The role of the complement system in determining the biocompatibility of candidate materials for medical implantable devices

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This Thesis dedidcated to my lovely wife Marina and daughter Sofia Linnea

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List of papers

This Thesis is based on the following papers:

I. **Sokolov A.**, Hellerud B.C., Pharo A., Johannessen E.A., Mollnes T.E. Complement activation by candidate biomaterials of an implantable microfabricated medical device. J Biomed Mater Res B Appl Biomater 2011;98B(2):323-9.

II. **Sokolov A.**, Hellerud B.C., Lambris J.D., Johannessen E.A., Mollnes T.E. Activation of polymorphonuclear leukocytes by candidate biomaterials for an implantable glucose sensor. J Diabetes Sci Technol., 2011, 5 (6):1490-1498

III. **Sokolov A.**, Hellerud B.C., Johannessen E.A., Mollnes T.E. Inflammatory response induced by candidate biomaterials of an implantable microfabricated sensor, J Biomed Mater Res A, In Press

IV. **Sokolov A.**, Hellerud B.C., Tønnessen T.I., Johannessen E.A., Mollnes T.E. Activation of coagulation and platelets by candidate membranes of implantable devices in a whole blood model without soluble anticoagulant. Submitted

Abbreviations

A2020 - araldite 2020

AAO – anodic aluminum oxide

ANOVA – analysis of variance

ASC – apoptosis-associated speck-like protein containing CARD

ASIC – application-specific integrated circuit

ASTM – American Society for Testing and Materials

BSA - bovine serum albumin

BTG – β-thromboglobulin

CM – cellulose ester membrane

Con A – concanavalin A

CR1 – complement receptor 1

CRIg – complement receptor of the immunoglobulin superfamily

CT – CeramTec GC

CTAD – Citrate-theophylline-adenine-dipyridamol buffer

DAF – decay accelerating factor

DAMP – danger-associated molecular pattern

DP – DuPont 951

EDTA – ethylenediaminetetraacetic acid

EGTA – ethyleneglycoltetraacetic acid

EIA – enzyme immunoassay

ELISA – enzyme-linked immunosorbent assay

ETek – Epo-Tek 353ND

FBGC – foreign body giant cells

HAIGG – heat aggregated immunoglobulin G

IgG – immunoglobulin G

IL-1β – interleukin 1β

IL-6 – interleukin 6

IL-8 – interleukin 8

ISF – interstitial fluid

ISO – International Standard Organization

LPS - lipopolysaccharide

LTCC – low temperature co-fired ceramic

MALDI-TOF – Matrix-assisted laser desorption/ionization - time-of-flight

MCP-1 – monocyte chemoattractant protein 1

Me – stainless steel 316L

MedFI – median fluorescence intensity

MFI – mean fluorescence intensity

MIP-1β – macrophage inflammatory protein-1β

MPO – myeloperoxidase

MWCO – molecular weight cut-off

Nlrp3 – NOD-like leucine-rich repeat-containing 3

OD – optical density

PA – polyamide

PAES – polyarylethersulphone

PAMP – pathogen-associated molecular pattern

PAR – polyamide reverse osmosis membrane

PATF – polyamide thin film membrane

PEG – polyethylene glycol

PBS – phosphate buffered saline

PC – polycarbonate

PDMS – polydimethylsiloxane

PMN – polymorphonuclear leukocyte

PRP – platelet-rich plasma

PTF 1+2 – prothrombin fragment 1+2

PU – polyurethane

ROI – reactive oxygen intermediate

S3140 – silicone 3140

S3145 – silicone 3145

Si – silicon

SiO_2 – silicon dioxide

TAT – thrombin-antithrombin complex

TCC – terminal complement complex

TF – tissue factor

TNF-α – tumor necrosis factor α

1. INTRODUCTION

1.1 Historical background

The introduction of nonbiological materials into the human body has been recorded far back in human prehistory. The Mayan people have been known to use sea shells as dental implants as early as 600 A.D. and iron dental implants dated around 200 A.D. have been found in Europe (1). Surgical sutures made from silk have been recorded back to the period of the Egyptian civilization, whereas catgut sutures were used in the Middle Ages in Europe. Bioartificial organs, such as the artificial kidney and heart, were introduced in the middle of the 20th century, but most implants prior to 1950 had a low probability of success because of the lack of understanding of the biocompatibility and sterilization of the biomaterials employed. Since the 1960s most of the materials used in the fabrication of medical devices have been designed specifically for their intended application. Silicones, hydrogels and different types of bioglass were all developed in this time period together with the substantial growth of knowledge gained about biocompatibility and material-body interactions. This fact led to a significant increase in the use of biomaterials in the field of medicine (1), and the application area has grown especially fast over the past decades and covers now almost all the fields of clinical medicine (Fig. 1, Table 1). Biosensor applications for interventional procedures and monitoring are considered the most challenging area of this field in which multiple biomaterials are applied at the same site in combination with special requirements for the material properties.

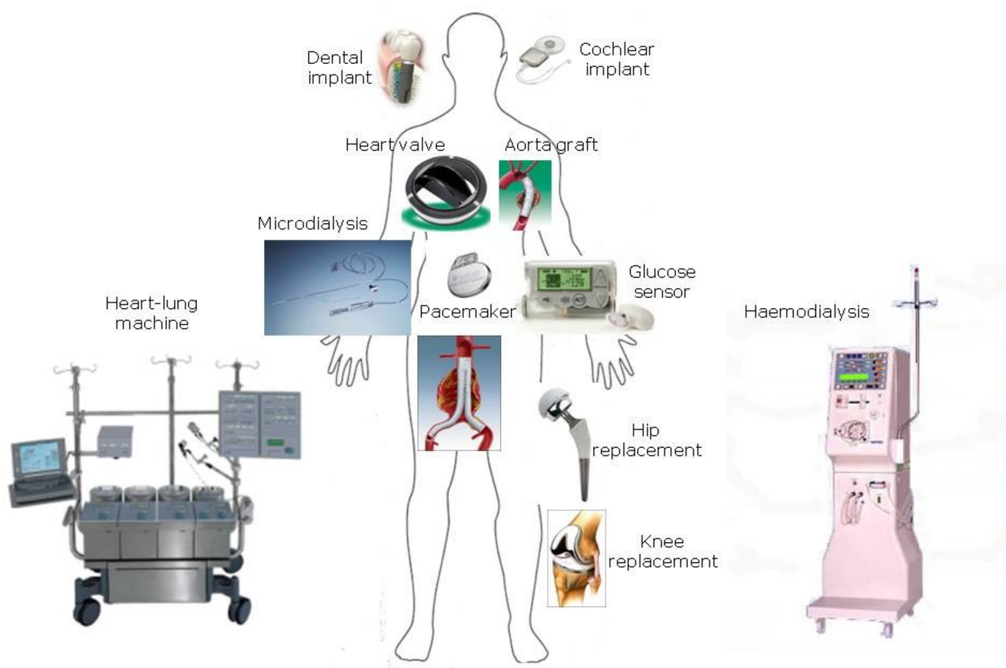

Fig. 1. *Clinical Applications of Biomaterials*

Biomaterials for long-term implantable devices should not just perform their intended function, but also be compatible with the host. The inflammatory response at the site of implantation is an important factor that may lead to sensor dysfunction. Inflammatory cells could affect the sensor signals by consuming substrate molecules that are also detected by the sensors (e.g. glucose), or attach to the device interface which may influence the membrane permeability. Biofouling of this sensor membrane is an important cause of sensor dysfunction (2). Proteins attached to the surface can impregnate the membrane and thus reduce the sensor performance by blocking access to the underlying transducer. In addition, cellular reaction to the implanted sensor will further enhance any sensor dysfunction and inactivation by

embalming the implant in a fibrous capsule that effectively separates the device from the rest of the body (2). Finally, the excretion of proteolytic enzymes and free radicals creates a local corrosive environment that may damage components of the sensor such as the polymeric membrane (2). In this context, the biocompatibility of biomaterials and the mechanisms involved in the inflammatory reaction to these biomaterials would be of paramount importance to understand considering the construction and intended function of the implantable device.

Table 1. *Some applications of synthetic materials and modified natural materials in medicine*

Application	Type of material
Intervention and monitoring	
Glucose sensors	Pt, Ag/AgCl, Nafion, polycarbonate, cellulose
Pacemakers	Titanium, stainless steel, polyurethane
Microdialysis	Polyarylethersulphone, polyurethane
Skeletal system	
Joint replacement (hip, knee)	Titanium, Ti-Al-V alloy, stainless steel, polyethylene
Bone plate for fracture fixation	Stainless steel, cobalt-chromium alloy
Bone cement	Polymethyl methacrylate
Bone defect repair	Hydroxylapatite
Artificial tendon and ligament	Teflon, Dacron
Dental implant for tooth fixation	Titanium, Ti-Al-V alloy, stainless steel, polyethylene, alumina, calcium phosphate
Cardiovascular system	
Blood vessel prosthesis	Dacron, Teflon, polyurethane
Heart valve	Reprocessed tissue, stainless steel, carbon
Catheter	Silicone rubber, Teflon, polyurethane
Organs	
Artificial heart	Polyurethane
Skin repair template	Silicone-collagen composite
Artificial kidney (hemodialyzer)	Cellulose, polyacrylonitrile
Heart-lung machine	Silicone rubber
Senses	
Cochlear replacement	Platinum electrodes
Intraocular lens	Silicone-acrylate, hydrogel
Corneal bandage	Collagen, hydrogel

1.2 Definition of biocompatibility

The International Standard ISO 10993 states that the basic biocompatibility requirements for a biomaterial is that it should be nontoxic, noncarcinogenic, nonantigenic, and nonmutagenic. Additionally, in applications that require direct contact with blood, the material must be of a nonthrombogenic nature that mitigates complications from thrombi and emboli (3;4). In clinical medicine some authors define biocompatibility of the dialysis membrane as the absence of any perturbation in the blood borne elements. This means in other words that there should be no adhesion or activation of blood cells or proteins absorption (5). Shaldon et al. defined biocompatibility as the quality of being mutually tolerant with life (6).

Biocompatibility has traditionally been concerned with implantable devices that are intended to remain in place inside the body for long periods of time. In accordance to the definition of D. Williams, biocompatibility refers to the ability of a biomaterial to perform its desired function with respect to a medical therapy, without eliciting any undesirable local or systemic effects in the recipient or beneficiary of that therapy, but generating the most appropriate beneficial cellular or tissue response in that specific situation, and optimizing the clinically relevant performance of that therapy (7). This definition applies for biosensors that are intended to measure metabolic parameters by relying on the intact diffusion of tissue fluids across a porous membrane.

1.3 Factors that influence the biocompatibility

Biomaterials differ in their capacity to induce (or prevent) a biological response and there are several factors that needs to be considered in this respect. Literature has suggested that the chemical properties of the materials are the main contributor towards the biocompatible properties. It has been shown that the inherent surface hydrophilicity or hydrophobicity is one of the key parameters that affects the activation of the immune system, and the tendency have

been that hydrophobic materials exhibit a less desirable response (8-10). The current evidence support a theory that surface nucleophiles in combination with the presence of NH_2 or hydroxyl groups on the material surface triggers a powerful activating potential. By contrast, negatively charged surfaces with the presence of COO^- , SO_{3-} and sialic acid appears to promote high-affinity association between the surface-bound C3b and the inhibitory factor H which prevents complement (and immune system) activation. Hence, the presence of chemical groups on the biomaterial surface determines its activation potential, which is currently evaluated by the degree of *in vitro* cytokines expression (11;12), *in vivo* cellular infiltration (13) and leukocytes activation with the corresponding expression of tissue factor (14). However, the surface topography of materials as well as the pore size of nanoporous membranes has also been found to contribute towards the biocompatibility. In this case the immune response is getting more potent as the pore size gets larger (15) whereas nanostructured surfaces shows a decrease in the immune system activation (16). The benefit of smaller pores (and nanostructured topography) is to limit the total accessible surface area that is available for protein absorption which in turn decreases the subsequent complement activation (9). Finally, the role of complement and the binding capacity of biomaterials towards factor H is another important feature that governs the biocompatibility. Surfaces that promote preferential binding of factor H and which does not favour binding of factor B will promote biocompatible properties (10;17-19).

1.4 Immune response to biomaterials

The immune system is responsible for the immediate recognition of foreign objects such as invading pathogens and artificial materials. The detection of pathogens is processed through a diverse set of pathogen-associated molecular patterns (PAMP). Because PAMPs are produced only by microorganisms, they are perceived by the innate immune system as a molecular

signature of infection, and their recognition leads to the induction of an immune response with a cascade activation and cell response (20;21). Non-microbial factors, such as damaged cells or cancer cells are recognized by alarmins, which together with PAMP are classified as danger-associated molecular patterns (DAMP). The activation of the innate immune system through these mechanisms provides an immediate immune response to pathogens or internal factors with subsequent activation of the inflammation cascade and the immune cells. One of these components, the inflammasome, have recently been related to biomaterials (22) since it appears to be a general mechanism for the initiation of inflammation in response to physical materials irrespective of their size and material properties. The activation of the inflammasome induces an inflammatory infiltrate that is dependent on the inflammasome components NOD-like leucine-rich repeat-containing 3 (Nlrp3), apoptosis-associated speck-like protein containing CARD (Asc), and caspase-1. These components (except Nlrp3) also orchestrate the development of a fibrous reaction in the late phases of inflammation. Hence, the removal of the inflammasome in laboratory knock-out mice hinders the progression of a fibrous reaction possibly through the attenuation of profibrotic signals governed by the inflammasome (22).

1.4.1 The complement system

The complement system is an important part of the innate immunity and serves as a first-line of defence after the invasion of a foreign object (23). Complement consists of more than 30 plasma and cell surface proteins (Fig. 2) and can be activated via the classical, the lectin and the alternative pathway (23;24).

