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Exploring the Complement Interaction Network Using Surface Plasmon Resonance

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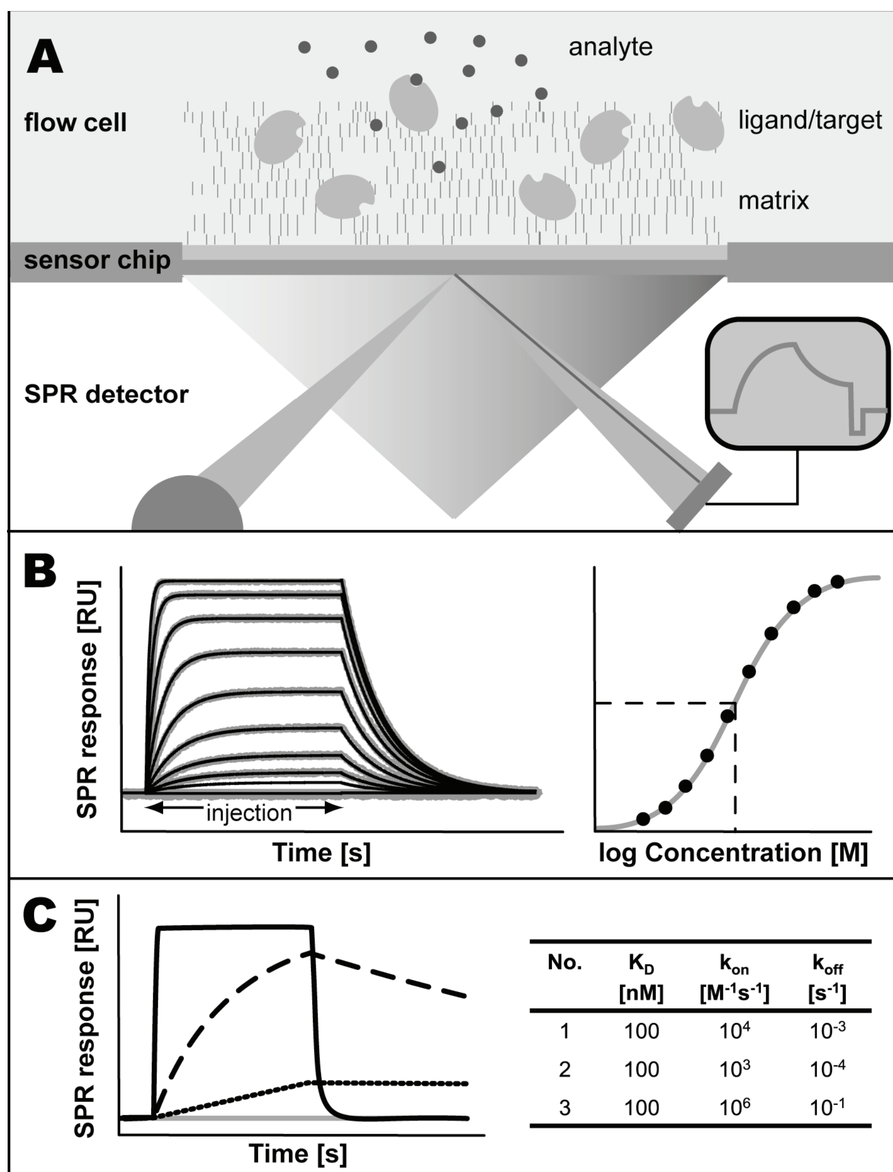
1 Introduction

The human complement system represents a well-balanced and tightly regulated network of protein interactions. Several cascades of soluble and membrane-bound proteins, enzymes, cofactors, and regulators guarantee the dynamic and specific answer to pathogenic conditions and make the complement system to the key part of innate immunity. However, even small changes in this network may lead to severe disorders (e.g. auto-immune diseases), and some pathogens (mis-)use complement proteins for entering human cells. A detailed knowledge about the individual molecular interaction is therefore essential for the understanding of the complement cascade and for the development of therapeutic interventions against complement-related diseases. Surface plasmon resonance biosensors are considered a key technology for characterizing such biomolecular interactions and are therefore used in complement research at a rapidly increasing pace. In addition to the label-free quantification of the affinity between two binding partners, SPR is able to generate kinetic profiles of these interactions. This review gives an introduction to SPR technology and shows its possibilities and applications followed by an illustration of its use in complement research on the basis of different examples.

2 Surface Plasmon Resonance – The Key to Kinetic Constants

During the past ten years, surface plasmon resonance (SPR) has emerged as a key technology for the characterization of biomolecular interactions. Nowadays, SPR biosensors are widely used in a variety of areas (Rich and Myszka 2000, 2001, 2002, 2003, 2005a, 2005b, 2006) from life sciences and biology to proteomics



No.	K_D [nM]	k_{on} [$M^{-1}s^{-1}$]	k_{off} [s^{-1}]
1	100	10^4	10^{-3}
2	100	10^3	10^{-4}
3	100	10^6	10^{-1}

Fig. 1. Schematic representation of a typical SPR experiment (A). A target protein is immobilized on a hydrogel matrix of a gold sensor chips. Analyte molecules in solution interact with the target and generate an SPR response. The resulting sensorgrams (B) can be either kinetically fitted (left) or the affinity can be evaluated from the steady state responses (right). Different kinetic profiles may result in the same affinity constant (C).

