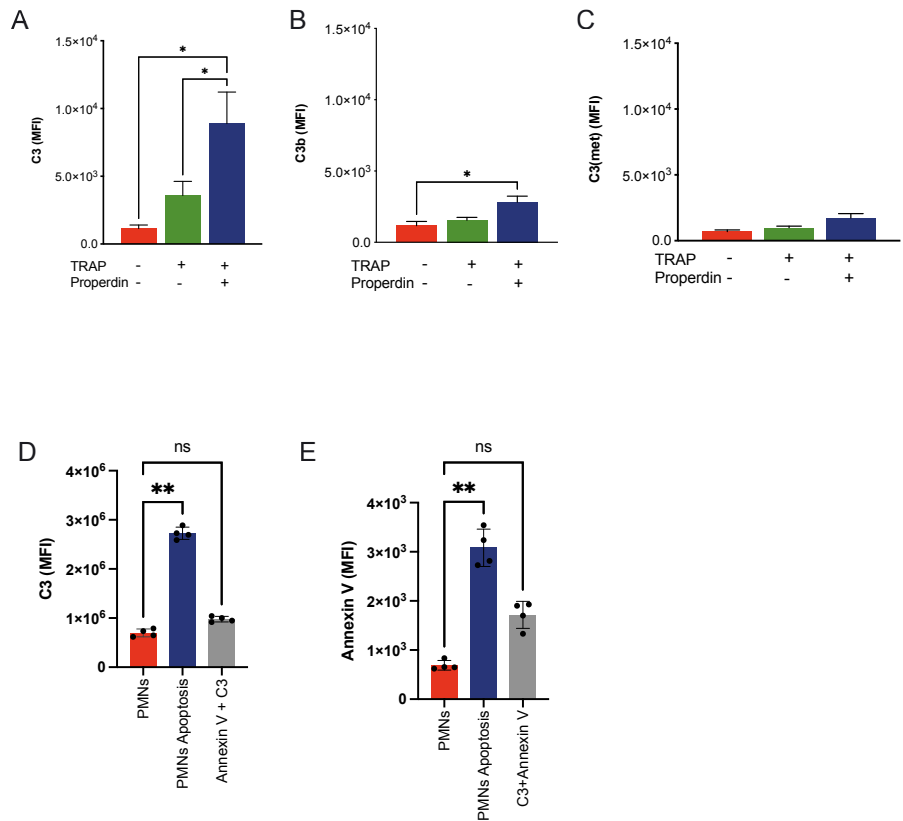
Thereafter, C3 binding and convertase formation on solid surfaces with poly(butyl methacrylate) a rigid plastic surface, poly(lauryl methacrylate) a soft plastic surface (156), polystyrene a hydrophobic surface, and SiO<sub>2</sub> a hydrophilic surface, was investigated using QCM-D. This to further elucidate surface properties that can trigger contact activation of C3. Native C3 adsorbed to all materials, however AP C3 convertase formation was only observed on the rigid plastic surface and the hydrophobic surface. In all the QCM-D experiments, properdin was essential to form an AP convertase, which is supporting earlier studies where observed AP C3 convertase formation on polymer surfaces was enhanced by properdin (27). With the observation in this study together with previous findings, the thioester bond is likely broken, due to the appearance of the neoepitopes related to C3 with a broken thioester (27, 111).

As of today there is no consensus reached on if the AP is initiated by a specific target recognition molecule (4), although since its discovery, properdin has been suggested as the initiating molecule (17). In this work we show that to

some degree that AP possesses target recognition functions, as C3's contact activation and ability to form an AP C3 convertase only occurs on some surfaces. The exact conformation and mode of binding remains to be investigated, and it is likely different mechanisms depending on the surfaces if C3 can bind covalently, or interact electrostatically and/or hydrophobically (149). The requirement for cholesterol in the membrane to activate C3, may be due to an interaction between the hydrophobic cholesterol and the hydrophobic pocket that protects the buried thioester in C3, making it accessible to nucleophilic attack by H<sub>2</sub>O (33, 150, 152), or possible to covalent attachment to the target surface if an -OH or -NH<sub>2</sub> group is in its immediate surroundings (33). Andersson *et al.*, have previously shown that contact activated C3 can bind covalently to proteins on a surface (27). The solid material surfaces used in paper II do not allow covalent binding via the thioester due to lack of reactive chemical groups such as -OH and -NH<sub>2</sub> groups on their surfaces; while the liposomes potentially could expose both -OH and -NH<sub>2</sub> groups. However, it is likely that the thiol ester not is engaged in the C3 attachment to the surface since both the solid surfaces and liposomes are able to bind large amounts of C3 and the conformational change induced by the surface, not seems to require exposure of reactive groups such as -OH or NH<sub>2</sub>. It is also assumed that not all adsorbed C3 is in an active conformation, there is likely a mixture of C3 with different conformations on the surfaces. And as for C3b, how contact activated C3 is oriented on the surface is likely very important for the subsequent C5 convertase formation (46). It is also a well-known fact that native C3 is sensitive for repeated F/T cycles, and the way native C3 is stored and handled will affect the amount of C3(H<sub>2</sub>O) present in the purified native C3 preparations (108). Therefore, in these experiments we cannot exclude that we in addition to contact activated C3 also detect a small fraction of preformed C3(H<sub>2</sub>O). However, due to the large C3 deposition observed on the surfaces, only a very small fraction of this could potentially be attributed to preformed C3(H<sub>2</sub>O).