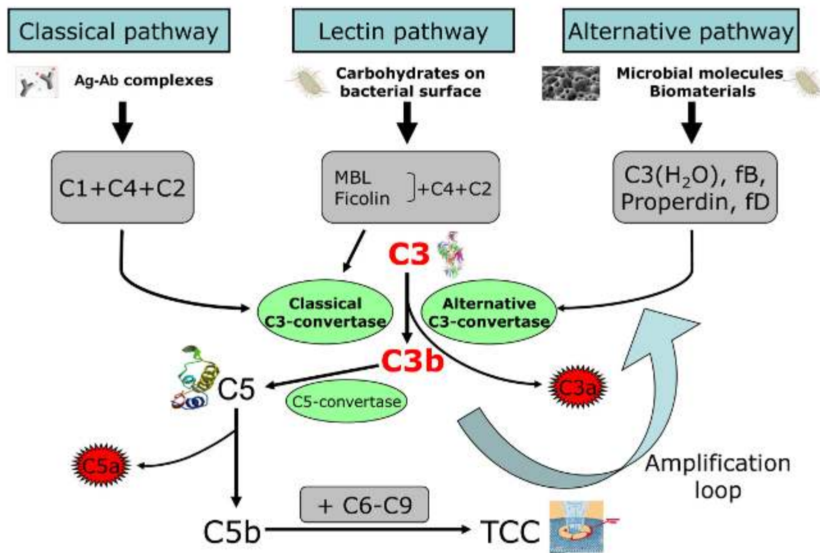

***Fig. 2**. The Complement System*

The classical pathway is activated by an antigen-antibody complex or by acute phase proteins, whereas the lectin pathway is activated by conserved sugar epitopes like mannose present on the surface of the invading pathogens. The alternative pathway serves mainly as an amplifier of the initial classical or the lectin pathway activation, but can also be directly activated by synthetic surfaces (24). All the initial pathways converge at the C3 level by the formation of the enzyme C3 convertase that cleaves C3 to C3a and C3b molecules. C3b is a key molecule of the complement cascade that acts as an important opsonin and mediates further activation of the complement cascade by activating the enzyme C5 convertase that splits C5 into the intermediate products C5a and C5b. The C5b molecule initiates formation of the terminal complement complex (TCC). TCC exists in the fluid phase, but can also be inserted into the bacterial cell membrane, which makes it permeable, causing the bacteria to succumb from

osmotic stress. Additionally, C3a and C5a are both potent anaphylatoxins because of their tissue-sensitizing activity and with properties to activate white blood cells with initiation of inflammation (25).

The activation of the complement system is tightly controlled by inhibitory molecules both in the soluble and membrane-associated form. Fluid phase complement regulators include factor H (an alternative pathways component that binds to C3b and competes for the binding site of factor B) and carboxipeptidase N (an anaphylatoxin inactivator) which inhibits all the three pathways. Soluble factors that regulate the classical and lectin pathways include the C1-inhibitor (binds with C1qrs complex), factor I (C3b and C4b inactivator) and the C4b-binding protein. The membrane of host cells is also protected with membrane-bound complement regulators including complement receptor 1 (CR1), CD46 (MCP), CD55 (DAF), CD59 (protectin) and complement receptor of the immunoglobulin superfamily (CRIg) (26-28) that prevent the complement system from attacking the cells of the body. The complement can be regarded as an early and fast recognition system that triggers inflammation processes at the site of implantation or pathogen invasion by mediating the immune cells activation.

1.4.2 Coagulation and platelet

The primary role of the haemostatic system is to arrest bleeding from injured blood vessels. However, the same process may produce adverse effects when an artificial material is introduced into human tissues and comes into contact with blood. The two main components of the haemostatic system, the coagulation cascade and platelets, do both equally participate in the inflammatory response to an implanted biomaterial, and the level of reaction may govern the function and lifespan of an implantable device. The process of coagulation proceeds through a cascade of reactions (Fig. 3) which converts inactive factors to enzymatically active ones. The coagulation cascade is traditionally divided up into the

intrinsic and extrinsic pathways. The intrinsic pathway initiates a cascade triggered by the surface contact with a biomaterial (surface-mediated reactions) whereas the extrinsic pathway is governed by TF and the proteolytic cleavage by other enzymes (1).

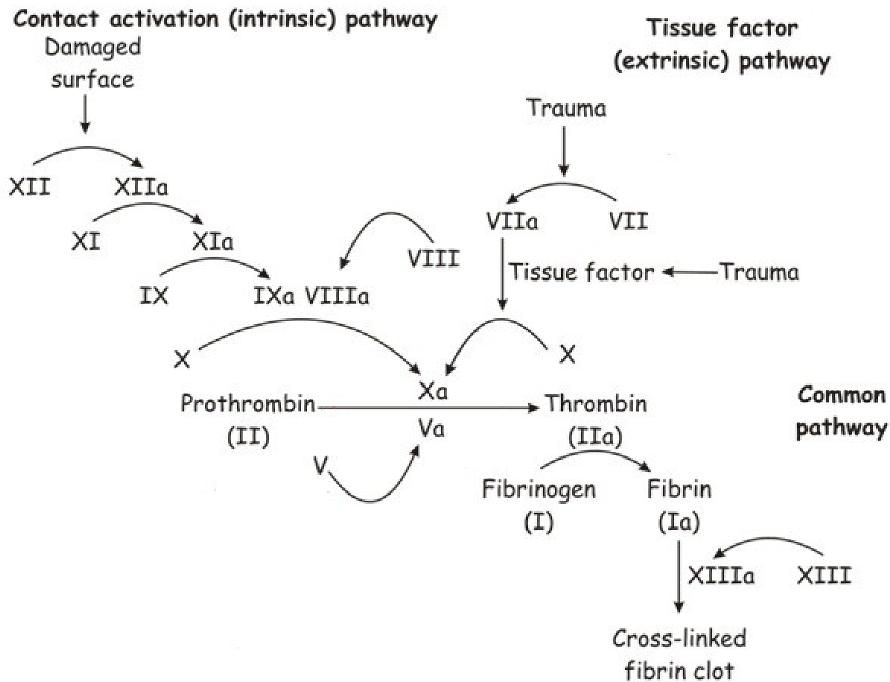

Fig. 3 *The Coagulation System*

Contact phase activation is thought to be the major trigger of the intrinsic pathway in the blood-biomaterial contact that occurs during the insertion of implantable devices, extracorporeal hemodialysis or artificial circulation as part of heart surgery (29). The components of the contact activation system such as prekallikrein, high molecular weight kininogen, coagulation factor XI and coagulation factor XII (Hageman factor) do all attach to foreign surfaces. Following attachment, the autoactivation of FXII to FXIIa cleaves

prekallikrein to kallikrein and initiates the FXII activation loop which activates the intrinsic pathway through the generation of the FXIIa.

The physiological initiator of the extrinsic pathway is TF, and the expression of TF on damaged cells at the site of vascular injury or by monocytes results in blood coagulation with fibrin formation at the site of injury or implantation (30-32). Factor VII (FVII) binds to TF on the cell membranes and is activated to FVIIa to form the TF-VIIa complex (33) which cleaves and activates Factor X. The two pathways converge into a common pathway at the FX level resulting in the formation of a fibrin clot with the aid of thrombin on fibrinogen (30). The two coagulation pathways are not independent and closely interact. The extrinsic enzymatic complex TF-FVII is responsible for the onset of the coagulation cascade while the contact phase is a major player in the propagation phase (30;34).

Platelets are nonnucleated cells that are produced in the bone marrow and circulate in the blood occupying approximately 0,3 % of the blood volume. The function of the platelets is to arrest bleeding by forming an initial platelet plug which is stabilized by catalyzing the coagulation reaction with the formation of fibrin (1). Platelet contain substantial quantity of muscle proteins that initiate platelet contraction, and three types of cytoplasmic storage granules (35;36):

- α-granules, which contains fibrinogen, coagulation factors V and VIII as well as the platelet-specific proteins platelet factor 4 (PF-4), β-thromboglobulin (BTG) and chondroitin sulfate A
- Dense granules containing adenosine diphosphate (ADP), calcium and serotonin
- Lysosomal granules containing hydrolytic enzymes

Consequently, the adhesion and activation of platelets on a biomaterial surface leads to the subsequent release of PF-4 and BTG from the intracellular α-granules. P-selectin, a cell-surface glycoprotein belonging to the selectin family, and which is released from the α-granules, mediates the adhesion of activated platelets to neutrophils and monocytes. All of these factors will in turn influence the activation of other platelets, as well as modulating the coagulation process and inflammation (32;37;38).

1.4.3 Polymorphonuclear leukocytes and monocytes

Neutrophils or polymorphonuclear leukocytes (PMN) represents 40-60% of the white blood cells and is an important part of the inflammatory response. PMN appears at the site of implantation within the first seconds (39;40) and the expression of adhesion molecules on a surface modulates the inflammatory response. An important group of adhesion molecules include the CD11/CD18 family. Inflammatory mediators like cytokines, chemokines and anaphylatoxins stimulate the rapid increase in these adhesion molecules on the leukocyte surface as well as increased PMN adhesion to biomaterial surface (39;41). The adhesion of PMN to material surfaces and the PMN's activation by the inflammatory molecules from the complement, coagulation and platelet systems triggers the release of a new set of bioactive molecules from the PMN which induce the production of reactive oxygen intermediates (ROI). ROI's are essential mediators of signalling by many cytokine and hormone receptors, such as those for insulin, platelet-derived growth factor, fibroblast growth factor, nerve growth factor, TNF and angiotensin. The azurophilic granules of PMN contains myeloperoxidase (MPO), a glycosylated protein which exerts microbicidal effects and which are able to convert the relatively innocuous H_2O_2 into the much more powerful hypochlorous acid, hypobromous acid and hypoiodous acid, which are all powerful bactericidal compounds (42;43). The PMN and monocytes does also produce a broad spectrum of cytokines and

chemokines that mediates further cells activation that amplifies the immune response at the site of implantation (42-44). Pro-inflammatory cytokines like tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and interleukin-1β (IL-1β) induce an acute phase response. The chemokines family also contribute to inflammation process by triggering the local accumulation of inflammatory cells at the site of implantation (45). Interleukin-8 (IL-8), macrophage inflammatory protein-1β (MIP-1β) and monocyte chemoattractant protein-1 (MCP-1) chemotactically attracts PMN, monocytes/macrophages and play an crucial role in the development of chronic inflammation through the recruitment of immune cells to the site of injury (12).

The close interaction between the complement system, haemostatic system and the cellular components of the innate immune system plays a collective role in determining the biocompatibility of a material. The contact activation system as well as thrombin and plasmin are able to cleave complement component *in vitro* (46;47). Complement activation leads to coagulation activation and the C5a-mediated upregulation of TF, which is the potent initiator of the extrinsic pathway of coagulation (48-50). Platelet activation during thrombotic events initiates the activation of complement (51) and the contact system (52), which in turn leads to inflammation. Thrombin receptor activated platelet are strong promoters of inflammation, since chondroitin sulfate A, released from the α-granules during platelet activation, activates complement in the fluid phase and generates anaphylatoxins that induce leukocyte activation (26;48;53;54). Thus it is important to evaluate different cascades and cells in a single test model to understand the overall mechanisms governing the biocompatibility of biosensors (30;55;56).

1.5 Immune system activation following materials implantation

1.5.1 Acute inflammation

The molecular mechanisms of acute inflammation are poorly understood. Yet, literature have identified the most important components such as the role that inflammasome activation, as well as plasma membrane cholesterol, and Syk signalling contributing to acute immune response to biomaterial implantation (22).

The immune response to artificial materials starts by coating the surface with a protein film composed from albumin, immunoglobulin G (IgG) and fibrinogen (17;57;58), that initiates complement activation. The study performed by Gifford (59) using MALDI-TOF mass spectrometry, showed that the mechanism of protein absorption was time-dependent with albumin being the first protein to be absorbed, followed by IgG and then fibrinogen. The absorbed proteins have been shown to activate the complement system through the classical pathway (60) in which the absorbed C3 protein, generated in this process, is able to serve as a nucleation point for the subsequent activation of the alternative pathway. Once C3b is generated and covalently bonded to the protein coat, the amplification loop of the alternative pathway can be triggered (18;61;62). Further, C3b that is attached to the material surface can physically shield the underlying proteins, including C1q and IgG, and thereby halting activation via the classical pathway (61).

The C3b and the anaphylatoxins C3a and C5a that is generated as a result of complement activation acts as ligands for the receptors on PMN and monocytes that can trigger inflammation and the release of pro-inflammatory cytokines and chemokines (62). The activation products of complement do also trigger inflammatory cell activation with expression of the adhesion molecules CD11/CD18 on the PMN surface and the expression of TF on monocytes. The activated PMN that adhere to complement- and immunoglobulin

coated biomaterial surfaces may release MPO and lactoferrin from their granules. The amount of enzymes that is released corresponds to the size of the material surface in which larger biomaterial implants have been found to release a great amount of enzyme from activated cells (39).

The acute inflammatory response to biomaterials is usually resolved within several days, depending on the extent of the injury at the implant site and the type of biomaterial used. The extent of tissue damage during implantation will lead to a longer acute inflammation period concomitant with an increasing amount of fibrous tissue formed at the site of injury (63).

1.5.2 Chronic inflammation

Chronic inflammation follows the acute phase and is identified by the presence of macrophages and lymphocytes at the site of implantation together with the proliferation of blood vessels and the formation of connective tissue (64;65). The macrophages are the most important cells governing chronic inflammation because they secrete biologically active products like chemotactic factors, reactive oxygen metabolites, complement proteins, coagulation factors and cytokines. The chronic inflammation is normally resolved within a limit period of time if this process is triggered by biocompatible materials (39). Classically, the development of granulation tissue has been considered as a part of the chronic inflammation process, but because of the unique tissue-biomaterials interaction it has become preferable to consider the formation of foreign body giant cells (FBGC) as a separate process that follows chronic inflammation together with the development of granulation tissue. On a molecular level chronic inflammation and formation of FBGC is mediated with the inflammasome as well as being dependent on Asc and caspase-1 (22).

