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ORIGINAL PAPER

Surface plasmon resonance biosensor for the detection of VEGFR-1—a protein marker of myelodysplastic syndromes

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Abstract The surface plasmon resonance (SPR) biosensor system with dispersionless microfluidics for the direct and label-free detection of a soluble vascular endothelial growth factor receptor (sVEGFR-1) is described. The detection approach takes advantage of an affinity interaction between sVEGFR-1 and its ligand, vascular endothelial growth factor (VEGF-A), which is covalently immobilized on the surface of the SPR sensor. The ability of the immobilized VEGF-A to specifically bind the sVEGFR-1 receptor is demonstrated in a buffer. The detection of sVEGFR-1 in 2% human blood plasma is carried out by using the sequential injection approach. The detection limit of 25 ng/mL is achieved. In addition, we demonstrate that the functional surface of the sensor can be regenerated for repeated use.

Keywords Surface plasmon resonance · Myelodysplastic syndromes · Vascular endothelial growth factor · Protein markers

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Introduction

Myelodysplastic syndromes (MDS) are a diverse group of clonal disorders of the hematopoietic stem cell. They are characterized by ineffective hematopoiesis, peripheral blood cytopenias, and a propensity to transform to acute myeloid leukemia (AML) and are manifested by a wide variety of clinical symptoms [1]. Therefore, a diagnosis of MDS presents a rather complex diagnostic task and a challenge for the current diagnostic methods. Thus, research toward gaining deeper understanding of MDS and the development of new diagnostic approaches is stimulated.

In the last decade, several protein markers of MDS have been identified. An increased expression of the soluble form of vascular endothelial growth factor receptor 1 (sVEGFR-1) was reported in patients with MDS and AML [2, 3]. VEGFR-1 is a transmembrane protein of 150 kDa, with tyrosine kinase activity existing in two forms—membrane-bound and soluble [4]. It has been suggested that the signaling pathways mediated by the VEGFR-1 receptor are triggered by a binding of their high-affinity ligand—vascular endothelial growth factor A (VEGF-A). VEGF-A (a homodimeric glycoprotein with a molecular mass of 46 kDa) is a member of the VEGF family, referred to as cytokines. The binding of VEGF-A to transmembrane VEGFR-1 gives rise to endothelial mitogenesis, proliferation, and the initiation of angiogenesis and vasculogenesis [5, 6]. In contrast, soluble VEGFR-1 inhibits angiogenesis. However, angiogenesis is a rather complex process, and the exact molecular

mechanism of sVEGFR-1's involvement has not been fully understood yet [7]. Moreover, plasmatic sVEGFR-1 may exist in complexes with other molecules, for example, with the soluble form of transmembrane protein neuropilin-1 [8].

The current diagnosis of MDS is mainly based on WHO/FAB (World Health Organization/French–American–British) assigned scores of heterogeneous groups, according to marrow blast cell percentage, karyotype, and degree of cytopenia. These parameters are found through invasive procedures such as bone marrow puncture and peripheral blood count [9]. It is expected that in future diagnosis of heterogeneous diseases such as MDS, methods based on high-throughput screening of molecular markers will play a crucial role. Although the microarray-based gene expression profiling of MDS has advanced substantially, the mRNA expression fails to consistently correlate with protein expression levels and does not characterize resulting protein functions influenced by such critical parameters as posttranslational modifications and splice variants. Therefore, characterization of MDS phenotypes by proteomic analysis presents an important step toward understanding the biological processes related to MDS [10].

Current approaches to the detection of sVEGFR-1 are based on immunological methods, such as enzyme-linked immunosorbent assay (ELISA), and use antibodies to capture the protein and fluorescent labels or enzymatic amplification to visualize the antibody–protein binding. The main drawbacks of these methods are the need for high-affinity antibodies, which are difficult and expensive to produce, as well as the limited ability to detect complexes of sVEGFR-1 with other molecules due to stochiometric and steric reasons [11, 12]. In addition, the detection of sVEGFR-1 via an antibody does not necessarily provide an accurate representation of the biological activity of the protein. Label-free biosensors allow for a direct real-time observation of molecular interactions, without the use of labels, and thus provide a promising alternative to conventional methods. Surface plasmon resonance (SPR) biosensors present a powerful optical label-free biosensor technology that has been widely applied to the investigation of biomolecular interactions and the detection of chemical and biological analytes [13]. In recent years, SPR biosensors were used to investigate interactions involving molecular biomarkers [14, 15] and measure their concentrations in bodily fluids [12, 16]. In addition, multichannel SPR biosensors [17] make it possible to observe large numbers of biomolecular interactions and identify and simultaneously quantify multiple biomarkers.