Contact activation of native C3 by various surfaces have been suggested by Pangburn to be a type of "enhanced tickover" (26). Here we suggest contact activated C3 as a potential targeted AP activation mechanism to maintain homeostasis in the body by e.g., recognize and clear of apoptotic cells. With these experiments we could show that native C3 binds and can generate an AP C3 convertase on activated platelets, whereas preformed C3(H<sub>2</sub>O), i.e., C3(met), was a poor activator of AP on the platelet surfaces. The binding of native C3 to specific surfaces lead to conformational changes within the molecule to a "C3b-like" molecule which could generate active AP C3 convertases in the presence of factor B, D, properdin and Mg<sup>2+</sup>. Annexin V and C3 competed for the same binding site on the surface of apoptotic cells, indicating that there is a direct interaction between C3 and phosphatidylserine. Further, it was demonstrated using QCM-D that native C3 can bind and form an active

AP convertase on negatively charged liposomes with cholesterol, poly(butyl methacrylate) a rigid plastic surface, and on hydrophobic surfaces with polystyrene. Thus, AP was initiated without prior proteolytic cleavage of C3 nor by preformed C3(H<sub>2</sub>O) on specific surfaces in a selective manner. To clarify what surface properties that are involved in the activation of the AP is important to expand our understanding of how the AP can be activated, as well as when designing immunological inert biomaterials.



**Figure 14.** Flowcytometry analysis of the binding of 10 µg/mL (A) native C3, (B) C3b and (C) C3(met) to isolated purified platelets in the absence and presence of 33 µM TRAP6 and 25 µg/mL properdin (n = 6). Apoptosis of PMNs induced by incubation with tunicamycin (15 µg/mL) for 2 h at 37 °C (D) Preincubation of PMNs with Annexin V (100 µg/mL) showed that C3, as measured by a polyclonal rabbit anti-human C3c-FITC antibody, bound to PMNs was reduced by Annexin V. (E) Preincubation of the PMNs with C3 (100 µg/mL) showed that the amount of Annexin V-FITC bound to the cells was reduced (n = 4). From paper II. FITC; fluorescein isothiocyanate, MFI; mean fluorescent intensity, PMN; polymorphonuclear leukocytes, and TRAP6; thrombin activating peptide-6.

## PAPER III

In paper II we demonstrated that the surface properties of artificial- and altered self-surfaces themselves can trigger complement activation through membrane flip-flop. The aims in paper III were to first synthesize synthetic phosphatidylcholine inspired polymer-lipids, to mimic the phospholipids in the outer layer of the cell membrane. And to investigate their interactions with plasma proteins albumin, C3, fibrinogen and anti-PEG-IgM antibodies, as well as evaluate the stability of PMPC-lipids when used as a liposome coating.

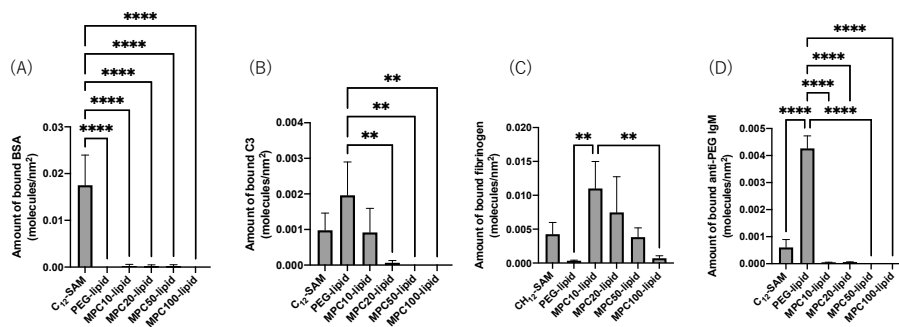
In paper II poly(2-methacryloyloxyethyl phosphorylcholine)-conjugated lipids (PMPC-lipids) with a polymerization degree ranging from 10-100 MPC units was successfully synthesized by activator regenerated by electron transfer-atom transfer radical polymerization to acyl chains with different lengths (C14, C16 and C18). The PMPC-lipids are referred to as MPC10-, MPC20-, MPC50- and MPC100-lipids based on the degree of polymerization. In parallel with our work, another group have also synthesized PMPC-lipids. However, we used lipids without phosphoryl group as an initiator to be able to polymerize longer PMPC chains (90).

Next, QCM-D was used to analyze the interaction between the different PMPC-lipids(C18) and bovine serum albumin (BSA), C3, fibrinogen and anti-PEG-IgM antibodies. Gold sensors prepared with C<sub>12</sub>-SAM was used for the incorporation of a monolayer of PMPC-lipids. Non-treated C<sub>12</sub>-SAM and PEG-lipids were used as controls. All PMPC-lipids could prevent the binding of BSA (Figure 15A). Subsequently BSA was used to prevent unspecific binding to the sensor surfaces. Small amounts of C3 were observed to bind to the surfaces modified with PEG-lipid, MPC10-lipid and MPC20-lipids (Figure 15B), whereas no adsorption of C3 was observed on surfaces modified with MPC50- and MPC100-lipids. Fibrinogen was adsorbed to surfaces modified with MPC10, MPC20- and MPC50-lipids. However, MPC100-lipids could almost completely suppress the binding of fibrinogen, similar to PEG-lipids (Figure 15C). The binding of C3 and fibrinogen to PMPC-lipids were associated with the degree of MPC polymerization, i.e., C3 and fibrinogen binding decreased for longer PMPC chains. Previous QCM-D studies on protein interactions with PEG-lipids with 1, 5 and 40 kDa PEG-chains shown that 5 kDa chains could completely prevent protein adsorption (157). Indicating that length of the polymer-chain influences the protein adsorption pattern for other polymers as well. However, since the PEG and MPC polymers are different, PMPC being more linear and rigid, the optimal length on the chain will likely differ from PEG-lipids.