The foreign body reaction to biomaterials is identified by the presence of FBGC and granulation tissue, which consist of macrophages and fibroblasts. Macrophage activation and

fusion have been identified as the critical cellular events leading to the formation of FBGC, including the induction of E-cadherin, Rac1 activation, and secretion of matrix metalloproteinase-9 (66). Both macrophages and FBGC can release mediators of degradation such as ROIs, oxygen free radicals and proteolytic enzymes. Both adherent macrophages and FBGC in the foreign body reaction are known to assist in the degradation of biomaterials, and as stressed earlier, both the shape, material and surface topography of the biomaterial determines the degree and severity of the foreign body reaction. A large amount of FBGC has been found for porous materials compared to smooth-surface implants, and which will result in fibrosis as a significant component at the implant site (39;63). Recently, though, it has been suggested that a textured rather than a smooth surface could lead to an improved long term sensor performance by increasing the vascularisation around the implant (67;68). Although compelling evidence links implant topology with the nature of the tissue response, a link between the surface texturing and sensor performance, particularly biofouling, remains poorly understood (69).

1.5.3 Fibrous encapsulation

The end-stage of the healing process related to a biomaterial implant is generally characterised by some degree of fibrosis that in part is dependent upon the extent of the injury or tissue defect that is created during the implantation procedure. Regeneration with replacement of granulation tissue by connective tissue leads to the formation of a fibrous capsule that encloses the layer of FBGC on the implant surface. The extent of this fibrous encapsulation as well as the degree and duration of chronic inflammation, foreign body reaction and the subsequent healing process, is determined by the acute inflammation and the presence of cytokines, chemoattractants, growth factors and macrophages.

In order to investigate these complex interactions between the different arms of the inflammatory network *in vitro*, it is necessary to apply a suitable model. There are several *in vitro* models that can be explored in biomaterial research. Serum and isolated cells are used to examine separate biological processes, whereas tissue cultures are a popular method to investigate macrophage reaction and the cytotoxicity effects of biomaterials. More complex model based on whole blood will facilitate the investigation of the interaction between cascades of innate immunity and the sequences of processes following material implantation.

1.6 Models used to investigate the biocompatibility of materials

1.6.1 Serum and isolated cells

Investigations of the initial complement activation and protein adsorption on a material surface requires the use of a simple *in vitro* serum model. Serum contains all the blood plasma proteins except fibrinogen, and since there are no cells present (i.e. no biological turnover), any activation product from complement, including C5a can be detected (70). Serum can also be used to examine the activation of different complement pathways. For instance, the role of the alternative pathway can be investigated by blocking the classical pathway with EGTA and Mg^{2+}. In contrast, a low concentration of the C3-inhibitor compstatin blocks the formation of the convertase and permits only the classical pathway to operate (61).

A separate evaluation of blood cells requires the use of an isolated cell model. Platelet-rich plasma (PRP) is the preferred option to evaluate either platelet adhesion to a materials surface, platelet activation, the release of granules as well as the interaction between platelet and plasma proteins (71-74). PMN’s that are isolated from whole blood and resuspended in a buffered solution can be used as a model to investigate PNM activation by biomaterials (40), whereas monocytes and lymphocytes can be used to examine cytokines and chemokines production respectively (12). However the activation of the coagulation cascade is also

dependent on the cross-talk between the contact activation system (32), blood cells and the complement system (30). Several studies have shown that thrombin plays an important part in the activation of platelets (75;76) and the use of the PRP model makes it impossible to explore the interaction between blood cells and platelet compared to the whole-blood model.

1.6.2 Cell and tissue cultures

The tissue culture method was introduced in 1965 as means to evaluate the toxicity of polymeric materials (77;78). It was later standardized by the American Society for Testing and Materials (ASTM) and is currently used to perform cytotoxicity screens *in vitro* by offering higher reported sensitivities to toxins than that of most animal models (79). Cell lines that have been developed for growth *in vitro* are preferred to primary cells (that are freshly harvested from live organisms), since they offer a comparable cell type that improves the reproducibility of the assays and thereby reduce the variability among the different laboratories (1). For instance, mouse fibroblast cells (L-929) exposed to biomaterials for several days is commonly used to test for leachable toxic substances in the material and for any residual solvents used during the material preparation (80). In accordance to the recommendation of the International Standard Organization ISO 10993-5 "Established cell lines are preferred and where used shall be obtained from recognized repositories" (81). Qualitative assessment of cytochemical staining can be used to assess changes in general morphology, vacuolization, cell lysis and membrane integrity (82). Quantitative tests measure the parameters of cell death, inhibition of cell growth, cell proliferation and colony formation (81;83).

Appropriate mammalian cell cultures can also be used to investigate the carcinogenicity and genotoxicity of a material. These cell cultures are assessed by the degree of gene mutations,

changes in chromosome structure (and number) as well as other DNA or gene related toxicities caused by the materials and/or their extracts (83;84).

1.6.3 Whole blood

To investigate the role of complement in the complex inflammatory network and to evaluate the role of blood cells, all the potential cellular and fluid-phase mediators needs to be present simultaneously (85). Such cross-talk can be achieved *in vitro* using a whole-blood model. Blood will readily coagulate as a result of protein adsorption to a material interface and the absence of regulators on the container walls requires the use of a soluble artificial anticoagulant. Both ethylenediaminetetraacetic acid (EDTA) and citrate are known to inhibit complement activation and should be avoided to investigate complement activation (70). Heparin is widely used in biocompatibility experiments although it inhibits complement activation in high concentration and enhance the activation at low concentrations (85). Heparin do also possess various direct effects on platelet and leukocytes (86;87), which excludes it as an optimal anticoagulant in models to study the inflammatory network. In the past decade, a highly specific recombinant thrombin inhibitor, lepirudin, has come into play with respect to *in vitro* blood research (85;88). Lepirudin binds to active thrombin, and irreversible inactivates it without any adverse effects to the rest of coagulation cascade and complement system (89). The addition of EDTA following the end of an experimental process prevents further activation of the blood sample during storage. This makes whole-blood anticoagulated with lepirudin the most native and suitable model used to investigate complement activation and the inflammatory processes induced by artificial materials.

In contrast, the best alternative that exists in order to evaluate the activation of the coagulation system and platelets, is to use native whole blood without any additives. As shown in several studies (75;76), thrombin have an important role in the propagation of coagulation and

platelets activation. The using of lepirudin as an anticoagulant blocks the thrombin generation that can have an important effect on a native haemostatic system *in vitro*. To prevent blood coagulation after contact with the container walls, different coating methods can be used. Heparin that is immobilized to an artificial surface has been shown to be the best acceptable candidate for this purpose used both in clinical practice (90;91) as well as research (92-96).

2. AIMS OF THE STUDIES

The aim of this project was to evaluate the *in vitro* biocompatibility of a selected group of candidate materials intended for use in a microfabricated implantable glucose sensor. The evaluated materials are also relevant for other implantable devices and therefore can be applied in different application areas. The material samples were incubated in standard 24 well polystyrene plates at 37^0C using either a serum, or a whole-blood model with a soluble anticoagulant. These models permitted the examination of both the complement system as well as the different cascades involving immune cells that closely mimic the *in vivo* environment. A novel whole blood model based on the incubation in heparin coated polystyrene wells permitted the investigation of complement related to the coagulation and platelet activation process without the use of a soluble anticoagulant.

The assays were selected in accordance to the International standard ISO 10993 and covered all the parts of the innate immunity cascades, the cells activation and the coagulation cascade.

The activation of complement, PMN's, the inflammatory response, the coagulation and the platelet system were evaluated in separate *in vitro* studies. The role of the complement system was examined by using specific complement inhibitors.

Study 1

The aim of this study was to evaluate complement activation by different candidate materials of an implantable microfabricated glucose sensor with the aid of a serum model.

Study 2

The aim of this study was to perform an *in vitro* analysis of PMN activation following exposure to selected candidate materials for an osmotic glucose sensor and to evaluate how

the activation of complement contributes to the activation of PMN in response to such materials.

Study 3

The aim of this study was to examine the activation of the *in vitro* inflammatory network in response to direct contact with candidate materials that were intended for use in microfabricated implantable glucose sensor, and the role of the complement system as a trigger of this process.

Study 4

The aim of this study was to evaluate the *in vitro* coagulation potential of selected membrane materials that is of interest for use in implantable microfabricated glucose sensor based on a modified whole blood model without soluble anticoagulant in heparin-coated wells. The role of complement in the activation of coagulation and platelet by biomaterials was explored.

3. MATERIALS AND METHODS

3.1 Sensor architecture

The biomaterials that were selected for investigation in this study were all candidate materials that would form part of a microfabricated osmotic glucose sensor under development by Lifecare AS. These materials were divided up into four different groups depending on which parts of the sensor they would be implemented (capsule, membrane, carrier and sealing). The sensor microimplant is built around a carrier of low temperature co-fired ceramics (LTCC) which is a multilayer ceramic platform that permit integration of microelectronic circuit components in 3 dimensions. This enables an ultracompact and thermally stable platform architecture that is currently used in the artificial pacemaker. Consequently, a choice of two LTCC materials that differ slightly in their processing parameters are included in this study, CeramTec GC (CT) and DuPont 951 (DP). The membrane and pressure transducer are attached to the LTCC using a two component epoxy resin Epo-Tek 353ND (ETek) that cures with temperature. This adhesive also acts as a sealing material that protects the electrical connections from moisture. However, the small distances (<250µm) between the electrical connections and the aqueous environment in the sensor cavity prompted two additional sealant materials to be considered; silicone 3140 (S3140) and 3145 (S3145) due to their good water repellent properties. A seal made of these materials will also act as a buffer between the sensor components and the external capsule. The membrane and control chip are all made from silicon (Si) and its glass derivative silicon dioxide (SiO_2). Additional membrane materials include anodic aluminium oxide (AAO) and the polymers cellulose ester (CM), polyamide (PAR, PATF) and polycarbonate (PC). These were selected from their nanoporous nature and chemical property as well as the potential for integration on a miniaturized silicon membrane frame. The whole sensor is enclosed by an external capsule that protects the

internal components from the external environment. Sylgard 184 is a polydimethylsiloxane (PDMS) that is commonly used in microfluidics and lab-on-a-chip devices, whereas araldite 2020 (A2020) is a two component epoxy resin that offer an additional degree of mechanical support. Both of these (alone or in combination) will be moulded around the sensor assembly and cured at room temperature. Type 316L stainless steel (Me) used to encapsulate prototype devices was included in these studies for comparison.

The sensor (Fig. 4) rests in the interstitial fluid (ISF) which represents an ultrafiltrate of blood, and which in accordance to previous studies has shown to mirror the glucose concentration in blood (97-100).

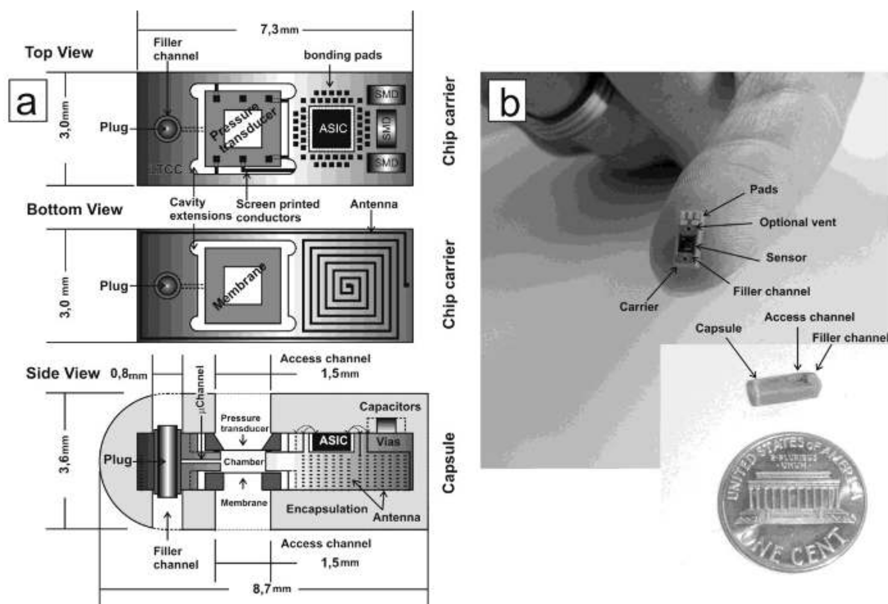

Fig. 5 *Computer-aided design depicting the architecture of the sensor implant **(a)**. The sensor chip, incorporating a differential pressure transducer located at the front, followed by the application-specific integrated circuit, ASIC (including temperature compensation) and components of the inductive powering and telemetry interface. The membrane and the inductive coil antenna are assembled on the reverse side. The whole unit is encapsulated in epoxy resin (A2020), with a filler channel enabling injection of the aqueous osmotic active solution prior to operation. **(b)** Assembled prototype carrier and capsule. An optional venting channel assisted filling in early versions (101)*

The glucose sensor records the osmotic pressure that is generated in a sensor cavity [1] enclosed by a nanoporous membrane [2] and a silicon pressure transducer [3]. The membrane and transducers are attached by an adhesive [4] to the sensor carrier [5] and sealed with a sealant material [6] forming a flexible buffer against the external capsule [7]. The sensor cavity maintains an active solution [8] based on the concanavalin A (Con A) - dextran affinity assay used to identify glucose from other components in blood (102;103). The membrane acts as an immunological barrier that protects the assay molecules against antibodies that are too large to pass through the nanopores. The membrane, sealant and encapsulant will be in direct contact with the ISF through the access channel [9], whereas the carrier, adhesive and transducer will be in indirect contact through the membrane. The osmotic pressure is generated by glucose diffusing through the membrane and displacing dextran from the Con A in a competitive manner. The release of dextran increases the free particle concentration inside the cavity. This particle increase changes the transmembrane concentration gradient which results in an increase in the osmotic pressure inside the cavity due to water diffusing through the membrane by the process of osmosis. This process is reversible and the free dextran will reattach back to Con A (reducing the net free particle concentration in the cavity) as the level of glucose is reduced.

