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Real-time label-free detection of complement activation products in human serum by white light reflectance spectroscopy

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ABSTRACT

We present a label-free, real-time sensor based on white light reflectance spectroscopy for quantitating the complement activation product C3b and its metabolites as a biomarker in human serum. Our novel sensor allows real-time monitoring of biomolecular reactions (in this case, antigen–antibody reactions) taking place on a reflective surface within a flow cell. Detection was based on monitoring the increase in film thickness caused by its immunoreaction with a specific antibody; this reaction was seen as a shift in the wavelength at which constructive interference was observed. Quantitation of C3b was achieved by immobilizing a specific mouse monoclonal antibody onto the refractive surface and monitoring the rate of the signal changes occurring during the first 60 s of the immunoreaction between the antibody and known concentrations of purified C3b or dilutions of complement-activated human serum. The lowest detectable concentration of purified C3b was 20 ng/mL, and complement activation products in human serum samples could be detected at dilutions as high as 6000-fold. The advantages of the method include its relatively low cost, short analysis time, and high assay sensitivity and reliability. Thus, this novel assay method can be used to monitor serum C3b produced as a result of complement activation in a variety of normal and pathologic conditions.

1. Introduction

The monitoring of biomolecular interactions is essential for many bioanalytical applications, including diagnostics, drug screening, environmental monitoring, and affinity sensing. The approach most widely employed for monitoring such interactions is to label one interactant with radioisotopes, fluorescent or chemiluminescent substances, or enzymes. Although the use of labels usually leads to an enhancement in detection sensitivity, there are several disadvantages to such approaches. In particular, the presence of the label or the labeling process itself can alter the binding affinity by masking binding sites, causing steric hindrance, or changing the conformation in the binding area. Therefore, it is highly desirable to develop label-free techniques that will enable the monitoring of biomolecular interactions without compromising detection sensitivity.

To address this need, a broad range of label-free technologies has been developed and used to monitor protein–protein, protein–DNA, antigen–antibody, and ligand–receptor interactions. These sensing techniques have thus far been based on surface plasmon resonance (SPR), ellipsometry, reflectometric interference spectroscopy (RIfS), grating couplers, or interferometric devices such as Mach–Zehnder interferometers (Gauglitz and Pröll, 2008). Compared to label-based techniques, these detection approaches offer the additional advantage of monitoring interactions in real time, thus providing information about the binding kinetics, reducing the analysis time, and minimizing sample requirements. SPR is the most widely used of the aforementioned approaches and is capable of highly sensitive detection. However, it is not the method of choice for applications such as diagnostics or environmental monitoring that require a substantial number of analyses to be performed on a daily basis because of its high instrument and analysis-associated costs. Although much effort has been devoted to the development of miniaturized SPR-based systems (Kim et al., 2007; Lee et al., 2007), these systems have not yet achieved the sensitivity levels of established SPR instruments.

RIfS is an alternative label-free detection approach that offers advantages in terms of simplicity of instrumentation and potential for miniaturization. In RIfS, detection involves monitoring the

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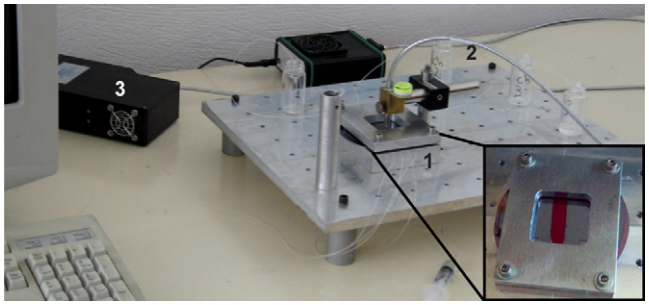

Fig. 1. Experimental setup of the sensor: (1) reaction cell, (2) optical fiber, and (3) spectrophotometer. The insert shows close-up picture of the measurement unit with a red-colored solution introduced in the flow cell. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

changes in the reflectance pattern of a white light beam on a thin transparent film that occur as a result of changes in optical thickness caused by interfacial molecular reactions (Hänel and Gauglitz, 2002; Lü et al., 2003; Spaeth et al., 1997). Recently, we described a methodology for monitoring biomolecular interactions that is based on white light reflectance spectroscopy (Zavali et al., 2006). Although our methodology is similar in principle to the RIfS technology, our approach differs with regard to the way in which the light probe is coupled to the sample and with regard to the thin film structure employed.