(Nedelkov and Nelson 2003; Buijs and Franklin 2005; Yuk and Ha 2005), food analysis (Bergwerff and van Knapen 2006; Gambari and Feriotto 2006; Rasooly and Herold 2006), or drug discovery (Myszka and Rich 2000; Cooper 2002; Keusgen 2002; Myszka and Rich 2003). The success of SPR is founded on its unique ability to simultaneously acquire data about both affinity and kinetics of a molecular binding event. In addition, no labeling of the analyte molecule is required and typical experiments consume comparatively small sample amounts. While the principles of SPR detection have been already described in the late 1950s (Turbadar 1959), the first commercial SPR instruments were only introduced in 1990 (Jonsson, Fagerstam, Ivarsson, Johnsson, Karlsson, Lundh, Lofas, Persson, Roos, Ronnberg, Sjolander, Stenberg, Stahlberg, Urbaniczky, Ostlin and Malmqvist 1991; Malmqvist 1993). Even though a variety of biosensors based on SPR or similar technologies (e.g. plasmon waveguide resonance) had been developed since then, the vast majority of today's SPR analyses are performed with instruments produced by Biacore in Sweden. As a consequence, the following introduction and the examples are based on Biacore biosensors.

In a typical SPR experiment, one of the interaction partners (i.e. the ligand or target) is immobilized on the surface of a sensor chip, while the other partner (i.e. the analyte) is injected in solution (Fig. 1A). The sensor chip consists of a glass slide covered with a thin layer of gold. In order to avoid immobilization-derived denaturation of target proteins, a hydrogel matrix consisting of carboxymethyl dextran chains is attached to the surface. In addition to keeping the protein in a quasi-solvent environment, the matrix allows the introduction of functional groups for diverse immobilization chemistries and increases the coupling capacity by providing a three-dimensional network (Johnsson, Lofas and Lindquist 1991). Binding of the analyte in solution to the immobilized target leads to a change in refractive index (i.e. electron density) around the gold surface, which is detected as a change in the absorbance angle of totally reflected light by the SPR detector (Jonsson et al. 1991; Nagata and Handa 2000). It has been shown that this change, quantified as arbitrary 'resonance units' (RU), can be correlated with a mass increase on the chip surface, with 1 RU = 1 pg/mm^2 (Stenberg, Persson, Roos and Urbaniczky 1991). Upon injection of the analyte, the binding event can be observed in real-time, and the time course of the SPR signal is referred to as a sensorgram (Fig. 1B). As long as analyte molecules are injected, the sensorgram represents a changing ratio of associating and dissociating molecules until a steady state is reached. After injection end, the pure dissociation can be observed and extracted. The kinetic association and dissociation rate constants (k_{on} and k_{off}, respectively) are calculated by globally fitting the sensorgrams at various analyte concentrations to mathematical equations describing different binding models (Morton, Myszka and Chaiken 1995; Roden and Myszka 1996). In addition, the affinity (equilibrium dissociation constant K_D) of the interaction can be either calculated from the rate constants ($K_D = k_{off}/k_{on}$) or by fitting a concentration-dependent plot of the steady state signals (Fig. 1B). Additional yet less frequent applications of SPR assays include the determination of thermodynamic parameters (de Mol, Dekker, Broutin, Fischer and Liskamp 2005; Wear and Walkinshaw 2006), concentration analyses (Fagerstam, Frostell-Karlsson, Karlsson, Persson and Ronnberg 1992; Kikuchi, Uno, Nanami, Yoshimura, Iida, Fukushima and

Tsuchiya 2005), and competitive experiments (IC_{50}) (Karlsson, Kullman-Magnusson, Hamalainen, Remaeus, Andersson, Borg, Gyzander and Deinum 2000).

Although there are other technologies available for determining affinity constants, they often rely on analyte labeling (e.g. radioactive or fluorescence-based direct binding assays) or require large sample amounts as in the case of isothermal titration calorimetry (ITC). Alongside with stopped-flow spectrophotometry, SPR offers the unique advantage of acquiring kinetic rate constants. As illustrated in Fig. 1C, an interaction may show the same affinity constant but completely different kinetic profiles. While fast association and dissociation kinetics might be advantageous for high-throughput screening processes, an enzyme inhibitor should ideally show rapid association and very slow dissociation rates. In addition, changes in the kinetic profile may also indicate structural alterations and differential exposition of binding sites. As a consequence, kinetic profiling becomes increasingly important both in biological sciences and drug discovery (Markgren, Schaal, Hamalainen, Karlen, Hallberg, Samuelsson and Danielson 2002; de Mol et al. 2005).