3.2 Materials description

A total of 18 different materials were investigated in this project. 14 of these were implemented in the first study (Study 1), 15 of these in Study 2 and 3 and eight materials in Study 4. All the materials were grouped in accordance to the construction of the device (Table 2). The commercial nanoporous membranes were chosen to have an average pore diameter ranging from below 1 nm (two different polyamide membranes PAR and PATF with molecular weight cut-off (MWCO) rated to zero), a cellulose ester membrane (CM) with pore

size 2.5 nm, anodic aluminium oxide membrane with pore size 5 nm and a polycarbonate membrane with a pore size of 15 nm (polycarbonate with a MWCO of 500 kDa).

Table 2. *Candidate materials for implementation in the glucose sensor*

	Material	Abbreviation	Specification	Manufacturer
Encapsulation materials	Sylgard 184	PDMS	Polydimethylsiloxane	Dow Corning Corp., Midland, MI
	Araldite 2020	A2020	Epoxy resin	Huntsman Ltd., Duxford, UK
	Stainless steel	Me	Corrosion resistant, Type 316L	Fosstech Engineering, Stokke, Norway
Membrane materials	Silicon	Si	Silicon with native 2-3 nm oxide surface	HiVe, Horten, Norway
	Silicon dioxide	SiO_2	Silicon with a 500 nm thick thermal oxidized surface	HiVe, Horten, Norway
	Cellulose ester	CM	Ultrafiltration membrane (MWCO 5000 Da, ~2.5 nm)	Spectrum Labs Europe B.V., Breda, Netherlands
	Polyamide	PAR	Reverse osmosis membrane (MWCO 0 Da, < 1 nm)	Sterlitech Corporation, Kent, WA
	Polyamide	PATF	Thin Film membrane (MWCO 0 Da, < 1 nm)	Sterlitech Corporation, Kent, WA
	Polycarbonate	PC	Track-etched membrane (MWCO 500 kDa, ~15 nm)	Whatman plc, Kent, UK
	Aluminum oxide	AAO	Anodic aluminum oxide (MWCO 50 kDa, ~ 5 nm)	Synkera Technologies Inc., Longmont, CO
	Polyarylethersulphone	PAES-1	Microdialysis membrane (MWCO 20 kDa)	Dipylon Medical AB, Solna, Sweden
	Polyarylethersulphone	PAES-2	Microdialysis membrane (MWCO 100 kDa)	Dipylon Medical AB, Solna, Sweden
	Polyurethane	PU	Microdialysis catheter, outer shaft	Dipylon Medical AB, Solna, Sweden
Sensor carrier materials	CeramTec GC	CT	Low temperature co-fired ceramic	Ceramtec AG, Plochingen, Germany
	DuPont 951	DP	Low temperature co-fired ceramic	Dupont, Wilmington, DE
Sealing materials	Silicone 3140 coating	S3140	Silicone-based polymer	Dow Corning Corp., Midland, MI
	Silicone 3145 adhesive	S3145	Silicone-based polymer	Dow Corning Corp., Midland, MI
	Epo-Tek 353ND	ETek	Epoxy resin	Epoxy Technol. Inc., Billerica, MA

A membrane with a pores size of 5 nm was the preferred candidate for implementation in the osmotic glucose sensor since it permits glucose to pass unhindered through the membrane and into the reference chamber (where the osmotic pressure is generated), while retaining the larger components of the affinity assay (101;103). Two commercially available membranes used in microdialysis catheters were included in Study 4 since these are approved biomaterials already in use by medical devices: polyarylethersulphone membrane PAES-1 with MWCO 20 kDa and polyarylethersulphone membrane mixed with polyamide PAES-2 with MWCO 100 kDa and a catheter shaft of polyurethane (PU) was used as the negative control for microdialysis membranes.

3.3 Experimental protocol

The experiments were performed in 24-wells polystyrene microtiter plates. The material samples with liquid properties (PDMS, A2020, S3140, S3145, ETek) was prepared by administering 0.5 mL of the uncured monomer in the respective wells and subsequently polymerized at room temperature (60^0C for ETek) for 24 hours in accordance to the recommendations of the manufacturer. Material samples with a solid structure (Si, SiO_2, CT, DP) were cut into pieces of 1 cm^2. The nanoporous membranes and metal parts were cut into circular structures with a surface area of 1 cm^2. The microdialysis membranes were delivered as catheters with a 30 mm long membrane part that was cut into 1 cm long units and collected in groups of 9 pieces that would correspond to the surface area of the other membranes.

All the material samples (except for the nanoporous membranes) were washed 3 times with ethanol and then rinsed with distilled water. The solid materials were additionally treated with an ultrasonic bath to remove residual microparticles left behind on the material surface as a result of the manufacturing process or dicing of samples. The nanoporous membranes were incubated in distilled water overnight in accordance to the manufacturer’s instruction and

stored in phosphate buffered saline (PBS). Microdialysis membranes were cut into the pieces before each experiment. All the materials used in this study were rinsed with PBS prior to use in the experimental protocols.

The serum model was based on a serum batch from five healthy volunteers (Study 1), whereas the whole blood models (with and without soluble anticoagulant) were based on whole blood from five healthy volunteers (Study 2, 3 and 4). Serum was prepared by allowing the blood to clot at room temperature for two hours. After centrifugation at 2500*g* for 15 min at 4^0C, the serum was collected and mixed to one batch before being stored at -70^0C. The whole blood was anticoagulated with the thrombin inhibitor lepirudin (Refludan®, Pharmion Germany GmbH, Hamburg, Germany) to a final concentration of 50 µg/mL (85;104) for use in Study 2 and 3. The whole blood without soluble anticoagulant (Study 4) was collected in heparin-coated tubes and immediately transferred to wells coated with heparin (Carmeda AB, Upplands Vasby, Sweden). Informed consent was obtained before blood donation and the study was approved by the local ethical committee.

The experiments were performed by first administering 0.25 mL of serum (Study 1) or 0.5 mL of whole blood (Study 2, 3 and 4) in each well containing the respective biomaterial or controls. The samples were then incubated at 37^0C in a climate room with the plate placed on a shaker platform in a sealed container. The incubation time depended on the different analysis methods and ranged from 20 min for the CD11b expression, 30 min for the complement activation study and coagulation assays, 60 min for the surface complement activation experiment, β-thromboglobulin (BTG) and MPO release, 120 min for the platelet count and finally 240 min for TF expression and cytokines analysis. The coagulation assays used native polystyrene wells as controls to validate the effect of the heparin-coated wells for incubation of blood without soluble anticoagulant. The plates were placed on a shaker

platform and incubated until clear signs of coagulation occurred or up to a maximum of 48 hours.

After the incubation was completed, serum (from Study 1) or whole blood (from Study 2, 3 and 4) was withdrawn from the wells, and immediately transferred on ice. The activation process was stopped by adding ethylenediaminetetraacetic acid (EDTA) to a final concentration of 20 mM (Study 1,2 and 3), or sodium citrate (Study 4, 1 part in 9 parts of whole blood). The serum samples were aliquoted and frozen in -70^{0}C, whereas the whole-blood samples required first to be centrifuged at 1400*g* for 15 min at 4°C to separate the plasma phase that was aliquoted and frozen at -70 °C. Blood used for subsequent platelet count (Study 3) was collected in EDTA tubes and delivered to the hospital laboratory for analysis. For the BTG assay, 450 µL of blood was aliquoted before adding 50 µL citrate-theophylline-adenine-dipyridamol (CTAD) buffer (105). The tubes were then immediately placed on ice and centrifuged at 2500*g* for 20 min at 4 °C. After centrifugation, one third of the plasma volume in the middle region was collected to prevent platelet contamination. The collected plasma was than centrifuged a second time at 2500*g* for 20 minutes at 4°C. The plasma was then collected as previously described and frozen at -70 °C.

The materials used for the detection of solid-phase complement activation in Study 1 were (after the incubation step) rinsed 3 times with PBS containing 0.1% Tween 20 before the TCC deposition was immediately analyzed by EIA.

3.4 Controls

Heat aggregated immunoglobulin G (HAIGG) and zymosan diluted to a final concentration of 1 mg/mL served as the positive control for complement activation. The thrombin receptor activation peptide (TRAP-6, Bachem AG, Bubendorf, Switzerland) diluted to a final concentration of 12.5 µM served as the positive control for the BTG-assay in Study 3 (106).

Ultrapure LPS in a concentration of 10 µg/mL was used as the positive control for cytokines, whereas wells coated overnight with 0.5 mL 50 µg/mL HAIGG and blocked with 1 mL 1% bovine serum albumin (BSA) were used as the positive control for surface complement activation. Empty polystyrene wells served as the negative control.

3.5 Assays

3.5.1 Enzyme immunoassays

Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assays (ELISA) based on monoclonal antibodies was used to measure the level of both solid-phase and fluid-phase complement activation (both in Study 1) since it recognizes the products from the different pathways. The ELISA was also used to assess the level of MPO (Study 2), BTG (Study 3 and 4), prothrombin fragment 1+2, PTF 1+2 and the thrombin-antithrombin complexes, TAT (Study 4).

Solid-phase complement activation

The deposition of TCC on the sample surface after incubation in serum was analyzed by incubating a monoclonal anti-TCC antibody (clone aE11) for 1 hour to permit reaction with a neoepitope exposed in the C9 when incorporated into the C5b-9 complex (107). In parallel experiments, the same material samples were incubated with an isotype control IgG2a antibody and the plates were then incubated with a biotinylated anti-mouse IgG2a monoclonal antibody (both obtained from BD Pharmingen, San Diego, CA) for 45 min. This was followed by incubation with streptavidin-conjugated horseradish peroxidase (Amersham Bioscience Inc, Piscataway, NJ) for 45 min. Finally, 2,2 azino-di(3-ethyl)-benzthiazoline sulphonate containing H_2O_2 was used as the substrate. The plates were washed 3 times between each incubation step in PBS containing 1% Tween 20. Duplicate wells were used in each experiment. The data acquired from different plates were compared by standardizing readouts

by the use of optical density (OD) in wells coated with HAIGG. All the results were recorded when the OD (wavelength of 405/492 nm in the microplate reader Asys Expert 96, Biochrom Ltd, Cambridge, UK) in wells coated with HAIGG was around 1.

Fluid-phase complement activation

The complement component Bb is an activation marker of the alternative pathway, whereas C4d represents the activation of both the classical and the lectin pathways. The intermediate product C3bc indicates activation of the common C3 precursor, while TCC represents activation of the terminal pathway.

The components C4d and Bb were quantified using ELISA based on mouse monoclonal antibody specific for human C4d and Bb according to the instructions from the manufacturer (Quidel, San Diego, CA). The C3bc concentration was measured by an ELISA based on the mouse anti-human C3bc antibody (clone bH6) reacting with a neoepitope exposed in C3b and C3c after activation of C3. The assay has been described in detail previously (108). The TCC concentration was measured by an ELISA based on the mouse anti-human TCC antibody (clone aE11) reacting with a neoepitope exposed in C9 when incorporated into C5b-9. This assay has also been described in detail previously (109), and was performed according to a later modification (110).

Myeloperoxidase

The MPO concentration in plasma was measured with a commercial EIA kit (Cat.no HK324, Hycult biotech, Uden, The Netherlands) in accordance to manufacturer's instruction.

β-thromboglobulin

The BTG level in plasma samples was quantified using an EIA based on a rabbit anti-human β-thromboglobulin antibodies according to the instructions from the manufacturer (Cat.no 00950, Diagnostica Stago, Asnieres, France).

Coagulation and platelet activation assays

PTF 1+2 and TAT were analyzed in plasma samples by an EIA kit (Enzygnost, both obtained from Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany) based on monoclonal mouse antibodies to human F1+2 and human thrombin.

Multiplex assay

Cytokine and chemokine concentrations in plasma

The plasma samples were first screened with a 27-plex multiplex cytokine assay (Bio-Plex Human Cytokine 27-Plex Panel, Bio-Rad Laboratories Inc., Hercules, CA). Of these, six cytokines and chemokines responded significantly and were chosen for further analysis: IL-6, TNF-α, IL-1β, IL-8, MCP-1 and MIP-1β. The analysis was performed according to the manufacturer's instructions.

3.5.2 Flow cytometry

Flow cytometry was used to measure the level of CD11b expression on PMN surface that was presented in Paper 2 and the TF expression on monocyte surfaces as presented in Paper 3.

CD11b expression

Blood was withdrawn from the wells containing the biomaterial candidate after a 20 min incubation period, and fixated using a 0.5% paraformaldehyde solution for 4 min at 37 °C. The blood cells were then protected from light (kept in the dark) and stained at room temperature with anti-CD11b-PE antibodies (Cat. no. 333142) or an isotype control IgG2a-PE antibodies (cat.no 349053), both obtained from Becton Dickinson (Franklin Lakes, NJ) for 15

min. The red blood cells were lysed and the samples centrifuged at 300*g* for 5 min at 4 °C. The supernatants were discarded and the cells were washed twice with 2 mL PBS before centrifugation at 300*g* for 5 min at 4 ºC. The cells were then resuspended in PBS containing 0.1% albumin and placed on ice in the dark until data acquisition by the flow cytometer (FACScan, Becton Dickinson, Franklin Lakes, NJ) was performed. The PMN were gated in a FSC/SSC-dotplot, and the mean fluorescence intensity (MFI) values for CD11b were calculated.