In our apparatus (Fig. 1), the incident light is directed through a glass window onto the substrate surface on which the biomolecular reactions take place. The substrate is composed of a highly reflective Si wafer surface, on top of which has been deposited a thick silicon dioxide layer, which acts as an interference spacer. A bifurcated optical fiber is used to deliver incident light to the surface and to collect the reflected light. The shift in the main interference peak to higher wavelengths that occur as a result of the reaction-mediated increase in the biomolecular layer thickness is monitored in real time. The presence of the silicon oxide layer on top of the Si substrate increases the number of interference fringes in the recorded spectrum by increasing the optical path of the light. Thereby, the fitting accuracy of the experimental spectrum with the reflectance equation, having the protein film thickness as the fitting parameter, is improved.

In this report, we document the application of our real-time, label-free detection method to quantitatively monitoring the production of an important biomarker, i.e., complement fragment C3b, in human serum. C3b is generated during activation of the complement cascade, which leads to the elimination of foreign cells by opsonization, direct lysis, pro-inflammatory signaling, and activation of immune cells. The enzymatic cleavage of complement component C3 into its active fragments, C3a and C3b, plays a central role in the initiation and amplification of the complement response. While the anaphylatoxin C3a is a potent immunomodulator, C3b becomes covalently attached to cell-surface antigens, leading to the opsonization of foreign cells and, finally, to stimulation of the adaptive immune response (Carroll, 2004; Sahu and Lambris, 2001). After activation, only 10% of C3b becomes deposited on surfaces (Law and Dodds, 1997), while 90% of the protein remains in circulation and is readily degraded to the inactive fragments iC3b and C3c.

In addition to being an essential part of immune response, C3 cleavage has been observed in several physiologic (Mastellos and Lambris, 2002) as well as pathologic conditions, including inflammatory, ischemic, and autoimmune diseases (Markiewski and Lambris, 2007; Ricklin and Lambris, 2007a). In the case of autoimmune diseases, monitoring complement activation via C3 cleavage is considered a valuable diagnostic approach for estimating the status of the disease and preventing acute events. In addition, there is considerable evidence that inhibiting complement activation, and particularly the cleavage of C3, can reduce or even prevent the occurrence of such events (Elliott et al., 2004; Holers and Thurman, 2004; Thurman et al., 2005). Diagnostic and therapeutic approaches related to complement activation are dependent on highly specific antibodies that can discriminate between the native C3 molecule and its active fragments (Aguado et al., 1985; Alsenz et al., 1990; DiLillo et al., 2006; Mastellos et al., 2004; Ricklin and Lambris, 2007a). Antibody C3-9 is of particular interest in this context, since it recognizes a neo-epitope that is generated during the large conformational rearrangements upon conversion of C3 into C3b (Nishida et al., 2006). This epitope remains exposed in the subsequent degradation products iC3b and C3c, which largely increases the detection probability. In the present study, we have utilized mAb C3-9 in our label-free assay based on white light reflectance spectroscopy, which allowed us to quantitatively assess C3b levels in human serum in real time.

2. Experimental

2.1. Reagents

A mouse monoclonal antibody (C3-9) specific for activation products of C3 (i.e., C3b, iC3b, and C3c) was developed and kindly provided by Dr. Eric Hack (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service). C3 was purified from human serum as previously described (Hammer et al., 1981). C3b was produced by limited proteolysis of purified C3 with 1% (w/w) trypsin for 2 min at room temperature, followed by gel filtration (Sephadex G-100) in 20 mM phosphate–buffered saline, pH 7.4. Bovine serum albumin (BSA) and zymosan A were purchased from Sigma (St. Louis, MO). All other reagents were from Merck (Darmstadt, Germany). Four-inch silicon wafers were from Wacker Chemie (München, Germany). An approximately 1000-nm-thick dioxide film was created on top of the wafers by wet oxidation at 1100 °C for 2 h in the clean room facility of the Microelectronic Institute of NCSR "Demokritos". The surface was further processed to allow the adsorption of a thin film (35–40 nm) of polymeric material appropriate for biomolecule immobilization. For this purpose, AZ-5214 photoresist (Clariant, Charlotte, NC) was applied by spin-coating and baked at 180 °C for 60 min to create a surface with high protein binding capacity (Petrou et al., 2007).

