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Diagnosis of Epstein–Barr virus infection in clinical serum samples by an SPR biosensor assay

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ABSTRACT

Label-free affinity biosensors offer a promising platform for the development of a new generation of medical diagnostic technologies. Nevertheless, when such sensors are used in complex biological media, adsorption of non-targeted medium components prevents the specific detection of the analyte. In this work, we introduce for the first time a biosensor assay based on surface plasmon resonance (SPR) capable of diagnosing different stages of Epstein–Barr virus (EBV) infections in clinical serum samples. This was achieved by simultaneous detection of the antibodies against three different antigens present in the virus.

To prevent the interference of the fouling from serum during the measurement, the SPR chips were coated by an antifouling layer of a polymer brush of poly[oligo(ethylene glycol) methacrylate] grown by surface-initiated atom transfer radical polymerization. The bioreceptors were then attached via hybridization of complementary oligonucleotides. This allowed the sensor surface to be regenerated after measurement by disrupting the complementary pairs above the oligonucleotides' melting temperature and attaching new bioreceptors. In this way, the same sensing surface could be used repeatedly. The procedure used in this work will serve as a prototype strategy for the development of label-free affinity biosensors for diagnostics in blood serum or plasma samples. This is the first example of detection of marker of a disease in clinical serum samples by an optical affinity biosensor.

1. Introduction

Label-free affinity biosensors, such as those based on surface plasmon resonance (SPR), quartz crystal microbalances, and Mach–Zehnder interferometers, offer the potential for the rapid detection of multiple biomarkers for diseases in clinical practice, which is expected to lead to a dramatic improvement in the quality of diagnostics and healthcare (Lechuga, 2012). SPR platforms in particular emerge as a promising biosensor technology as they allow the fast, direct quantification of analytes, even supporting automation of the measurement (Homola, 2008). These sensors have been the subject of intense research and remarkable progress has been made in their design achieving extremely low limits of detection of model analytes in simulated simple media and cheaper, more portable models (Fernández et al., 2010; Homola, 2006; Kvasnička et al., 2012; Slavík and Homola, 2007). Furthermore, sensor designs based on optical fibers that offer the potential for *in situ* and even *in vivo* measurements have been introduced (Jeong et al., 2013; Voisin et al., 2014; Slavík et al., 2002; Yanase et al., 2010). In addition further advances in photonics and signal processing have allowed the introduction of multiplex detection enabling the detection of a very large number of analytes at the same time (Homola, 2008).

However, in spite of their much acclaimed limits of detection in the order of pg mL^{-1}, so far this type of sensors has not been translated to clinical diagnostics or food safety. Several reports have presented the detection of disease markers and food pathogens, yet they have only dealt with simulated samples of analytes in buffer or samples that were spiked with the target analytes at concentrations that may not be biologically relevant (Besselink et al., 2004; Cao et al., 2006; Chung et al., 2005; Pimková et al., 2012). Nevertheless, label-free affinity biosensors cannot differentiate between the specific signal caused by analyte binding and the interfering signal due to the non-specific adsorption of components present in complex biological media on the sensing surface, known as fouling. It has prevented the advance of this promising technology into practical applications (Blaszykowski et al., 2012; Homola, 2008; Rodriguez-Emmenegger et al., 2011a, 2011b).

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In fact, the signal caused by fouling can be orders of magnitude higher than the specific signal from the analyte (Homola et al., 2002). In order to maximize the ratio between the specific biosensor response and the response to fouling, it is essential to immobilize the bioreceptors on a surface that can effectively prevent fouling (Blaszykowski et al., 2012; Rodriguez-Emmenegger et al., 2011b). Such surfaces are called "antifouling" and for clinical applications suppression of fouling from blood plasma and serum are of particular importance. Various surfaces resistant to non-specific protein adsorption from model plasma protein solutions have been prepared (Rodriguez-Emmenegger et al., 2009; Yu et al., 2011; Zamfir et al., 2013). Self-assembling monolayers (SAM) of alkanethiols have been used as antifouling layers capable of supporting bioreceptors but they have been restricted to the detection of analytes in model buffer solutions or diluted media spiked with high concentrations of analyte (Fernández et al., 2010; Rodriguez-Emmenegger et al., 2011a). However, only polymer brushes, such as those based on oligo(ethylene glycol) methacrylate, hydroxyethyl methacrylate, carboxybetaines, and *N*-(2-hydroxypropyl) methacrylamide, have been able to efficiently reduce or even completely suppress the fouling from blood plasma and serum (Kostina et al., 2012; Pop-Georgievski et al., 2013; Riedel et al., 2013; Rodriguez-Emmenegger et al., 2013, 2012b, 2011b, 2009). Nevertheless, the antifouling properties of the surfaces can be impaired by the bioreceptor immobilization procedure (Vaisocherová et al., 2013). In some model experiments, the response obtained on a detection surface to pooled plasma was subtracted from the response of the surface to plasma spiked with the studied analyte (Gao et al., 2010; Vaisocherová et al., 2007). Significant differences in the fouling from the plasmas collected from different donors or pooled plasma obtained from different commercial sources (Rodriguez-Emmenegger et al., 2009, de los Santos Pereira et al., 2014) preclude the application of such approach for diagnosing real clinical samples which contain an unknown analyte concentration and produce an unknown fouling. In order to achieve a biosensor suitable for diagnosis of clinical samples, the combination of an adequate antifouling layer, bioreceptor attachment procedure and assay protocol has to be optimized.