Tissue factor expression

The FITC-conjugated antibody against human TF (Cat.no. 4508CJ, American Diagnostica Inc., Stamford, CT) was prepared according to the manufacturer's instructions and used at a final concentration of 50 µg/mL. The IgG1-FITC antibodies (Cat.no. 345815) were used as the isotype matched control. Anti-CD14-PE antibodies (Cat.no. 345785, both obtained from Becton Dickinson, Franklin Lakes, NJ) was used as a marker of monocytes. After sample incubation, the antibody mixture was added and the samples incubated in the dark for 15 min at room temperature. The samples were then lysed with 1 mL EasyLyse (S2364, Dako Cytomation, Glostrup, Danmark) at room temperature for 15 min, centrifuged at 300*g* for 5 min at 4 ºC, washed twice, and analysed immediately with a flow cytometer with the threshold put on forward scatter (FACScan, Becton Dickinson, Franklin Lakes, NJ). The monocytes were gated in a PE/SSC-dotplot, and the results were recorded as median fluorescent intensity (MedFI).

3.6 Complement inhibition

As part of the process of evaluating the role that the complement system plays in determining the materials biocompatibility, it was required to inhibit part of the cascade process at the C3 and C5-level. The compstatin analog Ac-I[CV(1MeW)QDWGAHRC]T, which binds to and

inhibits cleavage of C3, was produced as previously described together with a control peptide (111). The analog was used in Study 2 at final concentrations of: 25 mM; 12.5 mM; 6.25 mM and 3.125 mM. Eculizumab (Soliris®, Alexion Pharmaceuticals, Cheshire, CT), a monoclonal antibody that specifically binds to the complement protein C5 was used in final concentrations of: 50 µg/mL; 25 µg/mL; 12.5 µg/mL and 6.25 µg/mL (Study 2), 50 µg/ml (Study 3), and 100 µg/ml (Study 4). The whole blood was pre-incubated with these complement inhibitors for 5 min. before the candidate materials were placed in the wells.

3.7 Statistical analysis

The results were statistically compared by one-way analysis of the variance between groups (ANOVA) with Bonferroni post test analysis. Data from the experiments with complement inhibitors in Study 2 were compared by one-way ANOVA in order to analyze the dose-dependent decrease of activation. The effect of complement inhibition in Study 3 and 4 was evaluated using a two-tailed t-test. Results with a p-value <0.05 was considered as statistically significant. All the statistical data were collected and calculated with GraphPad Prism version 5.01 (GraphPad Software, San Diego CA, USA).

4. SUMMARY OF THE MAIN RESULTS

4.1 Study 1

This study presents the complement-activating properties of the candidate materials used in the initial experiment (Step 1).

The solid-phase complement activation was measured by the degree of deposition of TCC on the material surfaces. Considering corrosion resistant stainless steel (Me), silicon (Si), silicon dioxide (SiO_2), cellulose ester membrane (CM), and the low temperature co-fired ceramics CeramTec GC (CT) and Dupont 951 (DP), the degree of deposition was higher compared to the negative control ($p<0.05$). In contrast, polydimethylsiloxane (PDMS) and silicone 3140 (S3140) showed a lower degree of deposition than the negative control ($p<0.05$). The deposition of TCC on araldite 2020 epoxy resin (A2020), Epo-Tek 353ND epoxy resin (ETek) and silicone 3145 adhesive (S3145) was comparable to the negative control (polystyrene). The isotype control antibody revealed a low degree of unspecific binding, except for the CM, which displayed high degree of unspecific binding.

Since the candidates for the membrane group were incompatible, three additional nanoporous materials, polyamide (PA), polycarbonate (PC) and anodic aluminium oxide (AAO), which exhibited different material composition and pore sizes, were selected for further investigation and comparison with the CM. The solid-phase complement activation was comparable for all the four membranes, and as evaluated by the deposition of TCC on the membrane surface, was found to be significantly higher than the negative control ($p<0.05$). The antibody used for the isotype control confirmed a particularly high unspecific binding to the CM and PA, whereas the other two materials, in particular PC, showed markedly less unspecific IgG binding.

Considering the fluid phase complement activation, the activation products C4d, Bb, C3bc and TCC re-confirmed CM to be highly incompatible, whereas PC was the only one of the membranes which did not induce any complement activation in the fluid phase.

4.2 Study 2

This study demonstrates the PMN activating properties of the candidate materials, as evaluated by the surface expression of CD11b and the release of MPO. The effect of complement inhibition on PMN activation was also investigated.

The expression level of CD11b after incubation with three of the membrane candidates (CM, PAR, and PATF) was significantly higher compared to the negative control ($p<0.05$). Similarly, the concentration of MPO in plasma after incubation with three of the membrane candidates (CM, PAR and PATF) was also significantly higher than the negative control ($p<0.05$). The candidate materials used for the encapsulation (PDMS, A2020, Me), carrier (CT and DP), sealing (S3140, S3145 and E-Tek) and the remaining four membrane candidates (Si, SiO_2, PC, AAO) did not induce CD11b expression and MPO release compared to the negative control.

The three membrane candidates which were shown to activate PMN, were chosen to investigate the effect of complement inhibition. Thus, complement inhibition by the C3-inhibitior compstatin led to a dose-dependent decrease in the level of CD11b expression and MPO release induced by all the three materials. Similarly, complement inhibition by the C5-inhibitor eculizumab decreased the CD11b expression on PMN and MPO release in a dose-dependent manner.

4.3 Study 3

This study demonstrates the importance of complement in inducing secondary inflammatory reactions related to exposure from a series of candidate biomaterials.

The complement activation products Bb, C3bc and TCC were significantly increased by three of the candidate membranes (CM, PAR and PATF) ($p<0.01$). These membranes were also found to significantly increase the concentration of the IL-6, TNF-α, IL-1β and MCP-1 ($p<0.05$ for all). The concentration of IL-8 was also significantly increased by these three materials as well as for two of the carrier candidates, CT and DP. The concentration of MIP-1β showed a slightly different pattern by being increased by only Me and CM.

The expression of TF was significantly higher for the same three membrane candidates CM, PAR and PATF. No difference was observed in the expression of TF with any of the other materials. The isotype matched control for PAR was, however, significantly higher than the negative control so the specificity could not be verified in this case.

CM, PAR and PATF were included in supplementary experiments to test the hypothesis that complement activation is the primary event leading to these secondary reactions. Complement inhibition led to a significant decrease in the TF expression on monocytes that were induced by PATF and CM, and virtually completely abolishing the expression. PAR showed a high level of unspecific binding of the TF antibodies and was therefore excluded from the experiment.

Complement inhibition with eculizumab showed a marked decreased in the pro-inflammatory cytokines IL-6, TNF-α and IL-1β after incubation with PATF, and IL-6 and TNF-α level after incubation with CM. The incubation with PAR had no effect on the cytokine level. Similarly,

complement inhibition significantly decreased the level of the chemokines IL-8 and MCP-1 after incubation with CM, PAR and PATF, but had no effect on the MIP-1β level.

4.4 Study 4

This study presents the coagulation and platelet activation by the membrane materials. The use of heparin coated wells permitted the use of a modified whole blood model without a soluble anticoagulant.

A whole blood model without a soluble anticoagulant was validated in an additional experiment that compared the effects of heparin-coated plates with native uncoated polystyrene. A complete coagulation of blood in the polystyrene wells was registered within 14 minutes (range 10 – 16 minutes, n=7). In contrast, whole blood from the same needle drained in heparin-coated wells (n=7) did not clot during 48 hours of incubation, when the experiment was terminated. Thus, the heparin-coated model was used in subsequent experiments.

The results showed that the PTF 1+2 was significantly increased after incubation with PAR in whole blood ($p<0.01$) and insignificantly increased after incubation with PATF. No increase in PTF 1+2 was seen after incubation with any of the other membrane materials chosen in the study. The concentration obtained with S3140, which was included in the experiments as a biocompatible material, was lower than the negative control. The level of TAT was significantly increased only after incubation with PAR in whole blood ($p<0.01$). An non-significant increase was observed by PATF. The level of BTG was significantly increased after incubation with five of the materials in whole blood, most pronounced for PAR, followed by PATF, PC, PAES-1 ($p<0.01$ for all) and PAES-2 ($p<0.05$)).

Complement inhibition with eculizumab had no effect on the concentration of PTF 1+2, TAT or BTG in the plasma samples. A TCC assay was performed to confirm that eculizumab inhibited the complement system. Consequently, the addition of eculizumab to whole blood completely abolished TCC production in the samples

5. DISCUSSION

The most important ethical goal for the use of artificial materials in medicine is to avoid any harmful effects on the human body. “Primarum non nocere”, the phrase known from Hippocrates oath concerning biomaterials meant that all interventions, including the implantation of artificial materials, should be safe for the patient and not elicit any dangerous reactions.

The safety of implantable devices does primarily depend on the biocompatible nature of the implant materials and the biological processes that is triggered at the site of implantation. The activation of biological cascades like the complement system, the coagulation system and the production of pro-inflammatory cytokines can both alter the sensor function and be harmful for the patient. This is especially important to consider regarding the potential use of implantable glucose sensors that would be capable of continuously monitoring the level of glucose in patients suffering from diabetes. Any infection or inflammatory reaction caused by the sensor implantation would have serious effects on the therapeutic treatment as well as the quality of life if the sensor was to malfunction or give faulty signals. In this project, we have evaluated a selection of candidate materials that were intended for use in an osmotic glucose sensor. The aim was to identify potential activating properties that could deem them acceptable or unacceptable for use in an implanted device. It was particular important to evaluate all the materials that would be incorporated, even if some of them would not be in direct contact with the interstitial fluid where the sensor would reside. Any mechanical damage to the sensor capsule would expose the underlying materials to the ISF and thus the potential incompatibility of these sensor components could lead to an inflammatory response.

Performing an *in vitro* analysis of the biocompatibility of a material is the first and absolutely necessary step to consider. In accordance to the International standard ISO 10993 part 4 (3) the appropriate methods were used to study the biocompatibility *in vitro*. The combined evaluation of the complement activation, the PMN activation, the cytokine secretion and the coagulation and platelet activation presented and almost complete picture of the *in vitro* immune response to implantable biomaterial candidates.

5.1 Complement activation on both solid and fluid phase

The complement system is considered to be the first line of defence of the immune system that is activated in response to the implantation of biomaterials. Literature have linked complement with the immune response to foreign materials and thereby their biocompatible nature (8;18;62;112;113). Therefore, the starting point of our initial study presented in Paper 1 was to use a serum model to investigate the activation potential of complement without having to consider any signals from other components of the immune system.

Complement activation by biomaterials is initiated by proteins that are adsorbed onto the material surface (48;61). This activation then leads to a spreading of this processes to the surrounding tissue, e.g. by the fluid phase. The degree of complement activation can clearly discriminate between biomaterials. Previous studies have suggested that both solid and liquid phases of complement activation are required to examine biomaterials (114). The initiation of the complement cascade at the surface of biomaterials with subsequent C3b absorption initiates formation of the enzyme C3-convertase that triggers activation of the alternative pathway (112). Moderately biocompatible biomaterials limit complement activation to the solid phase only, which makes it difficult to detect the activation products with the commonly known ELISA method. Biomaterials with a porous structure may additionally adsorb activation products that mask complement-activating properties of the material.

By evaluating both phases in study 1, the advantage of a combined approach was apparent by revealing different activating properties of the investigated materials. From the initial screening, two material candidates (the capsule candidate PDMS and the silicone adhesive S3140) activated complement to a lower degree than the negative control and was therefore regarded as biocompatible (Table 3). PDMS have been used in both implantable devices and biosensors (115) and its good biocompatibility have been confirmed both *in vitro* (116;117) and *in vivo* (118). Silicone-based polymers are also widely used as cosmetic implants as well as in contact lenses, and several studies have hence confirmed also these as biocompatible materials (119-121). The capsule candidate A2020, and the two sealing candidates ETek and S3145 were regarded as fairly biocompatible as the level of complement activation was comparable to the negative control. However, materials that activated complement in the solid phase only, was in this study considered as moderately biocompatible and hence deemed suitable candidates for implantation if further surface modifications are able to neutralise the observed immunological effects. Not a single candidate from the membrane group expressed the required biocompatible properties comparable to the negative control or better, which made this group a challenging issue, especially since the membrane forms the most central part of the sensors functional interface. The CM membrane as well as the additional membrane candidates (PA, AAO) expressed complement-activating properties in both the solid and the liquid phase. However, PC activated complement in the solid phase only and based on the results of the study could be regarded as the only membrane suitable for use in an implantable device (considering the limitations given above). The complement-activating properties of membrane candidates in literature have been related to the surface structure, the chemical composition (10) and the different pore sizes of the membrane materials (15). The membrane candidates that were examined in this project offer various pore sizes, as indicated by their molecular weight cut-off (MWCO, from zero for PA membrane to 5000 Da for CM).

The comparable magnitude of complement activation triggered by membranes with different pore size confirms the chemical structure and the degree of protein absorption on the surface as the major contributor of their complement-activating properties.

5.2 Activation of the immune cells and the inflammation cascade

The use of a whole blood model permitted further evaluation of these candidate materials as presented in Paper 2 and Paper 3 (85). This model allowed us to examine the interaction between the different inflammation cascades and the evaluation of blood cell activation. PMN is one of the first immune cells to appear at the site of implantation (39) and the primary role of these cells is to combat any infectious organism (43;44) with the release of various amount of cytokines, chemokines and enzymes. In the case of biomaterial implantation, the activation of this cell has a negative effect (40-42). The overactivation of PMN results in the release of an excessive amount of biologically active substances that does not only accelerate the healing process and combat infection, but could also lead to the damage of the implant and its materials. Our experiments identified a significant increase in PMN activation, TF expression on monocyte surface and cytokine concentrations after incubation with three of the membrane candidates (CM, PAR and PATF). The results are an agreement with study 1 and confirmed that these three materials are inappropriate candidates for use in an implantable device in their unmodified forms.