PMPC-lipids had no reactivity to anti-PEG-IgM antibodies (Figure 15D). Thus, PMPC-coated liposomes may be an alternative for PEG-sensitized

individuals, since PEGylated liposomes can rapidly be cleared from them through the ABC-phenomenon (158, 159). Studies have shown that the formation of a protein corona on liposomes cannot be fully suppressed. However, liposome characteristics such as size, charge, curvature, surface modification, and lipid- and cholesterol content will affect what type of proteins adsorb to the surface. This will in turn influence the subsequent biological response to the liposomes *in vivo*, which will differ depending on what type of proteins that are found in the protein corona (160, 161, 162, 163). For example, liposomes opsonized with C3 will be rapidly recognized and cleared by the innate immune system (73, 150, 164).

Liposomes were prepared with 1 mol% PMPC-lipids(C16 and C18) using the thin-film hydration method followed by hand-extrusion. Non-modified and PEGylated liposomes were used as controls. DLS characterization of the liposomes indicated that the liposomes were between 120-150 nm in diameter. PDI analysis showed that all liposome preparations were monodisperse (PDI <0.1). Non-modified liposomes had a slightly more negative zeta potential compared with PMPC-coated liposomes, which became more neutral with an increasing length of the PMPC-chain. The long-term stability of liposomes stored at 4 °C for 98 days was also analyzed by DLS. No change in liposome size, PDI and zeta potential was observed, indicating that surface modification with PMPC-lipids generates stable liposomes and prevents aggregation. No difference in liposome stability was observed between C16 and C18 PMPC-lipids. However, in theory long alkyl chains favor a more rigid membrane as the number of van der Waals interactions are increased (165). Therefore, we opted for the continued use of the PMPC-lipids(C18). With these experiments we could show that PMPC-lipids with different length on the acyl chains and polymerization degrees could be synthesised. And that the length of the polymer chain affected the protein adsorption in a pure system. As well as liposomes modified with PMPC-lipids were stable over time. Next, was to investigate how PMPC-lipids interact with proteins in a more complex system.



**Figure 15.** Quantitative QCM-D analysis of plasma protein interaction with PEG- and PMPC-lipids(C18) flowed onto a C<sub>12</sub>-SAM modified gold surface followed by the subsequent flowing of 1 mg/mL BSA, 50 µg/mL C3, 1 mg/mL fibrinogen or 10 µg/mL anti-PEG IgM. Calculated amount of (A) BSA (n=6), (B) C3 (n=3), (C) fibrinogen (n=3) and (D) anti-PEG IgM (n=3) bound to the polymer-modified surface (mean ± standard deviation). Non-modified C<sub>12</sub>-SAM surface was used as the control surface and BSA was used to block non-specific binding. From paper III. BSA; bovine serum albumin, C<sub>12</sub>-SAM; 1-dodecanethiol self-assembled monolayer, and QCM-D; quartz crystal microbalance with dissipation monitoring.

## PAPER IV

Paper IV was a continuation of paper III, and the aims of paper IV were to fabricate liposomes with different PMPC-lipids(C18) and study the interaction between modified liposomes and proteins in a more complex system using human plasma. EDTA plasma was used to investigate the passive adsorption of proteins since activation the complement and coagulation pathways are blocked. Lepirudin plasma was used to study the protein adsorption while the complement system remained fully active, while the coagulation system was inhibited. The connection between the length of PMPC-chain, molar ratio of polymer-lipid with protein adsorption, with a special focus on C3 binding was also evaluated.

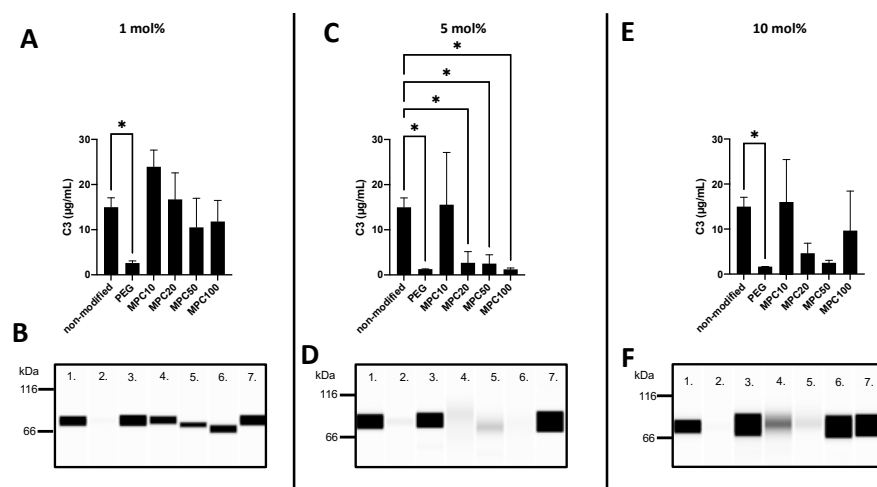
Liposomes were fabricated using the thin-film hydration method followed by hand-extrusion. The liposomes were modified with 1, 5 and 10 mol% PMPC-lipids with different molecular weights of MPC polymers (3 to 30 kDa, MPC10 to MPC100). Non-modified and PEGylated liposomes were used as controls. DLS analysis was performed before and after incubation of in EDTA plasma. The size of all the liposomes increased (6-21%) after incubation in plasma, which could be interpreted as a sign of protein adsorption. Alternatively, it could be an artefact of the purification method (ultracentrifugation) (160). Liposomes with a PDI value of <0.3 were considered monodisperse (i.e., not aggregated) (166). PEGylated and PMPC-coated liposomes had PDI values <0.09 before incubation in plasma, thus indicating highly

monodisperse liposome solutions. However, all liposome compositions were considered to be monodisperse before and after incubation in plasma even though the PDI-value increased for all formulations. The zeta potential of all the analyzed liposomes became more negative after plasma incubation, indicating that proteins had bound to all the liposomal surfaces irrespectively of polymer-modification.