2.2. Plasma and serum samples

Plasma from healthy human individuals was obtained by collection of blood into EDTA-coated tubes, following by centrifugation at 3500 rpm for 20 min. Serum was isolated from the same individuals by blood collection into glass tubes. The blood was kept for 60 min in an ice bath to allow clotting and then centrifuged at 3500 rpm for 20 min to recover the serum. Plasma and serum samples were stored in aliquots at −80 °C until used. Complement activation in serum and plasma was achieved by adding 1 mg/mL zymosan A and incubating the mixture for 30 min at 37 °C. Zymosan was removed by centrifugation, and the activated serum/plasma was used after appropriate dilution in 50 mM phosphate buffer, pH 7.4, containing 10 mg/mL BSA (assay buffer).

2.3. Instrumentation

The experimental setup used in this study is shown in Fig. 1. The optical portion of the device consisted of a VIS-NIR light source (Avantes BV, The Netherlands) and a splitter optical fiber that

divided the light beam in two equal parts, one of which was delivered to the slave channel of a double spectrophotometer (USB SD2000, Ocean Optics; Dunedin, FL), and the other was directed to a bifurcated optical fiber. The outer part of the optical fiber guided the light vertically to the surface on which the biomolecular reactions took place, while the reflected light was collected by the central part of the bifurcated optical fiber and guided to the master channel of the spectrophotometer. The measurement cell (Fig. 1; insert) is formed by combining the polymer-covered wafer surface with an open fluid compartment made of polymerized polydimethylsiloxane (PDMS) that incorporated embedded tubing for the fluid inlet and outlet. The fluid cell was closed by a flat glass plate, and the whole structure was secured in place by two aluminum holders. The cell volume was approximately 0.1 mL. A precision microsyringe pump was used to deliver fluids to the flow cell at a constant rate. All measurements were performed at room temperature using software developed in LabView (National Instruments Corp., Austin, TX). The reflectance and reference signals were monitored at each wavelength, and the two spectra were recorded every second. As the biomolecules bound to the surface, the effective thickness of the film increased, and a change in the interference spectrum was recorded that was correlated with the concentration of the bound protein.

2.4. Immobilization of anti-C3b antibody

A stable baseline value was achieved by introducing 50 mM phosphate buffer, pH 7.4 (washing buffer) into the flow cell at a constant rate of 30 μL/min. A 10 μg/mL solution of mAb C3-9 in washing buffer was injected, and the antibody adsorption onto the surface was monitored in real time. The antibody solution was left in the flow cell overnight to achieve stable binding to the surface. The antibody solution was replaced by washing buffer and the free protein-binding sites on the surface of the coated wafer were blocked by incubation with assay buffer. As soon as a steady state was achieved, the surface was washed to remove loosely bound protein. The coated and blocked reaction cell was either used immediately or kept filled with washing buffer at 4 °C until use.

2.5. Label-free determination of C3b/iC3b/C3c in buffer and serum

Purified C3b solutions and serum dilutions were prepared in assay buffer. Before the introduction of antigen solutions, the reaction cell was extensively washed with assay buffer to obtain a stable baseline. In case of complement-activated serum, a negative control was run before the introduction of the serum sample. This control consisted of a comparable dilution of plasma for which complement activation had been inhibited with EDTA. After completion of the immunoreaction, the flow cell was washed with assay and washing buffer, and the binding activity of the immobilized antibody was regenerated by a short wash (1–2 min) with 0.1 M glycine–HCl buffer, pH 2.5. A new baseline was obtained after extensive flushing with washing buffer. Kinetic evaluation of the initial binding rate was used for determining the detection range of the protein in buffer and serum. For this purpose, the signal increase within the first 60 s of injection was fitted by linear regression and plotted against the C3b concentration or serum dilution, respectively. In case of serum samples, the value of a corresponding dilution of non-activated plasma was subtracted from the serum slope in order to correct for non-specific binding.

2.6. Label-free detection of C3 activation products by SPR

C3 cleavage products in complement-activated human serum were also measured using an SPR-based biosensor (Biacore X; Biacore Inc., Piscataway, NJ) as described in Supplementary Methods.