Based on the results from study 1 we could hypothesize that the inflammatory reaction was mediated with complement. Literature have suggested that complement inhibition prevents the production of inflammatory cytokines and platelet activation (93;94), and our experiments with the C3-inhibitor compstatin and the C5-inhibitor eculizumab confirmed this hypothesis. The experiment with eculizumab (that blocks the complement at the C5-level), confirmed the primary role of the C5-dependent mechanism of the inflammatory response. The dose-

dependent decrease of PMN activation and inflammatory response (CM, PAR and PATF) with complement inhibitors, confirmed complement as the primary inducer of the secondary inflammation response.

Since the complement system determines the inflammatory response to implanted biomaterials, a novel approach to the evaluation of materials biocompatibility can be proposed. The complement activation by biomaterials can be used as a primary screen of the biocompatibility and can be used as a prognostic marker of inflammatory reactions to implantable devices. The use of TCC to screen the complement activation gives more advantages compared to another markers because of the stability of the TCC molecule *in vitro* compared to the highly labile C3 molecule (122).

Some of the materials that were claimed as incompatible in our study are known biomaterials that have been used in medical devices before. The CM membrane especially is a known complement activator. Clinical studies of dialysis membranes suggested (5;123-126) that cellulose is not biocompatible, and which was confirmed in our experiments. Polyamide that in clinical studies have showed good biocompatibility (127-129), triggered a substantial activation of complement in our study. This discrepancy can be explained by the different tests and models that were used as well as the different types of polyamides. Most of the literature studies on the biocompatible nature of polyamide membranes were performed in clinical conditions where patient-to-patient variations could significantly influence the results. The use of the *in vitro* whole-blood model in our studies decrease the variability of the results and allowed the interaction between the immune cells, the inflammatory network and the complement cascade to be examined.

Another component of the sensor, Con A, was not included in the study despite its known toxic activity. The liquid properties of Con A and the small volume used in the

microfabricated sensor cavity will prompt this component to dissolve into the surrounding tissue without harmful consequences (130).

5.3 Activation of coagulation and platelets

The activation of the coagulation cascade and platelets was further explored in study 4. Since thrombin is irreversibly blocked in a whole blood model that have been anticoagulated with lepirudin, the use of such a model for the evaluation of coagulation and platelets activation would be substantially limited. Our study used a modified whole blood model without soluble anticoagulant, and required incubation of the material samples in wells coated with heparin. This model permitted us to create conditions that were close to *in vivo* and to examine the interaction of the coagulation system and platelets with the complement system, blood cells and the inflammatory cascade. The clear difference in coagulation time between wells coated with heparin and polystyrene wells confirmed protective effect of heparin coating.

Both PAR and PATF activated the coagulation cascade as it was shown by the measured increase in the PTF 1+2 and TAT concentration in the plasma (although the difference with the negative control value was not statistically significant for PATF). In contrast, the cellulose membrane that was shown to be incompatible in our previous studies as well as in several clinical studies (5;123;124;126), did not induce coagulation or platelet activation. This finding is in accordance to clinical studies of haemodialysis membranes where a blood contact with polyamide membrane led to significantly higher levels of TAT compared to a modified cellulose membrane (131).

The platelet activation measured by BTG concentration in plasma was increased for five of the materials. In contrast, in Study 3 using a whole blood model with lepirudin as anticoagulant none of the candidate materials used activated platelets. Lepirudin irreversibly blocks thrombin and abrogate the influence of this molecule on platelets in this model. Since

thrombin is a well-known platelet activator (75;76), we modified the whole blood model by coating the wells with heparin and omitting the addition of lepirudin, thus leaving thrombin functionally active in the fluid-phase. In contrast to our data using lepirudin (Study 3), the five materials including the membranes used in the previous study, triggered platelet activation as show by the increased BTG concentration. These findings suggest that membrane-induced thrombin formation activate platelet and underscores the importance of selecting a suitable model in the studies of coagulation and platelets activation by biomaterials.

The polyamide membranes PAR and PATF that triggered coagulation and platelet activation in Study 4, was also shown to be incompatible materials from the results presented in Study 1, 2 and 3 (activation of coagulation, blood cells and secretion of cytokines). These processes were confirmed to be complement-mediated (PATF), and based on these results we could hypothesize that coagulation and platelet activation are also mediated by complement activation. However, additional experiments with the complement C5 inhibitor eculizumab showed that the complement system, at the level of C5, had no effect on the coagulation and platelet activation with the candidate membranes. Accordingly, we assume that complement-independent mechanisms are responsible for the pro-coagulant properties of the materials. Both in clinical studies (29) and *in vitro* (132) it has previously been shown that any contact of biomaterials with blood leads to the activation of the contact system with further thrombin generation or thrombosis formation as the result. The inhibition of complement at the level of C5 did not inhibit the contact system. Complement inhibition had no effect on platelet activation as well, and the increased concentration of BTG can be explained from a direct interaction of platelets with the surface of the materials with further amplification in the presence of thrombin (32).

5.4 Evaluating the performance of implantable devices

The *in vitro* evaluation of biocompatibility is a primary tool (133) used to select potential biomaterials for use in implantable devices. However, data from the literature shows that the results of *in vitro* assays does not always confirm to the results that have been observed using the same materials *in vivo* and is therefore not a guarantee of device biocompatibility (82;134). Still, several of the candidate materials used in our studies have been confirmed as biocompatible also *in vivo*. PDMS that in our studies was found to be biocompatible *in vitro* has also been confirmed as biocompatible *in vivo* (118;135) due to the absence of inflammatory markers at the site of implantation. The biocompatibility of silicone implants and silicon materials have also been confirmed *in vivo* with acceptable levels of tissue inflammatory response both for the silicone elastomer and the silicon nitride used in the study (120;136). The correlation between the *in vitro* and the *in vivo* cytokines profile for biomedical polymers was confirmed by Anderson (137), suggesting that the results from the *in vivo* studies that were similar in nature to our *in vitro* findings confirmed the use of a whole blood model as the closest *in vitro* experimental representative of the *in vivo* environment. The primary role of complement in initiating the PMN activating properties, TF expression and cytokine production in response to biomaterials exposure, renders complement as a preferred tool to use in order to identify suitable materials for subsequent *in vivo* studies.

6. FUTURE PERSPECTIVES

The material candidates investigated in Study 1-4 were classified as either biocompatible, fairly biocompatible, moderately biocompatible or incompatible (Table 3). Materials with biocompatible (level of complement, inflammation, PMN and coagulation activation lower than the negative control) and fairly biocompatible (level of activation comparable with the negative control) properties were proposed to be good candidates for use in implantable devices and for future *in vivo* studies. The materials that were classified as moderately biocompatible, activated complement in the solid phase or induced limited activation of the PMN, the inflammation cascade or the coagulation. These could only be considered as desirable candidates if they were subject to further improvement by for instance surface modifications. However, the materials that were found to be inbiocompatible (high degree of activation in several assays), were considered as unsuitable candidates for use in any implantable device.

Table 3. Classification of evaluated materials based on the results of Study 1-4

Material	Study 1	Study 2	Study 3	Study 4
PDMS	***Biocompatible***	Fairly biocompatible	Fairly biocompatible	NS
A2020	Fairly biocompatible	Fairly biocompatible	Fairly biocompatible	NS
Me	Moderately biocompatible	Fairly biocompatible	Moderately biocompatible	NS
Si	Moderately biocompatible	Fairly biocompatible	Fairly biocompatible	NS
SiO_2	Moderately biocompatible	Fairly biocompatible	Fairly biocompatible	NS
CM	**Incompatible**	**Incompatible**	**Incompatible**	Fairly biocompatible
PAR	**Incompatible**	**Incompatible**	**Incompatible**	**Incompatible**
PATF	**Incompatible**	**Incompatible**	**Incompatible**	**Incompatible**
PC	Moderately biocompatible	Fairly biocompatible	Fairly biocompatible	Moderately biocompatible
AAO	**Incompatible**	Fairly biocompatible	Fairly biocompatible	NS
PAES-1	NS	NS	NS	Moderately biocompatible
PAES-2	NS	NS	NS	Moderately biocompatible
PU	NS	NS	NS	Fairly biocompatible
CT	Moderately biocompatible	Fairly biocompatible	Moderately biocompatible	NS
DP	Moderately biocompatible	Fairly biocompatible	Moderately biocompatible	NS
S3140	***Biocompatible***	Fairly biocompatible	Fairly biocompatible	Fairly biocompatible
S3145	Fairly biocompatible	Fairly biocompatible	Fairly biocompatible	NS
ETek	Fairly biocompatible	Fairly biocompatible	Fairly biocompatible	NS

Abbreviations: NS – not included in the study

The main goal of surface modification is to shield the underlying artificial surface away from the immune system (138). Several strategies exist that improves the materials biocompatibility. These include surface modification with hydrogels, surfactants and heparin, as well as the modification of surface topography; and the application of flow-based systems that decreased biofouling and protein adsorbtion to a material surface (69). Since complement has been confirmed as the primary inducer of the inflammatory response to biomaterials, surface modifications with complement inhibitors or native regulators may represent a promising method to use. Polyethylene glycol (PEG) coating is found to decrease the nonspecific protein adsorption (69;139;140) and therefore assists in decreasing the activation of the coagulation system and the subsequent adhesion of platelets and cells (141). However, PEG-coated surfaces does not prevent the activation of complement (17;139). Instead, coating the surface with heparin may have a more potent effect on the immune system components. It has been shown to that heparin coating decreases level of coagulation activation and inflammatory reaction as well as that of complement activation (8;93;95). The adsorption of thrombomodulin on a material surface has been used to improve the blood compatibility and have showed promising effect on the coagulation and complement systems. However it demonstrated no effect on platelet activation (142). The immobilisation of apyrase effectively prevents platelet activation and adhesion, but this method had no effect on suppressing the complement activation (143). Factor H, a primary regulator of the alternative pathway activation (24) have showed promising results to prevent complement activation on material surface in literature studies (17;144) and presents a suitable method to improve the complement activating properties of moderately biocompatible materials.

Modification of material surface with adhesion ligands and growth factors that prevent fibrosis and controls neovascularisation is also possible, but the sensor interface is challenging to modify since the sensor should maintain appropriate analyte transport through the multi-

layer coating. For this reason, highly water-swollen hydrogels with minimal impact to sensor interface can be use for surface modification of implantable sensors.

All of these methods, alone or in combination, have the potential to reduce the immune response of implantable medical devices and their materials. As well as posing a direct effect on the immune system components, the immobilised layer would also need to maintain a high level of stability with time in order to have any noticeable impact on the lifespan of the device (145). Achieving long term operation of a medical implant will facilitate the continuous surveillance of key physiological parameters as part of an improved therapeutic regime. This will facilitate an early intervention that will help to decrease the consequences from acute physiological events (heart attach, stroke) as well as preventing long term complications from chronic diseases such as diabetes.

7. CONCLUSIONS

1. Discriminating between complement activation in the solid and the fluid phase illustrated the importance of investigating both phases to determine the level of biocompatibility of the material candidates in the serum model.

2. Materials that activated complement in both the solid and the fliud phase (CM, PAR and PATF) were regarded as incompatible and were considered as unsuitable candidates for implementation in an implantable device.

3. The incubation of three of the 15 materials (CM, PAR and PATF) in whole-blood with a soluble anticoagulant induced a significant inflammatory response with an increased level of CD11b expression on PNM, MPO release, TF expression on monocytes and an increased level of cytokines and chemokines. By activating this parameters they would not be considered as suitable candidates for use in implantable devices.

4. Complement inhibition completely abolished or significantly reduced the inflammatory response by CM, PAR and PATF in a dose-dependent manner that confirmed the complement system as a primary inducer of the inflammatory response to material candidates.

5. The use of a modified whole blood model without a soluble anticoagulant is considered to be a suitable *in vitro* assay for the investigation of the coagulation and platelet activation triggered by biomaterials.

6. The complement system is a primary event that is responsible of inducing an immune response to biomaterials. Nevertheless, the inclusion of haemostatic assays in whole blood without a soluble anticoagulant is required for to evaluate the biocompatibility, since it permits the study of complement-independent mechanisms

The biocompatibility of candidate materials in this project was studied using a wide spectrum of assays for complement, coagulation and PMN activation as well as cytokine production. The existing whole blood model was modified in order to study the coagulation cascade without using a soluble anticoagulant. The results presented in this Thesis suggest that complement activation is an important factor to consider as the initial screening marker of biocompatibility. This activation was to a great extent responsible for the inflammatory reaction induced by the biomaterial candidates in the other models that were investigated.

Since the *in vitro* environment can be different from the conditions *in vivo,* there is a possibility that the materials that do not activate complement and inflammatory cascades *in vitro*, might turn out to be incompatible *in vivo.* Future studies will seek to correlate the results from the *in vitro* assays with *in vivo* studies.

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9. Papers I-IV

II

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ORIGINAL ARTICLE

Activation of Polymorphonuclear Leukocytes by Candidate Biomaterials for an Implantable Glucose Sensor

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Abstract

Background:

Continuous monitoring of glucose by implantable microfabricated devices offers key advantages over current transcutaneous glucose sensors that limit usability due to their obtrusive nature and risk of infection. A successful sensory implant should be biocompatible and retain long-lasting function. Polymorphonuclear leukocytes (PMN) play a key role in the inflammatory system by releasing enzymes, cytokines, and reactive oxygen species, typically as a response to complement activation. The aim of this study was to perform an *in vitro* analysis of PMN activation as a marker for biocompatibility of materials and to evaluate the role of complement in the activation of PMN.