In this paper, we demonstrate label-free detection of protein biomarker of MDS disease, sVEGFR-1, via its physiological ligand VEGF-A, using a surface plasmon resonance biosensor and dispersionless microfluidics. VEGF-A molecules are immobilized on the sensing surface via amide coupling of the amino groups of VEGF-A protein and negatively charged carboxylic groups of alkanethiols. The negatively charged residues of VEGF-A, which are critical for the interaction of VEGF-A with the sVEGFR-1 receptor, do not participate in amide coupling and thus remain available for the binding of sVEGFR-1 via the physiological pathway. The ability of the immobilized VEGF-A to interact with the sVEGFR-1 receptor contained in the buffer is investigated. Finally, detection of sVEGFR-1 in diluted blood plasma is accomplished using the dispersionless microfluidics and sequential injection approach.

Materials and methods

Reagents

Human recombinant protein VEGF-A and VEGF receptor-1/Fc chimera were purchased from Sigma-Aldrich (St. Louis, MO). 11-Mercapto-tetra(ethyleneglycol)undecanol ($HSC_{11}(EG)_4OH$) and 16-mercapto-hexa(ethyleneglycol) hexadecanoid acid ($HSC_{11}(EG)_6OCH_2COOH$) were purchased from Prochimia (Gdansk, Poland). Bovine serum albumin (BSA) and sodium hydroxide (NaOH) were purchased from Sigma-Aldrich. Ethanolamine hydrochloride (EA), 3-(ethyliminomethylideneamino)-*N*,*N*-dimethylpropan-1-amine (EDC), and 1-hydroxypyrrolidine-2,5-dione (NHS), all included in the Amine Coupling Kit, were purchased from Biacore (Uppsala, Sweden). All other chemicals were of analytical grade. The buffers used were: PBS (0.01 M phosphate, 0.138 M sodium chloride, 0.0027 M potassium chloride, pH 7.4); SA (0.01 M sodium acetate, pH 5.0); PBNa (0.01 M phosphate, 0.75 M sodium chloride, 0.009 M potassium chloride, pH 7.4); 10% BSA/PBS (*w*/*w*); 0.2% BSA/PBNa (*w*/*w*); and ACD (0.08 M trisodium citrate dihydrate, 0.06 M acidum citricum monohydricum, 0.1 M glucose). All buffers were prepared using double glass-distilled and deionized water on Milli-Q50 (Millipore, Prague, Czech Republic).

Plasma samples

Human blood was drawn from healthy volunteers by venipuncture into polypropylene tubes containing 1.9% ACD (*w*/*v*). Plasma was obtained by centrifugation at 250×*g* at 37 °C for 15 min and subsequent centrifugation of the supernatant at 1,000×*g* at 37 °C for 10 min. All samples were obtained in accordance with the Regulations of the Ethical Committee of the Institute of Hematology and Blood Transfusion (Prague).

