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On the binding of complement to solid artificial surfaces *in vitro*

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Running Title: Binding of complement to solids

Abstract

Since the realization of a complement activation capacity by artificial surfaces upon contact with blood, a common belief has evolved that charged nucleophilic surface groups such as amine ($-\text{NH}_2$) and hydroxyl ($-\text{OH}$) react with and eventually bind to the internal thioester in complement factor 3 (C3). A covalent ester or amide linkage is thereby supposed to form between C3b and the surface itself. In this report, we present complement surface binding data by null-ellipsometry for two nucleophilic surfaces ($-\text{NH}_2$ and $-\text{OH}$), for surfaces with immunoglobulin G (IgG) covalently bound, and for IgG spontaneously pre-adsorbed to hydrophobic silicon. The results reveal that the plasma proteins that were deposited during complement activation became eluted by sodium dodecyl sulfate (SDS). Hence the direct covalent binding between C3 and solid nucleophilic surfaces seems to be only of moderate importance, at least during shorter serum incubations. This strongly suggests that the prevalent covalent linkage model between solid artificial surfaces and C3b is not accurate. Instead we suggest a more pronounced role for C3 associations to other adsorbed proteins and/or electrostatic and hydrophobic protein-surface interactions.

Keywords: Biomaterial; Complement activation; Protein adsorption; IgG; Hemodialysis

1. Introduction

More than a century ago blood was found naturally bactericidal and the complement system was described as a heat-labile component of serum, displaying a hemolytic ability “complementary” to the already discovered heat-stable factor (antibodies). Since then the complement system has been described more in detail and designated a number of important humoral and cell-dependent biological functions like lysis, opsonization and immune complex clearance [1].

The complement cascade is an effector system consisting of more than 30 soluble and membrane-bound proteins. It can be activated through at least three different pathways. The first described “classical” activation pathway is triggered by interaction of surface-bound immunoglobulins (IgG or IgM) with complement factor 1q, C1q, and the alternative pathway is set-off by a more or less non-specific surface binding of spontaneously activated C3b [2, 3]. The recently discovered lectin pathway is initiated by carbohydrates in the cell wall of certain microorganisms [4, 5].

In the field of artificial biomaterials there has been an uncertainty on whether the classical or alternative activation pathway is the most relevant. Contradictory reports have emphasized the importance of both pathways, but the literature tends to favor the alternative one [6-10]. The covalent binding of complement to surfaces is indeed valid in case of immune complexes and carbohydrates as well as for a wide range of “biological” surfaces, and the biocompatibility of biomaterials designed for direct blood-contacting applications will most certainly suffer from the activation of complement, for instance illustrated by leukopenia associated with hemodialysis [11]. Also, the question whether or not implants intended for tissue integration will benefit or not from complement activation is not yet answered. A large portion of the

biomaterial-complement research has utilized conventional radioimmunological or enzyme linked immunosorbent assays (RIA/ELISA) and/or often emphasized fluid phase products like the anaphylatoxins or soluble terminal complexes. However, also the direct surface analysis with regard to surface-bound complement proteins is of utmost importance in biomaterials research [12].

All the activation pathways form convertases that cleave native C3 into an anaphylatoxin, C3a, and the reactive and opsonizing C3b. After cleavage, the C3b molecule displays transiently a thioester bond that is susceptible to a nucleophilic attack by either surface hydroxyl or amino groups (forming ester or amide bonds) or attack by water [13-15]. Hereby C3b becomes surface-bound or remains in the fluid phase, respectively. Unlike its thioester-containing evolutionary relatives (α_2 -macroglobulin and C4), C3 has been postulated to preferentially form ester rather than amide bonds [14]. The surface immobilization probably also involves a concomitant change in the backbone of the molecule [16-18]. The oxygen ester bond in the surface-immobilized C3b is physiologically susceptible to slow hydrolysis by an “auto-esterase” activity performed by the C3b molecule itself [19-21]. *In vitro*, stronger nucleophiles such as hydroxylamine can be used to break this bond [14, 22].

The aim of the present study was to investigate the complement retaining capacity after activation by functionalized plain solid artificial model surfaces upon short or prolonged exposure to serum. In order to accomplish this we quantified adsorbed amounts by single wavelength null-ellipsometry and identified surface localized complement proteins with polyclonal antibodies specific for C3c or C1q, respectively.

