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# Solid-phase classical complement activation by C-reactive protein (CRP) is inhibited by fluid-phase CRP–C1q interaction

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**Abstract**

C-reactive protein (CRP) interacts with phosphorylcholine (PC), Fcγ receptors, complement factor C1q and cell nuclear constituents, yet its biological roles are insufficiently understood. The aim was to characterize CRP-induced complement activation by ellipsometry. PC conjugated with keyhole limpet hemocyanin (PC-KLH) was immobilized to cross-linked fibrinogen. A low-CRP serum with different amounts of added CRP was exposed to the PC-surfaces. The total serum protein deposition was quantified and deposition of IgG, C1q, C3c, C4, factor H, and CRP detected with polyclonal antibodies. The binding of serum CRP to PC-KLH dose-dependently triggered activation of the classical pathway. Unexpectedly, the activation was efficiently down-regulated at CRP levels >150 mg/L. Using radial immunodiffusion, CRP–C1q interaction was observed in serum samples with high CRP concentrations. We propose that the underlying mechanism depends on fluid-phase interaction between C1q and CRP. This might constitute another level of complement regulation, which has implications for systemic lupus erythematosus where CRP is often low despite flare-ups.


*Keywords:* C-reactive protein; C1q; Complement; Inflammation; Opsonization; Pentraxins; Systemic lupus erythematosus

The human complement system is composed of at least 30 soluble or membrane-associated proteins, and forms a core of the innate immune system. The classical pathway can be activated by target-bound antibodies, lipopolysaccharide, C-reactive protein (CRP) or DNA–histone complexes [1]. The alternative pathway can be triggered by the classical pathway or by direct contact with a variety of foreign molecules, protein aggregates/particles [2] and the lectin pathway is activated by mannose-binding lectin [3]. The physiological relevance of complement is demonstrated by recurrent infections in patients lacking certain components, as well as the overrepresentation of systemic lupus erythematosus (SLE) or SLE-like disease in individuals with deficiencies of the early complement factors [4]. On the other hand, in SLE as well as other inflammatory disorders, e.g., cardiovascular disease, rheumatoid arthritis, and multiple sclerosis, complement activation may also contribute to the pathogenesis [2,5,6].

Together with serum amyloid P component (SAP) and pentraxin-3, CRP belongs to the phylogenetically ancient and highly conserved family of pentraxins, which share a common structure with five identical subunits linked by weak non-covalent bonds and arranged in cyclic symmetry. Due to its dramatic rise within the initial 24–72 h in response to pro-inflammatory stimuli or tissue damage, CRP is an important marker of ongoing systemic inflammation [7].

*Abbreviations:* CRP, C-reactive protein; PC, phosphorylcholine; KLH, keyhole limpet hemocyanin; mCRP, monomeric CRP; RID, radial immunodiffusion; VB, veronal buffer; *vs*, versus.

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Circulating CRP is essentially derived from hepatocytes, but small amounts may also be produced locally by other cells [8]. The synthesis is mainly regulated at the transcriptional level through IL-6- and IL-1β-directed induction of the CRP gene, located on the long arm of chromosome 1, by activation of NF-κB and transcriptional factor NF-IL-6/CAAT-enhancer binding protein (C/EBP) family members C/EBPβ and C/EBPδ [7,9].

The calcium-dependent binding of CRP to phosphorylcholine (PC) exposed on bacterial cell walls, damaged cell membranes or apoptotic blebs, offers an explanation to the antibody-like properties of CRP, which is opsonizing by its Fc gamma receptor (FcγR) affinity [7,8,10,11]. Minor CRP elevations have been shown to reflect a low-grade vascular inflammation, and to predict coronary events in patients with angina pectoris as well as in apparently healthy subjects [8,12]. More substantial elevations of circulating CRP are a useful sign of systemic inflammation, e.g., in bacterial infections. In SLE, however, the CRP response is often limited or absent despite high disease activity and high circulating IL-6 levels [13].

Activation of the complement cascade is regarded as one of the main physiological functions of CRP, originally described by Kaplan and Volanakis who demonstrated consumption of hemolytic complement components in acute-phase sera mixed with pneumococcal C-polysaccharide, and by Siegel et al. using CRP–protamine complexes [14,15]. In the presence of calcium, each CRP subunit is able to complex with a PC-containing structure. Since all subunits of the CRP pentamer are targeted in the same direction, a recognition face is formed on the PC-bearing surface. C1q binds to the opposite face of the pentamer, thereby inducing complement activation via formation of the classical C3 convertase, which assembles in a fashion similar to that initiated by antibody–antigen complexes [7]. In contrast to IgM- and IgG-mediated complement activation, the CRP-mediated activation appears to be essentially limited to the initial stage involving C1–C4 with less consumption of the terminal complement proteins C5–C9 [16]. The difference in complement activation by CRP and immune complexes (ICs) has been suggested to be due to a stronger direct interaction between CRP and factor H, leading to inhibition of the alternative complement pathway C3 and C5 convertases [17,18].

Concomitantly with the release of acute-phase reactants, neutrophils infiltrating an inflammatory locus may become activated, discharging hydrolytic enzymes and reactive oxygen species, and contributing to an acidic local microenvironment. Under these conditions *in vitro*, there is evidence that CRP undergoes structural changes with dissociation of its subunits [19,20]. These monomeric CRP subunits (mCRP) expose epitopes not present on pentameric CRP and display properties distinct from those of native CRP. For instance, mCRP has been shown to interact with ICs, affect neutrophil–platelet adhesion and neutrophil aggregation, augment platelet activation and stimulate platelet generation [21–24]. In contrast to native CRP, the dissociation of monomers has been suggested to mediate pro-atherosclerotic effects on human endothelial cells [12]. However, the complement activating properties of mCRP have not yet been fully elucidated [25,26].

The aim of the present study was to analyze CRP-induced complement activation on model PC-surfaces with focus on protein deposition over time and CRP concentration in serum.