SPR biosensor platform

In this work, a four-channel laboratory SPR sensor platform (PLASMON IV) was used, developed at the Institute of Photonics and Electronics (Prague). The sensor is based on the Kretschmann geometry of the attenuated total reflection method and spectral modulation. In this SPR sensor, a polychromatic light beam passes through an optical prism interfaced with a chip with a thin metal layer and excites surface plasmons at the interface between the metal layer and a liquid sample. The excitation of surface plasmons gives rise to a narrow dip in the spectrum of reflected light. The wavelength at which the dip occurs (i.e., the resonant wavelength) depends on the refractive index in the proximity of the SPR metal layer surface. If a biomolecular recognition element is immobilized on the sensor surface, the binding of the analyte molecule to the biomolecular recognition element gives rise to a change in the local refractive index. The amount of captured analyte can be quantified by measuring the change in the resonant wavelength. The SPR sensor platform used in this work consists of a polychromatic light source, an optical setup for excitation and modulation of surface plasmons (sensor head), and a four-channel spectrograph. Light from the light source (halogen lamp, HL-2000-HP, Ocean Optics, USA) is brought to the sensor head via an optical fiber (BFL22-365, Thorlabs, USA). The sensor head comprised an input collimator (custom built, consisting of two cylindrical lenses with 20- and 50-mm effective focal lengths) producing a large-diameter parallel beam of polychromatic light, a BK7 glass prism with an attached SPR chip, a dichroic polarizer (Polarcor 800-HC from Corning, USA), and an output four-channel collimator. The output collimator incorporates four graded-index lenses (GT-LFRL-180-024-20-NC, Grintech, Germany) which receive light reflected from four areas of the sensor surface and couple the light into four optical fibers (BFL22-365, Thorlabs) which are connected to the inputs of a four-channel spectrograph unit (consisting of four spectrometer boards S2000, Ocean Optics, and custom-made electronics). In our experiments, we used replaceable SPR sensor chips produced by coating a BK7 glass substrate with an adhesion-promoting titanium layer (thickness, ~2 nm) and a gold layer (thickness, 50 nm) by e-beam evaporation in vacuum. The optical contact between the SPR chip and the prism was established using refractive index matching fluid (Cargille Laboratories, USA). A flow cell with four separate flow chambers was interfaced with the chip. Each flow chamber was aligned to cover one sensing channel. A multichannel peristaltic pump was used to deliver liquid samples to the flow cell. All the experiments were performed using a recently developed dispersionless microfluidics [18]. This microfluidics allows for a fast switching between the analyzed sample (e.g., blood plasma) and the running buffer, nearly eliminating dispersion and intermixing before the sample reaches the sensing area. The SPR system exhibits a baseline noise of 0.9 pm, which corresponds to a refractive index resolution of 1.3×10^{-7} refractive index unit. All experiments were performed at a temperature of 25 °C and a flow rate of 30 μL/min (unless otherwise stated).

Immobilization of VEGF-A on SPR chip

The immobilization method is expected to provide robust attachment of VEGF-A molecules to the surface while preserving their structure and biological activity. As described by Keyt et al. [5], negatively charged acidic residues (Asp^{63}, Glu^{64}, and Glu^{67}) in the active site of VEGF-A are critical for the binding of sVEGFR-1 receptors. Therefore, in our work, VEGF-A was immobilized via amide coupling of the amino groups of VEGF-A protein with the negatively charged carboxylic groups of alkanethiols, leaving the negatively charged residues of VEGF-A accessible for binding sVEGFR-1 via the physiological pathway. The blocking of residual carboxylic groups of alkanethiols on the surface with EA is essential for lowering nonspecific interactions with sVEGFR-1.

Prior to the attachment of VEGF-A, the surface of the SPR chip was functionalized with a mixed self-assembled monolayer (SAM) of alkanethiols, following the procedure given in [19]. Initially, the surface of the gold-coated SPR chip was washed by deionized water and dried by a stream of nitrogen. Then, the chip was put into an UV ozone cleaner (UVO-cleaner 42–220, Jelight Company, USA) for 10 min to remove organic contaminants. Subsequently, the chip surface was washed with deionized water and absolute ethanol and dried by a stream of pure nitrogen. Then, the surface was functionalized with a mixed SAM, with a mixture (7:3) of $HSC_{11}(EG)_4OH$ and $HSC_{11}(EG)_6OCH_2$-COOH alkanethiols (total concentration, 200 μM). After immersing the chip in a mixed thiol solution and heating it to a temperature of 40 °C for 10 min, the chip was stored in darkness at room temperature for up to 1 day. $HSC_{11}(EG)_6OCH_2COOH$ alkanethiols were used to anchor ligand (VEGF-A) by amino coupling, while $HSC_{11}(EG)_4OH$ alkanethiols were used to form a nonfouling background. The activation of the carboxylic terminal groups on the sensor surface was accomplished in situ in the SPR sensor by injecting deionized water followed by a (1:1) mixture of NHS and EDC for 5 min and deionized water again (flow rate, 20 μL/min). After the activation, the surface of the chip was incubated with PBS buffer. Immobilization of VEGF-A to the activated surface was carried out in PBS (VEGF-A concentration, 2 μg/mL) for 10 min. Then, buffers were injected in the

following order—PBS, SA buffer, PBS, and SA buffer—to test the effect of the buffers on the stability of the immobilized VEGF-A. In order to increase the resistance of the surface to the nonspecific adsorption, BSA was covalently immobilized to the areas of the surface not coated with VEGF-A. For that purpose, the sensor surface was incubated with 0.5 mg/mL BSA in SA for 15 min. The non-covalently bound VEGF-A and BSA were removed through the use of a high ionic strength PBNa buffer, which coursed through the flow cell for 5 min. Finally, the sensor surface was treated with 1 M EA for 5 min to deactivate the carboxylic groups of alkanethiols. Reference surfaces only coated with BSA were used to check the specificity of the VEGF-A-coated surface.