2. Materials and methods

2.1. Reagents, sera and buffers

All reagents were of analytical grade and delivered from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated. Blood was drawn from non-medicated apparently healthy volunteers and fresh normal human serum (NHS) was prepared by standard procedures and cooled on melting ice. NHS from at least two different donors were routinely pooled after preparation and stored below -70°C. Small vials of NHS were thawed at 37°C immediately before each experiment and diluted in Veronal-buffered saline (VBS; 5 mM 5,5'-diethylbarbituric acid with its corresponding sodium salt and 145 mM sodium chloride, pH 7.4), supplemented with 0.15 mM CaCl₂ and 0.5 mM MgCl₂ (VBS²⁺). In control experiments, the complement activation in NHS was inhibited by the addition of 10 mM ethylene-diamine-tetraacetic acid (EDTA, Merck, Darmstadt, Germany) to the VBS (no Mg²⁺ or Ca²⁺ present). Reagents potentially sensitive to the presence of divalent cations were diluted in Phosphate-buffered saline (PBS; 10 mM sodium hydrogen phosphate, 10 mM potassium dihydrogen phosphate and 0.15 M sodium chloride, pH 7.4). All buffers contained 0.25 mM sodium azide.

2.2. Model surfaces

The model surfaces were methylated silicon (hydrophobic), amine-silanized silicon or gold with adsorbed mercaptoglycerol. Some important features of the various surfaces are summarized in Table 1 and Fig. 1.

2.2.1. Hydrophobic surfaces

Silicon wafers (Okmetic LMTD, Esbo, Finland) were cleaved to 5 x 10 mm pieces in the (100) crystal direction and cleaned for 5 minutes at 80°C in five parts of distilled (Milli-Q) water, one part of hydrogen peroxide (30% v/v) and one part of NH₄OH (25% v/v) followed by a thorough rinsing in distilled water. Thereafter, the wafers were “boiled” for 5 minutes at 80°C in six parts of distilled water, one part of hydrogen peroxide (30% v/v) and one part of hydrochloric acid (37% v/v), resulting in a thin hydrated surface layer of SiO₂ [23, 24]. The water/surface static contact angle (θ_w) was less than 10° (Ramé-Hart NRL Model 100 goniometer, USA). Some of the hydrophilic wafers were washed in distilled water, technically pure ethanol and xylene (Merck, Darmstadt, Germany), and then incubated for 5 minutes at room temperature in 1% (v/v) dichlorodimethylsilane (Cl₂(CH₃)₂Si) in xylene. Remaining silane was removed through sequential rinsing in ethanol, xylene and finally sonicated for one minute in a Sondrex RK100 (Bandelin Electronics, Berlin, Germany). The methylated hydrophobic ($\theta_w > 90^\circ$) silicon wafers were stored in xylene and used within one week.

2.2.2. Amine and glutaraldehyde surfaces

Clean and dry silicon surfaces were aminated through first 10 minutes baking of the surfaces at 60°C in vacuum (approximately 6 mtorr) followed by inlet of 200 µl amino-propyl-triethoxysilane (APTES, Merck, Darmstadt, Germany) into the chamber and finally baked for 60 minutes at 150°C in vacuum. The wafers were subsequently rinsed in ethanol, xylene and sonicated for one minute. The APTES layer ellipsometric ($n_{\text{film}} = 1.5$; see below) thickness was approximately 2nm and the surfaces were stored in xylene until use. A layer of approximately 0.4nm glutaraldehyde (GA) was added [25, 26] on top of the amine-functionalized surfaces by 30 minutes of incubation at room temperature in 6% glutaraldehyde (Merck, Darmstadt, Germany) in Tris buffer (pH 9) followed by triplicate rinsing in distilled water.

2.2.3. Mercaptoglycerol surfaces

Two hundred nanometers of gold on 9 x 9 mm glass slides (Biacore AB, Uppsala, Sweden) were hydrophilized by 5 minutes of cleaning in a Model 42-220 UVO-cleaner (Jelight Company Inc., Irvine, CA) and immediately transferred to 2 mM 3-mercapto-1,2-propanediol (mercaptoglycerol, MG, Janssen Chimica, Belgium)

in VBS²⁺ at room conditions. After 30 minutes of incubation, the samples were thoroughly rinsed in distilled water and dried under nitrogen before use. The approximately 0.5nm thick hydrophilic ($\theta_w < 10^\circ$) glycerol layer exposes a carbon backbone and 2 hydroxyl end groups per molecule facing out from the surface and is known to spontaneously activate both the classical and alternative activation pathways [27, 28]. The MG was not resolved from the gold when surfaces were incubated in NHS or in detergents.