## Materials and methods

*Preparation of aminated silicon.* Due to its even optical properties during ellipsometry, silicon was chosen as the model substrate. The wafers were cleaved in the (100) crystal direction, cleaned in a basic peroxide solution of 5:1:1 parts of distilled water (Milli-Q quality), hydrogen peroxide (30% v/v), and $NH_4OH$ (25% v/v), respectively, at 80 °C for 5 min [27]. The wafers were rinsed five times in distilled water followed by washing with an acidic peroxide solution with 6:1:1 parts of distilled water, hydrogen peroxide (25% v/v), and hydrogen chloride (37% v/v), at 80 °C for 5 min [28]. Finally, the surfaces were rinsed three times in distilled water and dried in flowing nitrogen. This treatment resulted in a thin hydrated silicon dioxide layer with an advancing water contact angle of less than 5°. The surfaces were placed in a low vacuum chamber at 6 mtorr pressure and 200 μL 3-aminopropyl triethoxy silane (APTES; Sigma–Aldrich, St. Louis, USA) was let into the chamber and evaporated. The APTES coated surfaces were heat-treated inside the chamber at 60 °C for 10 min, and at 150 °C for 60 min. The prepared slides were rinsed three times in xylene (Merck GmbH, Darmstadt, Germany) at room temperature, and stored in xylene until use within 8 h. The static water contact angle ($\theta$) of aminated silicon was $<45°$ ($n = 5$; Rame-Hart goniometer, USA).

*Preparation of phosphorylcholine surfaces.* Six % glutaraldehyde (GA) in phosphate-buffered physiological saline (PBS), pH 9, was used to link the first layer of fibrinogen (Haemochrom Diagnostica, Mölndal, Sweden) onto APTES on silicon. The surfaces were incubated with GA for 30 min followed by extensive rinsing in distilled water. The APTES–GA samples were incubated for 30 min at room conditions in 1 mg/mL fibrinogen in PBS, pH 7.4, and rinsed in PBS. The fibrinogen-coated surfaces were incubated in a fresh mixture of *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide HCl:*N*-hydroxysuccinimide (EDC:NHS; Sigma–Aldrich, 37.5 mg/mL:5.75 mg/mL in PBS, pH 5.5, for 30 min), followed by incubation in the fibrinogen solution. The EDC:NHS surface activation was repeated prior to incubations in fibrinogen solutions until three layers of fibrinogen and an outer layer of PC hapten (*p*-diazonium phenylphosphorylcholine) conjugated to keyhole limpet hemocyanin (PC-KLH; Biosearch Technologies, Novato, USA) were covalently immobilized. The PC-KLH layer was deposited during a 60 min incubation of EDC:NHS activated cross-linked fibrinogen layers in 0.1 mg/mL PC-KLH in veronal buffer containing 0.15 mg/mL $CaCl_2$ and 0.5 mg/mL $MgCl_2$ ($VB^{2+}$).

Since mCRP has poor affinity for PC [29], approximately 13 Å mCRP was pre-immobilized by EDC:NHS chemistry on two layers of fibrinogen on APTES–GA as described above.

*Reagents.* Aqueous solutions (20 mM Tris–HCl, pH 7.8–8.2, containing 280 mM NaCl, 5 mM $CaCl_2$, and 0.1% sodium azide) of at least 99% pure human pentameric plasma CRP (stock concentration 2.5 mg/mL; Sigma–Aldrich) or human serum from seven individuals (CRP levels and complement profile given in Table 1) were used as source of CRP. The CRP concentrations were determined using high sensitivity turbidimetry technique (Bayer HealthCare, Advia 1650, NY, USA). Irreversible dissociation of purified CRP (mCRP) was obtained by acid-treatment (pH 2.0) of pentameric CRP prior to neutralization [19].

Fresh serum samples were stored at 4 °C or frozen at −70 °C within 3 h for less than 5 days. In some experiments, CRP was added to sera and plasma samples to final concentrations up to 500 mg/L, and allowed between 5 and 60 min of pre-equilibration at room temperature. In other

Table 1
Given diagnoses, and CRP, C3, and C4 levels in patient sera

| Sample | Diagnosis | CRP (mg/L) | C3 (mg/L) | C4 (mg/L) |
|---|---|---|---|---|
| 1 | Healthy | 0.4 | 830 | 140 |
| 2 | Healthy | 4 | 910 | 155 |
| 3 | Thrombosis | 106 | 890 | 225 |
| 4 | Erysipelas | 184 | 1500 | 300 |
| 5 | Sepsis | 218 | 920 | 265 |
| 6 | Sepsis | 331 | 883 | 132 |
| 7 | Pneumonia | 420 | 1221 | 251 |

experiments sera were serially diluted in threefold steps in $VB^{2+}$-buffer and incubated for 5 min. Where indicated, final concentrations of 10 mM bis-(aminoethyl)-glycolether-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA; Merck) and 2.5 mM $MgCl_2$, or only 10 mM disodium ethylenediaminetetraacetic acid (EDTA; Merck) were added to serum. $VB^{2+}$-buffer was used for the dilutions and rinsings in experiments with normal serum and $VB^{2-}$-buffer was used for experiments with EGTA–$Mg^{2+}$-containing sera.

*Antibodies.* The following antibodies were used: rabbit anti-human IgG, rabbit anti-human C1q, rabbit anti-human C3c, rabbit anti-human C4, rabbit anti-human fibrinogen, rabbit anti-human C-reactive protein, and sheep anti-human factor H (from DAKO, Glostrup, Denmark). All antibodies were polyclonal IgG-fractions (4–17 g/L). No unspecific binding of antibodies was observed in ellipsometric control experiments when human proteins, i.e., IgG (Biovitrum, Stockholm, Sweden), human serum albumin (Sigma–Aldrich), fibrinogen (Haemochrom Diagnostica) or high molecular weight kininogen (Binding Site, Birmingham, UK), were immobilized as antigens. In addition, control experiments with pre-adsorbed bovine serum showed no unspecific adsorption of the antibodies. To distinguish the two forms of CRP, two monoclonal antibodies specific for either native CRP or mCRP (3H12 and 2C10; generously provided by Dr Lawrence A Potempa) were used [30].