After immobilization of VEGF-A on the surface of the sensor, the interaction of VEGF-A and sVEGFR-1 proteins in buffer was investigated. Prior to the injection of sVEGFR-1, PBS buffer was flowed across the sensor surface until a stable baseline was established. Then, sVEGFR-1 at a concentration ranging from 25 to 500 ng/mL was flowed across the sensing surface for 5 min. Following that, the surface was flushed with PBS buffer. In order to evaluate the specificity of the VEGF-A-coated surface, a control experiment was performed in which a solution containing sVEGFR-1 at concentrations ranging from 50 to 500 ng/mL was flowed over the reference surface (only coated with BSA).

Detection of sVEGFR-1

For the detection of sVEGFR-1 in diluted blood plasma, the sequential injection approach was used [18]. The sequential injection approach is based on a sequential injection of the sample and a special buffer, which removes the nonspecifically adsorbed molecules from the surface while not affecting analyte molecules bound to the immobilized ligand. This minimizes the nonspecific sensor response and allows for the detection of low concentrations of analyte, even in complex samples. In our experiments, a series of blood plasma samples (2% in BSA/PBS) spiked with different concentrations of sVEGFR-1 (25–500 ng/mL) were used. The addition of BSA molecules to blood plasma samples was found to reduce the nonspecific interaction of plasma proteins with the sensing surface in each experiment. Therefore, the detection experiments were carried out in the BSA/PBNa running buffer, which was designed in such a way as to remove nonspecifically adsorbed proteins while not disrupting the binding of sVEGFR-1 to VEGF-A immobilized on the sensor surface (data not shown). Blood plasma sample and the running buffer were introduced to the sensing surface sequentially. A total of ten 30-s cycles of blood plasma sample and running buffer were carried out. Therefore, the total time during which the blood plasma sample was in contact with the sensor surface was 5 min. Finally, the running buffer was flowed through the flow cell until equilibrium was reached. To provide a reference, 2% blood plasma, diluted with BSA/PBS and no addition of sVEGFR-1 (blank sample), was flowed through one of the sensing channels.

Results and discussion

Immobilization of VEGF-A on SPR chip

A sensorgram illustrating the immobilization of VEGF-A and BSA is shown in Fig. 1. The SPR sensor response to the immobilized VEGF-A is about 7 nm (the shift in the resonant wavelength), which corresponds to the surface concentration of VEGF-A of ~119 ng/cm^2. The difference between the refractive index of the PBS and SA buffer caused a change in the resonant wavelength of about 7 nm. After the sensor response to the immobilization of VEGF-A (in PBS buffer) reached 7 nm (~119 ng/cm^2), the surface was incubated with BSA (in SA buffer) to minimize nonspecific adsorption to the sensing surface. It is known that BSA molecules tend to bind to each other and form a multilayer. Our previous experiments indicate that the formation of a full BSA monolayer produces a resonant wavelength shift of 11 nm. We allowed adsorption of BSA to the areas of the surface not coated with VEGF-A to proceed until the total resonant wavelength shift (for VEGF-A and BSA combined) reached 11 nm to avoid the formation of a multilayer. Longer incubations with BSA have been found to result in a lower sensor response to the subsequent binding of sVEGFR-1 to VEGF-A. We suppose

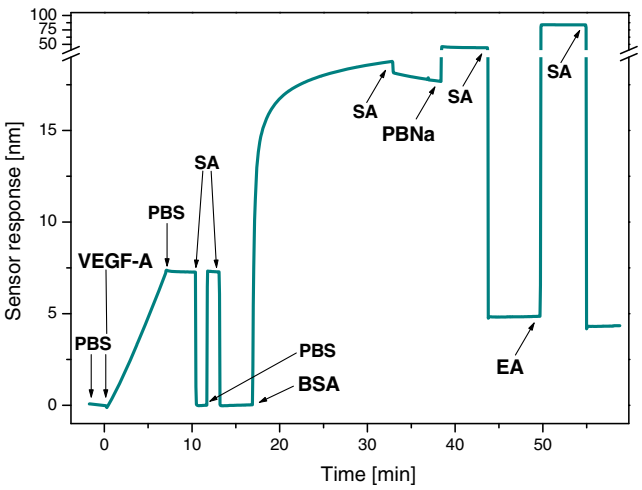

Fig. 1 SPR sensor response to the immobilization of vascular endothelial growth factor A (*VEGF-A*) and BSA on the SPR sensor surface