2.3. Preparation of IgG-coated surfaces

Methylated hydrophobic surfaces were washed twice in PBS and a monolayer of normal human IgG (Gammaglobulin 165 mg/ml, Pharmacia & UpJohn, Stockholm, Sweden) was spontaneously adsorbed during half an hour of incubation at room temperature in 1 mg/ml of IgG in PBS, followed by duplicate rinsing in PBS. Hereby a > 2nm thick monolayer of IgG was formed. IgG was covalently bound to APTES + GA surfaces through a similar incubation. IgG immobilized by both procedures has proven able to activate the classical pathway of complement [29, 30] and to induce leukocyte frustrated phagocytosis *in vitro* [31, 32].

2.4. Ellipsometry

The flat sample surfaces were illuminated with elliptically polarized light from a HeNe $\lambda=632.8\text{nm}$ laser at a 70° angle of incidence. Two ellipsometric angles, Δ and ψ , were recorded at the minimum intensity of the light after reflection. By finding new angles ψ and Δ after adsorption where the intensity in the analyzer is zero (“null”), the thickness of the surface film was iterated from the angle changes according to the McCrackin evaluation algorithm [33]. The film thickness was converted to amount of mass per unit area according to the algorithm by de Feijter *et al* [34]. The adsorption experiments were performed at 37°C and the samples rinsed gently four times between each type of incubation. The resulting organic films on the hydrophobic (methylated), APTES and APTES + GA surfaces were analyzed in air assuming organic film refractive index $n_{\text{film}} = 1.465$ with a Rudolf Research AutoEl III (NJ, USA) and the silicon background was routinely subtracted. On Au-MG, both the incubations and film analyses were performed *in situ* (Fig. 1E) on samples mounted in a 37°C glass cuvette with assumed ambient refractive index $n_{\text{buffer}} = 1.335$ [27, 35]. The duration and concentration of the NHS (5 minutes in 10% NHS or 45 minutes in 67%, respectively) and antibody (1/50 for 30 minutes in VBS²⁺) incubations were selected on an empirical basis from previous studies [27, 30, 36]. The antibodies were IgG-fractions of polyclonal rabbit anti-human A 0062 (C3c Complement), A 0136 (C1q Complement) and A 424 (IgG, Specific for γ -Chains)

from Dakopatts (Glostrup, Denmark) and contained protein concentrations of 10, 3.7 and 9.3 g/l respectively.

2.5. Stability tests on adsorbed protein layers

In order to characterize the binding of adsorbed proteins after incubations in NHS, the samples were incubated for 30 minutes at 37°C in sodium dodecyl sulfate (SDS, 10.4 mM) or 0.1% (v/v) Tween 20 (poly-oxyethylene sorbitan monolaurate, Kebo LabAB, Stockholm, Sweden) in PBS. SDS is capable to remove non-covalently adsorbed proteins from both hydrophobic and hydrophilic surfaces and Tween 20 is more efficient on hydrophobic than hydrophilic surfaces [37]. After elutions, the surfaces were blocked with 1 mg/ml L-lysine (30 minutes, 37°C in VBS²⁺) to inhibit a non-specific antibody binding. Possible disulfide bonds between surfaces and proteins were reduced with 5% (v/v) 2-mercaptoethanol (Janssen Chimica, Belgium) during 30-min incubations at 37°C in PBS, and ester bonds hydrolyzed during 2.5-hour incubations at 37°C in 1 M hydroxylamine in a 0.2 M Tris buffer, pH 9.0.

2.6. Statistics

Data are presented as the average values $\pm$ standard errors of the means (SEM). The differences in NHS layer thicknesses and subsequent antibody

depositions before and after surface treatments were analyzed using the Student's two-tailed paired t -test. When $p < 0.05$, data were regarded as significantly different and divided into $p < 0.05$, $p < 0.01$ or $p < 0.001$ categories. All sample measurements (except for single measurements on the Au-MG surfaces) were performed at least in triplicate and n always indicates the number of independent experiments, in general ≥ 6 (except some control experiments).