While a label-free detection principle offers many advantages to the researcher, there are also some limitations and pitfalls to consider. Since SPR biosensors detect any change of electron density around the gold surface of the sensor chip, it may be hardly possible to discriminate between specific and non-specific signals. Along with secondary binding sites on the target protein itself, impurities of both target and analyte molecules may lead to further heterogeneities. Careful sample preparation is therefore a prerequisite for reliable SPR results. Even more, conformational changes of the target or contributions from the hydrogel matrix (e.g. by electrostatic effects) may be visible and overlaid with the binding signal (Winzor 2003). The choice of an appropriate reference surface and a critical evaluation of the raw data usually help avoiding such artifacts. Since the SPR response is directly correlated to the mass of the analyte, screening of small molecules often features unfavorable signal-to-noise ratios. However, improvements in both instrumental sensitivity and data processing expanded the range to lower molecular weight (< 100 Da) significantly (Myszka 2004). While no labeling of the analyte molecule is required, the experimental setup usually requires the target protein to be covalently attached to the surface. A variety of coupling chemistries is available for this purpose (Johnsson et al. 1991), most of which lead to a random and heterogeneous orientation on the surface. Dependent on the number and location of attachment sites, this may largely affect the target activity and the signal quality (Catimel, Nerrie, Lee, Scott, Ritter, Welt, Old, Burgess and Nice 1997; Peluso, Wilson, Do, Tran, Venkatasubbaiah, Quincy, Heidecker, Poindexter, Tolani, Phelan, Witte, Jung, Wagner and Nock 2003). Capturing by antibodies, streptavidin, or metal affinity surfaces (e.g. Ni2+-NTA) is principally possible but often lacks surface stability or requires the expression of specific tags on the protein. Recently, a novel approach for the covalent yet oriented immobilization using a suicide enzyme reaction has been reported (Kindermann, George, Johnsson and Johnsson 2003), which might offer an alternative approach for coupling-sensitive proteins. The requirement of target attachment to a solid support raised some skepticism in the research community if SPR-derived binding data are comparable to solution-based methods such as ITC. Meanwhile, a series of comparative studies could clearly demonstrate that SPR profiles are not only highly reproducible but

indeed very comparable to data from ITC, stopped-flow spectrophotometry, or ultracentrifugation (Cannon, Papalia, Navratilova, Fisher, Roberts, Worthy, Stephen, Marchesini, Collins, Casper, Qiu, Satpaev, Liparoto, Rice, Gorshkova, Darling, Bennett, Sekar, Hommema, Liang, Day, Inman, Karlicek, Ullrich, Hodges, Chu, Sullivan, Simpson, Rafique, Luginbuhl, Westin, Bynum, Cachia, Li, Kao, Neurauter, Wong, Swanson and Myszka 2004; Katsamba, Navratilova, Calderon-Cacia, Fan, Thornton, Zhu, Bos, Forte, Friend, Laird-Offringa, Tavares, Whatley, Shi, Widom, Lindquist, Klakamp, Drake, Bohmann, Roell, Rose, Dorocke, Roth, Luginbuhl and Myszka 2006; Papalia, Leavitt, Bynum, Katsamba, Wilton, Qiu, Steukers, Wang, Bindu, Phogat, Giannetti, Ryan, Pudlak, Matusiewicz, Michelson, Nowakowski, Pham-Baginski, Brooks, Tieman, Bruce, Vaughn, Baksh, Cho, Wit, Smets, Vandersmissen, Michiels and Myszka 2006).

3 Applications in Complement Research

As a first line of human defense to pathogens and principal component of innate immunity, the complement system has to react dynamically and rapidly to changes in its environment. While the complement cascade is always kept on a low level of alertness (i.e. tickover), activation of the system leads to a subsequent amplification of the response. In order to minimize the stress for host cells and tissues, the complement system has to be able to differentiate between healthy and pathogenic structures and to down-regulate the response after eliminating the threat. This complex and complicated task is fulfilled by a tailored and carefully balanced network of various soluble and membrane-bound proteins, which react with each other, with pathogenic proteins, or with surface structures (Walport 2001). For example, the complement component C3 alone is known to interact with more than 25 different molecules, making it one the most multifunctional and versatile proteins described so far (Lambris, Sahu and Wetsel 1998). During the regulation of the complement cascade, large conformational changes (as e.g. in the conversion from C3 to C3b), competition for binding sites, affinity enhancement through cofactors, and the formation of multi-molecule complexes play a crucial role. A detailed knowledge of the individual interactions is therefore essential for the understanding of complement function and regulation. In addition, these findings may build the base for the development of therapeutic interventions for many diseases that are connected with an erroneous regulation of the complement system (e.g. systemic lupus erythematosus, age-related macular degeneration). SPR technology is considered an ideal tool for investigating and describing interactions between complement components, since it allows not only a qualitative but also quantitative description of binding events without labeling requirements and with comparatively low sample amounts.

Comparable to many other research areas, SPR experienced increasing importance in the characterization of the complement system in recent years. As of writing this review, nearly 100 studies using SPR for investigating binding of

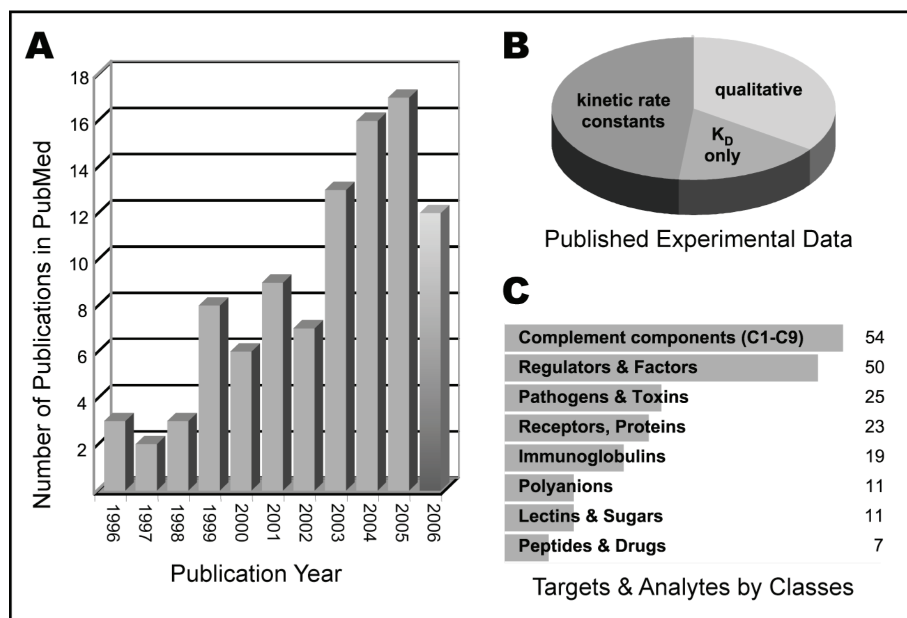


Fig. 2. Complement-related SPR studies in the PubMed database (as of November 2006) grouped by publication date (A), type of binding study (B), and by investigated classes of interacting molecules (C).