Protein adsorption to liposomes post incubation in EDTA and lepirudin plasma from three different donors was quantified using a Micro BCA kit. Liposomes modified with 1 mol% PMPC-lipids could not suppress protein adsorption. However, liposomes coated with higher molar ratios of polymer lipids and/or longer PMPC-chains were observed to suppress the binding of proteins. In particular liposomes modified with 5 and 10 mol% MPC50- and MPC100-lipids were seen to suppress protein adsorption. SDS-PAGE analysis of liposomes incubated in EDTA plasma demonstrated similar protein binding patterns for all liposome compositions with the exception of two bands, one at 25 kDa and the other at 180 kDa. The 25 kDa band was more abundant for liposomes coated with higher molar ratio and longer polymer-chains of PMPC-lipids. In contrast, the 180 kDa band was only observed under reduced conditions with non-modified-, PEGylated- and PMPC-coated liposomes with a lower molecular weight and at lower molar ratios of PMPC-lipids. Using liquid chromatography with tandem mass spectrometry (LC-MS/MS) analysis and western blot analysis the 25 kDa band was identified as apolipoprotein A-I and the 180 kDa band as  $\alpha_2$ M. These experiments indicated that the surface coverage and molecular weight of PMPC are important to obtain the optimal shielding effect to reduce protein adsorption, and also influences what type of proteins that bind to the surface. It is also worth to note that using SDS-PAGE we could clearly see that all liposomes, no matter polymer-coating, had some type of proteins on their surface. While on some liposomes no protein adsorption was observed using the Micro BCA kit. Thus, this indicates a limitation in protein detection on liposomes using the Micro BCA assay (140).

Lastly, Wes experiments were performed to quantify the binding of C3 to the liposomes following incubation in freshly collected human lepirudin plasma from three healthy blood donors. Liposomes modified with 1 mol% PMPC-lipids could not suppress C3 binding (Figure 16A and B). However, the C3 binding was reduced for liposomes modified with 5 mol% MPC20-lipid, MPC50-lipid and MPC100-lipid (Figure 16C and D). For liposomes modified with 10 mol% MPC20-lipid and MPC50-lipid there was a clear trend in reduction of C3 binding (Figure 16E and F). With these experiments we also observed an association between MPC-polymer-chain length and C3 binding. The short MPC10-lipid was insufficient to inhibit C3 binding, whereas both 5 and 10 mol% MPC20- and MPC50-liposomes suppressed C3 binding as well as total protein to the liposomes.

Liposomes, including PEGylated liposomes, exposed to a biological fluid will have proteins adsorbed to the surface and thus forming a protein corona. The composition of the protein corona will influence the fate of liposomes *in vivo*. The synthetic identity (composition, surface charge, size etc.) of the liposomes influences what proteins are adsorbed to the surface, thus giving liposomes their biological identity (167, 168). In addition to the ABC-effect observed for PEG, PEGylated liposomes and nanoparticles have also been shown to cause complement activation and a phenomenon called complement activation-related pseudoallergy (CARPA) (169, 170, 171). Therefore, the development of new liposome surface coatings is needed. One approach being to try to suppress the activation of the immune system against liposomes by recruiting specific proteins to the liposomal surface, for example complement regulators.



**Figure 16.** Wes immunoassay, under reduced condition with DTT, using a specific monoclonal antibody against C3  $\beta$ -chain for detection of the active binding of C3 to liposomes modified with 1, 5 and 10 mol% polymer-lipids after incubation in lepirudin plasma ( $n=3$ ). **(A, C and E):** quantification of amount of surface bound C3 to liposomes at a lipid concentration of 4 mM (mean  $\pm$  standard deviation). **(B, D, F):** representative Wes virtual blots of lane 1; non-modified liposomes, lane 2; PEGylated liposomes, lane 3; MPC10-, lane 4; MPC20-, lane 5; MPC50-, lane 6; MPC100-liposomes and lane 7; 5  $\mu$ g/mL native C3 (for reference). From paper IV.

## PAPER V

With the lessons learned from paper I-IV with regard to AP activation, and desirable properties of phospholipid surfaces to reduce complement activation, the aim in paper V was to study the regulation of innate immune system on lipid bilayer surfaces using liposomes coated with fHep-lipids, which mimics the glycocalyx of host-cell surfaces. We investigated fHep-lipids ability to

recruit complement regulators factor H and C4BP as well as AT to modified liposomes.