Methods:

Fifteen candidate materials of an implantable glucose sensor were incubated in lepirudin-anticoagulated whole blood. The cluster of differentiation molecule 11b (CD11b) expression on PMN was analyzed with flow cytometry and the myeloperoxidase (MPO) concentration in plasma was analyzed with enzyme-linked immunosorbent assay. Complement activation was prevented by the C3 inhibitor compstatin or the C5 inhibitor eculizumab.

Results:

Three of the biomaterials (cellulose ester, polyamide reverse osmosis membrane, and polyamide thin film membrane), all belonging to the membrane group, induced a substantial and significant increase in CD11b expression and MPO release. The changes were virtually identical for these two markers. Inhibition of complement with compstatin or eculizumab reduced the CD11b expression and MPO release dose dependently and in most cases back to baseline. The other 12 materials did not induce significant PMN activation.

Conclusion:

Three of the 15 candidate materials triggered PMN activation in a complement-dependent manner and should therefore be avoided for implementation in implantable microsensors.

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Abbreviations: (A2020) araldite 2020, (AAO) aluminium oxide, (ANOVA) analysis of variance, (CD11b) cluster of differentiation molecule 11b, (CM) cellulose ester, (CT) ceramTec GC, (ConA) concanavalin A, (DP) DuPont 951, (ETek) epo-Tek 353ND, (ISF) interstitial fluid, (LTCC) low-temperature cofired ceramic, (Me) stainless steel, (MFI) mean fluorescence intensity, (MPO) myeloperoxidase, (MWCO) molecular weight cut off, (PAR) polyamide reverse osmosis membrane, (PATF) polyamide thin film membrane, (PBS) phosphate buffer saline, (PC) polycarbonate, (PDMS) polydimethylsiloxane, (PMN) polymorphonuclear leukocyte, (ROI) reactive oxygen intermediate, (S3140) silicone 3140, (S3145) silicone 3145, (Si) silicon, (SiO_2) silicon dioxide

Keywords: biocompatibility, biomaterials, complement, implantable device, polymorphonuclear leukocyte

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Introduction

Global prevalence of diabetes mellitus is increasing, with an estimated 250 million people now suffering from the condition according to the latest survey by the American Diabetes Association. Latest figures released in January 2011[1] confirm that the number of diagnosed cases in the United States have risen to 18.8 million people, whereas another 7 million may live with the condition undiagnosed. Therefore, the main goal for an effective treatment of diabetes is the maintenance of blood glucose values within what is considered normal values for a healthy subject. Such tight control of glucose can best be achieved by continuous monitoring of the glucose level either directly in blood or in the interstitial fluid (ISF) representing an ultrafiltrate of blood.[2]

Continuous glucose measurements will track the glucose level in real time and thereby predict upcoming hypo- or hyperglycemic events at an early stage. To realize this potential, implantable glucose sensors should be able to measure the concentration of glucose within tolerances that are as good as that of current state-of-the-art *ex vivo* sensors. They should exhibit as small a drift as possible, be unobtrusive, and exhibit biocompatible properties with the tissues at the implantation site.

Biocompatibility, by definition, is the ability of a material to perform with an appropriate host response in a specific application,[3] and which in clinical medicine have been extended to a quality of being mutually tolerant with life.[4] Since the complement system plays a critical role in the inflammation response to implantable devices, it is reasonable to refer to complement compatibility as an initial important factor in predicting biocompatibility of a material.[5–7]

The complement system is an important part of innate immunity, and can be activated through the classical, the lectin, or the alternative pathway. These complement pathways converge at the C3-level, which results in formation of C3b. This is a key molecule that governs further activation of C5 to C5a and C5b, as well as triggering an amplification loop for the alternative pathway. C5a is a potent anaphylatoxin, whereas C5b participate in the formation of the terminal complement complex, which is the end product of complement activation.[8,9]

Transcutaneous glucose sensors approved for continuous glucose monitoring are based on enzyme technology, and the most long-lived sensors are qualified for use up to 7 days.[10] We propose that application of a microfabricated osmotic sensor technology in combination with selective biomaterials can extend the lifetime operation of the glucose sensor *in vivo*. The sensor makes use of an affinity assay, which does not consume any reagents or generate any toxic by-products that are detrimental for sensor operation with time.[11] Because it will be essential to avoid any biological responses that may interfere with detection of glucose, the sensor should not elicit any significant inflammatory response following implantation *in vivo*.[12–15] Hence, the purpose of these *in vitro* studies was not to accurately predict sensor performance *in vivo*,[16] but to enable a preliminary screen of biocompatibility in order to remove materials that would otherwise elicit a detrimental immunological response inside the body.[5]

Polymorphonuclear leukocytes (PMN) can be activated by invading pathogens as a favorable event to combat infection as well as by artificial materials, then often with unfavorable effects damaging the surrounding tissue with impairment of the device function. They play a key role in the inflammatory system through the release of enzymes and reactive oxygen species as well as producing proinflammatory cytokines, in particular, chemokines generating chemotactic signals attracting other leukocytes. Polymorphonuclear leukocytes are the predominant cells causing local inflammation in response to biomaterials during the first days after implantation.[17] Activation of PMN involves alterations of membrane receptors such as cluster of differentiation molecule 11b (CD11b) and L-selectin (CD62) that lead to an increased adhesiveness to artificial surfaces and initiation of degranulation. Polymorphonuclear leukocytes do also produce highly reactive oxygen intermediates (ROI) through a process known as oxidative burst. This is another potent mechanism of defense, but overproduction of these substances could also damage surrounding tissues as well as the implanted biomaterials.[18,19] The action of ROI is further augmented by myeloperoxidase (MPO) released from azurophilic granules during PMN activation.[18,20] All these processes can significantly alter the function of implantable devices and lead to sensor dysfunction. Thus, it is important to determine the PMN-activating properties of implantable materials.[21]

An important part of PMN activation is governed by the complement system of the innate immunity.[22,23] We examined the biomaterials intended for use in an

osmotic glucose sensor with respect to their complement activation potency, and found that the membrane materials activated complement through the alternative pathway.[24] Such activation leads to generation of anaphylatoxins C3a and C5a. These are known to stimulate PMN with subsequent degranulation and to generate production of ROI and up-regulation of membrane receptors.[22,23,25]

The aim of this study was therefore to perform an *in vitro* analysis of PMN activation following exposure to selected candidate materials for an osmotic glucose sensor and to evaluate how the activation of complement contributes to activation of PMN in response to such materials.

Materials and Methods

Material Preparation and Incubation

This study emphasizes candidate materials from a sensor produced by microfabrication, and consequently takes reference in the materials available for this sensor technology. Fifteen potential candidate materials were selected in which the biocompatibility's relation to activation of PMN has yet to be assessed (**Table 1**). The materials were divided into four groups depending on which part of the sensor they would be implemented (capsule, membrane, carrier, or sealing).

The sensor microimplant is built around a carrier of low-temperature cofired ceramic (LTCC), which is a multilayer ceramic platform that permits integration of microelectronic circuit components in three dimensions. This enables an ultracompact and thermally stable system architecture that is used in the artificial pacemaker. Consequently, a choice of two LTCC materials that differ slightly in their processing parameters are included in this study, CeramTec GC (CT) and DuPont 951 (DP). The membrane and pressure transducer are attached to the LTCC using a two-component epoxy resin, Epo-

Table 1.
Candidate Materials for Implementation in the Glucose Sensor

	Material	Abbreviation	Specification	Manufacturer
Encapsulation materials	Sylgard 184	PDMS	polydimethylsiloxane	Dow Corning Corp., Midland, MI
	Araldite 2020	A2020	Epoxy resin	Huntsman Ltd., Duxford, UK
	Stainless steel	Me	corrosion resistant, Type 316L	Fosstech Engineering, Stokke, Norway
Membrane materials	Silicon	Si	Silicon with native 2–3 nm oxide surface	Vestfold University College, Horten, Norway
	Silicon dioxide	SiO_2	Silicon with a 500 nm thick thermal oxidized surface	Vestfold University College, Horten, Norway
	Cellulose ester	CM	Ultrafiltration membrane (MWCO 5000 Da, ~2.5 nm)[a]	Spectrum Labs Europe B.V., Breda, Netherlands
	Polyamide	PAR	Reverse osmosis membrane (MWCO 0 Da, <1 nm)	Sterlitech Corporation, Kent, WA
	Polyamide	PATF	Thin Film membrane (MWCO 0 Da, <1 nm)	Sterlitech Corporation, Kent, WA
	Polycarbonate	PC	Track-etched membrane (MWCO 500 kDa, ~15 nm)	Whatman plc, Kent, UK
	Aluminum oxide	AAO	Anodic aluminum oxide (MWCO 50 kDa, ~5 nm)	Synkera Technologies Inc., Longmont, CO
Sensor carrier materials	CeramTec GC	CT	Low-temperature cofired ceramic	Ceramtec AG, Plochingen, Germany
	DuPont 951	DP	Low-temperature cofired ceramic	Dupont, Wilmington, DE
Sealing materials	Silicone 3140 coating	S3140	Silicone-based polymer	Dow Corning Corp., Midland, MI
	Silicone 3145 adhesive	S3145	Silicone-based polymer	Dow Corning Corp., Midland, MI
	Epo-Tek 353ND	ETek	Epoxy resin	Epoxy Technol. Inc., Billerica, MA

[a] Molecular weight cut-off

Tek 353ND (ETek), which cures with temperature. This adhesive also acts as a sealing material that protects the electrical connections from moisture. However, small distances between the electrical connections and the aqueous environment in the sensor cavity prompted two additional sealant materials to be considered: silicone 3140 (S3140) and 3145 (S3145), because of their good water-repellent properties. These will also act as a buffer between sensor components and the external capsule. The membrane and control chip are made from silicon (Si) and its glass-derivative silicon dioxide (SiO_2). Additional membrane materials include anodic aluminum oxide (AAO) and the polymers cellulose ester (CM), polyamide (PAR, PATF), and polycarbonate (PC). These were selected for their nanoporous nature and chemical property as well as the potential for integration on a miniaturized silicon membrane frame. The whole sensor is enclosed by an external capsule that protects internal components from the external environment. Sylgard 184 is a polydimethylsiloxane (PDMS) that is commonly used in microfluidics and lab-on-a-chip devices, whereas araldite 2020 (A2020) is a two-component epoxy resin that offers an additional degree of mechanical support. Both of these (alone or in combination) will be molded around the sensor assembly and cured at room temperature. Type 316L stainless steel (Me) used to encapsulate prototype devices was included in these studies for comparison.

The sensor (**Figure 1**) rests in the ISF and detects osmotic pressure generated in the sensor cavity (1) enclosed by the nanoporous membrane (2) and silicon pressure transducer (3). The membrane and transducers are attached by the adhesive (4) to the sensor carrier (5) and sealed with sealant materials (6), forming a flexible buffer against the external capsule (7). The sensor cavity maintains an active solution (8) based on the concanavalin A-dextran affinity assay,[11,26] which is protected against antibodies by the nanoporous membrane. Thus, the membrane, sealant, and encapsulant will be in direct contact with the ISF through the access channel (9); whereas the carrier, adhesive, and transducer will be in indirect contact through the membrane.

The material samples with liquid properties (PDMS, A2020, S3140, S3145, ETek) were prepared by administering 0.5 ml of uncured monomer in respective wells of a 24-well polystyrene plate and subsequently polymerized at room temperature (60 °C for ETek) for 24 hours in accordance with the recommendations of the manufacturer. Material samples with solid structure (Si, SiO_2, CT, DP) were cut into pieces of 1 cm^2. The nanoporous membranes and metal parts were cut into circular structures with

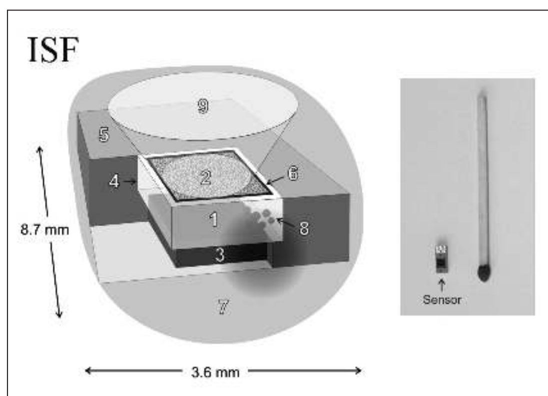

Figure 1. Conceptual illustration (left) of the microfabricated implantable osmotic glucose sensor, and the capsule less prototype (right). The whole packaged device measures 3.6 × 8.7 mm and resides in the ISF of subcutaneous adipose tissue. The unit samples glucose as an ultrafiltrate from blood through the open access channel of the capsule that provides direct access to the integrated 2 × 2 mm^2 osmotic pressure sensor. The labels are related to (1) chamber, (2) membrane, (3) pressure transducer, (4) adhesive, (5) carrier, (6) sealant, (7) capsule, (8) bioparticles, and (9) access channel. See text for details.

a surface area of 1 cm^2. Empty polystyrene wells were used as the negative control.

All materials, except the nanoporous membranes, were washed three times with ethanol and then rinsed with distilled water. Solid materials were additionally treated with an ultrasonic bath to remove residual microparticles that may persist on the material surface as a result of the manufacturing process. Nanoporous membranes were incubated in distilled water overnight in accordance with the manufacturer's instructions, and stored in phosphate buffer saline (PBS). All materials used in this study were rinsed with PBS solution prior to the experiment.

