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Hydrogel-Terminated Photonic Crystal for Label-Free Detection of Angiopoietin-1

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***Abstract*— We report on a label-free, time-resolved biosensing technique based on refractometric measurements. A one-dimensional photonic crystal (1DPC) constituted by a planar stack of dielectric layers is used for supporting Bloch Surface Waves (BSWs). These electromagnetic surface waves probe the aqueous environment within few hundreds of nanometers from the photonic crystal surface, which is functionalized with a hydrogel binding matrix. Therein, selected antibodies are grafted for the specific recognition of the tumoral biomarker Angiopoietin-1. A model bioassay is established demonstrating a detection of Antiopoiegin-1 as low as picomolar concentrations.**

***Index Terms*— photonic crystals, dextran hydrogel, label-free biosensing, surface waves.**

I. Introduction

Surface plasmon resonance (SPR) biosensors represent an established technology for detection and interaction analysis of chemical and biological species [1,2]. It typically relies on heterogeneous assay format with biomolecular recognition elements attached to the sensor surface in order to specifically capture target analyte from analyzed liquid sample. In order to increase binding capacity of the sensor surface, a binding matrix based on carboxymethyl dextran brush exhibiting thickness of about 100 nm was developed [3]. More recently, a dextran-based crosslinked polymer network that can be prepared with much larger thickness was implemented to SPR-based biosensors in order to serve as large binding capacity hydrogel binding matrix [4]. As a result, a significantly improved limit of detection (LOD) was reported by using sensor modalities exploiting hydrogel optical waveguide waves and surface wave-enhanced fluorescence [5,6]. In an attempt to further improve on the sensitivity achieved with SPR-based label-free biosensing, an alternative method based on dielectric photonic crystals sustaining Bloch Surface Waves (BSW) have been proposed [7–9]. BSWs are surface electromagnetic waves that can be coupled at the distal interface of a properly designed dielectric multilayer (also called one-dimensional photonic crystals—1DPC) [10]. Similarly to surface plasmons on flat metallic thin films, BSW can be optically coupled by means of a prism in the so-called Kretschmann configuration and is manifested by a very narrow resonance. In this work, we demonstrate the working principle of a SPR-like bio-affinity assay based on a BSW-sustaining dielectric 1DPC functionalized with a water-permeable hydrogel matrix layer (abbreviated 1DPC), such that the overall number of available receptor sites is significantly increased compared to a planar 2D receptor architecture utilizing surface-attached monolayers.

We test our sensing approach by using a model assay for detection of Angoipoietin-1 (Ang-1). It is established that Ang-1 plays an essential role in tumor progression, however, the exact effects of angiopoietin on tumor angiogenesis is under debate [11]. Experimental and clinical studies have demonstrated that increased expression of Ang-1 promotes or inhibits tumor angiogenesis [12]. Much attention is paid to the detection of such biomarker at low concentrations, because Ang-1 is a pro-angiogenic factor that promotes endothelial cell survival and tumor angiogenesis, especially in the presence of vascular endothelial growth factor. Ang-1 is present in blood of healthy patient with concentrations of 0.1–1.5 ng/ml. In cancer patients the concentration is between 1.5 and 30–60 ng/ml [13].

II. Materials and Methods

The experimental configuration is sketched in Fig. 1a. A stack of alternating dielectric layers made of Ta_2O_5 and SiO_2 is

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evaporated on a glass substrate by means of a plasma ion-assisted deposition under high-vacuum conditions (APS904 coating system from Leybold Optics). Specifically, six layers of alternating Ta_2O_5 and SiO_2 are obtained, with thickness 125 nm and 270 nm respectively. However, the last SiO_2 layer is only 230 nm thick and carries a dextran-based hydrogel layer, which is covalently functionalized with Angiopoietin-1-specific antibody receptors, as specified below (chemical structure in Fig. 1b). Externally, a PMMA microfluidic cell provided with a PDMS retaining ring, an inlet and an outlet channel (not shown), is used to contact the upper surface of the 1DPC with the aqueous solution containing the analytes.