*Ellipsometry.* The thickness of the organic layers on silicon substrates was determined by null ellipsometry, which is a highly sensitive optical method with a practical resolution during protein adsorption measurements of about 1 Å [28,31]. The measurements yielded two angles, Ψ and Δ, which were used to iterate the protein film thickness. The serum and complement activation experiments ($n \geqslant 5$) were performed as follows. Silicon samples of size $10 \times 5$ mm with immobilized PC were placed in a 1.5 mL plastic incubation trough containing CRP-enriched or unmodified serum. In other experiments, the sera were diluted in $VB^{2+}$-buffer. The incubation times varied between 1 and 60 min and all experiments were performed at 37 °C. Some of the serum-incubated surfaces were rinsed in $VB^{2+}$-buffer and distilled water, dried in flowing nitrogen and the total serum deposition quantified. The other serum-incubated surfaces were rinsed in $VB^{2+}$ alone, and without drying transferred to antibody solutions (1:50 dilutions in $VB^{2+}$-buffer) and incubated for 30 min at 20 °C. The surfaces were finally rinsed in distilled water, dried as described above, and the thicknesses determined. The Auto-Ell III ellipsometer (Rudolph Research, Fairfield, NJ, USA) was programmed for measurements on silicon, and this option was used. The protein film thickness was calculated according to the McCrackin evaluation algorithm [32] and the adsorbed amount per unit area calculated by De Feijter's formula [33]. The refractive indices used were 1.335 and 1.465 for the buffer and the adsorbed APTES and proteins, respectively [34,35]. One nanometer in thickness of dried deposited organic film equals approximately 120 ng/cm$^2$ in mass [36]. See also Supplementary data.

*Adsorption assay and RID.* Increasing amounts of commercial CRP (Sigma–Aldrich) were added to a human normocomplementemic low-CRP serum sample (sample 1, Table 1) resulting in six tubes with different CRP concentrations (0, 50, 100, 150, 250, and 500 mg/L) and a volume of 0.5 mL, respectively. Fifty microliter of polyclonal anti-CRP antibody (DAKO) was added, the tubes were mixed and equilibrated for 15 min at room temperature. One-hundred microliter of protein G–Sepharose (GE Healthcare, Uppsala, Sweden) was added and the tubes mixed again. The tubes were then allowed to sediment before centrifugation during 10 min at 850*g*. The supernatants were transferred to new tubes.

Each sample was applied to commercial RID kits (Binding Site, Birmingham, UK) measuring C1q, C2, and C4. The enclosed protocols were followed. Two independent readers evaluated the results. The results are presented as percentage of a control serum, which was exposed to buffer alone instead of CRP solution but otherwise treated exactly as the other samples.

*Statistics.* The ellipsometric data are presented as mean values ± SEM of at least five independent experiments where each experiment was the mean value of five measurements. The RID data originate from three separate experiments in duplicate. Evaluation was performed using the two-tailed Student's *t*-test and the conventional significance levels of $p < 0.05$, $p < 0.01$, and $p < 0.001$ were used.

*Ethics.* Informed consent was obtained from each patient and the study protocol was approved by the ethics committee at Linköping University.

## Results

### *Complement activation on PC- and IgG-coated surfaces*

In order to compare complement activation on IgG- versus (*vs*) PC-coated surfaces, IgG was adsorbed to hydrophobized silicon and PC-KLH immobilized to pre-fabricated cross-linked fibrinogen layers. The surfaces were subsequently incubated for 0–60 min in normal serum (s-CRP 4 mg/L) with/without EGTA, or in normal serum supplemented with CRP (s-CRP 75 mg/L). Fig. 1a illustrates the total serum protein deposition onto the different surfaces whereas Fig. 1b shows anti-C3c-binding after the serum incubations in each case. Apparently, the IgG-coated surfaces activated the classical pathway strongly, followed by activation of the alternative pathway (effector pathway) in normal serum. Previously, we have shown that the total serum protein deposition is dominated by surface C3b and its degradation fragments [36]. The amount was larger on IgG than on PC-surfaces and increased steadily throughout the 60 min incubation. Upon inhibition of the classical pathway by calcium chelation, the IgG-coated surfaces showed an approximately 10 min delay until serum protein begun to deposit, and now only via the alternative pathway. The deposited amounts increased in this case rapidly during alternative activation after 30–60 min of incubation [36]. The total serum protein deposition from normal serum (s-CRP 4 mg/L) was significantly lower onto PC-KLH-surfaces than onto IgG-surfaces, and could only be moderately increased through the addition of CRP (s-CRP 75 mg/L). The lowest protein deposition was observed on PC-surfaces after incubations in EGTA-sera with s-CRP 4 or 75 mg/L, indicating that PC-coated surfaces did not activate the alternative pathway *per se*. The net deposition of anti-C3c (Fig. 1b), on top of serum proteins (Fig. 1a), suggests activation on PC-surfaces of the classical pathway also at s-CRP levels below 10 mg/L. No anti-C3c bound to PC-surfaces after exposure of EGTA-sera with s-CRP levels 4 or 75 mg/L. Thus, in contrast to IgG-coated surfaces, the alternative pathway seemed not to be involved in the activation process on PC-surfaces.

In order to measure the initial activation and protein deposition kinetics, the PC-surfaces were incubated in diluted patient sera with s-CRP levels ranging from 0.4 to 106 mg/L

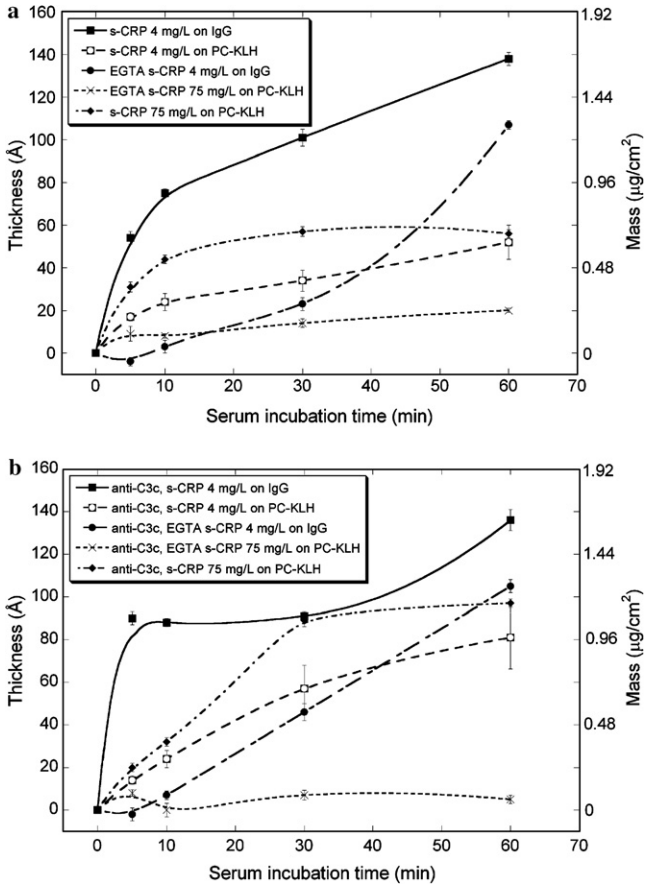


Fig. 1. Comparison of protein deposition from normal serum and EGTA-serum onto PC- and IgG-coated hydrophobic silicon (positive reference) surfaces during 60 min of incubation at 37 °C. The sera containing 4 mg/L were also supplemented up to 75 mg/L s-CRP (a). Anti-C3c-binding to surfaces is demonstrated here. Observe the low anti-C3c deposition to PC-surfaces incubated in EGTA s-CRP 75 mg/L serum (b).