3. Results

3.1. Spontaneous complement activation by mercaptoglycerol on gold

The Au-MG surfaces (Fig. 1A), were previously shown to spontaneously activate the complement system *in vitro* [27, 28] and to induce a strong inflammatory response *in vivo* [38]. Substantial amounts of NHS ($8.1\text{nm} \pm 0.2\text{nm}$) deposited in our experiments onto this surface after 5 minutes of exposure to 10% NHS (Fig. 2A-B, group I). Ellipsometric detection of the concomitant binding of anti-C1q or anti-C3c revealed a strong C1q- (Fig. 2A, group I) and C3c- (Fig. 2B, group I) signal ($2.6\text{nm} \pm 0.4\text{nm}$ and $8.5\text{nm} \pm 0.5\text{nm}$, respectively) already after 5 minutes of incubation in NHS. When normal human IgG was used instead of the specific antibodies after the NHS-incubations, no thickening of the organic film was observed on top of the NHS film. After 45 minutes of incubation in 67% NHS (Fig. 2C) the average NHS deposition increased to $9.8\text{nm} (\pm 0.2\text{nm})$ and the C1q-signal was abolished, probably due to the subsequent complement protein deposition [27]. Surprisingly, the anti-C3c binding decreased upon the prolonged NHS exposure ($6.3\text{nm} \pm 1.0\text{nm}$; Fig. 2C, group I), perhaps due to inhibitors or an auto-esterase activity performed by the surface-bound C3b molecule itself. Control experiments with samples incubated for 45 minutes in EDTA-NHS indicated an approximately 6nm NHS deposition. Roughly 2nm of this remained after the SDS-elution and no anti-C3c bound in to this remaining layer (not shown).

The complement retaining capacity of the Au-MG surfaces after serum incubations was tested. SDS was able to remove almost totally the surface localized NHS proteins (Fig. 2A-B, group II) and at average only 0.7nm ($\pm$ 0.3nm) out of approximately 8nm remained on surfaces that were pre-incubated for 5 minutes in 10% NHS. The result indicates that only few direct covalent associations were formed between the surface itself and the plasma proteins (Fig. 2A-B, group II). However, lower amounts of anti-C3c or anti-C1q (29% and 61% of the respective original antibody signals) still bound in to the remaining protein layer, although the signals were likely overestimated after the elution step since the washing procedure may facilitate antigen-antibody contacts (a phenomena also suggested by Benesch *et al.* [39]). Alternatively, the blocking agent L-lysine failed to inhibit non-specific binding on the relatively clean surfaces, a theory supported by the observation that more than 1nm of normal human IgG deposited onto NHS-incubated surfaces after the SDS-elutions although pre-blocked with L-lysine (not shown). After 45 minutes of incubation in 67% of NHS (Fig. 2C, group II), a thicker protein film remained after the SDS-treatment and the anti-C3c deposition decreased only by approximately one third compared to the non-eluted sample (Fig. 2C, group I). A general observation in this study was that the longer the surfaces were exposed to NHS, the more stable were the adsorbed layers against detergents and reducing agents.

The native C3 molecule consists of two subunits (α and β) linked together by disulfide bonds [40]. We investigated the effect of disulfide bond breaking with 2-mercaptoethanol for the stability of the adsorbed protein layer (Fig. 2A-C, groups III-IV). The treatment decreased the protein film thickness with more than 40% and 36% after 5 minutes in 10% NHS and 45 minutes in 67% NHS, respectively. Subsequent anti-C1q and anti-C3c depositions remained high after mercaptoethanol treatments,

86% of the original anti-C1q signal (after 5 minutes in 10% NHS) and 74% / 63% (5- / 45-min NHS incubations) of the anti-C3c deposition. When the mercaptoethanol treated protein films were subjected to SDS incubations, the layer reduced further by 43% and 36% (at 5- / 45-min NHS incubations, respectively). After this, only the anti-C3c deposition remained significant on surfaces that were incubated for 45 minutes in NHS ($p < 0.01$; Fig. 2C, group IV). Surprisingly, the remaining layer appeared somewhat thicker than on surfaces that were treated with SDS only after the serum incubation ($p < 0.01$ for 5-min incubations, not significant after 45-min incubations, compare groups II and IV). The ester bond hydrolysis with hydroxylamine on the NHS coated Au-MG samples; either pre- or post-treated with SDS, revealed results qualitatively similar to those obtained with mercaptoethanol, i.e. a low reducing capacity. The Tris buffer washing effect on NHS-coated Au-MG surfaces (2.5 hours in 37°C) was of the same magnitude as the reduction performed by hydroxylamine (not shown).