The detection of markers of an infection by the Epstein–Barr virus (EBV), found in highly fouling blood serum, by means of a biosensor presents a particular challenge. Vaisocherová et al. (2007) introduced a biosensor capable quantifying very high concentrations of anti-EBV antibodies (much higher than biologically relevant) spiked in buffer or diluted blood serum, but applications to diagnosis of clinical samples remain elusive. This disease is of considerable clinical importance, as it is one of the most common viruses which attack the immune system of humans. EBV is present in all populations and infects over 90% of individuals within the first decades of life. Clinical symptoms differ according to the immune status of the patients. Manifestations of a primary infection vary from asymptomatic to various diseases such as infectious mononucleosis and lymphoproliferative diseases. EBV follows a productive lytic infection and establishes a latent infection in the host (Rickinson and Kieff, 2001). EBV has been linked to some kinds of oncogenic diseases including Burkitt's lymphoma, nasopharyngeal carcinoma and some cases of Hodgkin's lymphoma. It may also be associated with rheumatoid arthritis, systemic lupus erythematosus, Sjogren's syndrome and multiple sclerosis (Ascherio and Munger, 2010; Thorley-Lawson and Gross, 2004; Toussirot and Roudier, 2008).

In the course of the infection different anti-EBV antibodies are produced. The specific EBV serological profile distinguishes between three possible diagnoses: acute infection, past infection, and no infection. These diagnoses are based on the presence of antibodies against viral capside antigen (anti-VCA), Epstein–Barr nuclear antigen (anti-EBNA), and early antigen (anti-EA). Currently, typical tests for EBV infections include: heterophile antibody test, indirect fluorescent antibody assay (IFA), Western blot, enzyme linked immunosorbent assay (ELISA) and PCR analysis. All of these multi-step methods require highly trained personnel, expensive reagents, take from several hours to a few days to yield results. Furthermore, these indirect methods provide mostly qualitative results, as they are based on subsequent steps that cannot be monitored individually (Hess, 2004; Odumade et al., 2011). Since 95% of adults have been infected with EBV, most adults will show antibodies to EBV from infection years earlier. Symptoms of acute infectious mononucleosis such as fever, sore throat, and swollen lymph glands, are similar to those of various other diseases. The multiplex testing for antibodies to several EBV-associated antigens can distinguish promptly the acute EBV infection from the infection in the past or from other diseases. Similarly, a fast and direct simultaneous detection of various biomarkers is highly necessary for the unambiguous diagnosis of many other diseases.

In this work, an SPR biosensor capable of real time recognition of the different stages of an Epstein–Barr virus (EBV) infection in blood serum samples was developed for the first time. A low serum fouling and high level of capture of targeted antibodies specific for the EBV infection were obtained by a multi-step technique for the immobilization of the respective antigens onto antifouling polymer brushes of hydroxy-functional poly[oligo (ethylene glycol) methacrylate]. The results of biosensor analysis corresponded with serological studies by ELISA. This work presents the first example of direct detection of disease biomarkers in non-spiked clinical samples with an optical affinity biosensor and paves the way to the practical application of affinity label-free biosensors for medical diagnostics. Furthermore, it can be envisioned that the combination of the technology developed in this work with patterning techniques and multiplexing detection will lead to sensors capable of the simultaneous detection of a large number of analytes in complex biological media. Moreover, the application of this technique to fiber optics based SPR sensors holds the potential for *in vivo* operation capabilities.

2. Materials and methods

2.1. Materials

The set of biorecognition elements used (BRE) included streptavidin (STA) (Thermo Scientific, Czech Republic) and four oligonucleotides conjugated with biotin (ON-1: 5′-CTACTGAAATACA CAGAA-3′; ON-2: 5'-GTGTAGATCTTAACGAGT-3′; ON-3: 5′-GGTAC-TATACTATGGTCTC-3′; ON-4: 5′-TTCTGTGTATTTCAGTAG-3′) and their complementary oligonucleotides (Generi Biotech) covalently attached to bovine serum albumin conjugated with Epstein–Barr nuclear antigen (EBNA-1, M_w=27 kDa) or viral capside antigen (VCA, M_w=23 kDa) antigens and complementary oligonucleotide conjugated (Generi Biotech) with early antigen (EA, M_w= 44 kDa) antigen (CON-1-VCA, CON-2-EA, CON-3-EBNA-1), stored in 50% glycerol. The conjugates were prepared at VIDIA s.r.o. The functionality of the conjugates was tested by ELISA tests using anti-VCA, anti-EBNA and anti-EA positive and negative sera. Copper (II) bromide (99.999%), copper (I) chloride (99.995%), *N*,*N*′-disuccinimidyl carbonate (DSC), triethylamine, 4-(dimethylamino)pyridine (DMAP), *N*,*N*-dimethylformamide biotech grade (DMF, 99.9%), oligo(ethylene glycol) methacrylate (HOEGMA, average $M_n \sim 526$ g mol^{-1}), 2,2′-bipyridine (99%), and human serum albumin (HSA, 99% by electrophoresis) were acquired from Sigma-Aldrich. The polymerization initiator (ω-mercaptoundecyl bromoisobutyrate) was synthesized according to a method published earlier (Rodriguez-Emmenegger et al., 2009). The buffers used were: phosphate buffered saline (PBS, 10 mM disodium hydrogen

phosphate, 2 mM potassium phosphate, 137 mM sodium chloride, 2.7 mM potassium chloride, pH 7.4); phosphate buffered saline containing 1% BSA solution (PBS_{BSA}); Borate buffer (BB, 10 mM sodium borate, 10 mM boric acid, pH 8.5); Tris buffer (TB, 10 mM Tris–HCl, 150 mM sodium chloride, 1 mM EDTA, pH 7.2).

Serum samples were obtained from healthy individuals and from patients with clinically suspected infection. The sera were tested for presence of anti-EBV antibodies with CE certified ELISA kits from Vidia s.r.o.