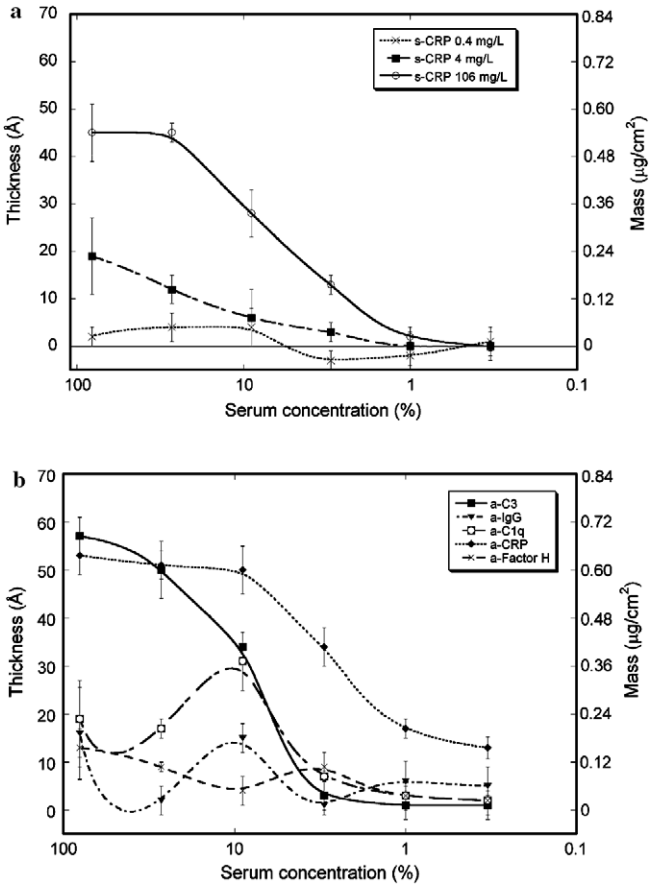


Fig. 2. Normal serum deposition from a dilution series on PC-surfaces from three individuals with different levels of CRP (0.4, 4 and 106 mg/L, respectively). The incubation time was 5 min (undiluted serum: s-CRP 0.4 mg/L *vs* 4 mg/L, $p < 0.05$) (a). Binding of anti-C3c, anti-IgG, anti-C1q, anti-CRP, and anti-factor H to surfaces in Fig. 2a is demonstrated for s-CRP concentration 106 mg/L (b).

for 5 min (Fig. 2a), followed by incubations in selected antibody solutions. In addition to anti-C3c, the binding of anti-C1q and anti-IgG was measured at each dilution. As expected, sera with higher s-CRP deposited significantly more serum proteins than those with low s-CRP (undiluted serum, s-CRP 0.4 mg/L *vs* 4 mg/L, $p < 0.05$). Antibody binding at 106 mg/L CRP concentration is shown in Fig. 2b.

*Spontaneous adsorption of CRP on silicon*

Very low amounts of anti-CRP bound to hydrophilic and hydrophobic reference surfaces after incubations in CRP-containing sera or plasma. The obvious indication is that, independent of the CRP concentration, hydrophilic negatively charged surfaces and hydrophobic surfaces *per se* possess low attraction to serum CRP, and hence induce low complement activation during elevated CRP conditions.

*CRP levels higher than 300 mg/L inhibit complement activation on PC*

Plasma and sera were prepared from blood of five individuals displaying elevated CRP levels, ranging from 106 to 420 mg/L (Table 1). The total serum protein (Fig. 3a) and anti-C3c (Fig. 3b) depositions were significantly lower in the two patient samples exceeding 300 mg/L s-CRP compared to samples with concentrations below 220 mg/L. The sample containing s-CRP 218 mg/L showed high complement activation and a partial hemolysis during serum collection. A similar pattern was found when we compared normal sera with either low s-CRP (0.4–4 mg/L) or acute-phase sera exceeding s-CRP 300 mg/L. As shown in 3b, PC-surfaces exposed to sera with s-CRP 331 or 420 mg/L bound virtually no anti-C3c after 5 min of serum incubation (undiluted sera, s-CRP 420 mg/L *vs* 184 mg/L, $p < 0.001$; 1:3 dilution $p < 0.001$; 1:9 dilution $p < 0.05$). In addition, control experiments showed that plasma and sera from same individuals displayed the same capacity to activate complement on IgG-surfaces, i.e., fibrinogen seemed not to be involved in the process.

The results from patient sera and plasma urged us to make experiments with CRP-supplemented sera. Fig. 4a shows the increase in anti-C3c accumulation on top of the serum layer was most dramatic in the s-CRP interval between 4 and 10 mg/L, i.e., levels that in numerous studies have been