3. Results and discussion

The binding of the protein to the surface was monitored by observing how the spectrum of the reflected light changed during interfacial reactions. Despite the fact that the whole spectrum should generally be considered in this type of assay, it was necessary to avoid unwanted effects on the reflected spectrum as a result of variations in the light source spectrum. Therefore, we used a rather short spectral segment to perform the final fitting between the experimental spectrum and the equation that correlates the spectral changes with the effective layer thickness. The spectral segment used was limited to the area around the main constructive interference peak, since this approach has been found to provide to highest signal-to-noise ratio (Zavali et al., 2006). The main interference peak for the 1000-nm thick silicon oxide layer used in the study was in the range of 610–670 nm. The analytical performance of the system was first evaluated using solutions of purified C3b fragments, followed by human serum samples appropriately processed for complement activation.

3.1. C3b calibration curve

C3 is the most abundant complement protein in human plasma (~1.3 mg/mL), and its cleavage into C3a and C3b is a nodal point for complement activation. A successful immunochemical determination of complement activation is dependent on the use of antibodies that are highly specific for activation products. The monoclonal antibody used in this study, mAb C3-9, is specific for C3b and its degradation products (i.e., iC3b and C3c) but does not recognize native C3 (Becherer et al., 1992; Hack et al., 1988). Its immobilization onto a reflective surface was accomplished through physical adsorption (Fig. 2A). Although plateau values were obtained after 2–3 h, overnight incubation was preferred because the resulting antibody surfaces were found more stable during repetitive regeneration cycles. After blocking with BSA (assay buffer), a solution of purified C3b was allowed to flow through the reaction cell, and the sensor response was monitored in real time (Fig. 2B). The shifts observed in the constructive interference maximum after running purified C3b (0.01–2 μg/mL) for 15 min through the reaction cell ranged from 0.1 to 1.7 Å. However, we found that the analysis time was significantly reduced by employing kinetic instead of end-point measurements. Therefore, we calculated the initial reaction rate for the first minute of the interaction. This approach yielded a linear relationship for the entire range of C3b concentrations, with a detection limit of 20 ng/mL (Fig. 2C).

C3b concentrations in the human serum of patients with autoimmune diseases are significantly elevated when compared to those in serum from healthy individuals, with activation levels often reaching some 2–12% (corresponding ~25–150 μg/mL C3b) (Tamerius et al., 1985). The detection limit achieved for our immunosensor is therefore highly adequate for covering the expected serum concentrations of C3b in such applications. However, given its many constituents and the complexity of serum as a fluid matrix, this biological fluid can greatly affect the performance of the assay. Therefore, an appropriate dilution should be defined in order to minimize sample consumption and to avoid any non-specific effects of serum components on the systems' performance.

3.2. Detection of C3b fragments in human serum

For evaluating the specificity of our system in human serum, we treated serum from healthy individuals with zymosan A from *Saccharomyces cerevisiae* to induce complement activation and generate C3 cleavage products (Fearon and Austen, 1977). In order to determine the effect of serum matrix on the sensor response, unprocessed plasma was used as a negative control. Both the spe-