complement components are reported in the PubMed database. While first complement-related SPR studies appeared relatively late (more than 5 years after the introduction of the technique), a clear and steady increase in the number of publications could be observed in recent years (Fig. 2A). Almost half of the published studies took full advantage of the method and reported kinetic rate constants, while another 15% published affinity constants and a third of the publications only included qualitative descriptions of binding events (Fig. 2B). This ratio closely reflects the trend observed in the collectivity of SPR studies (Rich et al. 2005a). As expected, the majority of the investigated proteins belong the to the complement components C1 to C9 and their fragments (Fig. 2C), with a clear emphasis on component C3 (more than 40%). Regulators of complement activation (RCAs) and complement factors contribute to a similar amount followed by various pathogenic as well as intrinsic proteins and receptors. Immunoglobulins and fragments thereof were often used for capturing, blocking, or identifying other components. However, some publication also screened antibodies for therapeutic purposes (see chapter 2.4). Besides the classical investigation of protein-protein interactions, SPR had also been used for characterizing the binding of complement components to carbohydrates (i.e. mannose-binding lectin (MBL) and MASPs of the lectin pathway), to polyanionic structures (e.g. heparin) and surfaces, or to drugs and therapeutic peptides (e.g. compstatin). In the following paragraphs, we illustrate the usefulness of SPR

biosensors for answering crucial questions about the structure and function of proteins involved in the complement cascade.

3.1 Making connections – old and new ones

Obtaining biophysical profiles for individual interaction in the complement network has been the major aim for a large fraction of the published studies. While target immobilization is considered a major deviation from natural conditions for many biosensor experiments, the contrary is true in the case of C3 and its fragments. Upon activation of C3 to C3b, a previously buried thioester site is cleaved and exposed to the solvent. For a short period after activation, C3b can be covalently attached to polyhydroxyl surfaces, e.g. of bacterial cell walls, before the thioester site gets hydrolyzed. Coupled C3b is then able to form the C3 convertase and amplify the complement, finally leading to the formation of the membrane attack complex (MAC) and lysis of the cell. It has been shown that this mechanism can be mimicked on the sensor chip by in-situ formation of the convertase and that the carboxymethyl dextran matrix is accepted as a coupling surface for active C3b (Nilsson, Larsson, Hong, Elgue, Ekdahl, Sahu and Lambris 1998).

Given its central role in the regulation of complement activation, it is not surprising that factor H (fH) has been investigated in large detail. This soluble plasma glycoprotein is mainly responsible for the down-regulation of complement activity on host surfaces (Pangburn 2000). As a member of the RCA family, fH consists of 20 modular structure elements called short consensus repeats (SCR) (Kirkitadze and Barlow 2001). Various binding sites for C3b, C3d, heparin, or pathogenic proteins are distributed over these SCRs leading to a complex binding behavior. In a series of mainly qualitative studies, SPR assays were used for dissecting and localizing individual binding sites on fH. For this purpose, truncated forms of fH containing only a limited number of SCRs were expressed and screened against different C3 fragments, heparin, and other polyanions (Jokiranta, Hellwage, Koistinen, Zipfel and Meri 2000; Jokiranta, Westin, Nilsson, Nilsson, Hellwage, Lofas, Gordon, Ekdahl and Meri 2001; Hellwage, Jokiranta, Friese, Wolk, Kampen, Zipfel and Meri 2002; Jokiranta, Cheng, Seeberger, Jozsi, Heinen, Noris, Remuzzi, Ormsby, Gordon, Meri, Hellwage and Zipfel 2005; Cheng, Hellwage, Seeberger, Zipfel, Meri and Jokiranta 2006). Combining these data, it was possible to detect or confirm three binding sites for each C3b (SCR 1-4, 8-15, 19-20) and heparin (SCR 7, 8-15, 20) on fH. Recently, Bernet et al. further characterized the fH-C3b interaction by kinetic means and detected a complex binding mode with a K_D of 50 nM for the primary binding site (Bernet, Mullick, Panse, Parab and Sahu 2004).

Heparin is not only known to interact with fH but also with a variety of other complement proteins. This makes this sulfated polysaccharide a potent regulator of both the classical and alternative complement pathway itself. In one of the most comprehensive kinetic profiling of a single target, Yu et al. studied the binding of a plethora of proteins (C1-C9, C1 inhibitor, factors B, H, I, and properdin) to immobilized, biotinylated porcine heparin (Yu, Munoz, Edens and Linhardt 2005). All of the screened components showed affinity constants in the nanomolar range, from 2 nM for factor B up to 320 nM in case of C2. Interestingly, the dissociation rate

constant showed a nearly ten-times higher variability than the association rate. Since some of the investigated analytes showed a strong non-specific background signal on the streptavidin-carboxymethyl dextran chip, a novel sensor chip surface based on polyethylenglycol chains was developed (Munoz, Yu, Hallock, Edens and Linhardt 2005) and used for this study.