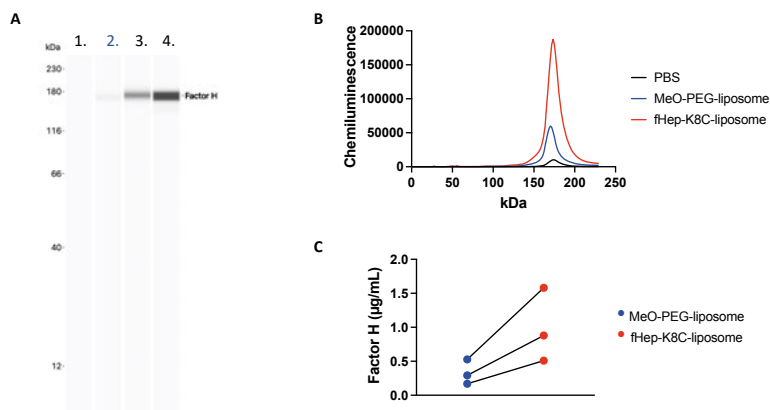
QCM-D was used to study the interaction between fHep-lipids and factor H, AT and C4BP. The lipids were flowed onto gold sensors coated with C<sub>12</sub>-SAM, thus the lipids oriented themselves in a monolayer with the hydrophobic carbon tails towards the hydrophobic C<sub>12</sub>-SAM sensor surface. BSA was flowed to block unspecific binding. Methoxy (MeO)-PEG-lipid was used as a negative control. The more heparin chains conjugated to the lipids, the more factor H and C4BP were bound, which was demonstrated by the fact that fHep-K8C-lipids recruited the most factor H and C4BP in the QCM-D experiments. However, even though fHep-K8C-lipid had almost double the number of fHep-chains conjugated to lipid, it did not recruit twice as much factor H when compared to fHep-K4C-lipids. Presumably, all the binding sites are not fully accessible due to the high density of heparin at the end of lipids.

When factor H followed by AT was flowed over fHep-K1C-lipids and fHep-K8C-lipids of different molar ratios of fHep-lipid:MeO-PEG-lipid, 0:100, 25:75, 50:50, 75:25 and 100:0, we observed that with increasing concentrations of fHep-lipids the more factor H and AT could bind to the coated surface. Again, fHep-K8C-lipids bound most factor H and AT. It was also observed that when more factor H that was bound to the fHep-lipids, less AT could bind. These results indicated that factor H either bound close to the binding site of AT on fragmented heparin, or sterically hindered the binding of AT.

Next, since fHep-K8C-lipids were the most promising with regard to factor H recruitment in the QCM-D experiments, we decided to pick up this lipid for modification of liposomes to study regulator recruitment in a more complex system. Liposomes were successfully coated with fHep-K8C-lipids and size, PDI and zeta potential was stable up to 60 days. Liposome aggregation was observed after 90 days for the modified liposomes, PDI >0.1. This may be the limitation of the spontaneous incorporation technique of the fHep-lipids, thus not generating high enough concentrations of fHep-lipids on the liposomes to prevent aggregation up to 90 days. However, compared to non-modified liposomes, 60 days should be considered a relatively long and stable storage time for the liposomes. Liposomes modified with MeO-PEG-lipids were used as a negative control. Liposomes modified with fHep-K8C-lipids could recruit factor H in a pure system, as well as from human EDTA plasma which was analyzed using Wes (Figure 17). AT could also be recruited from plasma. This was in line with what we observed in the QCM-D, that both factor H and AT can bind to the fHep-K8C-lipid modified liposomes. This was an interesting result, as the QCM-D experiments were a competitive test between factor H and AT in a pure system, and still both could bind selectively to fHep-liposomes incubated in a more complex system as human plasma. Finally, we

investigated if the factor H recruited to the surface remained active. Which was confirmed by the cleavage of C3b to iC3b in the presence of factor I, which generated a 40 kDa fragment (Figure 6, C3dg).

As previously discussed, the ABC-phenomenon is a concern for liposomes used as a drug-delivery system. And since the fHep-lipids consist of fHep-chains conjugated to a PEG-lipid, it is possible that fHep-liposomes may also trigger an ABC reaction upon repeated injections (82, 172). It would be of interest to conjugate the fHep-chains to another polymer, e.g., PMPC to investigate the use of fHep-lipids as a potential complement regulating liposomal drug delivery system. Since the AP is the major contributor in the amplification of complement, the recruitment of factor H, the main fluid phase AP regulator, is a promising approach for complement regulation on surfaces. In comparison to other coating strategies using heparin and complement binding peptides, the fHep-lipids have the advantage of achieving heparin modification of a lipid surface in a single-step (94, 96). In comparison, to e.g., a factor H binding peptide, such as 5C6-peptide (52), fHep-lipids have a dual effect, as it can regulate complement as well as they have the ability to regulate the coagulation system through the binding of AT.



**Figure 17.** Wes immunoassay measurements of factor H recruitment to the surface of MeO-PEG-lipid (blue) and fHep-K8C-lipid (red) modified liposomes incubated in human EDTA plasma for 60 min at 37 °C at 20 rpm, followed by removal of unbound plasma proteins using ultracentrifugation. A sample with only PBS and no liposomes, treated the same way as the liposome containing samples were used as a negative control. A goat-anti-human factor H antibody was used as the detection antibody. **(A)** Representative Wes virtual blot, **lane 1:** negative control with PBS sample, **lane 2:** MeO-PEG-liposomes, **lane 3:** fHep-K8C-liposomes, and **lane 4:** positive control with 0.625  $\mu$ g/mL factor H. **(B)** Representative chromatogram of the peak area of recruited factor H, and **(C)** quantification of peak area of factor H recruited to MeO-PEG-liposomes and fHep-K8C-liposomes ( $n = 3$ ). From paper V. MeO-PEG; methoxy-polyethylene glycol, fHep; fragmented heparin.

# Conclusions

## PAPER I

C3(H<sub>2</sub>O) is not an effective initiator of the AP in the fluid phase. Thus, indicating that the hydrolysis of C3 via the tickover theory is not the main mechanism of AP activation.

## PAPER II

Specific surfaces, such as activated platelets, apoptotic cells and negatively charged liposomes can activate AP via contact activation of native C3 in a surface selective manner.