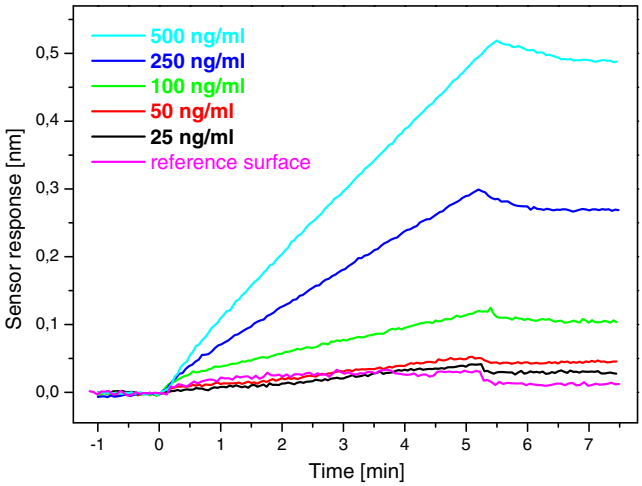

Fig. 2 Detection of sVEGFR-1 in buffer. Response of the sensor to five different concentrations of sVEGFR-1 interacting with VEGF-A, immobilized on the surface of the sensing channel and to reference surface coated only with BSA

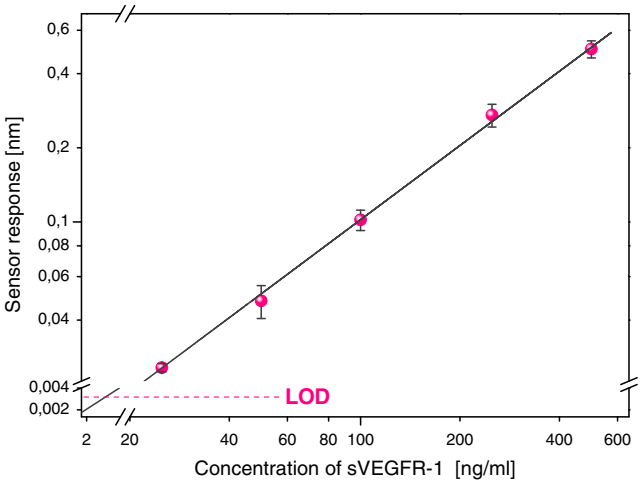

Fig. 3 Calibration curve for the detection of sVEGFR-1 in buffer. The sensor response corresponding to the limit of detection is noted by a *horizontal line*

that it is because the BSA multilayer reduced the access of sVEGFR-1 to the binding sites of VEGF-A. The covalently bound BSA (not removed with PBNa buffer) provided a sensor response of 3.5 nm, which corresponds to ~60 ng/cm^2. Therefore, the resulting surface coverage ratio of VEGF-A and BSA was approximately 2:1. In addition, the stability of the immobilization of VEGF-A in PBNa was tested. No decrease in sensor response was observed when PBNa was injected (data not shown), which indicates that the attachment of VEGF-A is stable in PBNa.

Development of assay

In order to evaluate the interaction between sVEGFR-1 and VEGF-A immobilized on the sensor surface, different concentrations of sVEGFR-1 in PBS buffer (in the range of 25–500 ng/mL) were introduced to the sensor. The resulting sensor response to sVEGFR-1 was normalized to the amount of VEGF-A immobilized on the sensor surface. As follows from Fig. 2, the binding of sVEGFR-1 to VEGF-A is concentration-dependent, stable, and specific—the nonspecific binding to the BSA-coated surface (reference surface) gave rise to a rather minor sensor response (within 5 standard deviations of the baseline noise). The largest nonspecific sensor response was observed for the concentration of sVEGFR-1 of 500 ng/mL and is shown in Fig. 2. The detection experiment was repeated using three sensing channels of a single chip for each concentration of sVEGFR-1, and on-chip reproducibility was determined to be between 85% and 96%. Sensor responses determined for different concentrations of sVEGFR-1 in buffer, collected in the form of a calibration curve, are shown in Fig. 3. The limit of detection (LOD) for sVEGFR-1 in buffer, defined as a concentration of sVEGFR-1 that corresponds to the sensor response equal to 3 standard deviations of the baseline noise (3×0.9 pm), was estimated to be 3 ng/mL.