In control experiments (not shown), clean APTES (amine-terminated; Fig. 1B) and clean hydrophobic (methylated) surfaces were incubated for 45 minutes in 67% NHS. Only a moderate (approximately 3nm) NHS deposition was observed on both surfaces. Anti-C1q or anti-C3c did not deposit to NHS-coated hydrophobic surfaces, but a low anti-C3c deposition was observed on the NHS-coated APTES surfaces ($\geq 1.6\text{nm}$). The NHS deposition on clean APTES was roughly halved by the SDS-elution and the subsequent anti-C3c deposition reduced by approximately 70%.

3.2. IgG-triggered complement activation

Hydroxyl-terminated surfaces are expected to activate complement via the alternative pathway. However, it was previously shown that the Au-MG surfaces activated spontaneously through the classical pathway, a phenomenon that was manifested by an early and transient detectability of IgG and C1q during NHS incubations [27]. With the above in mind we performed model experiments where complement activation was provoked with either an IgG-coating on hydrophobic silicon (non-covalent association) or with IgG covalently attached to glutaraldehyde (APTES + GA surfaces).

3.2.1. Non-covalent IgG-coating

IgG-coated hydrophobic surfaces (Fig. 1C) deposited $2.0\text{nm} \pm 0.3\text{nm}$ of plasma proteins after 5 minutes incubation in 10% NHS (Fig. 3A-B, group I). Both C1q and C3c were clearly detectable with respective antibody ($1.6\text{nm} \pm 0.3\text{nm}$; $p < 0.01$ and $4.3\text{nm} \pm 0.4\text{nm}$; $p < 0.001$). After 45 minutes of incubation in 67% NHS (Fig. 3C, group I), the additional protein deposition was massive ($10.3\text{nm} \pm 0.3\text{nm}$, p

< 0.001) and incubation in anti-C3c resulted in a large additional mass deposition ($10.1\text{nm} \pm 0.5\text{nm}$; $p < 0.001$).

The subsequent SDS incubations reduced the NHS layers to below the IgG-monolayer levels after both the 5-min ($1.4\text{nm} \pm 0.1\text{nm}$; $p < 0.001$) and 45-min NHS incubations ($2.3\text{nm} \pm 0.1\text{nm}$; $p < 0.001$), but failed to completely remove the proteins (Fig. 3A-C, group II). The relative anti-C1q deposition to the 5 minutes in NHS surfaces was reduced by 80% (Fig. 3A, group II) and only 9% (at 5-min, Fig. 3B, group II) or 19% (at 45-min, Fig. 3C, group II) of the anti-C3c-binding remained after the SDS elutions. Mercaptoethanol removed 44% of the 5-min and 57% of the 45-min NHS depositions, respectively. Subsequently, no anti-C1q bound to the surfaces. The remaining anti-C3c deposition, however, was 52% (5 minutes in NHS) and 61% (45 minutes in NHS) compared to depositions without SDS treatment (group I). Pre-incubation of the NHS-coated samples in mercaptoethanol before SDS and antibody incubations did not facilitate SDS elution (rather the opposite) and the anti-C1q or anti-C3c depositions remained similar to those obtained after SDS treatment only.

Since SDS could not completely remove the deposited NHS proteins, another detergent known to disrupt the lateral stability of adsorbed protein layers, Tween 20, was introduced (Fig. 3D, groups II and IV). This resulted in only a low reduction of the 45-min in NHS layer, and the washing capacity was modest. The anti-C3c deposition remained high after the washings and Tween 20 could neither affect the NHS layer thickness nor the anti-C3c signal following the mercaptoethanol treatment. In order to test if the failure of SDS to totally clean the surfaces was due to “residence time effects” of adsorbed proteins, we tested the elution capacity of both detergents on IgG monolayers (Fig. 3E, groups I and II). Apparently, SDS was able to remove the

major part (but not all) of the pre-adsorbed IgG-monolayer even after prolonged residence time in buffer (no significant difference between the 1-minute and 45-min samples). When the IgG-coated samples were exposed to NHS, a decrease in the elutability by SDS was observed and the remaining layer increased from $1.2\text{nm} \pm 0.1\text{nm}$ to $2.6\text{nm} \pm 0.2\text{nm}$ (Fig. 3E, groups III and IV). The already observed modest washing effects of Tween 20 declined further with increased residence time on both IgG only and on IgG surfaces after incubation in NHS.