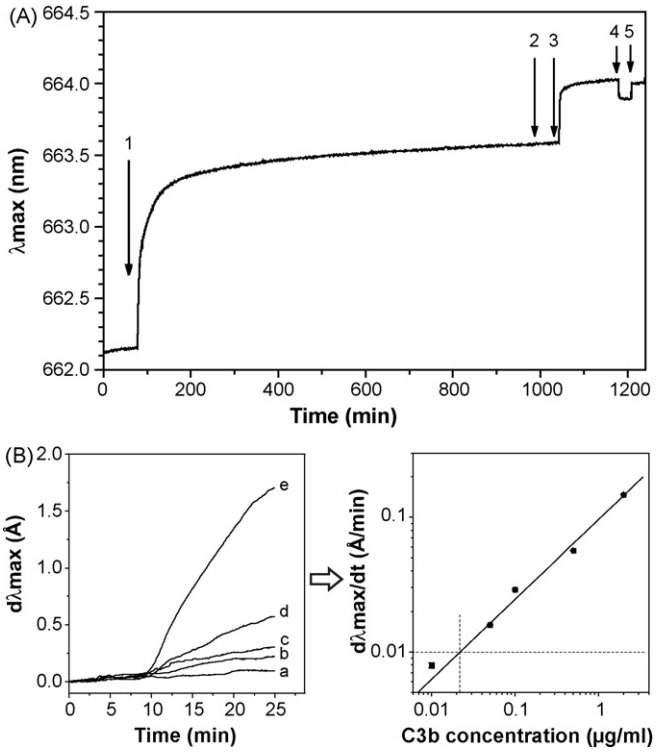

Fig. 2. (A) Monitoring of antibody immobilization and subsequent blocking on the sensor surface. The solutions allowed to flow through the fluid cell were: (start) 50 mM phosphate buffer, pH 7.4 (washing solution); (1) anti-C3b antibody (mAb C3-9) solution in washing solution; (2) washing solution; (3) 50 mM phosphate buffer, pH 7.4 containing 10 mg/L BSA (assay buffer); (4) washing solution; (5) assay buffer. (B) Real-time signals for purified C3b on immobilized mAb C3-9 (left). The C3b concentrations tested were: 0.01 μg/mL (a), 0.05 μg/mL (b), 0.1 μg/mL (c), 0.5 μg/mL (d), and 2 μg/mL (e) in assay buffer. The signal slope during the initial 60 s was plotted against the C3b concentration (right). Each point is the mean value of three measurements; error bars represent ±1SD. The dashed lines indicate the limit of detection value (20 ng/mL), which was defined as the concentration corresponding to +3SD of 10 zero analyte concentration replicates.

cific and non-specific sensor responses increased as the serum dilution was decreased from 1:3000 to 1:100 (Fig. 3A; left). However, even at the highest serum concentration, there was a distinct difference between the two signals. In order to determine whether the observed signal was a result of the plasma matrix itself or to partial complement activation during blood collection, we coated a reaction cell with a control antibody (goat anti-mouse IgG) and tested the sensor with both activated serum and plasma dilutions. The responses on channels coated with control antibody were almost identical for the activated serum and plasma samples (Supp. Fig. 1). Furthermore, it also corresponded to the response seen in the specific antibody-coated channel when the corresponding plasma dilution was added. These results indicate that the observed plasma dilution signals are not generated by either specific or non-specific binding of plasma components to mAb C3-9 but may have rather resulted either from the direct adsorption of serum/plasma components onto the reflective surface or the absorption or scattering of the incident or reflected light by serum/plasma components.

As was true for purified C3b, the level of C3b/iC3b/C3c in serum could be determined by kinetic evaluation of the initial 60 s of binding. For each serum dilution, the value for the respective negative control (plasma) was subtracted from the value for the serum dilution (Fig. 3A; right). The detection limit was defined as the highest serum dilution that provided a specific signal that was statistically different ($p < 0.001$) from the non-specific signal, taking into account the variation in the baseline slope. Thus, a serum dilution of 1:6000 was determined to be the detection limit of the system. This high sensitivity would allow detecting and reliably measuring low levels of complement activation.

3.3. Comparison to SPR

Not only is SPR an established method for monitoring of biomolecular interactions and the extraction of kinetic rate constants, but it can also be used to measure analyte concentrations in complex mixtures (Jason-Moller et al., 2006). SPR-based approaches have also been widely used for characterizing interactions within the complement system, including those involving activation products of C3 (Ricklin and Lambris, 2007b). We therefore validated our newly developed method by comparing it to the results obtained with an SPR-based instrument. By using standard immobilization methods, we achieved a surface density of 10,000 resonance units for mAb C3-9 (i.e., ~10 ng per flow cell). The detection limit for purified C3b was determined as 5 ng/mL (Supp. Fig. 2). Non-specific binding with diluted plasma samples was suppressed by subtracting the signals of an unreacted flow cell and by increasing the Tween-20 concentration and adding dextran sulfate to the running buffer.

By using the same mAb, a comparable buffer system and a similar dilution range of zymosan-activated plasma, the SPR instrument detected activated C3 fragments in plasma samples diluted as much as 1:12,800 (Fig. 3B; left). When the initial slope of the association phase was plotted, the plasma dilution series showed a linear correlation between 1:100 and 1:12,800 (Fig. 3B; right). In comparison with our newly developed sensor, both the sensitivity and the correlation of the kinetic reaction rate of the SPR instrument were found to be very similar (Fig. 3). This good agreement clearly validates our method and demonstrates the high sensitivity of both instruments for the detection of C3b/iC3b/C3c in human samples.