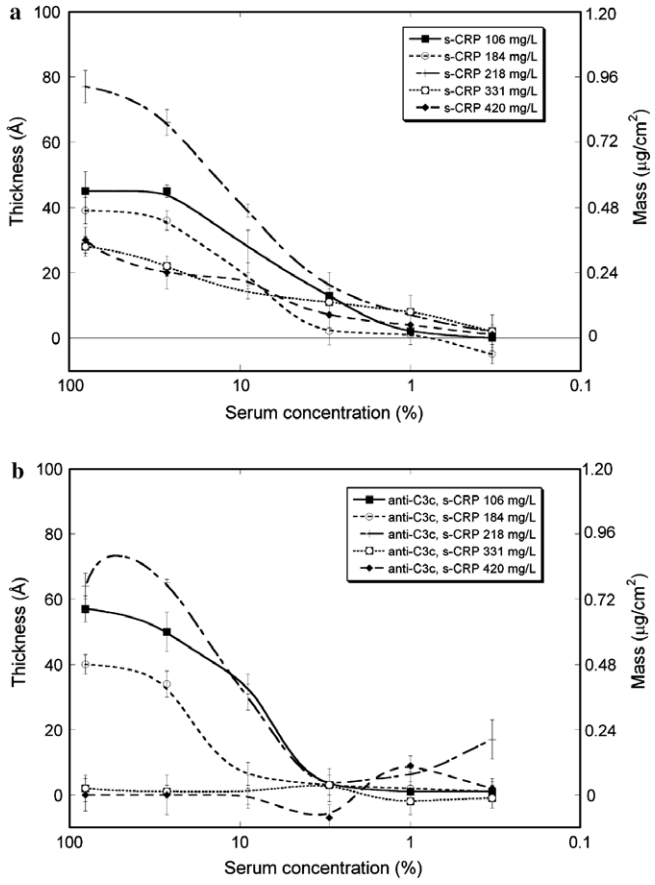


Fig. 3. Protein deposition using sera with elevated CRP levels in dilution series onto PC-surfaces. Incubation time 5 min (a). Anti-C3c-binding to surfaces in Fig. 3a (undiluted serum, s-CRP 331 mg/L or 420 mg/L *vs* 184 mg/L, $p < 0.001$; 1:3 dilution, s-CRP 331 mg/L *vs* 184 mg/L, $p < 0.001$, s-CRP 420 mg/L *vs* 184 mg/L, $p < 0.01$; 1:9 dilution, s-CRP 420 mg/L *vs* 184 mg/L, $p < 0.05$) (b).

shown to predict coronary events [8,12]. The largest amounts of anti-C3c bound to surfaces exposed to serum with s-CRP ≈150 mg/L, whereas virtually no anti-C3c bound to surfaces exposed to serum with s-CRP >300 mg/L (Fig. 4a). The supplemented normal serum with native pentameric CRP confirmed the result of low complement activation on PC-surfaces in patient sera with high CRP. The total serum deposition after 5 min incubation increased up to a concentration of approximately 250 mg/L and decreased thereafter.

Comparison of the complement activation capacity by high s-CRP (500 mg/L) on IgG- and PC-surfaces showed that elevated CRP concentrations did not abolish the IgG-mediated complement activation and surface C3b deposition during a 5 min incubation.

### *Complement activation by mCRP*

Matrices with covalently bound mCRP were incubated with normal human serum (CRP concentration 0.4 mg/L). Anti-C3c was then applied and the amount of antibody deposited on top of the total serum protein deposition quantified to indicate the degree of complement activation.

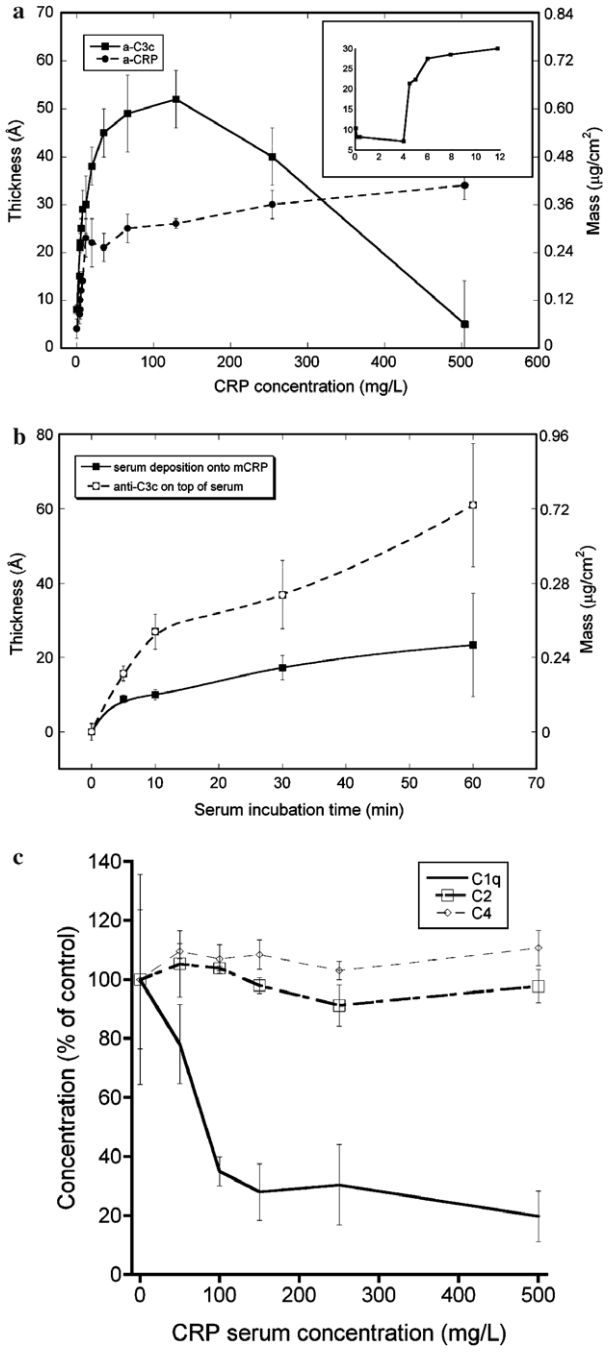


Fig. 4. Protein deposition onto PC-KLH-surfaces after addition of 0–500 mg/L CRP to serum containing 4 mg/L CRP. The insert shows anti-C3c binding at low s-CRP concentrations (a). Anti-C3c binding on top of the total serum deposition on pre-immobilized mCRP during 60 min of incubation at 37 °C. Significant differences between serum deposition and anti-C3c binding were seen at 5 min ($p < 0.05$), 10 ($p < 0.01$), 30 ($p < 0.05$), and 60 ($p < 0.05$) min of incubation (b). Results from the adsorption assay and radial immunodiffusion. C1q, C2, and C4 were measured by RID. The results indicate a clear-cut concentration dependent fluid-phase interaction between CRP and C1q (in comparison with control serum; $p < 0.01$ at 50 mg/L, $p < 0.001$ at 100, 150, 250, and 500 mg/L) (c).