3.2.2. Covalently bound IgG

Surfaces with covalently bound IgG (Fig. 1D) were incubated for 5 or 45 minutes in NHS, and deposited $2.3\text{nm} \pm 0.2\text{nm}$ and $10.0\text{nm} \pm 0.3\text{nm}$ (respectively) of proteins on top of the IgG layer (Fig. 4A-C, group I). Upon a subsequent incubation in anti-C1q the thickness increased by $2.1\text{nm} \pm 0.2\text{nm}$ (5-min incubation; Fig. 4A, group I). The anti-C3c binding was significantly larger, $4.9\text{nm} \pm 0.2\text{nm}$ after the 5-min incubation (Fig. 4B, group I) and $8.4\text{nm} \pm 0.5\text{nm}$ after the 45-min incubation (Fig. 4C, group I).

SDS reversed the 5-min NHS deposition almost back to the IgG-background level although the washing effect was smaller for the 45-min NHS layer (22%, $p < 0.001$). The subsequent anti-C1q-deposition was significant ($p < 0.05$) but low (a couple of Ångströms, Fig. 4A, group II). The anti-C3c signal became lower after the SDS-elution, but remained at a fairly high level, $1.0\text{nm} \pm 0.1\text{nm}$ after 5 minutes of

NHS incubation (Fig. 4B, group II) and $3.9\text{nm} \pm 0.3\text{nm}$ after the 45-min NHS incubation (Fig. 4C, group II). Treatment with mercaptoethanol after the NHS incubation gave results similar to those for the above-described IgG-coated hydrophobic surface. Interestingly, the combined washing effect of mercaptoethanol followed by a SDS treatment completely abolished the anti-C3c binding. Incubation for 30 minutes at 37°C in VBS^{2+} showed only a small (approximately 10%) rinsing effect after 45 minutes of NHS deposition and the subsequent anti-C3c binding was not reduced (not shown).

The relatively powerful reducing agent hydroxylamine was capable to remove up to 56% of the NHS layer that was formed after 45 minutes of incubation in 67% NHS ($p < 0.001$) and decreased the subsequent anti-C3c-signal by 19% (Fig. 4D, group III). In comparison, upon 2.5 hours of incubation in the corresponding Tris buffer 39% of the NHS was removed ($p < 0.001$), and the anti-C3c deposition was almost unaffected (not shown). The combination of hydrolysis by hydroxylamine and SDS elution was very effective but did not reset the complement/NHS protein layer down to IgG-levels. The remaining layer thickness was $3.1\text{nm} \pm 0.3\text{nm}$, not significantly different from the IgG layer only, and some anti-C3c could be deposited onto this remaining layer (19% of the reference anti-C3c deposition, Fig. 4D, group I).

4. Discussion

Studies that are made to elucidate the mechanisms behind frustrated leukocyte phagocytosis as a consequence of complement activation at the blood – biomaterial interface are complex. The reason for the violent inflammatory cell reaction is probably multi-factorial and involves several humoral and cellular systems. For instance, C1q, C3-fragments and IgG act independently with leukocyte cell surface receptors, and they dramatically increase the adhesion and respiratory burst events in synergy [41, 42]. Therefore we cannot determine the properties of the surface stimuli solely by concluding that frustrated phagocytosis occurs at an interface. We demonstrate in the present, and in earlier studies that the deposition of both anti-C1q and anti-C3c was extensive and significant when Au-MG surfaces were exposed to NHS. Qualitatively similar results were obtained when IgG was pre-adsorbed or covalently bound to surfaces. The specific polyclonal antibodies that were used are large molecules (150 kDa) and these are unlikely to penetrate through the supposedly dense layer of packed plasma proteins, indicating that complement proteins were immunologically accessible on top of the adsorbed protein layers. This does not, however, give us information on whether the detected C1q and C3 molecules were firmly bound or in direct association with the actual surface or not. Therefore, incubations in detergents or reducing solutions provided in this case valuable information about the characteristics of protein binding after complement was activated on the present known activator surfaces.