SPR-based instruments offer advantages in terms of high sensitivity, reduced analysis time, and low sample consumption. However, the cost of the instrument and individual analyses is still prohibitive for routine use in clinical diagnostics. In comparison, the sensor developed here is a rather simple and inexpensive instrument, yet its sensitivity for C3 activation products was found to be similar to that of the SPR assay. Another advantage of our new system over SPR methods is the ease of immobilization: Whereas coupling onto SPR chips requires covalent bonding of the reagent itself or of a capture molecule the surface used for biomolecule immobilization in our system can be adapted for immobilization either by covalent binding (e.g., via aminosilanization) or by physical adsorption. This second option further reduces the cost by eliminating the need for coupling reagents while facilitating the use of the sensor.

3.4. Stability of the sensor response

The stability of the sensor response was assessed by using a number of different substrates as reflective/adsorbing surfaces and also by using the same substrate in combination with a four-channel fluid cell that permitted the sequential determination of four immunoreactions on the same substrate. The variation in the responses obtained for 10 individual surfaces tested on 10 consecutive days with various dilutions of the same serum sample was 7.4%, whereas the variation obtained for the same substrate on a single day was about 4.8%. The low variability among the different substrates was not surprising, since the software developed for signal processing included the determination of the main interference peak at the beginning of each recording in order to achieve a higher signal-to-noise ratio for each measurement.

The stability of the immobilized antibody on a single sensor was assessed after multiple assay/regeneration cycles (Fig. 4A) on the same day or on different days. The antibody response was very

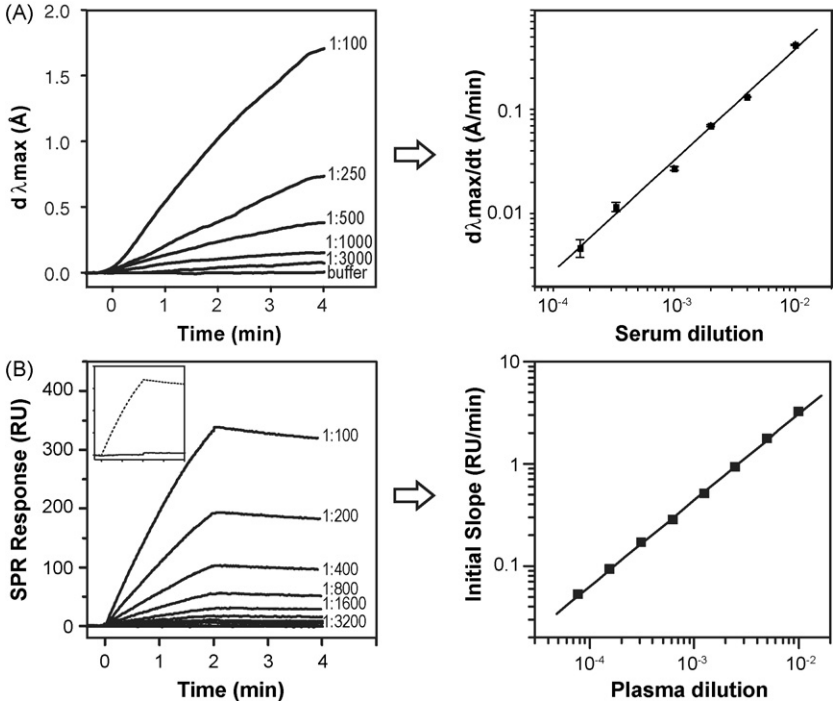

Fig. 3. (A) Signal versus dilution of zymosan-activated human serum (left). The dilutions tested were 1:3000, 1:1000, 1:500, 1:250, and 1:100 in assay buffer. The signals from the corresponding dilutions of non-activated plasma have been subtracted. The baseline obtained by running the assay buffer is also provided. Initial reaction rate versus dilution of activated serum (right). Each point is the mean value of three measurements; error bars represent ±1SD. The linear regression equation is $y = 38.30x - 0.01$; $r = 0.991$; $p < 0.0001$. (B) The same monoclonal anti-C3b antibody used in the sensor assays was immobilized on a SPR chip, and a 2-fold dilution series (1:100–1:12,800) of zymosan-activated plasma was injected for 2 min. All dilutions of activated plasma showed concentration-dependent binding signals (left). In contrast to zymosan-activated plasma (dashed line), the same dilution (1:100) of EDTA-inhibited plasma (solid line) only showed a negligible response (inset). The initial reaction rate was plotted against the dilution factor and showed a linear correlation for plasma dilutions from 1:100 to 1:12,800 ($r = 0.998$; right).