2.2. Preparation of antifouling surfaces

Polymer brushes of HOEGMA (poly(HOEGMA)) were prepared on the surfaces of SPR sensor chips by surface-initiated atom transfer radical polymerization (ATRP) as previously reported (Rodriguez-Emmenegger et al., 2012a, 2012b). Briefly, a self-assembled monolayer of ATRP initiator was firstly prepared by immersing freshly ozone-cleaned (Jelight) gold-coated chips in a 1 mM solution of ω-mercaptoundecyl bromoisobutyrate at room temperature for 24 h. The chips were rinsed with ethanol and water, dried with nitrogen and transferred to glass reactor under Ar atmosphere. To a degassed solution of $CuBr_2$ (8.1 mg, 36.4 μM), 2,2′-dipyridyl (145 mg, 930 μM) and oligo(ethylene glycol) methacrylate (HOEGMA) (10 g, 19 mM) in 10 mL of water, CuCl (37 mg, 375 μM) was added under Ar protection. The polymerization mixture was transferred to the reactor containing the initiator-coated substrate. The polymerization was carried out at 30 °C to obtain 30-nm-thick polymer brushes (refer to the Supporting information for details on the characterization of the surface). Data on the control and kinetics of the polymerization can be found elsewhere (Rodriguez-Emmenegger et al., 2011a).

2.3. SPR spectroscopy

Surface plasmon resonance (SPR) was conducted using an instrument based on the Kretschmann geometry and spectral interrogation developed at the Institute of Photonics and Electronic of the Academy of Sciences of the Czech Republic. Broadband light from a halogen lamp (Mikropack) was collimated, polarized, and coupled with the SPR sensing element using a prism. The SPR chip consisted of a glass plate coated with an adhesion titanium film (thickness 2 nm) and a gold film (thickness 50 nm). Incident light beam excited surface plasmons at a wavelength about 750 nm at gold surface located in four independent chambers of a flow cell pressed on the SPR chip. The light reflected from the four spots was collected into four optical fibers and coupled to an Ocean Optics spectrophotometer. The acquired spectra were analyzed in real time and the resonant wavelength was determined for each sensing channel. The refractive index resolution was 3×10^{-7} RIU. A low volume (1 μL) flow-cell with four separate chambers facing each of the sensing spots was used.

The tested solutions were driven by a peristaltic pump with a flow rate of 5 μL min^{-1} through four independent channels (volume 1 μL) of a flow-cell in which changes in the SPR resonant wavelength, λ_{res}, induced by changes in the mass immobilized or adsorbed at the sensor surface and/or by changes in refractive index of the adjacent medium were measured simultaneously (Homola, 2008). The flow cell was equipped with a temperature controller enabling to access temperatures in the range of 5–50 °C with a stability of 0.01 °C (Rodriguez-Emmenegger et al., 2012b; Vaisocherová et al., 2008). The immobilization and detection experiments were performed in the flow cell at 25 °C while regeneration was carried out at 45 °C. The times of individual experiments observed *in situ* by SPR are specified below.

2.4. Attachment of biorecognition elements

The sensing platform was prepared by immobilization of STA directly to the brushes which was subsequently exploited for immobilization of biotin-functionalized oligonucleotides. Firstly, the hydroxyl groups of the poly(HOEGMA) brushes were activated with a solution of DSC (153 mg, 0.3 mM) and DMAP (72 mg, 0.6 mM) in anhydrous DMF at room temperature for 24 h.

Freshly activated samples were quickly rinsed with DMF and water, dried with nitrogen and immediately plugged to the SPR flow cell. The cell was filled with PBS and biorecognition elements (BRE) were attached step-by-step by exchanging the solutions pumped over the sensor surface. The process was monitored *in situ* by measuring λ_{res}. Each step was started by the replacement of the buffer with a BRE solution. After λ_{res} reached a steady state value, the BRE solution was replaced with the original buffer, followed by the buffer used in the next step. Streptavidin (STA) was covalently attached from a 50 μg mL^{-1} solution in PBS, by forming carbamate bonds between its amino groups and the activated brush. After washing with PBS, residual succinimidyl carbonate active groups were deactivated by BB for one hour. After washing with PBS and TB, biotinylated oligonucleotides ON-1, ON-2, ON-3, or ON-4 (0.4 μM in TB) were attached via biotin–streptavidin interaction. In the last step, CON-1-VCA, CON-2-EA, CON-3-EBNA-1, or CON-4 were attached from 4 μg mL^{-1} solutions in TB via hybridization of their oligonucleotide parts with the immobilized ON-1, ON-2, ON-3, or ON-4, respectively. The multistep procedure resulted in the formation of detection surfaces, termed poly (HOEGMA)-STA-ON-#-CON-#-antigen.

2.5. Detection of anti-EBV antibodies

Blood serum in PBS or in PBS_{BSA} buffer was flowed through the channels of the SPR biosensor to determine the presence of anti-VCA, anti-EA and anti-EBNA-1 antibodies. The associated diagnosis of an EBV infection was based on the presence of different antibodies against the EBV antigens. The presence of anti-VCA antibodies could indicate both a primary or a past infection. Anti-EA antibodies are usually found in the presence of anti-VCA antibodies and they help to indicate a primary infection. The presence of anti-EBNA-1 IgG, both in presence or absence of the previous antibodies, is a good marker of a past infection. The absence of all antibodies indicates no infection (Hess, 2004; Klutts et al., 2009). The specific biosensor “reference-compensated” response to the capture of targeted antibodies from a serum sample was obtained by subtracting the response in a reference channel where no antigens were present (poly(HOEGMA)-STA-ON-#) from the response in the measuring channel. The mass of irreversibly adsorbed proteins or antibodies (anti-EBNA-1, anti-VCA or anti-EA) captured by the immobilized antigens was evaluated after pumping the serum for 30 min and subsequent washing of the surface with PBS or PBS_{BSA} until stable baseline, λ_{res}, was achieved. An incubation time of 30 min was selected as a reasonable standard for real biosensor applications.