It is well known that SDS denatures soluble proteins, and the surface binding of C3b involves a denaturation similar to that accompanying an SDS treatment [16, 18]. Traditionally, SDS is used to remove non-covalently bound proteins [14]. Cornelius and Brash [43] demonstrated that C3 was present in SDS eluates after plasma incubations of a number of hemodialysis membranes. In accordance with this, we show in the present study that SDS has a dramatic washing effect on different complement activator surfaces. Also, SDS-elutions often substantially decreased the subsequent C3c and C1q antibody binding into the remaining protein layer. Upon SDS-treatment of the NHS exposed Au-MG surface, the protein layer was almost completely removed, and a similar low NHS layer thickness was obtained on non-charged hydrophobic (methylated) surfaces. We therefore find it unlikely that the present complement activator surfaces had a large number of direct covalent bonds to complement proteins. In addition, in this study, the ester linkage disruption by hydroxylamine as well as disulfide breaking by mercaptoethanol was only of partial importance.

It should be emphasized that the chemical surface functionality has a great impact on its complement activation capacity [44]. The role of a direct amide or ester linkage between an amine- or hydroxyl-terminated surface and C3b seems in light of the present results to be overestimated in the literature. Cheung *et al.* [8, 45] and Gachon *et al.* [44] showed that the complement activation potential of hemodialysis membranes is not directly correlated to the number of potential surface activation sites for C3b. Hence, and for other reasons reviewed by Janatova *et al.* [46], the covalent C3 binding model, that may be relevant to biological surfaces, cannot be directly transferred to artificial surfaces.

The C3-opsonization of the present model surfaces, and most likely also of other blood contacting artificial surfaces, probably occurs through multiple interactions between C3 and other rapidly adsorbed proteins. The molecular weight of C3 is 185 kDa [40] and therefore it diffuses therefore relatively slowly compared to *e.g.* albumin, IgG, *etc.* to surfaces. In other studies, strong covalent associations between albumin and C3-fragments have been reported [47, 48]. Furthermore, IgG and C1q are transiently detectable at the Au-MG surfaces after contact with NHS [27], and IgG enhances the alternative pathway activation via the classical pathway [3]. Surprisingly, the SDS elutability of NHS was dramatically lower on the surface with covalently immobilized IgG. This surface probably behaves similar to immune aggregates that bind covalently to C3b, probably via an amide bond [49]. The amide bond is not sensitive to nucleophilic attack [20], this may explain why the anti-C3c binding was not totally abolished on surfaces with covalently attached IgG after incubations in hydroxylamine and SDS. In contrast, IgG has been shown to possess a C3 binding site between Val-134 and Lys-156 on the CH1 domain of IgG₁, which probably forms an ester by reacting with the C3 thioester [50]. However, this covalent IgG-C3b ester association has been demonstrated sensitive to hydroxylamine, thus suggesting further investigations. An alternative and less likely explanation for the high deposition of anti-C3c on NHS layers could be that fluid phase C3c (that lacks the thioester) is present at the surfaces due to a passive protein adsorption [8, 45]. Thus, our suggestion is that leukocytes probably encounter rather an opsonized protein layer than an opsonized surface during the frustrated phagocytosis process upon contact with a flat solid artificial surface.

It should also be emphasized that C3b likely binds to glycosylated proteins [51], and the length of polysaccharide chains on biological particles affects the C3b

affinity for the soluble complement inhibiting factor H [52, 53]. In an ellipsometric study that was recently performed in our laboratory with HS(CH₂)₁₅CONH((CH₂)₂O)₆H self assembled on gold, only 15% of the NHS deposition after 30 minutes of incubation in 67% NHS could be removed by SDS [39]. This indicates that the surface - protein molecule penetration depth is of importance for the complement retaining capacity of an artificial surface.

5. Conclusions

We suggest that complement upon activation at solid artificial surfaces primarily associates to other adsorbed proteins. The extent of covalent C3 surface binding is minor and the deposition of C3b most likely occurs via electrostatic and hydrophobic interactions. The elutability of adsorbed NHS proteins decreases with time.

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LEGENDS TO TABLE AND FIGURES

Table 1. Summary of some characteristics of the model surfaces. See also Materials and methods.

Fig. 1. Schematic illustrations of the four complement activating surfaces (A-D) and the *in situ* ellipsometric measurement set-up (E, modified from [35]). In (B), the silane also polymerizes laterally [26]. For details, see Materials and methods (abbreviations: IgG = immunoglobulin G, GA = glutaraldehyde, E = electric field, s = perpendicular, p = parallel, i = incident light, r = reflected light).