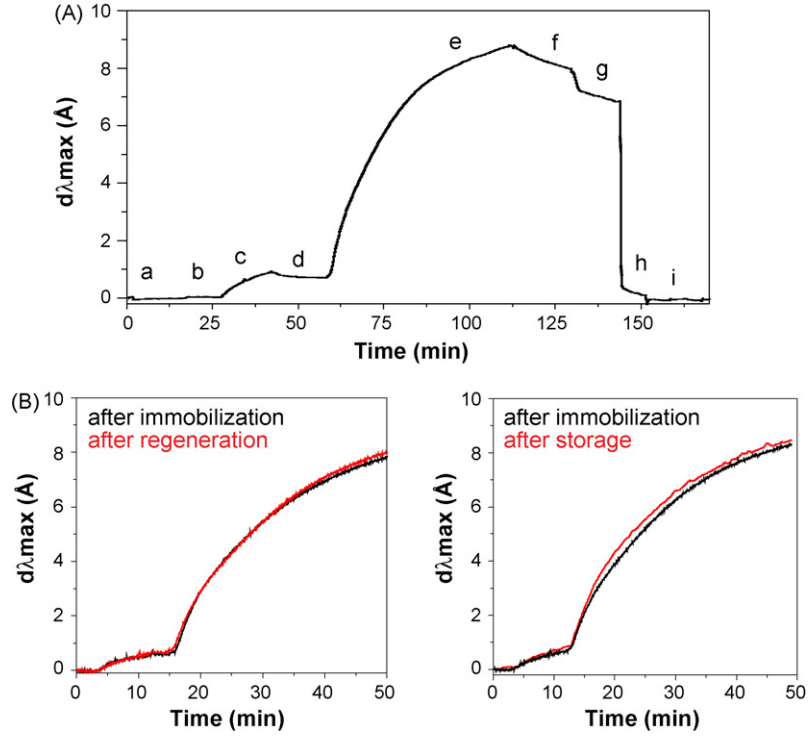

Fig. 4. (A) Regeneration of immobilized antibody. Real-time signal evolution during washing with 50 mM phosphate buffer, pH 7.4 (a), assay buffer (b), plasma diluted 100-fold with assay buffer (c), washing with assay buffer (d), addition of activated serum diluted 100-fold with assay buffer (e), washing with assay buffer (f), washing with 50 mM phosphate buffer, pH 7.4 (g), regeneration buffer (h), and washing with 50 mM phosphate buffer, pH 7.4 (i). (B) Stability of the immobilized mAb C3-9. Real-time signals obtained for activated serum (1:100 dilution) immediately after antibody immobilization (black lines), after three sequential regeneration cycles (red line; left), and after one regeneration cycle and storage of the immobilized antibody for 2 weeks at 4 °C (red line; right). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

stable during repetitive assay/regeneration cycles on a single day (Fig. 4B; left), as well as when the regenerated antibody-coated surface was used after storage for 2 weeks at 4 °C (Fig. 4B; right). The stability of the immobilized antibody during the regeneration process is advantageous for the calibration of the system during use or after storage for an extended period of time. This stability contributes to the system's reliability in terms of future diagnostic applications, since it allows for single-point calibration.

Another important characteristic of the proposed system, and advantage over SPR-based instruments, is that it does not require strict temperature control. In case of refractometric detection methods such as SPR, the measured changes in refractive index are highly dependent on the temperature. In contrast, reflectometric instruments measure optical thickness and are rather insensitive to variations in temperature (Gauglitz and Pröll, 2008). The negligible effect of daily temperature variation on the sensor response was clearly indicated by the stability of the maximum plateau signal during the overnight coating with mAb C3-9 (Fig. 2A). Instantaneous deviations of the response as result of the occasional occurrence of air bubbles could be suppressed by averaging the signal, e.g., every 10 s, without affecting the final output signal.

4. Conclusions

We have established a method for real-time, label-free determination of C3b and its degradation products by white light reflectance spectroscopy as a means of monitoring complement activation in human serum and have evaluated this method in terms of its analytical sensitivity and reliability. This newly developed sensor makes it possible to perform kinetic measurements, therefore significantly reducing the analysis time without decreasing the detection sensitivity. The sensor showed high sensitivity towards C3b/iC3b/C3c in human serum, which covered a significant range of clinically observed complement activation levels and was highly comparable to an SPR-based assay. In this respect, the sensor we have developed is an important step towards a sensitive, flexible and cost-effective immunodiagnostic system for both clinical and research applications.

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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.bios.2009.04.040.

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