Upon 5 min incubation, complement activation was observed with kinetics similar to that of pentameric PC-captured CRP, i.e., classical activation. Although the binding

of polyclonal anti-C3c onto the serum layer displayed some fluctuation using mCRP compared to PC-captured CRP, a significant difference between serum deposition and anti-C3c binding were seen at all time points (Fig. 4b).

*Fluid-phase interaction between CRP and C1q*

We evaluated the possibility of fluid-phase interaction between CRP and one or more of the classical complement components as an underlying mechanism of the powerful inhibition on complement activation seen when high CRP concentrations were used. A normocomplementemic low-CRP serum was supplemented with increasing amounts of CRP. After equilibration, CRP was captured with a polyclonal antibody and eliminated by protein G–Sepharose. C1q, C2, and C4 were measured in the supernatants with radial immunodiffusion (RID). Less C1q was detected with increasing concentration of CRP whereas C2 and C4 were not affected at all (Fig. 4c).

## Discussion

Although it is known since 75 years that CRP is a major acute-phase protein with a variety of biological activities, its physiological/pathophysiological roles remain unclear. One of the first identified properties of native CRP was its ability to activate the complement cascade by sequestration of C1q. In previous studies on CRP–complement interactions, indirect techniques, such as hemolytic activity [14,15], enzyme immunoassays [37] or immunohistochemistry [38] have been used. In the present study, we chose null ellipsometry to analyze the CRP-mediated complement deposition onto model PC-surfaces, a method that is direct, non-destructive, quantitative, and allows the detection of early transient actors of the classical pathway, e.g., C1q, at interfaces with low serum dilutions and short time serum incubations [28,31,36]. Ellipsometry allows the detection of surface-adsorbed protein layers with a practical resolution of about 1 Å in biological environments with sensitivity similar to radioimmunoassays [35,36]. Herein, we demonstrate classical activation of the complement system by CRP that interacts with surface-bound PC. The main indications for actual activation are the increasing protein deposition from normal and EGTA sera with added CRP (Fig. 1a) as well as the concomitant surface binding of C1q and C3 in the serum dilution series (Fig. 2b).

Numerous studies have recognized an association between a minor elevation of CRP and future cardiovascular disease. Furthermore, there is considerable evidence suggesting that complement activation occurs in atherosclerotic lesions [2,38]. In addition, elevation of the highly pro-inflammatory complement component C5a has been reported to increase the risk of cardiovascular events in patients with advanced atherosclerosis [39]. In the present study, we show that purified CRP and CRP in patient sera have similar capacity to activate complement on surface immobilized PC in a dose-dependent manner. Most interestingly, CRP proved to be a powerful complement activator even at concentrations below 10 mg/L (insert, Fig. 4a). This finding has implications both for the normal innate immune homeostasis and for cardiovascular disease where high sensitivity analysis of CRP is nowadays a widely used risk marker.

Our findings are in line with reports suggesting that the CRP-mediated complement activation on PC proceeds via the classical pathway [16–18,25]. CRP-mediated complement activation could contribute to chronic inflammatory reactions in atherosclerosis and rheumatic diseases or represent an anti-inflammatory response that limits the deleterious effects of otherwise complete complement cascade. Possibly, the presence of PC-expressing structures, such as oxidized low-density lipoprotein (LDL), bacteria and apoptotic cells in the vascular environment induces generation of CRP, which together with C3 fragments opsonize the structures and facilitate their removal by phagocytes [40]. In support of this, CRP, LDL, and complement factors have been shown to co-localize in early atherosclerotic lesions [38].

*In vitro* studies have suggested that CRP binds factor H, a serum protein binding to immobilized C3b and inhibiting the alternative complement pathway, thereby regulating the complement activation and providing a possible explanation to the prevention of terminal complement complex formation [16–18,41]. In contrast to many previous studies using purified proteins, we used complete serum. The antibody deposition pattern illustrated for the 106 mg/L serum in Fig. 2b suggests the involvement of both IgG and C1q during the initiation of the activation process whereas low but detectable levels of anti-factor H also bound onto this surface. The involvement of anti-PC antibodies can be excluded since no deposition of IgG occurred with any of the sera at CRP concentrations below 4 mg/L.

Very intriguingly, a dramatic fall in C1q-mediated complement activation occurred at high CRP levels, both in CRP-supplemented serum (s-CRP >150 mg/L; Fig. 4a) and in high-CRP patient sera (s-CRP >300 mg/L; Fig. 3). This phenomenon, which has not been described previously, urged us to evaluate the hypothesis that soluble CRP prevents classical complement on surfaces by fluid-phase consumption. Indeed, this was substantiated by an inverse relationship between CRP concentration and C1q level, whereas no CRP-interactions were found with C2 or C4 (Fig. 4c). Thus, we suggest that CRP binds C1q in fluid-phase and may have a dual role in classical activation by restricting potential damage on tissue surfaces in acute-phase sera with high CRP levels. Lack of adequate CRP-mediated complement activation could possibly contribute to IC-mediated tissue damage in SLE [41,42], where the CRP response often appears to be lacking or is in disharmony with other acute-phase reactions [43]. Interestingly, CRP supplementation to mice with lupus nephritis induces a sustained remission and prolongs the survival [44]. We have previously reported the occurrence of autoantibodies

to mCRP in SLE [45]. Although this does not explain the deficient CRP-reaction in SLE, it correlates to disease activity and may have relevance to the pathogenetic process [8,46].

Monomeric CRP has poor solubility in aqueous media but is found in normal vascular tissues as well as at sites of inflammation [8,20]. We prepared mCRP by acid-treatment and the capacity of mCRP to activate complement was investigated in comparison with native pentameric CRP. A previous study by Miyazawa and Inoue showed that the pH optimum for CRP-mediated complement activation was 6.3, which however, is not low enough to cause complete dissociation of pentamers to monomers [47]. Vaith and coworkers studied complement activation on HEp-2 cells and showed that mCRP binds distinct filamentous cytoplasmic structures but was not followed by deposition of complement components [26]. Our results support the hypothesis that immobilized mCRP may activate the complement system. The reason for previous studies failing to display the deposition of C3 onto mCRP may be found in difficulties to obtain a reproducible deposition of anti-C3 after serum incubation. Similarly to our findings, a recent study by Ji et al. using mCRP indicated different effects on classical activation dependent on whether mCRP was in fluid-phase or bound to C1q [48].