The close relationship between complement activation, inflammation, and wound healing makes a connection between the complement and the coagulation network not only possible but very likely. However, the mechanisms how these cascades might be linked to each other, are still poorly understood and just at the beginning of investigation (Markiewski, Nilsson, Nilsson-Ekdahl, Mollnes and Lambris 2007). Again, SPR studies have the potential to detect and describe important connections and bring more light into the pending questions. One of the most promising advances in this area was made by del Conde and coworkers (Del Conde, Cruz, Zhang, Lopez and Afshar-Kharghan 2005). By combining SPR with immunoblotting and flow cytometry, the authors could demonstrate an interaction between C3b and P-selectin, which is primarily expressed on platelets. In another study (Vaziri-Sani, Hellwage, Zipfel, Sjoholm, Iancu and Karpman 2005), factor H was found to interact with platelets via the GPIIb/IIIa receptor or thrombospondin, a glycoprotein known to induce platelet aggregation. SPR studies showed a direct interaction of fH with thrombospondin (K_D = 49 nM), which could be localized to the heparin binding area on SCR 20 by differential fH fragment binding and heparin-inhibition assays. These findings were connected by the authors to the pathogenesis of atypical hemolytic uremic syndrome (aHUS), where mutation in the C-terminal part of fH may lead to an insufficient down-regulation of the complement system on platelets. Finally, a series of studies used SPR to investigate the interaction between the C4-binding protein (C4BP) and the vitamin K-dependent anticoagulant protein S (He, Shen, Malmborg, Smith, Dahlback and Linse 1997; Evenas, Garcia De Frutos, Linse and Dahlback 1999; Giri, Linse, Garcia de Frutos, Yamazaki, Villoutreix and Dahlback 2002).

An involvement of the complement cascade in the pathogenesis of prion diseases, most likely through the classical pathway, has long been discussed. In a recent SPR-based study, Blanquet-Grossard et al. indeed reported a direct interaction between mouse prion protein (PrP) and human C1q (Blanquet-Grossard, Thielens, Vendrely, Jamin and Arlaud 2005). When PrP was covalently immobilized on the sensor chip, both full-length C1q and its isolated globular domain showed binding in the nanomolar range. However, the affinity of globular domain was reduced by more than a factor of 40, which could be almost exclusively attributed to a slower kinetic on rate. This effect was explained by an avidity effect, since intact C1q is a hexameric protein whereas the globular domain is monomeric. Interestingly, the binding affinity was essentially lost when soluble PrP was injected over immobilized C1q. While large differences upon reversal of the interaction partners usually indicate a problem in the assay format, they may indeed be attributed to changes in functionality in this case. By performing a series of ELISA the authors found evidence that soluble PrP undergoes significant conformational changes upon immobilization. Addition of Cu^{2+} ions (but not Mg^{2+}, Mn^{2+}, Zn^{2+}, or Ni^{2+}) led to a large increase in binding signal intensity, while the kinetic rate constants and the resulting affinity remained largely unchanged. These findings were consistent with a model that copper ions induce a

pathogenic conversion of PrP and that more ‘active’ molecules are available on the chip surface upon addition of Cu^{2+}. Based on these SPR data, the authors therefore concluded that C1q may represent a natural sensor of converted prion protein.

3.2 The good and evil side of complement: interaction with pathogens

A central task of the complement system is the elimination of pathogenic intruders from the human body, which it usually fulfills dutifully and efficiently. However, some of the pathogens have found ways to avoid or even mis-use the complement system for their own fate. Cell-bound complement components and receptors may serve as target structures to some microorganisms for entering human cells (Lindahl, Sjobring and Johnsson 2000; Walport 2001). For example, the Eppstein-Barr virus (EBV) uses an interaction between its envelope glycoprotein 350 (gp350) and the complement receptor 2 (CR2) for this purpose (Nemerow, Mold, Schwend, Tollefson and Cooper 1987; Tanner, Weis, Fearon, Whang and Kieff 1987). As a consequence, B cells, which predominantly express CR2, are the major target of this virus. Using kinetic SPR profiles, the binding behavior of gp350 could be elucidated and compared to the natural CR2 ligands iC3b and C3d (Sarrias, Franchini, Canziani, Argyropoulos, Moore, Sahu and Lambris 2001). In contrast to the C3 fragment, which showed complex binding kinetics and affinities in the µM range, EBV gp350 featured 100 to 1000 times stronger affinities and the interaction followed a simple 1:1 binding model indicating a single gp350 binding site on CR2. Both the association and dissociation rate constants were responsible for the remarkable increase in affinity. In a recent SPR study, interferon α (IFN-α) and the immunoregulatory protein CD23 were also identified as ligands for CR2 (Asokan, Hua, Young, Gould, Hannan, Kraus, Szakonyi, Grundy, Chen, Crow and Holers 2006). Since both the infection with EBV (Ioannou and Isenberg 2000) and a therapeutic administration of IFN-α (James, Kaufman, Farris, Taylor-Albert, Lehman and Harley 1997) have been discussed as triggers for the autoimmune disease systemic lupus erythematosus (SLE), these findings may contribute to a better understanding of the pathological mechanisms behind SLE.