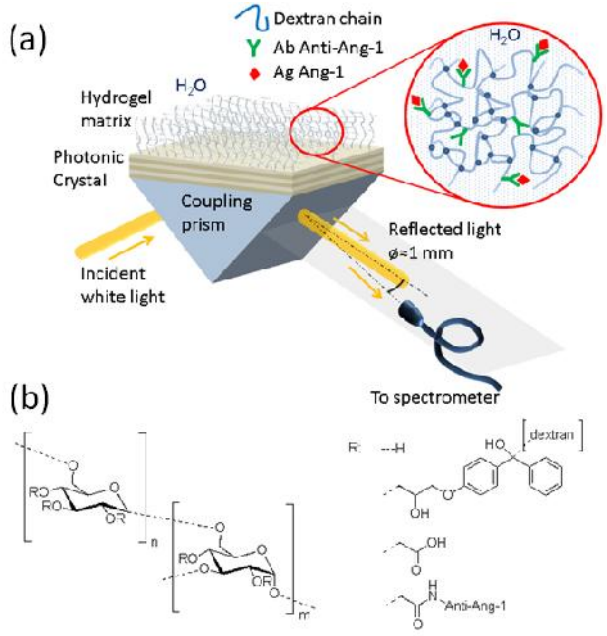

Fig. 1. (a) Sketch of the one-dimensional photonic crystal (1DPC) detection scheme for Bloch Surface Wave (BSW)-assisted detection of Angiopoietin-1 (Ang-1). The spectrometer fiber is angularly displaced apart from the directly reflected beam by about 1 deg. (b) Chemical structure of the dextran-based (branching structure simplified) hydrogel network carrying free carboxyl groups, the photocrosslinking unit (based on benzophenone photochemistry) and the Angiopoietin-1-specific antibody (Anti-Ang-1).

The hydrogel network is formed by a dextran derivative, which contains carboxymethyl groups for further chemical functionalization and benzophenone moieties as a photocrosslinkable unit [14]. The films are prepared on the silica-terminated 1DPC surfaces, by first chemisorbing an organosilane adhesion layer of (4-(3-triethoxysilyl) propoxy benzophenone) from 5 mM ethanolic solution at room temperature for 24 h. After chemisorption, the surfaces are cleaned extensively with ethanol and acetone and dried with nitrogen. Then dextran is deposited by spin-coating of 5% wt dextran-water solution with 2000 rpm for 2 min, obtaining a film thickness of about 150 nm in dry condition evaluated with AFM measurements. The sample is dried at 60 °C overnight and crosslinked by UV light (λ = 365 nm with a dose of 4 J/cm^2).

The -COOH groups in the hydrogel layer are activated with a solution of 0.1 M Sulfo-NHS (N-hydroxysulfosuccinimide) and 0.4 M EDC (1-ethyl-3(3dimethylamino propyl) carbodiimide hydrochloride) in 0.1 M MES conjugation buffer (2-(N-morpholino) ethanesulfonic acid) with 0.9% NaCl at pH 4.7 for 15 minutes. After the hydrogel activation step, a solution 50 μg/ml of specific antibody (Anti-Ang-1—product number A0604) in Dulbecco phosphate buffer saline D-PBS, pH 7.4, 150 mM NaCl, is reacted with the sensor surface for 1 h at room temperature. Then, a rinsing step is performed in order to remove any excess of antibodies and side products.

For the construction of the sensor setup the bottom side of the glass carrying the 1DPC is oil-contacted with a glass prism (refractive index 1.5) and then optically interrogated with an incident quasi-collimated white light beam extracted from a halogen lamp. Detection is performed by means of a fibered spectrometer (Acton SP-300i) equipped with a 2400 g/mm blazed grating. In the standard Kretschmann configuration, a dip in the angular spectrum of reflected beam intensity corresponding to the surface-mode resonance is detected when a mode coupling condition is fulfilled. In the present case, however, the dip corresponding to the BSW might appear as rather shallow, because of the low loss materials constituting the 1DPC [15,16]. In addition, the low collimation level of the white light beam might produce a smearing of the resonance dip due to the well-known convolution effect between the resonance and the incident beam angular profile.

In order to overcome this effect, the detection is performed by collecting the radiation that is resonantly scattered according to a BSW-driven effect. It is well-known that the power scattered by dielectric multilayers is greatly enhanced when the illumination angle is such that a resonantly coupling to the guided modes supported by the structure occurs [17]. In the case of BSW coupling, the surface defects of the 1DPC and the overlying hydrogel layer can scatter light which finally leaks out of the prism at the very same incidence angle. We placed the spectrometer input fiber at the leakage angle, with an additional lateral displacement (about 1 mm) that is sufficient to exclude the directly reflected beam. Practically, the spectrometer fiber is angularly separated from the reflection direction by about 1 deg. (see Fig.1(a)). In such a configuration, a peak rising from an almost zero background can be easily identified. At increasing distances (>1 mm) of the spectrometer fiber, the scattered light shows a decrease in intensity, the spectrum shape being preserved.