In order to minimize the non-specific interaction of the clinical serum samples and to optimize the ratio between specific and non-specific responses, the sensor response for each antigen was measured as a function of the concentration of a positive serum (0.05 to 10%). Additionally, the dilution was performed with PBS or with PBS containing 1% BSA.

The regeneration experiments were carried out directly after a sensing routine in the SPR. After detection of the biomarkers, the temperature in the flow-cell was risen over the melting point (45 °C) of oligonucleotides for 30 min in PBS buffer. The increase in temperature disturbs the hydrogen bonds holding the two oligonucleotide strands which unzips, regenerating the pristine sensor.

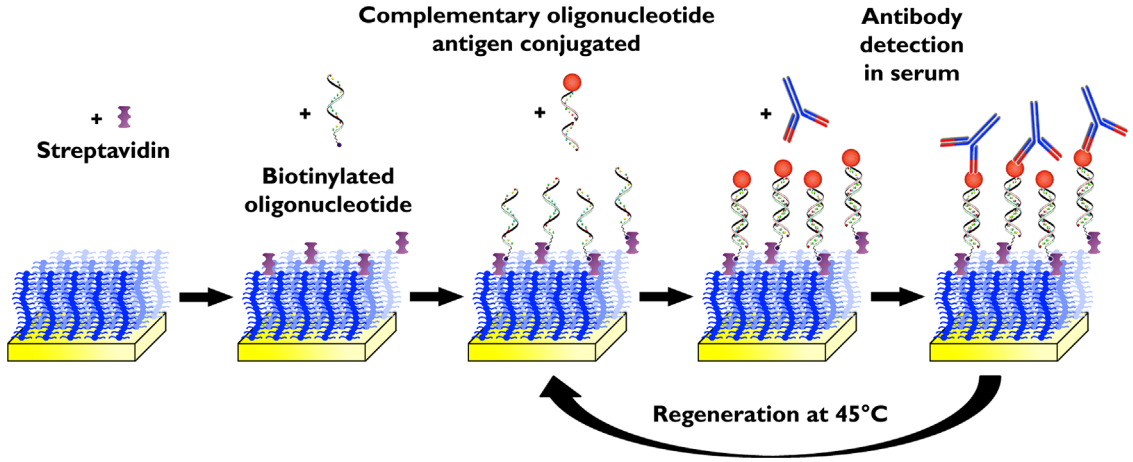

Fig. 1. Scheme showing the preparation of the biosensor for detection of the EBV infection in clinical samples.

Subsequently, temperature was left to stabilize at 25 °C and fresh CON-#-antigen was injected over the surface. Finally, blood serum was injected and the detection was carried out as explained above. The regeneration was repeated 2 times to evidence the robustness of the system. All measurements have been carried out at least in triplicate.

3. Results

3.1. Biosensor preparation

The multistep procedure resulting in the formation of a biosensor capable of real time detection of anti-EBV antibodies is shown in Fig. 1. The mass of streptavidin attached to poly (HOEGMA) via activated hydroxyl groups resulted in a shift in the resonant wavelength, λ_{res}, of about 8 nm which corresponds to *ca.* 1200 pg mm^{-2} (Rodriguez-Emmenegger et al., 2009). The shifts associated with the attachment of biotinylated oligonucleotides to streptavidin, $\Delta\lambda_{res,}$ were about 1 nm. The attachment of antigen conjugates resulted in $\Delta\lambda_{res}$ of about 2 nm for CON-1-VCA and CON-3-EBNA-1 and 1 nm for CON-2-EA hybridized with immobilized ON-1, ON-3, and ON-2, respectively. On the other hand when a non-complementary CON-1-VCA was contacted with an ON-4 surface, no immobilization was observed (dotted curve in Fig. 2). This clearly shows the high specificity of oligonucleotides hybridization and its potential for high throughput sensors. The sharp increase and decrease of the λ_{res} are due to the differences in the refractive indices of the TB and the CON-# solution. The other curves in Fig. 2 reflect both responses to the difference in the refractive index of the bulk solutions and the attachment of the antigen conjugated via hybridization.

3.2. Optimization of the biosensor response

The poly(HOEGMA) brushes were able to reduce the fouling from undiluted blood serum by more than 90% with respect to bare gold (see Supporting information). Fig. 3 shows the reference-compensated biosensor responses to sera S-1 and S-5 diluted with PBS_{BSA} and the ratios between the specific response (difference between detection and reference channels) and fouling (reference channel alone) response for S-5. It can be seen that the specific responses increase with increasing serum concentrations, however, the ratio between the specific response and fouling decreases (Fig. 3 inset, dotted curve). A noticeable saturation in the specific response at higher serum concentrations was probably due to the saturation of the accessible immobilized antigens with targeted

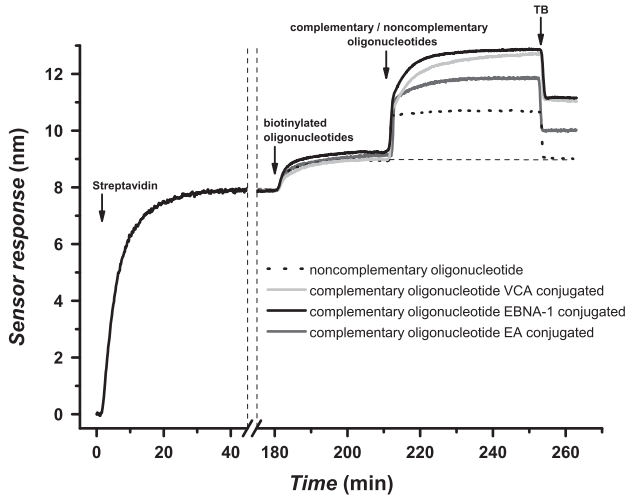

Fig. 2. SPR sensogram of the preparation of the biosensor: immobilization of streptavidin from PBS, pH 7.4, on activated poly(HOEGMA) brush, deactivation of the brush in BB, pH 8.5 for 2 h, attachment of biotinylated oligonucleotides, ON-1, ON-2, ON-3, ON-4, from TB, the attachment of CON-1-VCA (light grey), CON-2-EA (grey), CON-3-EBNA-1 (black) from TB via hybridization with immobilized ON-1, ON-2, ON-3, respectively. There was no attachment of CON-1-VCA to the surface with immobilized ON-4 (dotted curve).