Due to its ability to differentiate between pathogenic and host surfaces, factor H is a major target for many bacterial intruders. Since an enrichment of fH on the bacterial surface leads to the same down-regulation of the complement cascade as on host surfaces, some bacteria (e.g. streptococci) produce fH-binding surface proteins as a part of their complement evasion strategy (Rautemaa and Meri 1999). SPR assays served as an important tool to describe or verify interaction between factor H and many pathogenic surface proteins (Table 1). In the case of the streptococcal protein Hic, kinetic profiles for the fH-Hic interaction were already included in the initial description of the novel protein (Janulczyk, Iannelli, Sjoholm, Pozzi and Bjorck 2000). By using a panel of fH fragments, the Hic binding site could be localized within SCR

Table 1. Pathogenic surface proteins that were analyzed by SPR for their interaction with fH.

Protein	Organism	Reference
Bac (β protein)	Streptococcus agalactiae	(Jarva et al. 2004)
Fba	Streptococcus pyrogenes	(Pandiripally et al. 2003)
Hic	Streptococcus pneumoniae	(Janulczyk et al. 2000) (Jarva et al. 2002) (Jarva et al. 2004)
LfhA	Leptospira interrogans	(Verma et al. 2006)
OspE	Borrelia burgdorferi	(Hellwage et al. 2001) (Alitalo et al. 2004)
OspE	Borrelia granii	(Alitalo et al. 2005)
PspC	Streptococcus pneumoniae	(Dave et al. 2004)

8-11 (Jarva, Janulczyk, Hellwage, Zipfel, Bjorck and Meri 2002). Finally, it was demonstrated that Hic binds to more than one site in the middle part of fH (SCR 8-11, 12-14) and that its recognition is comparable to the structurally related streptococcal protein Bac (Jarva, Hellwage, Jokiranta, Lehtinen, Zipfel and Meri 2004). However, competition experiments with heparin suggested a slightly different binding mode since only Bac but not Hic binding was inhibited by heparin.

A viral strategy for evading an attack of the complement system is the production of RCA-like molecules (Mastellos, Morikis, Isaacs, Holland, Strey and Lambris 2003). Similar to the natural complement regulators, these proteins are also composed of SCR domains and are therefore able to act as cofactors or to interfere with complex formation. One of those proteins, the vaccinia virus complement control protein (VCP), has been extensively studied. While VCP was previously known to bind C3b, C4b, and heparin, SPR studies demonstrated that all four SCR domains are required for interaction with C3b/C4b, whereas heparin only bound to the C-terminal part of VCP (Smith, Sreenivasan, Krishnasamy, Judge, Murthy, Arjunwadkar, Pugh and Kotwal 2003). A more detailed SPR study by Bernet *et al.* allowed an even deeper insight in the molecular recognition pattern of VCP (Bernet et al. 2004). Despite some indications for multiple C3b/C4b binding sites in previous studies, the authors could demonstrate that VCP binding to these molecules follows a simple 1:1 binding mode with a nearly 5-fold stronger affinity to C4b. Competition experiments showed that both C3b and C4b interact within the same region of VCP. Furthermore, the VCP binding sites could be localized in the C4c and C3dg fragment, respectively. The study also illustrates the importance of the assay design. While an oriented immobilization of C3b/C4b on the sensor chip led to homogeneous signals following a simple 1:1 kinetic model, the reversed assay with randomly immobilized VCP resulted in significant heterogeneities and decreased affinity. Finally, the authors could demonstrate a large influence of the charge state by performing binding studies in buffers of increasing ionic strength. Latter was further investigated by Sfyroera *et al.*, who used hybrid mutants between VCP and the smallpox inhibitor of complement enzymes (SPICE) combined with electrostatic modeling (Sfyroera, Katragadda, Morikis, Isaacs and Lambris 2005). These two molecules differ by only 11 amino

acids but show a 1000-fold different regulatory activity. The study demonstrated that the activity is indeed largely influenced by modulating the electrostatic potential of the hybrid protein.

The majority of the available SPR studies rely on isolated surface proteins of the pathogens for the binding experiments. However, in a series of publications about the interaction of echoviruses with the decay accelerating factor (DAF), whole virus particles were immobilized on the flow cell (Lea, Powell, McKee, Evans, Brown, Stuart and van der Merwe 1998; Goodfellow, Evans, Blom, Kerrigan, Miners, Morgan and Spiller 2005; Pettigrew, Williams, Kerrigan, Evans, Lea and Bhella 2006). Generally, all tested echoviruses featured rapid kinetic rate constants and K_D values in the low micromolar range. All steady state data sets could be fitted to a single-site binding model.

3.3 The complexity of complex formation

The ability to observe molecular interactions in real-time makes SPR technology an ideal tool for studying the formation of multi-molecular complexes. Typically, one central component is immobilized on the sensor chip, while the additional components are injected sequentially or as a mixture. Changes in signal intensity or dissociation half-times provide information about the formation and stability. By varying buffer conditions or adding cofactors, additional details about the essential requirements or driving forces of the complex formation can be obtained.