## PAPER III

PMPC-conjugated lipids with degree of MPC polymerization ranging from 10 to 100 were successfully synthesized. Surfaces coated with PMPC-lipids could suppress albumin, C3 and fibrinogen binding. PMPC-lipids with longer polymer-chains were better at suppressing protein binding. PMPC-lipid modified surfaces did not interact with anti-PEG IgM antibodies. The size, PDI and surface charge PMPC-lipid-modified liposomes was maintained over a long period (98 days). Thus, our findings suggest that PMPC-lipids can be used for liposomal coating, and in the future, it might be an alternative liposome coating strategy for patients with anti-PEG antibodies.

## PAPER IV

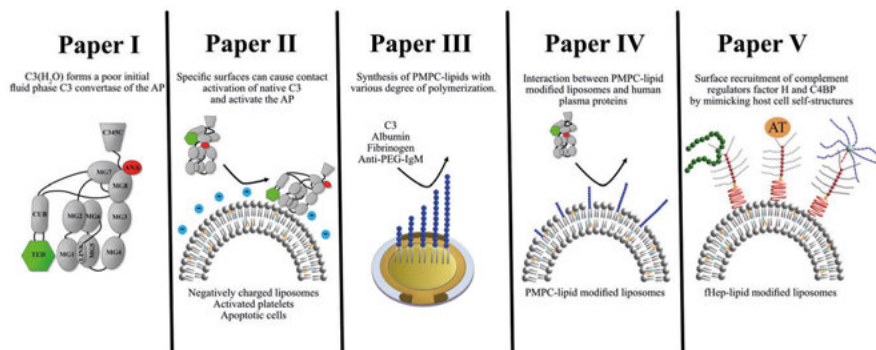
Liposomes were produced with PMPC-lipids with different length of PMPC-chain and molar ratio of polymer-lipid (1-10 mol%). Surface coating with 1 mol% polymer-lipid was not sufficient to suppress protein adsorption. Out of all the evaluated PMPC-lipids, the PMPC-lipids with a molar ratio of 5 or 10 mol% and a polymerization degree of 20 or 50 were the best at suppressing protein adsorption and C3 binding when incubated in human EDTA or lepirudin plasma. Thus, surface coverage of the polymer layer and polymer chain

length are important parameters for liposome preparation which are likely to influence the immunological response against PMPC-liposomes *in vivo*.

## PAPER V

The fHep-lipids were shown to have the ability to bind to complement regulators factor H and C4BP, and the binding increased with increasing numbers of fHep-chains. Liposomes were successfully modified with fHep-K8C-lipids and size, PDI and zeta potential were stable over 60 days. Factor H and AT could also be recruited to the liposomes incubated in plasma. The factor H bound to the modified surface were still active, as it has the ability to act as a cofactor in the presence of factor I to cleave C3b to iC3b. Thus, fHep-lipids could be a new biomaterial coating for complement regulation which can work on liposomes (Figure 18).

## Graphical summary of paper I-V



**Figure 18.** Graphical summary of the main findings in paper I-V. In this thesis we investigated how the AP can be initiated, as well as developed new liposomal coatings for immune suppression and regulation. **Paper I;** C3(H<sub>2</sub>O) has been described as a molecule with “C3b-like” conformation and properties, but with an intact C3 α-chain. In paper I we demonstrated that C3(H<sub>2</sub>O) was a poor initiator of the AP, thus suggesting that the slow spontaneous hydrolysis of the internal thioester in C3, via the tick-over theory, likely is not the main activation pathway of the AP. **Paper II;** here we showed that specific surfaces could bind native C3 and trigger conformational changes within the C3 molecule to a C3b-like structure without prior proteolytic cleavage. Active C3 convertases of the AP could be generated on these surfaces, suggesting contact activated C3 as a mechanism of targeted AP activation. **Paper III;** as a new alternative liposome coating to PEG, which can activate the immune system and the complement cascade upon repeated injections into the body, we synthesized PMPC-lipids with different degrees of MPC polymerization, i.e., different length of the MPC-polymer chains. We then evaluated the protein adsorption to the polymer-lipids in QCM-D. PMPC-lipids with a longer polymer-chain were better at suppressing protein binding, and anti-PEG-IgM did not bind to PMPC-lipids. **Paper IV;** Liposomes were coated with 1-10 mol% of PMPC-lipids. The protein binding, with focus on C3, to coated liposomes in human plasma was evaluated. Liposomes coated with 5 and 10 mol% PMPC-lipids with a polymerization degree of 20 and 50, were determined as the most efficient in suppressing protein and C3 binding. Thus, PMPC-lipids could be an alternative liposome coating to PEGylation. **Paper V;** fHep-lipids were synthesized and studied for their ability to regulate complement, by recruiting complement regulators factor H and C4BP, as well as AT to liposome surfaces. Here we could demonstrate that fHep-lipids can bind factor H and C4BP, as well as AT. Where fHep-lipids with increasing numbers of fHep-chains binds the most factor H and C4BP. Liposomes coated with fHep-lipids, with nine conjugated fHep-chains, could recruit factor H and AT from human plasma. In addition, surface recruited factor H could regulate complement activation on the surface, by working as a cofactor in the degradation of C3b to iC3b. AP; alternative pathway, AT; antithrombin, C4BP; C4b-binding protein, fHep; fragmented heparin, PEG; polyethylene glycol, and PMPC; poly(2-methacryloyloxyethyl phosphorylcholine)-conjugated lipids.