To conclude, we present new *in vitro* data on CRP-mediated C1q-dependent complement activation on surfaces. Complement is activated already at low-CRP levels with a concentration-dependent increase of C3 surface deposition up to a CRP level of approximately 150 mg/L, followed by a marked down-regulation of C3 deposition at higher CRP concentrations. We suggest that the underlying mechanism to this phenomenon depends on fluid-phase interaction between CRP and C1q. An adequate CRP-response in acute inflammation may therefore be essential for normal immune homeostasis, cardiovascular disease, and the pathogenesis of many inflammatory conditions.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.11.013.

## References

[1] H. Gewurz, S.C. Ying, H. Jiang, T.F. Lint, Nonimmune activation of the classical complement pathway, Behring. Inst. Mitt. 93 (1993) 138–147.
[2] F. Niculescu, H. Rus, Complement activation and atherosclerosis, Mol. Immunol. 36 (1999) 949–955.
[3] K. Ikeda, T. Sannoh, N. Kawasaki, T. Kawasaki, I. Yamashina, Serum lectin with known structure activates complement through the classical pathway, J. Biol. Chem. 262 (1987) 7451–7454.
[4] A.G. Sjöholm, G. Jönsson, J.H. Braconier, G. Sturfelt, L. Truedsson, Complement deficiency and disease: an update, Mol. Immunol. 43 (2006) 78–85.
[5] E.T. Molenaar, A.E. Voskuyl, A. Familian, G.J. van Mierlo, B.A. Dijkmans, C.E. Hack, Complement activation in patients with rheumatoid arthritis mediated in part by C-reactive protein, Arthritis Rheum. 44 (2001) 997–1002.
[6] D.A. Compston, B.P. Morgan, A.K. Campbell, P. Wilkins, G. Cole, N.D. Thomas, B. Jasani, Immunocytochemical localization of the terminal complement complex in multiple sclerosis, Neuropathol. Appl. Neurobiol. 15 (1989) 307–316.
[7] J.E. Volanakis, Human C-reactive protein: expression, structure, and function, Mol. Immunol. 38 (2001) 189–197.
[8] C. Sjöwall, T. Bengtsson, T. Skogh, CRP and anti-CRP autoantibodies in systemic lupus erythematosus, Curr. Rheumatol. Rev. 1 (2005) 81–89.
[9] R. Kleemann, P.P. Gervois, L. Verschuren, B. Staels, H.M. Princen, T. Kooistra, Fibrates down-regulate IL-1-stimulated C-reactive protein gene expression in hepatocytes by reducing nuclear p50-NFκB-C/EBP-β complex formation, Blood 101 (2003) 545–551.
[10] D.E. Manolov, C. Röcker, V. Hombach, U. Nienhaus, J. Torzewski, Ultrasensitive confocal fluorescence microscopy of C-reactive protein interacting with FcγRIIa, Arterioscler. Thromb. Vasc. Biol. 24 (2004) 2372–2377.
[11] S.S. Taskinen, M. Hyvönen, P.T. Kovanen, S. Meri, M.O. Pentikäinen, C-reactive protein binds to the 3β-OH group of cholesterol in LDL particles, Biochem. Biophys. Res. Commun. 329 (2005) 1208–1216.
[12] S. Verma, P.E. Szmitko, E.T. Yeh, C-reactive protein: structure affects function, Circulation 109 (2004) 1914–1917.
[13] C. Gabay, P. Roux-Lombard, P. de Moerloose, J.M. Dayer, T. Vischer, P.A. Guerne, Absence of correlation between interleukin 6 and C-reactive protein blood levels in systemic lupus erythematosus compared with rheumatoid arthritis, J. Rheumatol. 20 (1993) 815–821.
[14] M.H. Kaplan, J.E. Volanakis, Interaction of C-reactive protein complexes with the complement system. I. Consumption of human complement associated with the reaction of C-reactive protein with pneumococcal C-polysaccharide and with the choline phosphatides, lecithin and sphingomyelin, J. Immunol. 112 (1974) 2135–2147.
[15] J. Siegel, R. Rent, H. Gewurz, Interactions of C-reactive protein with the complement system. I. Protamine-induced consumption of complement in acute phase sera, J. Exp. Med. 140 (1974) 631–647.
[16] S. Berman, H. Gewurz, C. Mold, Binding of C-reactive protein to nucleated cells leads to complement activation without cytolysis, J. Immunol. 136 (1986) 1354–1359.
[17] H. Jarva, T.S. Jokiranta, J. Hellwage, P.F. Zipfel, S. Meri, Regulation of complement activation by C-reactive protein: targeting the complement inhibitory activity of factor H by an interaction with short consensus repeat domains 7 and 8–11, J. Immunol. 163 (1999) 3957–3962.
[18] E. Giannakis, T.S. Jokiranta, D.A. Male, S. Ranganathan, R.J. Ormsby, V.A. Fischetti, C. Mold, D.L. Gordon, A common site within factor H SCR 7 responsible for binding heparin, C-reactive protein and streptococcal M protein, Eur. J. Immunol. 33 (2003) 962–969.
[19] L.A. Potempa, J.M. Zeller, B.A. Fiedel, C.M. Kinoshita, H. Gewurz, Stimulation of human neutrophils, monocytes, and platelets by