The C3 convertase is unquestionably the most important complex in the complement cascade. Upon hydrolysis of a buried thioester group in the native C3 molecule, the protein undergoes a large conformational change to C3(H_2O). During this process, binding sites for factor B are exposed, which leads to a C3(H_2O)B complex. The serine protease factor D, which is able to cleave complex-bound but not soluble factor B in its Ba and Bb fragments, leads to the formation of the initial convertase C3(H_2O)Bb and activation of a serine protease site on Bb. This complex cleaves native C3 (184 kDa) into the anaphylatoxin C3a (9 kDa) and C3b (175 kDa). The majority of C3b is covalently bound to polyanionic surfaces (e.g. bacterial cell walls) and forms the final C3 convertase (C3bBb) using factors B and D. The generation of additional C3b by the C3 convertase leads to the amplification of the response (amplification loop). The formation of the alternative pathway convertase on a SPR sensor chip was initially described by Nilson and coworkers for investigating the influence of the complement inhibitor compstatin (Nilsson et al. 1998). The authors immobilized purified C3b covalently on a carboxymethyl dextran sensor chip and sequentially injected factor B and D in the presence of Ni^{2+}. When native C3 was injected over the chip-bound complex, a signal increase caused by the deposition of newly formed C3b on the sensor chip via its thioester moiety could be observed (Fig. 3A). As proposed, no C3b deposition occurred when the C3 inhibitor compstatin was added. The in-situ convertase formation for the oriented immobilization of C3b was further developed and compared with standard amine coupling (Jokiranta et al. 2001). While probably closest to physiological conditions, this in-situ method is rather time

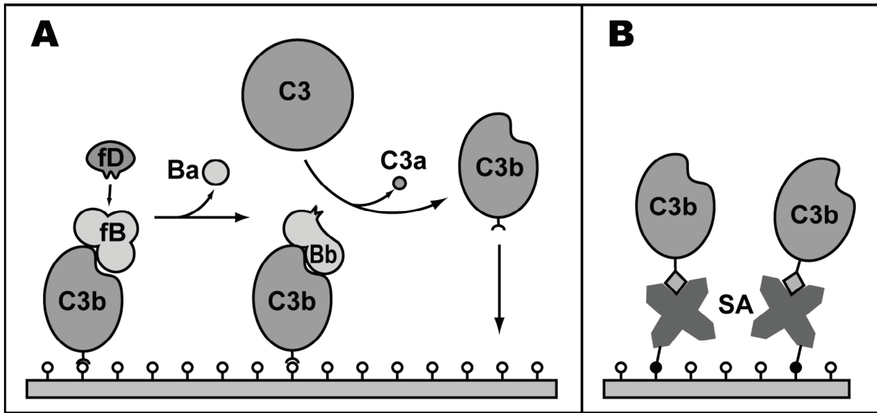


Fig. 3. Oriented immobilization of C3b via its thioester moiety by on-chip assembly of the C3 convertase (A) or by in vitro biotinylation and coupling to a streptavidin (SA)-coated sensor chip (B).

and protein consuming. Therefore, an alternative approach has been suggested by Sarrias et al., in which the thioester domain of C3 is selectively biotinylated after activation and coupled to streptavidin-coated sensor chips (Sarrias et al. 2001). Biotinylated C3 samples can be further cleaved, e.g. to biotinylated C3b, stored, and freshly immobilized in an oriented manner (Fig. 3B).

The ability of SPR to monitor the assembly and stability of complexes in real-time was further used to study the influence of different regulators on the C3 convertase formation with an emphasis on either DAF (Harris, Abbott, Smith, Morgan and Lea 2005) or properdin (Hourcade 2006). Using immobilized C3b, it could be shown that the stability of the convertase complex (C3bBb) is significantly larger than the proenzyme complex (C3bB) in the presence of Mg^{2+} (Harris et al. 2005). Interestingly, only C3bB but not C3bBb was sensitive to removal of magnesium by EDTA indicating that Mg^{2+} is only important in the initial convertase formation step. On the other hand, only the fully assembled convertase was destabilized by injection of soluble DAF. The authors also demonstrated that DAF interacts with both C3b and factor B (via its vWF-A domain) in the micromolar affinity range. Kinetic analysis of the interaction between C3b and factor B indicated a complex binding mode, which might include a conformational change. In contrast to de-stabilizing proteins like DAF or factor H, properdin is the only known physiological promoter of the C3 convertase (Schwaeble and Reid 1999). In a recent publication (Hourcade 2006), Hourcade found evidence that properdin not only stabilizes pre-formed convertase but also promotes its formation. The author suggested that the multimeric properdin molecule is able to form a lattice, where multiple stages of the convertase (C3b, C3bB, C3bBb) are bound and stabilized. It was even found that iC3b, which was considered as deactivated C3b, may still contribute to convertase formation due to its affinity to properdin. A similar on-chip assembly approach has also been performed to investigate the formation of the membrane attack complex (Thai and Ogata 2005).

3.4 Developing therapeutic interventions

Despite the frequent use of SPR technology in drug discovery and development, only a very limited number of applications in the field of complement therapeutics is reported so far. Almost all of them involve macromolecules such as peptides and monoclonal antibodies. Only a single publication presents SPR data involving a small molecular therapeutic, the antibiotic drug chloramphenicol, as an inhibitor of the interaction between the E. coli adhesin DraE and decay accelerating factor (DAF) (Pettigrew, Anderson, Billington, Cota, Simpson, Urvil, Rabuzin, Roversi, Nowicki, du Merle, Le Bouguenec, Matthews and Lea 2004).