Subsequently, the reusability of the sensor and the reproducibility of the measurements in blood plasma were studied. To investigate the reusability of the sensor, the captured sVEGFR-1 was removed from the sensor surface with 10 mM NaOH and the sensor was used for another detection cycle. The experiments (Fig. 4) revealed that even after five detection–regeneration cycles, the binding capacity was maintained and that the reproducibility of the sensor response was better than 92%. Chip-to-chip reproducibility was examined using three independent chips for each concentration in the concentration range 25–100 ng/mL and was determined to be between 88% and 96%.

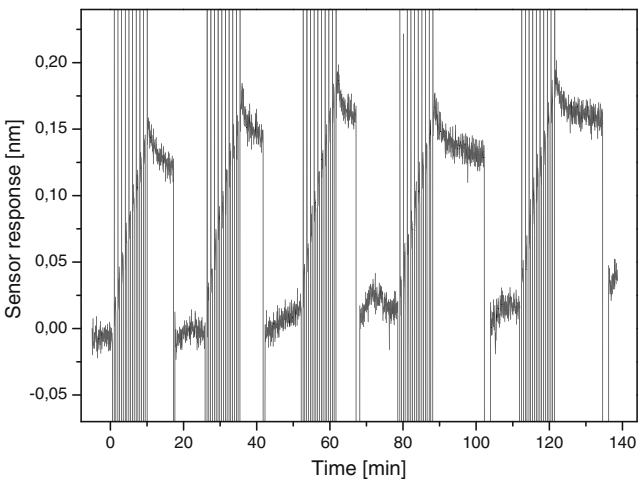

Fig. 4 Regeneration of sensor with NaOH and subsequent detection of blood plasma sample, spiked with 50 ng/mL of sVEGFR-1, in five cycles

Detection of sVEGFR-1

The binding data for sVEGFR-1 in diluted blood plasma obtained using the sampling method are shown in Fig. 5. As illustrated in the inset of Fig. 5, switching between the running buffer and the blood plasma sample (injection cycle) results in a great change in the sensor response (caused mainly by changes in the bulk refractive index). Therefore, the kinetic curves provided in Fig. 5 only contain the values obtained at the end of each injection cycle. As follows from Fig. 5, for each concentration of VEGFR-1, the sensor response to diluted blood plasma spiked with sVEGFR-1 increased nearly linearly over time. The total amount of the captured sVEGFR-1 is proportional to the concentration of sVEGFR-1. The specific sensor responses to the tested concentrations of sVEGFR-1 in blood plasma (obtained by subtracting the blank sample response) agree well with those measured in buffer. Using the sensor responses obtained for different concentrations of sVEGFR-1, the calibration curve for the detection of sVEGFR-1 in 50× diluted blood plasma was established (Fig. 6). As the sensor surface is regenerable, the sensor response to each concentration was determined from at least three measurements on different chips. The average sensor response to the blank sample (2% blood plasma diluted with BSA/PBS with no sVEGFR-1 added) was 0.097± 0.008 nm, which corresponds to the surface concentration as low as 1.7 ng/cm^2. This ultimate level of nonspecific adsorption from the blank sample, using the sequential injection approach, is comparable with the nonspecific adsorption levels achieved using advanced non-fouling surfaces (e.g., zwitterionic polymers). The low nonspecific

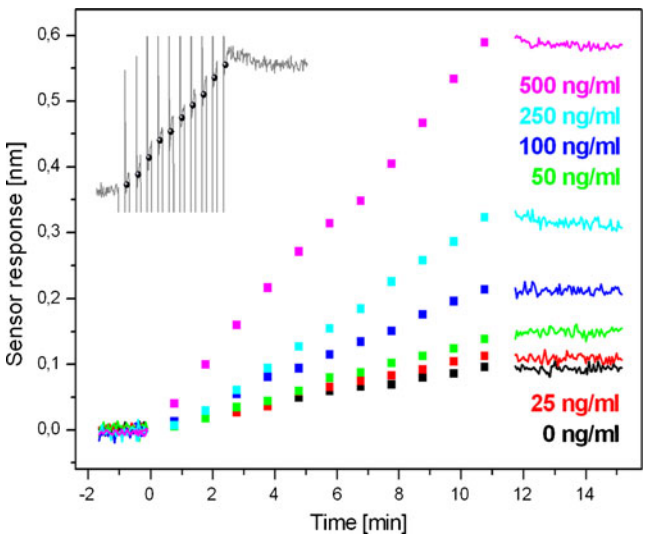

Fig. 5 Sensor response to plasma samples spiked with different concentrations of sVEGFR-1. The *inset* illustrates the sequential injection of plasma sample and running buffer