antibodies. A serum concentration of 1% was selected as the optimum for the detection experiments. Responses to this concentration were sufficiently below the saturation while they were far above the measurement noise level ($\Delta\lambda_{res}$~0.005 nm). A significant decrease in the fouling was achieved when the sera were diluted with 1% BSA in PBS (PBS_{BSA}) instead of only PBS. The responses to fouling from 1% and 5% serum S-5 solutions prepared by dilution with PBS_{BSA} were 0.3 nm and 1 nm respectively, while the response to fouling from S-5 diluted to the same concentrations with PBS was 0.7 nm and 2.75 nm.

3.3. Detection of anti-EBNA-1, anti-VCA, and anti-EA antibodies in clinical samples

The biosensor was used for the analysis of 7 human clinical serum samples collected from patients with different diagnoses of EBV infection. The samples were diluted with PBS_{BSA} to obtain 1% serum solutions. The presence of anti-EBNA-1, anti-VCA, or anti-EA antibodies in a tested serum sample was evaluated as negative, weakly positive, positive, and strongly positive if the reference-compensated responses on surfaces with the corresponding

immobilized antigens were below 0.1 nm, 0.1–1.0 nm, 1.0–3 nm, and above 3 nm, respectively (Table 1). Three times the standard deviation of the blank (negative sera) was used as the cut-off between positive and negative samples. The positive samples were arbitrarily divided into the three grades similar to those used for evaluation of ELISA tests to yield semi-quantitative results. This approach is usually implemented by each particular analytical laboratory to estimate the progress of the disease with time from repeated testing of the patient. Fig. 4 shows a representative biosensor response to serum S-3. The clear positive response for anti-VCA and anti-EA antibodies together with the absence of anti-EBNA-1 markers indicates that the donor of serum S-3 was in the primary phase of the infection with EBV. The same measurements were performed on all samples. Table 1 shows the results obtained for all the sera tested and the associated diagnosis (Hess, 2004). The results are in agreement with serological studies by ELISA conducted by an independent laboratory.

Regeneration of the original poly(HOEGMA)-STA-ON-1 coating was done by incubating the sensor in PBS at 45 °C for 30 min and by the attachment of a new CON-1-VCA (Fig. 5). The regeneration did not affect the sensor response.

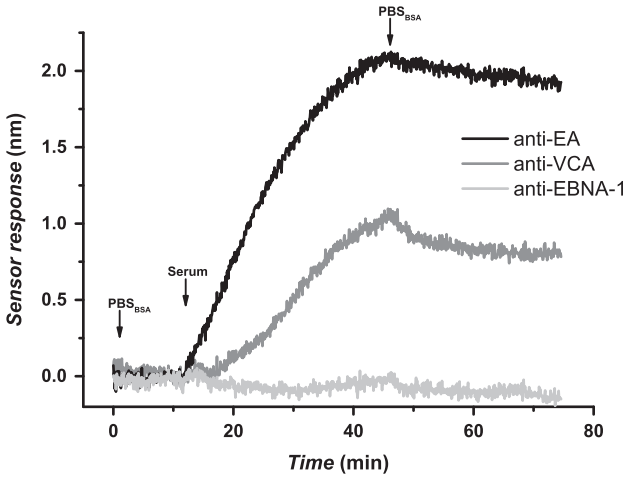

Fig. 4. Reference-compensated responses to serum S-3. Black, grey, and light grey curves show the reference-compensated responses of surfaces functionalized with EA, VCA and EBNA-1 antigens to S-3 (black, grey, and light grey curve, respectively). The positive signal for anti-EA and anti-VCA antibodies and the negative signal for anti-EBNA-1 antibodies indicate that the patient was in the primary phase of the infection.

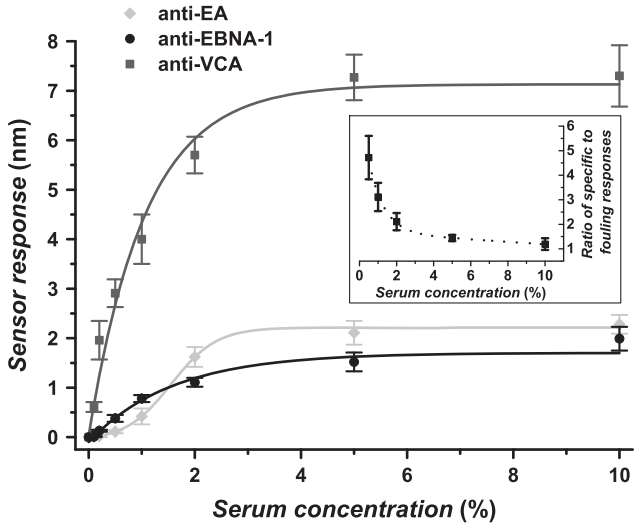

Fig. 3. The effect of serum dilution with PBS$_{BSA}$. Reference-compensated responses of biosensors with immobilized CON-1-VCA (grey curve), CON-2-EA (light grey curve), and CON-3-EBNA-1 (black curve) to sera S-1, S-1, and S-5, respectively. The inset shows the ratio of the specific response to the fouling from S-5 measured in the reference channel. Each point represents a mean value obtained from three independent measurements and the error bars represent the standard deviation.