A whole series of independent publications deals with the neutralization of the anaphylatoxins C3a and C5a. Those small protein fragments are released from C3 and C5, respectively, upon complement activation and are known to induce various inflammatory and anaphylactic reactions. Specific blockage of these anaphylatoxins is therefore believed to be of substantial therapeutic significance. In one study, a panel of specific and non-specific IgG antibodies was screened on immobilized C3a and C5a (Basta, Van Goor, Luccioli, Billings, Vortmeyer, Baranyi, Szebeni, Alving, Carroll, Berkower, Stojilkovic and Metcalfe 2003). Surprisingly, all of the non-specific control antibodies also showed significant binding towards the anaphylatoxin. Since no interaction was detected with isolated Fc fragments and the specific antigens did not compete with C3a/C5a, the binding site was attributed to the constant region of the Fab fragment. Fung *et al.* described and characterized a C5a-neutralizing antibody, which detects both free C5a as well as the C5a region within C5 with subnanomolar affinity (Fung, Lu, Fure, Sun, Sun, Shi, Dou, Su, Swanson and Mollnes 2003). A different approach was taken by Fujita et al., who designed C5a-complementary peptides by using a molecular modeling (Fujita, Farkas, Campbell, Baranyi, Okada and Okada 2004). One of these peptides showed a strong binding towards immobilized C5a.

One of the most potent and best characterized complement inhibitors so far is the cyclic tridecapeptide compstatin. While the initial discovery through screening of phage display libraries was performed by ELISA (Sahu, Kay and Lambris 1996), SPR data were involved in most stages of later development. For example, Nilsson could demonstrate that the on-chip formation of the C3 convertase (see also chapters 2.1 and 2.3, Fig. 3A) was successfully inhibited by compstatin (Nilsson et al. 1998). In a later study, detailed kinetic profiles for the interaction with C3, C3(H_2O) as well as their fragments C3b, C3c, and C3d have been evaluated (Sahu, Soulika, Morikis, Spruce, Moore and Lambris 2000). While binding to C3c could not be reliably detected by ELISA, SPR analysis showed a clear but much weaker interaction compared to native C3 (9 µM vs. 130 nM). Since compstatin did not bind to C3d at all, the authors concluded a limited exposition of the binding site upon cleavage of C3. Qualitative binding studies further revealed specificity towards component C3, with no binding to C4 and C5, as well as species specificity for primate C3 (human and baboon), while no binding was detected for mouse and rat C3 (Sahu, Morikis and Lambris 2003). Finally, a combination of SPR with various other assays was used for the characterization of different compstatin analogs (Soulika, Morikis, Sarrias, Roy, Spruce, Sahu and Lambris 2003).

4 Conclusions & Perspectives

Despite the comparatively small number of complement-related SPR publications, they cover an impressive range of applications; from qualitative screening up to detailed kinetic profiles, from small molecules and peptides up to multimolecular complexes and virus particles, and from picomolar up to millimolar affinity ranges. Besides their key application of measuring binding constants, SPR instruments are extensively used in complement research for the localization of binding sites. The reason for this may be due to the modular structure of many complement regulators (e.g. RCA) and the distinct fragmentation pattern of many complement components (e.g. C3). One of the major advantage of SPR technology for these applications is the ability to get a fairly detailed pictures of changes in binding behavior upon cleavage or mutation, even with single injections and limited sample amounts. Another advantage is that the same assay can be easily repeated under different experimental conditions (e.g. buffer composition, pH, temperature). Since many of the interactions within the complement cascade are driven by electrostatic interactions, SPR assays were often used for detecting different binding pattern in buffers of increasing ionicity. However, the large involvement of charge effects may also lead to method-related artifact when non-specific binding to the negatively charged carboxymethyl dextran matrix are involved. Careful assay design, control experiments and cross-validation with other methods are therefore highly important. The same is true for the coupling of the target molecule, which might significantly influence the homogeneity and affinity of the interaction. Fortunately, some of the complement components can be immobilized in a near-natural way with high activity.

With the recent publications of several crystal structures of complement proteins, such as C3 (Janssen, Huizinga, Raaijmakers, Roos, Daha, Nilsson-Ekdahl, Nilsson and Gros 2005), C3b (Abdul Ajees, Gunasekaran, Volanakis, Narayana, Kotwal and Krishna Murthy 2006; Janssen, Christodoulidou, McCarthy, Lambris and Gros 2006; Wiesmann, Katschke, Yin, Helmy, Steffek, Fairbrother, McCallum, Embuscado, Deforge, Hass and van Lookeren Campagne 2006), C3c (Janssen et al. 2005), C3d (Nagar, Jones, Diefenbach, Isenman and Rini 1998), or factor B and its fragment Bb (Ponnuraj, Xu, Macon, Moore, Volanakis and Narayana 2004), the mapping and characterization of interaction sites becomes even more exciting. SPR may play a major role in linking these static images to dynamic functions. While powerful as a technology by itself, combinations of SPR with other interaction technologies such as ITC may further potentiate its value. Increasing knowledge about the complement network and the availability of novel crystal structure are also believed to bring new fuel to the development of complement therapeutics, which may be another key area of SPR.

The almost unique ability to obtain kinetic profiles is a clear advantage of SPR. Especially when comparing similar analytes, differences in the kinetic on- and off-rates may e.g. indicate differential accessibility of a binding site or the formation of secondary contacts. However, even though nearly half of all complement-related SPR studies contained kinetic data, very few of them interpreted or connected the results in respect of structural or functional properties. Here again, the availability of crystal

structures and the combination with complementary methods may help tapping the full potential of SPR technology.

The recent challenges in elucidating the structure and connections of the complement network seem to be a perfect match to the capabilities of SPR biosensors, and we are strongly looking forward to a continuous increase of related articles in the future.

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