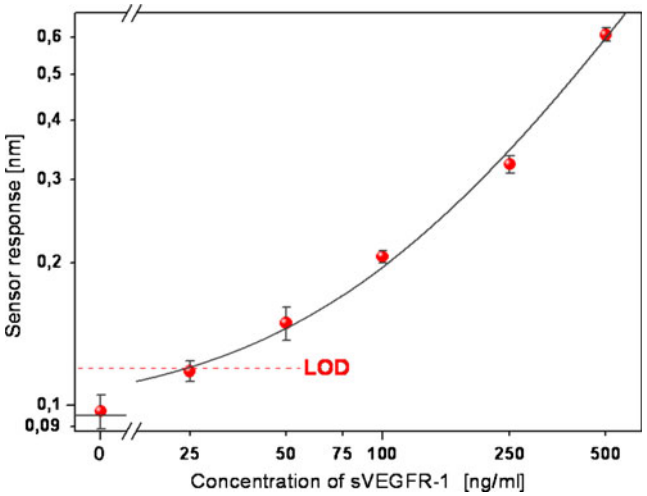

Fig. 6 Calibration curve for the detection of sVEGFR-1 in blood plasma. The sensor response corresponding to the limit of detection is noted by a *horizontal line*

adsorption observed is probably due to the fact that high ionic strength running buffer removes the nonspecifically adsorbed molecules during each sampling cycle before they can form a solid layer to enable secondary nonspecific adsorption on previously adsorbed biomolecules. Different types of surfaces were compared with respect to the nonspecific adsorption from blood plasma (blank sample). It was found that in comparison with the VEGF-A surface coated with covalently attached BSA, the surface coated only with VEGF-A and the surface coated only with BSA exhibited a nonspecific adsorption which was higher by a factor of 1.8 and lower by a factor of 50, respectively (data not shown). The LOD was determined as the concentration corresponding to the sensor response to a blank sample, increased by 3 standard deviations of the sensor response to a blank sample (0.097+3×0.008 nm). Using the calibration curve (Fig. 6), the LOD for the reported sVEGFR-1 biosensor was found to be 25 ng/mL.

To our knowledge, this is the first attempt to measure sVEGFR-1 levels using an SPR biosensor. Moreover, there is no commercial method for the diagnosis of MDS via the detection of sVEGFR-1. Conventional approaches for the detection of sVEGFR-1 are based on ELISA [20–25]. For instance, Hu et al. [2] measured the plasmatic levels of sVEGFR-1 using ELISA and found them to be about 0.02 ng/mL and up to 0.5 ng/mL for healthy individuals and MDS patients, respectively. While the reported SPR biosensor does not yet reach this level of sensitivity, it only requires one biorecognition element to carry out the measurement and thus may be better able to detect sVEGFR-1 in complexes with other proteins. As the molecular mechanisms involving sVEGFR-1 are not yet fully understood, the potential to detect sVEGFR-1 in complex with other (possibly unknown) proteins may be an

important feature both in terms of unveiling the complex role of sVEGFR-1 and in the diagnosis of MDS.

Conclusions

We demonstrated the SPR biosensor-based detection of sVEGFR-1, the plasmatic protein marker of myelodysplastic syndromes (MDS), via interaction with its high-affinity counterpart VEGF-A, immobilized on the sensor surface. The conditions for the immobilization of VEGF-A and interaction with sVEGFR-1 were optimized in buffer using an SPR sensor system with special dispersionless microfluidics. The attached VEGF-A was found to form a stable layer, with a sufficient number of binding sites demonstrating preserved biological activity. Subsequently, the detection of sVEGFR-1 in 2% human blood plasma was performed. Using the improved surface functionalization with reduced nonspecific adsorption and the sequential injection approach, we achieved the detection limit of 25 ng/mL for sVEGFR-1. This study suggests a model for future protein microarray for MDS diagnosis based on protein–protein interactions under physiological conditions, which could help clarify the molecular pathogenesis of the disease.

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