## Future perspectives

Several interesting findings have been observed in our studies regarding C3(H<sub>2</sub>O) and contact activated C3 and their involvement in AP initiation. In future studies it would be of interest to investigate what form(s) of C3(H<sub>2</sub>O) that exist in humans. As well as to investigate how contact activated C3 is bound to a surface, with or without involvement of the thioester. For example by substituting the thioester bond to e.g., disulfide bridge (173). And/or investigate if the thiol group is accessible, thus then indicating non-covalent binding. An option could be to try to label contact activated C3 with e.g., a fluorophore with a maleimide end group (174). Future studies should also involve studying how efficient a contact activated convertase is in comparison to a covalently bound C3bBb, as well as studying its ability to form a C5 convertase. In addition, studying C3 in organisms with a more ancient immune system where the AP is likely playing a more central role in the immune defence would also be interesting to gain more insight into the role of C3 and C3(H<sub>2</sub>O) (175).

The evaluation of PMPC-modified liposomes will be continued. The next step is to incubate them in an *ex vivo* human whole blood model to further evaluate their interaction with blood proteins with a special focus on the complement activation. As well as investigate if the PMPC-lipids also have an ABC-effect *in vivo*. It would also be of interest to explore PMPC-liposomes as an alternative to PEGylated liposomes, in patients with anti-PEG-antibodies. It may be of even more importance now since many of the COVID-19 vaccines contains PEGylated lipid nanoparticles, which may lead to a wide spread anti-PEG antibody production in the general population (171).

Next step is also to investigate if the fHep-lipid modified liposomes can suppress complement activation in human whole blood. Giulimondi *et al.*, have suggested pre-coating liposomes with an artificial protein corona containing human plasma proteins as a potential strategy to increase liposome circulation time *in vivo* (161). Therefore, to investigate if fHep-liposomes pre-incubated in human factor H can enhance the suppression of complement activation on the surface and increase the circulation time of the fHep-liposomes needs to be further investigated as an alternative to only recruiting the factor H available in plasma. In the future, it would also be interesting to investigate

multicomponent liposome and cell surface coating strategies using several different polymers and peptides together.

# Populärvetenskaplig sammanfattning

Immunsystemet är ett väldigt komplext biologiskt system i vår kropp, som skyddar kroppen mot främmande ämnen. Det består av barriärer, såsom vår hud, immunceller, samt cellbundna och cirkulerande proteiner och andra molekyler. Immunsystemet kan göra skillnad på friska celler som hör hemma i kroppen, skadade och döende celler, samt kroppsfrämmande ämnen. Det innebär att immunsystemet kan skilja på "själv" och "icke-själv". Detta är baserat på att vi har ämnen/strukturer på våra celltor som känns igen av immunsystemet som "själv", och dessa celler lämnar immunsystemet i fred. Men när något känns igen som "icke-själv", på grund av att de uttrycker något på sin yta som är främmande eller förändrat eller avsaknad av "själv"-strukturer, så kommer immunsystemets aktörer starta en kollektiv och koordinerad immunattack för att tillsammans angripa och bekämpa det främmande ämnet. Vilka komponenter som är inblandade i immunreaktionen, det vill säga vilken försvarslinje som aktiveras och hur snabbt det främmande ämnet bekämpas, beror dels på vilket ämne, var i kroppen ämnet återfinns, och om kroppen tidigare exponerats för ämnet.

Immunsystemet är indelat i det medfödda och det förvärvade immunförsvaret. Det medfödda immunförsvaret är i princip förprogrammerat vid födseln, och känner igen generella strukturer på främmande ämnen. Det reagerar likadant under hela livet, även vid upprepade exponeringar av samma kroppsfrämmande ämne. Det medfödda immunsystemet reagerar snabbt genom att starta en inflammation och/eller bombardera och klä in det främmande ämnet med molekyler som hjälper kroppen att bryta ner och eliminera hotet. Det förvärvade immunförsvaret består av specialiserade immunceller som utvecklas under hela livet. Dessa specialiserade immunceller, T- och B-celler, tränas upp att vid upprepade exponeringar av samma ämne bli bättre på att känna igen specifika strukturer för just det ämnet. Exempelvis bildar B-celler specifika antikroppar mot ämnet. Första gången det förvärvade immunförsvaret träffar på ett nytt ämne är det relativt långsamt i att mobilisera en immunattack, men vid upprepade exponeringar, blir det förvärvade immunförsvaret successivt snabbare och kraftfullare i att generera en immunattack. Detta beror på att T- och B-celler bildar minnesceller för det specifika ämnet. Det medfödda och förvärvade immunförsvaret samarbetar för att tillsammans skydda och bekämpa kroppen mot "icke-själv".

Komplementsystemet är en central del av det medfödda immunförsvaret och finns i blodplasma, vävnader och celler. Komplementsystemet består av över 50 plasmacirkulerande och cellbundna proteiner och regulatorer. Det kan aktiveras via tre olika aktiveringsvägar; den klassiska-, lektin- eller den alternativa vägen. Vilken väg som aktiveras beror på vad det är för ämne som startar aktiveringen, men när väl komplementsystemet aktiveras, startar en kedjereaktion som förstärker sig själv. Alla tre aktiveringsvägarna leder till: lokal inflammation, att det främmande ämnet kläs in med olika molekyler som signalerar att det ska tas upp och brytas ner av immunceller, samt att de kan göra hål i membran på bakterier och celler vilket orsakar lysis och celldöd. Det mest centrala proteinet i komplementsystemet är komplementprotein 3 (C3).