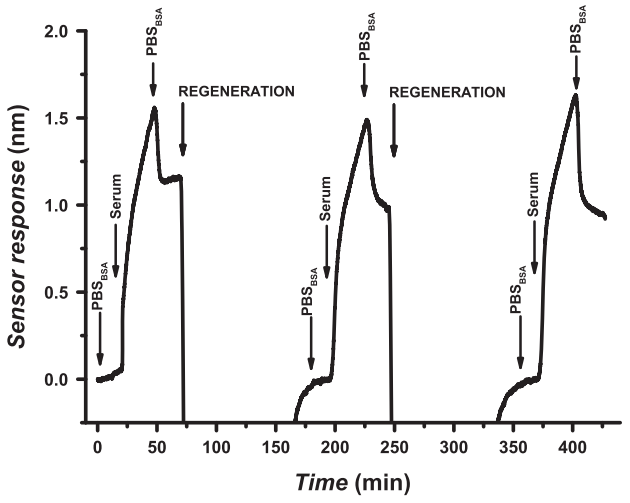

Fig. 5. Measurement of the responses to anti-VCA-positive serum S-1 with a repeatedly regenerated biosensor (Not reference-compensated). After each measurement, the original poly(HOEGMA)-STA-ON-1 coating was recovered by incubating the sensor in PBS at 45 °C for 30 min and the biosensing surface was regenerated by the attachment of a new bioreceptor CON-1-VCA.

Table 1
Assignment of sensor response to the presence of antibody in the tested serum and diagnosis of EBV infection in clinical samples using the developed SPR biosensor according to Klutts et al. (2009).

Sensor response (nm)	Characteristic	Symbol
0–0.09	Negative	–
0.1–1.0	Weakly positive	+
1.0–2.99	Positive	++
≥3	Strongly positive	+++

Serum sample	Anti-EBNA-1	Anti-VCA	Anti-EA	Presumed phase of infection
S-1	++	+++	+	Past infection
S-2	–	++	–	Primary infection/past infection
S-3	–	++	++	Primary infection
S-4	++	+	–	Past infection
S-5	+	+	–	Past infection
S-6	–	–	–	No infection
S-7	–	–	–	No infection

4. Discussion

4.1. Biosensor preparation

The covalent attachment of streptavidin (STA) onto the poly (HOEGMA) provides a versatile surface onto which any biotinylated compound can be immobilized. The amount of attached STA ($\Delta\lambda_{res}$=8 nm, 1200 pg mm^{-2}) was similar to the saturated concentration of BSA adsorbed physically on a bare gold surface ($\Delta\lambda_{res}$=7 nm, 1050 pg mm^{-2}). Assuming that SPR responses, $\Delta\lambda_{res}$, are roughly proportional to the mass attached to the sensor surface, the relative amounts of biorecognition elements (BRE) attached in the following steps of the biosensor preparation can be estimated by comparing the SPR responses divided by the molecular weight of the respective attached BRE (streptavidin 52.8 kDa; ON-1, ON-2, ON-3, and ON-4 5.5 kDa; CON-1-VCA 90 kDa, CON-2-EA 50 kDa, and CON-3-EBNA-1 93 kDa). The average numbers of immobilized BRE per one STA molecule were 1.2 biotinylated oligonucleotides and 0.12 hybridized CON-#-antigen. It is worth noting that the same amounts of each antigen conjugate were able to be immobilized on the surfaces. The attachment of antigens via hybridization made it possible to release CON-#-antigen conjugates by increasing temperature above the melting point of the oligonucleotides ON and CON pairs. Fig. 5 shows the repeated measurements of the response to anti-VCA-positive serum S-1 with a biosensor regenerated two times. After each measurement the original poly(HOEGMA)-STA-ON-1 coating was recovered by incubating the sensor in PBS at 45 °C for 30 min and the biosensing surface was regenerated by the attachment of a new CON-1-VCA. The regeneration did not affect the sensor response. Neither the specific response nor the response to fouling were changed after regeneration at 45 °C, which can be attributed to the thermal stability of streptavidin–biotin complex (González et al., 1999) and the poly(HOEGMA) brush structure respectively. The ease of the regeneration under mild conditions is an important advantage of the biosensor conferred by the multistep preparation method presented in this work. This technique could be particularly convenient for the preparation of multi-spot sensing arrays (Boozer et al., 2004). A variety of biotinylated oligonucleotides could be spotted on selected sites of a single poly(HOEGMA)-STA coating and various bioreceptors conjugated with the complementary oligonucleotides would be subsequently hybridized to the respective spots.

4.2. Optimization of the biosensor

Important differences in fouling (40–630 pg mm^{-2}) on poly (HOEGMA) brushes from blood plasma collected from various donors and pooled plasma from different sources, respectively, were reported earlier (de los Santos Pereira et al., 2014). Similarly, considerable differences of up to 1 nm in the responses of the reference surfaces poly(HOEGMA)-STA-ON-# to sera from different donors were observed here. Evidently, the response of a detection surface poly(HOEGMA)-STA-ON-#-CON-#-antigen to a serum from a healthy donor cannot be used to compensate the unknown fouling from a positive serum sample. On the other hand, there were no significant differences in the responses of the detection and the reference surfaces to negative sera. The non-specific responses were assumed to be only due to the serum fouling. Thus, the response of the respective reference surface to a tested serum sample was subtracted from that of the detection surface to obtain the specific response to the targeted antibodies. It should be noted that the serum fouling observed on these brushes is an order of magnitude lower than on SAMs, which is critical to the success of the approach (Rodriguez-Emmenegger et al., 2009). The increase in the ratio of specific to fouling responses with increasing dilution of the serum samples could be due to different kinetics of the antibody capture and serum fouling and/or a competition between the two processes for the free area on the sensing surface. The fouling decreased significantly if 1% BSA in PBS was used to dilute the sera instead of pure PBS. A negligible amount of BSA ($\Delta\lambda_{res}$=0.1–0.2 nm) remained adsorbed on the surfaces after 30 min incubation with 1% BSA and washing with PBS indicating that an irreversible BSA adsorption or covalent attachment to some residual activated functional groups on the surfaces did not play a significant role. Thus, a reversible interaction of BSA with the surface or with adsorbing serum proteins could be supposed to reduce the fouling from serum.