Whole blood from five healthy volunteers was used in the experiments. Informed consent was obtained before blood donation, and the study was approved by the local ethical committee. The blood was anticoagulated with the thrombin inhibitor lepirudin (Refludan®, Pharmion Germany GmbH, Hamburg, Germany) in a final concentration of 50 μg/ml.[23,27]

The experiments were performed by first administering 0.5 ml of blood in each well containing the respective candidate materials, prior to incubation at 37 °C in a climate room with the plate placed on a shaker platform. After 20 min, 45 μl of blood was withdrawn from the wells and used in flow cytometry in accordance to the

research protocol. The rest of the blood was incubated for 60 min, before the incubation was stopped by adding ethylenediaminetetraacetic acid to a final concentration of 20 mM. The samples were immediately transferred on ice and then centrifuged at 1400*g* for 15 min at 4 °C. The collected plasma was then centrifuged a second time at 1400*g* for 15 min at 4 °C, and the plasma was finally aliquoted and frozen at -70 °C. Whole blood incubated in empty polystyrene wells was used as the negative control.

Complement Inhibition

Materials that triggered an increased level of CD11b expression and MPO production were selected for further experiments using complement inhibitors. Compstatin analog Ac-I[CV(1MeW)QDWGAHRC]T, which binds to and inhibits cleavage of C3, and a control peptide were produced as described.[28] These were used in the experiments to a final concentration of: 25, 12.5, 6.25, and 3.125 mM. Eculizumab, (Soliris®, Alexion Pharmaceuticals, Cheshire, CT), a monoclonal antibody that specifically binds to the complement protein C5, was used in final concentrations of: 50, 25, 12.5, and 6.25 μg/ml. Whole blood was preincubated with these complement inhibitors for 5 min before the candidate materials were placed in the wells.

CD11b Expression

The CD11b expression on PMN was determined by flow cytometry. Blood was withdrawn from the wells containing the material candidate after a 20 min incubation period, prior to fixation using a 0.5% paraformaldehyde solution for 4 min at 37 °C. The cells were then stained for 15 min with anti-CD11b-PE antibodies (cat. no. 333142) or an isotype control IgG2a-PE antibodies (cat. no 349053), both obtained from Becton, Dickinson and Company (Franklin Lakes, NJ). Red blood cells were lyzed in accordance to protocol, washed twice, and cells were then resuspended in PBS containing 0.1% albumin and placed on ice in the dark until data acquisition was performed by the flow cytometer (FACScan, Becton, Dickinson and Company, Franklin Lakes, NJ). The PMNs were gated in a forward-scatter/side-scatter dotplot, and mean fluorescence intensity (MFI) values for CD11b were calculated.

Myeloperoxidase

Myeloperoxidase concentration in plasma was measured with a commercial enzyme-linked immunosorbent assay kit (cat. no HK324, Hycult Biotech, Uden, The Netherlands) in accordance to the manufacturer's instructions.

Statistics

Results from the CD11b and MPO experiments were statistically compared by one-way analysis of variance (ANOVA) between groups with Bonferroni post-test analysis. Data from the experiments with complement inhibitors were compared by one-way ANOVA in order to analyze the dose-dependent decrease of activation. All statistical data were collected and calculated with GraphPad Prism version 5.01 (GraphPad Software, San Diego CA). A p value <0.05 was considered as statistically significant.

Results

Activation of PMN

Expression of CD11b

The expression level of CD11b on the surface of PMN was measured with flow cytometry (**Figure 2**). The expression level of CD11b after incubation with three of the membrane candidates (CM, PAR, PATF) was significantly higher compared to the negative control (MFI 431, 511, and 462 vs MFI 240, $p < 0.05$). Material candidates used for the encapsulation, carrier, sealing, and the remaining four membrane candidates did not induce an expression of CD11b compared to the negative control.

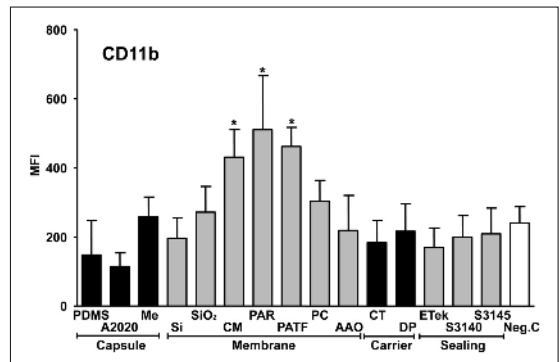

Figure 2. Expression of CD11b on PMN surface. The expression level of CD11b on PMN is shown with bars representing mean values with 95% confidence interval (n = 12). *p < 0.05 compared with the negative control. Neg.C, negative control.

Myeloperoxidase Release

Similar to CD11b, the concentration of MPO (**Figure 3**) in plasma after incubation with three of the membrane candidates (CM, PAR, PATF) was significantly higher than the negative control (500, 421, and 268.3 μg/ml vs 83.7 μg/ml, $p < 0.05$). Material candidates used for the encapsulation, carrier, sealing, and the remaining

four membrane candidates did not induce MPO release compared to the negative control.

Effect of Complement Inhibition on the Polymorphonuclear Leukocyte Activation

The effect of complement inhibition on the activation of PMN was evaluated by measuring the CD11b expression and the MPO concentration in lepirudin-anticoagulated whole blood incubated with materials after addition of compstatin and eculizumab. Based on the results from previous experiments examining CD11b expression on PMN and MPO concentration, the three membrane candidates that activated PMN were chosen to investigate the effect of complement inhibition.

CD11b Expression in Whole Blood with Complement Inhibitors

Complement inhibition by the C3-inhibitior compstatin led to a significant and dose-dependent decrease in the level of CD11b expression induced by all three materials (**Figure 4**). Maximum effect was obtained at 25 mM compstatin, where the expression of CD11b was reduced to the negative control value for all three materials.

Similarly, complement inhibition by the C5-inhibitor eculizumab significantly decreased the CD11b expression on PMN and dose dependently (**Figure 4**). The maximum was obtained at 50 μg/ml eculizumab, where the expression of CD11b was reduced to the negative control value for all three materials.

Unspecific binding of antibodies to the surface of PMN was measured in blood incubated with the isotype IgG2a control antibody. No unspecific bind was observed (**Figure 4**).

MPO Concentration in Whole Blood with Complement Inhibitors

Complement inhibition by compstatin led to a significant and dose-dependent decrease in the level of MPO secretion induced by all three materials (**Figure 5**). Maximum effect was obtained at 25 mM compstatin, where MPO concentrations for two of the three materials were reduced to negative control values.

Complement inhibition by eculizumab led to a dose-dependent decrease in the level of MPO secretion induced by all three materials (**Figure 5**). Maximum effect was obtained at 50 μg/ml eculizumab, where MPO concentrations for two of the materials were reduced to negative control values (**Figure 5**).

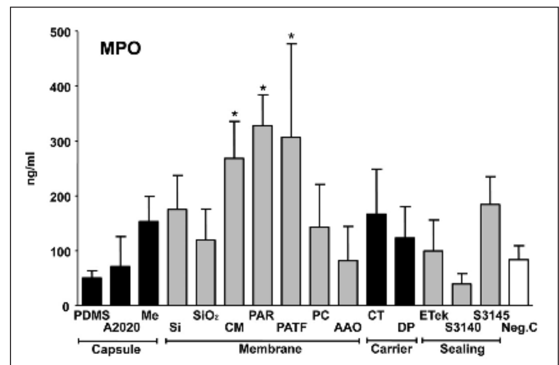

Figure 3. MPO concentration in plasma. The concentration of MPO in plasma after incubation with the 15 candidate materials. The bars represent mean values with 95% confidence interval (n = 8). *$p < 0.05$ compared with the negative control. Neg.C, negative control.

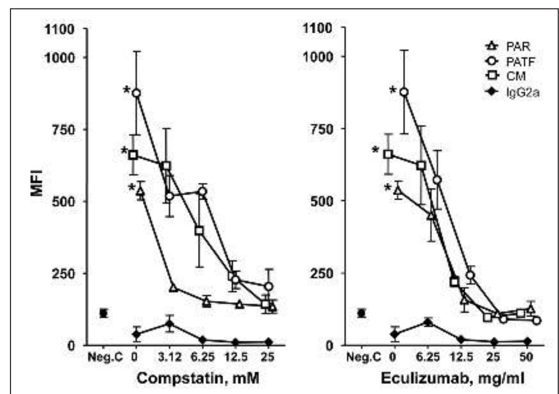

Figure 4. Expression of CD11b on PMN in whole blood with and without complement inhibitors. CD11b expression level for three material candidates is shown for different concentrations of compstatin and eculizumab. The bars represent standard error mean values (n = 4). *$p < 0.05$ compared by ANOVA for identifying the significance of the dose-dependent decrease. Neg.C, negative control; IgG2a, isotype control antibody

Discussion

This study documented a substantial variation in the PMN-activating properties of different candidate biomaterials intended for use in an implantable glucose sensor.

Assays used in this study were selected on the basis of international standard ISO 10993-4, which recommends the use of both complement assays and flow cytometry to detect increased leukocyte markers such as CD11b as a testing method to evaluate implantable devices.[29]

The 12 material candidates that did not show any significant increase in the CD11b expression and the MPO release were considered as biocompatible, and hence deemed possible candidates for use in other *in vivo* studies. The glucose-sensitive solution enclosed in the sensor chamber (concanavalin A and dextran) was not investigated in this study. Although concanavalin A is reported to exhibit a toxic response, the amount present in tiny biosensor implants is so small that in the case of membrane rupture this would not possess any overall toxic effect for the organism.[30] Further, the liquid properties of the affinity assay will permit it to dilute away from the sensor implant in contrast to the remaining sensor materials that may persist in generating a potential inflammatory response.

Three membrane candidates (CM, PAR, PATF) caused a virtually identical pattern of increase in the CD11b expression and the MPO release from the PMN. The results obtained for the cellulose membrane in our study are well correlated with literature. Although cellulose is a well-known material that has been used in dialysis membranes, the literature has suggested that this material is not biocompatible.[31–33] Different polyamide membranes have been widely used in implantable devices, but the reported studies concerning the biocompatibility of this material is controversial. Some clinical and *in vitro* studies (cell cultures) show a good biocompatibility of polyamide,[34–36] whereas other studies have claimed this material to be only moderately biocompatible.[37] Our studies showed that the polyamide membranes (PAR and PATF) activated PMN. These discrepancies can be explained by the different assays used to examine biocompatibility as well as the different types of polyamide used.

Since a study demonstrated that the same three membrane materials did activate complement through the alternative pathway,[24] we hypothesized that the current activation of PMN was also mediated by complement. Consequently, by blocking complement activation with either the C3 inhibitor compstatin or the C5 inhibitor eculizumab before incubation of the materials in whole blood, a significant decreased CD11b expression and release of MPO by PMN was found. The results were virtually identical for the two inhibitors, indicating that the effects were mediated by C5 activation, since compstatin blocks C3 and subsequent C5 activation, whereas eculizumab is highly specific for C5. This is consistent with the well-known effect of C5a on PMN activation.[27] This finding underscores the role of the complement system as a primary inducer for leukocyte activation, since the latter was markedly attenuated by inhibition of complement, irrespective of the material in question. This novel approach in biocompatibility testing does not only correlate the activation of different systems but also shows the causal relationship between them.

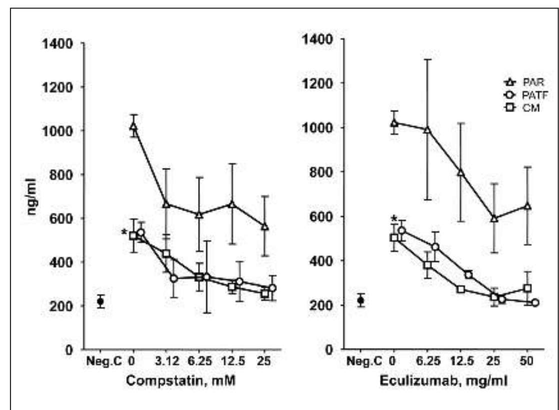

Figure 5. MPO concentration in plasma after incubation of the materials in whole blood with and without complement inhibitors. The concentration of MPO in plasma is shown for different concentrations of compstatin and eculizumab. The bars represent standard error mean values ($n = 4$). $*p < 0.05$ compared by ANOVA for identifying the significance of the dose-dependent decrease. Neg.C, negative control.

The role of complement as a primary inducer of the expression of CD11b was confirmed, since complement inhibition completely abolished the CD11b expression for all the three materials. However, a complete MPO inhibition was obtained for only two of these (PATF and CM). Since some MPO release still occurred for PAR despite an optimal inhibition of complement, a direct activation of the PMN by the surface of the material[25] or by activated platelets[21] might also contribute to PMN degranulation. The membrane materials that were examined have various pore sizes, as indicated by their molecular weight cut-off (MWCO), from zero for PAR and PATF to 5000 Da for CM). The comparable magnitude of PMN activation triggered by these three materials suggests that the chemical structure and the degree of protein absorption on the surface of the materials may have a major contribution to their potential to activate complement and PMN.[38]

Collectively, however, the present data indicate that complement activation is the main inducer of PMN activation by the biomaterials investigated in this study. The abolished PMN activation for CM and PATF by complement inhibition shows the insignificant role of direct surface activation of the PMNs for these two

tested material candidates. Irrespective of the mechanism leading to complement activation by these surfaces, a main goal to improve biocompatibility would be to reduce the primary complement activation in order to avoid the secondary activation of PMN.

Conclusion

Examination of 15 material candidates for an implantable microfabricated glucose sensor identified 3 out of 7 membrane candidates as potent complement-dependent PMN activators that make them undesirable candidates for use in an implantable device. The remaining materials that were examined were rendered biocompatible in terms of PMN activation and suggested to be good candidates for use in an implantable glucose sensor. The results underscore the important role of complement in mediating immune system-triggered responses to a foreign material and should be included as an essential component in the future portfolio of immune system markers when studying the mechanisms involved in the biocompatible nature of a material. Because the possibility cannot be excluded that materials that do not activate complement and PMNs *in vitro* might turn out to be incompatible *in vivo,* future studies will seek to correlate the results from *in vitro* assays with *in vivo* studies.

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