Liposomer är små, runda, konstgjorda fettdroppar bestående av lipider och kolesterol, vilket är samma beståndsdelar som våra cellmembran är uppbyggda av. De används kliniskt som bärare av läkemedel, men också inom forskning för att härma enkla biologiska cellmembran. Lipider och kolesterol är amfifila molekyler, vilket betyder att de har en vattenhatande och en vattenälskande del. Om man blandar lipider och kolesterol med vatten så kommer de bilda ett runt dubbellager som omsluter en kärna av vatten, då deras vattenhatande delar vänder sig mot varandra för att skydda sig mot vattnet, medan de vattenälskande delar interagerar med varandra och är vända mot vattnet. Liposomer kan bära vattenälskande molekyler i den omslutna vattenfasen, medan vattenhatande (fettlösliga) molekyler kan transporteras i det vattenhatande dubbellagret av lipider. Liposomer kan användas för att transportera och skydda känsliga läkemedel som annars snabbt hade brutits ner i kroppen. Men de används också för att skydda kroppens friska celler från att ta skada när man administrerar celldödande läkemedel, så som vid cancerbehandling. I teorin anses liposomer ha stor potential som läkemedelssystem, däremot känns liposomer igen av immunsystemet som "icke-själv" och bryts snabbt ner av det medfödda immunförsvaret. En strategi för att skydda liposomer mot en immunattack är att klä in dem med stora syntetiska molekyler bestående av en eller flera upprepade enheter, s.k. polymerer, för att minska inbindning av plasmaproteiner till liposomernas yta. Polyetylenglykol (PEG) är den mest använda polymeren för att klä in liposomer. En välkänd tillämpning av PEGylerade lipid-nanopartiklar är Modernas och BioNTech/Pfizers mRNA vaccin, Spikevax® respektive Comirnaty®, som använts globalt vid vaccinering av SARS-CoV-2. Dock har man observerat att anti-PEG-antikroppar bildas vid upprepade doser av PEGylerade liposomer, vilka då snabbt känns igen och bryts ner av immunsystemet.

Denna avhandling är baserad på fem delarbeten där vi dels på detaljnivå undersökt hur C3 i den alternativa vägen av komplement kan aktiveras i lösning och på olika artificiella och biologiska ytor. Vi har även framställt nya material för att klä in liposomer med avsikten att undvika och reglera

aktivering av immunsystemet, med fokus på komplementsystemet, på de inklädda ytor.

I **arbete I** studerade vi hur den alternativa vägen i komplementsystemet aktiveras i lösning. Den generella uppfattningen sedan 1980-talet har varit att den alternativa vägen aktiveras spontant via formförändrat C3, s.k. C3(H<sub>2</sub>O), i lösning. I detta arbetet studerade vi olika former av C3(H<sub>2</sub>O) och kom fram till att C3(H<sub>2</sub>O) i lösning är en dålig aktivator av den alternativa vägen, och är sannolikt inte den främsta aktiveringsvägen av den alternativa vägen under normala förhållanden.

I **arbete II** undersökte vi om C3 kan formförändras och aktiveras via kontakt med ytor och på så sätt aktivera den alternativa vägen på specifika ytor, och inte via spontan aktivering i lösning. Här kunde vi visa att aktiverade trombocyter, döende celler och negativt laddade liposomer alla kunde binda och formförändra C3, samt aktivera den alternativa vägen på sina ytor. Då vi visade att C3 kan binda direkt till dessa ytor, aktiveras och initiera den alternativa vägen utan att först formförändras i lösning eller klyvas till den aktiva produkten C3b, föreslår vi detta som en ny alternativ och specifik aktiveringsväg.

I **arbete III och IV** framställde vi nya membranlipid-inspirerade polymerlipider, bestående av poly(2-metakryloyloxyetyl fosforylkolin)-konjugerade lipider (s.k. PMPC-lipider). PMPC-lipiderna framställdes med olika längd på PMPC-kedjan och vi undersökte om vi kunde klä in liposomer med dem. Vi undersökte även hur de klädda liposomerna interagerade med uppenade proteiner och proteiner i plasma, med fokus på C3. PMPC-lipider med en längre polymer-kedja och vid högre koncentrationer var bäst på att hindra proteinadsorption till ytor och inklädda liposomer.

Slutligen, i **arbete V** så undersökte vi om lipider, kopplade till fragmenterat heparin (fHep-lipider) för att efterlikna ytan på blodkärlens insida, kan användas för att klä in liposomer samt reglera komplementsystemet på ytan. Heparin kan i teorin binda komplementregulatorerna faktor H och C4b-bindande protein, och i detta arbete undersökte vi om det fragmenterade och lipidkopplade heparinet fortfarande hade dessa egenskaper. Vi kunde visa att faktor H och C4b-bindande protein kunde binda till fHep-lipider, samt att fHep-lipid klädda liposomer kunde rekrytera faktor H till ytan från blod-plasma (från människa). Faktor H bundet till fHep-liposomer kunde även reglera komplementsystemet genom att hjälpa till med att bryta ner den centrala aktiveringsprodukten C3b.

Sammantaget var syftet med **arbete I-V** att få en djupare förståelse i hur komplementsystemet aktiveras i lösning och på ytor. Detta för att vidare kartlägga hur komplementsystemet kan initieras, samt för att få kunskap om hur man

kan designa material för att minska risken att de känns igen som "icke-självt" i (den mänskliga) kroppen. Vi syntetiserade nya polymer-lipider för att klä in liposomer, med syftet att utveckla ett alternativ till PEGylerade liposomer. Liposomer kläddes även in med molekyler som liknar kroppens egna ytor för att på så sätt undvika att immunsystemet ska känna igen dem och aktiveras. Komplementregulatorer rekryterades även till fHep-liposomer från plasma som ytterligare ett led i att minska immunaktiveringen och reglera komplementaktiveringen på liposomytan. Målet är att i framtiden kombinera dessa två strategier med avsikten att klä in celler för transplantation, för att på så sätt minska risken för avstötning.

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