4.3. Detection of anti-EBNA-1, anti-VCA, and anti-EA antibodies in clinical samples

The diagnosis of EBV infection is based on the presence of three specific serological antibody markers (anti-VCA, anti-EA, and anti-EBNA-1 antibodies) and can be correlated with different stages of the EBV infection (Klutts et al., 2009). During the primary infection IgM antibodies against VCA are raised, followed by IgG antibodies against VCA. Contrary to anti-VCA IgM antibodies, produced only briefly and not necessarily in all patients, anti-VCA IgG antibodies have lifelong persistence. Even though the direct SPR detection does not differentiate the IgG and IgM antibodies, a secondary antibody can be used to distinguish between the IgG and IgM types of EBV antibodies if needed. In particular, for serum S-2 such approach would discriminate between primary and past infection. Anti-EBNA-1 IgG are produced later in the course of the infection. Typically, they are not detectable until six to eight weeks after the onset of symptoms, and in most cases they persist lifelong. The presence of anti-EBNA-1 IgG is thus a good marker of a past infection. However, up to 5% of individuals do not produce anti-EBNA-1 IgG and under some circumstances, such as immunosuppression, the level of anti-EBNA-1 IgG can be decreased or completely lost (Bauer, 2001). Anti-EA IgG are produced early after primary infection and, together with the presence of anti-VCA antibodies and the absence of anti-EBNA-1 antibodies, are a good marker of an ongoing EBV infection. Negative results for anti-VCA, anti-EA and anti-EBNA-1 antibodies indicate that the individual has not been infected.

The label-free biosensor assay presented herein is the first example of an optical affinity biosensor capable of real time detection of analytes in clinical samples of blood serum. Previous reports dealt usually with model detection in pooled serum and plasma spiked with a targeted analyte at concentrations orders of magnitude higher than biologically relevant (Gao et al., 2010; Vaisocherová et al., 2007). Compared to other methods for diagnosis this SPR assay requires only a fraction of the time (herein 30 min) of automatic measurement. The procedures used in this work could serve as a prototype strategy for the development of label-free affinity biosensors for diagnostics of blood serum or plasma samples. The choice of a reference surface, which exhibits the same fouling from tested samples as the detection surface with immobilized specific bioreceptors, is a crucial condition for the detection in complex biological media. An antifouling polymer brush with covalently bound streptavidin could provide both a reference surface and a convenient substrate on which other biorecognition elements can be immobilized without changing the fouling by further surface activation. The dilution of tested samples with albumin makes it possible to decrease the fouling and to optimize the ratio of specific to fouling responses. The immobilization of bioreceptors via oligonucleotide hybridization enables the regeneration of the sensor under mild conditions and, potentially, the preparation of sensor multi-arrays functionalized by μ-contact printing.

5. Conclusion

A label-free SPR biosensor assay capable of real time recognition of different stages of the Epstein–Barr virus (EBV) infection in blood serum samples was developed for the first time. The biosensing surface was prepared utilizing a modular strategy including the coating of the SPR chip with a poly(HOEGMA) brush and successive attachment of streptavidin, biotinylated oligonucleotides (ON), and complementary oligonucleotide (CON)-antigen conjugates. The specific response could be obtained by subtracting the response on a reference surface without the antigens from the measuring channel. This was supported by the fact that fouling from a non-infected donor was the same on both surfaces. The dilution of serum samples with 1% BSA made it possible to decrease the fouling and to optimize the ratio of specific to fouling responses. The biosensor could be repeatedly used after disrupting ON–CON pairs at 45 °C and attachment of new antigen conjugates. The stages of EBV infection were diagnosed by estimating the relative titer of three different anti-VCA, anti-EBNA-1 and anti-EA antibodies in the tested sera from patients. This is the first example of a label-free affinity biosensor capable of detecting disease markers at their natural concentration in real clinical serum samples and paves the way for the development of label-free affinity biosensors for diagnostics in blood serum or plasma samples. The immobilization of bioreceptors via oligonucleotide hybridization makes it possible to regenerate sensors under mild conditions and, potentially, to prepare sensor multi-arrays functionalized by μ-contact printing.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2013.12.011.