Fig. 2. Spontaneous complement activation induced by the hydroxyl-containing mercaptoglycerol on gold (Au-MG). The Au-MG-surfaces were mounted in a heat-controlled (37°C) measurement cell and the protein adsorption from normal human serum (NHS) was measured *in situ* by null ellipsometry (Fig. 1E). The samples were rinsed gently four times between each treatment and all measurements were performed in veronal buffered saline supplemented with Mg^{2+} and Ca^{2+} (VBS²⁺). Antibodies were added at 1/50 dilutions in VBS²⁺ for 30 minutes. The surfaces were blocked for non-specific binding with 1 mg/ml L-lysine (15 minutes, VBS²⁺) in (A) and (B). The bars indicate standard errors of the means (SEM, $n = 6$).

(A) 5 minutes in 10% NHS. Subsequent binding of anti-C1q before and after sodium dodecyl sulfate elutions (SDS, 10.4 mM in phosphate buffered saline, PBS) and before and after reduction with 5% (v/v) mercaptoethanol (in VBS²⁺).

(B) As (A) but shows the binding of anti-C3c.

(C) 45 minutes incubation in 67% NHS. Subsequent binding of anti-C3c before and after SDS elutions and before and after reduction with mercaptoethanol.

Fig. 3. Complement activation triggered by IgG-precoated hydrophobic silicon. The protein deposition from normal human serum (NHS) onto a spontaneously adsorbed monolayer of immunoglobulin G (IgG) was measured with null ellipsometry in air. All incubation steps were performed sequentially with four rinsing steps in between, at 37°C. Triplicate film measurements were performed at different spots.

(A) 5 minutes in 10% NHS. Subsequent binding of anti-C1q before and after sodium dodecyl sulfate elutions (SDS, 10.4 mM in phosphate buffered saline, PBS, followed by blocking with 1 mg/ml L-lysine for 15 minutes in veronal buffered saline, VBS²⁺) and before and after reduction with 5% (v/v) mercaptoethanol (in VBS²⁺). (*n* = 6).

(B) As (A) but shows the binding of anti-C3c. (*n* = 6).

(C) 45 minutes incubation in 67% NHS. Subsequent binding of anti-C3c before and after SDS elutions and before and after reduction with mercaptoethanol. (*n* = 9).

(D) As (C) but with SDS exchanged for 0.1% (v/v) Tween 20 (poly-oxyethylene sorbitan monolaurate). (*n* = 6).

(E) Elutability of NHS on IgG layers after 1- or 45-minute incubations. (*n* = 6).

Fig. 4. Complement activation by covalently immobilized IgG on amino-propyl-triethoxysilane (APTES) + diglutaraldehyde (GA) on silicon. The samples were exposed to normal human serum (NHS) diluted in veronal buffered saline supplemented with Mg²⁺ and Ca²⁺ (VBS²⁺). The protein depositions were studied by

null-ellipsometry in air. The samples were kept at 37°C throughout the various incubations and gently rinsed (x 4) in VBS²⁺ before analysis.

(A) 5 minutes in 10% NHS. Subsequent binding of anti-C1q before and after sodium dodecyl sulfate elutions (SDS, 10.4 mM in phosphate buffered saline, PBS, followed by blocking with 1 mg/ml L-lysine for 15 minutes in VBS²⁺) and before and after reduction with 5% mercaptoethanol (in VBS²⁺). ($n = 10$).

(B) As (A) but shows the binding of anti-C3c. ($n = 10$).

(C) 45 minutes incubation in 67% NHS. Subsequent binding of anti-C3c before and after SDS elutions and before and after reduction with mercaptoethanol. ($n = 6$).

(D) Effect of reduction with 1 M hydroxylamine (2.5 hour in 0.2 M Tris buffer, pH 9.0) on the NHS layer after 45 minutes of incubation in 67% NHS. ($n = 7$).

TABLE 1

<i>Model coating</i>	<i>Bulk substrate</i>	<i>Medium for optical analysis</i>	<i>Surface water contact angle (θ_w)</i>	<i>Terminal functionality</i>
Hydrophobic	Si	Air	$> 90^\circ$	-CH ₃
Amino-propyl-triethoxysilane (APTES)	Si	Air	$< 10^\circ$	-NH ₂
Amino-propyl-triethoxysilane + diglutaraldehyde (APTES + GA)	Si	Air	$40^\circ < \theta_w \leq 60^\circ$	-CHO
Mercaptoglycerol (Au-MG)	Au (on glass)	VBS ²⁺	$< 10^\circ$	-OH

FIGURE 1

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FIGURE 2

FIGURE 3

E. Elutability change with residence time

FIGURE 4