modified C-reactive protein (CRP) expressing a neoantigenic specificity, Inflammation 12 (1988) 391–405.
[20] H.W. Wang, S.F. Sui, Dissociation and subunit rearrangement of membrane-bound human C-reactive proteins, Biochem. Biophys. Res. Commun. 288 (2001) 75–79.
[21] M. Motie, S. Brockmeier, L.A. Potempa, Binding of model soluble immune complexes to modified C-reactive protein, J. Immunol. 156 (1996) 4435–4441.
[22] T. Khreiss, L. Jozsef, L.A. Potempa, J.G. Filep, Opposing effects of C-reactive protein isoforms on shear-induced neutrophil–platelet adhesion and neutrophil aggregation in whole blood, Circulation 110 (2004) 2713–2720.
[23] K. Miyazawa, S. Kiyono, K. Inoue, Modulation of stimulus-dependent human platelet activation by C-reactive protein modified with active oxygen species, J. Immunol. 141 (1988) 570–574.
[24] L.A. Potempa, M. Motie, K.E. Wright, B.L. Crump, J.A. Radosevich, N. Sakai, G. Lai, K. Tanaka, E. Kojima, A. Tsuboi, Stimulation of megakaryocytopoiesis in mice by human modified C-reactive protein (mCRP), Exp. Hematol. 24 (1996) 258–264.
[25] H.X. Jiang, J.N. Siegel, H. Gewurz, Binding and complement activation by C-reactive protein via the collagen-like region of C1q and inhibition of these reactions by monoclonal antibodies to C-reactive protein and C1q, J. Immunol. 146 (1991) 2324–2330.
[26] P. Vaith, V. Prasauskas, L.A. Potempa, H.H. Peter, Complement activation by C-reactive protein on the HEp-2 cell substrate, Int. Arch. Allergy Immunol. 111 (1996) 107–117.
[27] W. Kern, D.A. Puotinen, Cleaning solutions based on hydrogen peroxide for use in silicon semiconductor technology, RCA Rev. 31 (1970) 187–206.
[28] R.M. Azzam, N.M. Bashara (Eds.), Ellipsometry and Polarized Light, second ed., Elsevier, Amsterdam, 1987.
[29] L.A. Potempa, B.A. Maldonado, P. Laurent, E.S. Zemel, H. Gewurz, Antigenic, electrophoretic and binding alterations of human C-reactive protein modified selectively in the absence of calcium, Mol. Immunol. 20 (1983) 1165–1175.
[30] S.C. Ying, E. Shephard, F.C. de Beer, J.N. Siegel, D. Harris, B.E. Gewurz, M. Fridkin, H. Gewurz, Localization of sequence-determined neoepitopes and neutrophil digestion fragments of C-reactive protein utilizing monoclonal antibodies and synthetic peptides, Mol. Immunol. 29 (1992) 677–687.
[31] L. Vroman, A. Lukosevicius, Ellipsometer recordings of changes in optical thickness of adsorbed films associated with surface activation of blood clotting, Nature 204 (1964) 701–703.
[32] F.L. McCrackin, A fortran program for the analysis of ellipsometer measurements, NBS Tech. Note No. 479, Washington DC, 1969.
[33] J.A. De Feijter, J. Benjamins, F.A. Veer, Ellipsometry as a tool to study adsorption behaviour of synthetic and biopolymers at air–water interface, Biopolymers 17 (1978) 1759–1772.
[34] M. Stenberg, H. Nygren, A receptor–ligand reaction studied by a novel analytical tool—the isoscope ellipsometer, Anal. Biochem. 127 (1982) 183–192.
[35] J. Benesch, A. Askendal, P. Tengvall, Quantification of adsorbed human serum albumin at solid interfaces: a comparison between radioimmunoassay (RIA) and simple null ellipsometry, Colloids Surf. B, Biointerfaces 18 (2000) 71–81.
[36] P. Tengvall, A. Askendal, I. Lundström, Temporal studies on the deposition of complement on human colostrum IgA and serum IgG immobilized on methylated silicon, J. Biomed. Mater. Res. 35 (1997) 81–92.
[37] G.J. Wolbink, M.C. Brouwer, S. Buysmann, I.J. ten Berge, C.E. Hack, CRP-mediated activation of complement *in vivo*: assessment by measuring circulating complement—C-reactive protein complexes, J. Immunol. 157 (1996) 473–479.
[38] W.K. Lagrand, H.W. Niessen, G.J. Wolbink, L.H. Jaspars, C.A. Visser, F.W. Verheugt, C.J. Meijer, C.E. Hack, C-reactive protein colocalizes with complement in human hearts during acute myocardial infarction, Circulation 95 (1997) 97–103.
[39] W.S. Speidl, M. Exner, J. Amighi, S.P. Kastl, G. Zorn, G. Maurer, O. Wagner, K. Huber, E. Minar, J. Wojta, M. Schillinger, Complement component C5a predicts future cardiovascular events in patients with advanced atherosclerosis, Eur. Heart J. 26 (2005) 2294–2299.
[40] M.K. Chang, C.J. Binder, M. Torzewski, J.L. Witztum, C-reactive protein binds to both oxidized LDL and apoptotic cells through recognition of a common ligand: phosphorylcholine of oxidized phospholipids, Proc. Natl. Acad. Sci. USA 99 (2002) 13043–13048.
[41] C. Mold, H. Gewurz, T.W. Du Clos, Regulation of complement activation by C-reactive protein, Immunopharmacology 42 (1999) 23–30.
[42] D. Gershov, S. Kim, N. Brot, K.B. Elkon, C-reactive protein binds to apoptotic cells, protects the cells from assembly of the terminal complement components, and sustains an antiinflammatory innate immune response: implications for systemic autoimmunity, J. Exp. Med. 192 (2000) 1353–1364.
[43] D.A. Hesselink, L.A. Aarden, A.J. Swaak, Profiles of the acute-phase reactants C-reactive protein and ferritin related to the disease course of patients with systemic lupus erythematosus, Scand. J. Rheumatol. 32 (2003) 151–155.
[44] W. Rodriguez, C. Mold, M. Kataranovski, J. Hutt, L.L. Marnell, T.W. Du Clos, Reversal of ongoing proteinuria in autoimmune mice by treatment with C-reactive protein, Arthritis Rheum. 52 (2005) 642–650.
[45] C. Sjöwall, P. Eriksson, S. Almer, T. Skogh, Autoantibodies to C-reactive protein is a common finding in SLE, but not in primary Sjögren's syndrome, rheumatoid arthritis or inflammatory bowel disease, J. Autoimmun. 19 (2002) 155–160.
[46] C. Sjöwall, A.A. Bengtsson, G. Sturfelt, T. Skogh, Serum levels of autoantibodies against monomeric C-reactive protein are correlated with disease activity in systemic lupus erythematosus, Arthritis Res. Ther. 6 (2004) R87–R94.
[47] K. Miyazawa, K. Inoue, Complement activation induced by human C-reactive protein in mildly acidic conditions, J. Immunol. 145 (1990) 650–654.
[48] S.R. Ji, Y. Wu, L.A. Potempa, Y.H. Liang, J. Zhao, Effect of modified C-reactive protein on complement activation: a possible complement regulatory role of modified or monomeric C-reactive protein in atherosclerotic lesions, Arterioscler. Thromb. Vasc. Biol. 26 (2006) 935–941.