References

Ascherio, A., Munger, K.L., 2010. J. Neuroimmune Pharmacol. 5, 271–277.
Bauer, G., 2001. Clin. Lab. 47, 223–230.
Besselink, G.A.J., Kooyman, R.P.H., van Os, P.J.H.J., Engbers, G.H.M., Schasfoort, R.B. M., 2004. Anal. Biochem. 333, 165–173.
Blaszykowski, C., Sheikh, S., Thompson, M., 2012. Chem. Soc. Rev. 41, 5599–5612.
Boozer, C., Ladd, J., Chen, S., Yu, Q., Homola, J., Jiang, S., 2004. Anal. Chem. 76, 6967–6972.
Cao, C., Kim, J.P., Kim, B.W., Chae, H., Yoon, H.C., Yang, S.S., Sim, S.J., 2006. Biosensors Bioelectron. 21, 2106–2113.
Chung, J.W., Kim, S.D., Bernhardt, R., Pyun, J.C., 2005. Sensors Actuators B: Chem. 111–112, 416–422.
de los Santos Pereira, A., Rodriguez-Emmenegger, C., Surman, F., Riedel, T., Bologna, A., Brynda, E., 2014. RSC Adv. 4, 2318–2321.
Fernández, F., Hegnerová, K., Piliarik, M., Sanchez-Baeza, F., Homola, J., Marco, M.P., 2010. Biosensors Bioelectron. 26, 1231–1238.
Gao, C.L., Li, G.Z., Xue, H., Yang, W., Zhang, F.B., Jiang, S., 2010. Biomaterials 31, 1486–1492.
González, M., Argaraña, C.E., Fidelio, G.D., 1999. Biomol. Eng. 16, 67–72.
Hess, R., 2004. J. Clin. Microbiol. 42, 3381–3387.
Homola, J., 2006. Surface Plasmon Resonance Based Sensors. Springer, Berlin Heidelberg(Springer Series on Chemical Sensors and Biosensors).
Homola, J., 2008. Chem. Rev. 108, 462–493.
Homola, J., Dostálek, J., Chen, S., Rasooly, A., Jiang, S., Yee, S.S., 2002. Int. J. Food Microbiol. 75, 61–69.
Jeong, H.-H., Erdene, N., Park, J.-H., Jeong, D.-H., Lee, H.-Y., Lee, S.-K., 2013. Biosensors Bioelectron. 39, 346–351.
Klutts, J.S., Ford, B.a., Perez, N.R., Gronowski, A.M., 2009. J. Clin. Microbiol. 47, 3204–3210.
Kostina, N.Y., Rodriguez-Emmenegger, C., Houska, M., Brynda, E., Michálek, J., 2012. Biomacromolecules 13, 4164–4170.
Kvasnička, P., Chadt, K., Vala, M., Bocková, M., Homola, J., 2012. Opt. Lett. 37, 163–165.
Lechuga, L.M., 2012. Anal. Bioanal. Chem. 404, 2795–2796.
Odumade, O.A., Hogquist, K.A., Balfour, H.H., 2011. Clin. Microbiol. Rev. 24, 193–209.
Pimková, K., Bocková, M., Hegnerová, K., Suttnar, J., Cermák, J., Homola, J., Dyr, J.E., 2012. Anal. Bioanal. Chem. 402, 381–387.
Pop-Georgievski, O., Rodriguez-Emmenegger, C., de los Santos Pereira, A., Proks, V., Brynda, E., Rypáček, F., 2013. J. Mater. Chem. B 1, 2859–2867.
Rickinson, A.B., Kieff, E., 2001. In: Knipe, D.M., Howley, P.M. (Eds.), Fields Virology. Lippincott Williams & Wilkins, Philadelphia, PA, pp. 2575–2627.
Riedel, T., Riedelová-Reicheltová, Z., Májek, P., Rodriguez-Emmenegger, C., Houska, M., Dyr, J.E., Brynda, E., 2013. Langmuir 29, 3388–3397.
Rodriguez-Emmenegger, C., Avramenko, O., Brynda, E., Skvor, J., Bologna Alles, A., 2011a. Biosensors Bioelectron. 26, 4545–4551.
Rodriguez-Emmenegger, C., Brynda, E., Riedel, T., Houska, M., Šubr, V., Bologna Alles, A., Hasan, E., Gautrot, J.E., Huck, W.T.S., 2011b. Macromol. Rapid Commun. 32, 952–957.
Rodriguez-Emmenegger, C., Brynda, E., Riedel, T., Sedlakova, Z., Houska, M., Bologna Alles, A., 2009. Langmuir 25, 6328–6333.
Rodriguez-Emmenegger, C., Hasan, E., Pop-Georgievski, O., Houska, M., Brynda, E., Bologna Alles, A., 2012a. Macromol. Biosci. 12, 525–532.
Rodriguez-Emmenegger, C., Houska, M., Bologna Alles, A., Brynda, E., 2012b. Macromol. Biosci. 12, 1413–1422.
Rodriguez-Emmenegger, C., Preuss, C.M., Yameen, B., Pop-Georgievski, O., Bachmann, M., Mueller, J.O., Bruns, M., Goldmann, A.S., Bastmeyer, M., Barner-Kowollik, C., 2013. Adv. Mater. 25, 6123–6127.
Slavík, R., Homola, J., 2007. Sensors Actuators B: Chem. 123, 10–12.
Slavík, R., Homola, J., Brynda, E., 2002. Biosensors Bioelectron. 17, 591–595.
Thorley-Lawson, D.a, Gross, A., 2004. N. Engl. J. Med. 350, 1328–1337.
Toussirot, E., Roudier, J., 2008. Best practice and research. Clin. Rheumatol. 22, 883–896.
Vaisocherová, H., Mrkvová, K., Piliarik, M., Jinoch, P., Steinbachová, M., Homola, J., 2007. Biosensors Bioelectron. 22, 1020–1026.
Vaisocherová, H., Ševců, V., Adam, P., Špačková, B., Hegnerová, K., de los Santos Pereira, A., Rodriguez-Emmenegger, C., Riedel, T., Houska, M., Brynda, E., Homola, J., 2013. Biosensors Bioelectron. 51, 150–157.
Vaisocherová, H., Yang, W., Zhang, Z., Cao, Z., Cheng, G., Piliarik, M., Homola, J., Jiang, S., 2008. Anal. Chem. 80, 7894–7901.
Voisin, V., Pilate, J., Damman, P., Mégret, P., Caucheteur, C., 2014. Biosensors Bioelectron. 51, 249–254.
Yanase, Y., Araki, A., Suzuki, H., Tsutsui, T., Kimura, T., Okamoto, K., Nakatani, T., Hiragun, T., Hide, M., 2010. Biosensors Bioelectron. 25, 1244–1247.
Yu, Q., Zhang, Y., Wang, H., Brash, J.L., Chen, H., 2011. Acta Biomater. 7, 1550–1557.
Zamfir, M.E., Rodriguez-Emmenegger, C., Bauer, S., Barner, L., Rosenhahn, A., Barner-Kowollik, C., 2013. J. Mater. Chem. B 1, 6027–6034.