This peak is the spectral signature of the BSW resonance used to monitor the refractive index changes occurring within the hydrogel matrix.

An angular-resolved spectral map of the scattered radiation $S(\lambda,\theta)$ can be collected in an off-axis θ–2θ goniometric configuration [18] once the hydrogel-functionalized 1DPC is contacted to the microfluidic cell filled with recirculating water. Here, θ is the incidence angle of light at the bottom side of the glass substrate and λ is the corresponding wavelength. After few minutes of rinsing any remaining non-crosslinked residues are completely removed from the hydrogel matrix and the system stabilizes in such a way that measurements can be performed. The here demonstrated 1DPC sensor structure can sustain BSW at the swollen hydrogel/water interface,

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wherein the BSW electric field vector is parallel to the planar surface of the 1DPC. For this reason, the illumination is provided as an s-polarized light. The BSW dispersion curve is observed as a narrow peak of scattered light that is spectrally dispersed at angles larger than the glass/water critical angle (Fig. 2a). At fixed incidence/collection angles, the detected scattering peak has a spectral width of about 3 nm as the mode-assisted scattering peak is not dependent on the angular spread of the illumination radiation.

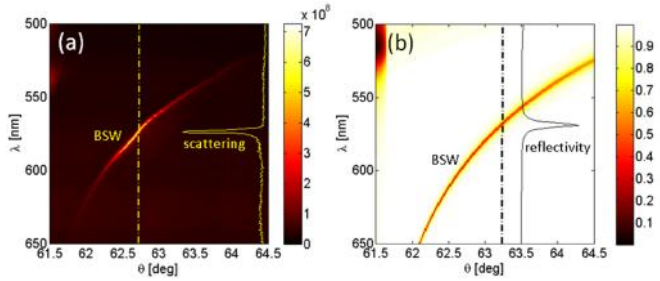

Fig. 2. (a) Measured scattering map S(λ,θ) with white light illumination in Kretschmann θ–2θ configuration. Scattering intensity is in arbitrary units. (b) calculated reflectivity map R(λ,θ) of the photonic crystal, as illuminated from the glass substrate. In both cases, incident light is s-polarized.

For comparison, the s-polarized reflectivity map R(λ,θ) can be calculated with a transfer matrix algorithm [19]. The refractive index of the hydrogel in the swollen state in water was estimated as 1.348 based on previous work [6]. It should be noted that this value may be affected by variations in the irradiation dose for crosslinking. In addition, a fictitious absorption coefficient as high as k=2e-4 has been given to the last layer of the stack. The value introduced here is overestimated as compared to previous works [20] in order to take into account the scattering losses by the hydrogel and enhance the visibility of the BSW dispersion in the reflectivity map. The best match with experimental data is obtained for a calculated thickness of the swollen hydrogel layer of roughly 500 nm in water. A reflectivity dip dispersed in a spectral range between 500 nm and 650 nm is then obtained, according to the same dispersion of the scattered peak experimentally measured (Fig. 2b).

Similarly to SPR, when the refractive index of the outer medium close to the 1DCP surface is increased, the BSW resonance shifts toward longer wavelengths. In our working conditions, the hydrogel matrix is able to fully contain the evanescent tail of BSW penetrating into the outer medium (water). As a result, the refractive index changes occurring in the hydrogel matrix due to the uptake of target molecules will strongly perturb the BSW and result in a large spectral shift of the resonance. As the sensitivity to refractive index changes within the hydrogel matrix is concerned, we estimated about 2200 nm/RIU by calculations. Although the sensitivity expressed in nanometers/RIU is generally smaller as compared to the SPR case, BSWs show a larger figure of merit that provides several advantages, including a smaller measurement uncertainty.

III. Results and Discussion

In order to detect Ang-1 the 1DPC sensor was equipped with the Anti-Ang-1-modified hydrogel matrix, as discussed above. Recognition experiments are carried out in a microfluidic cell with the specific target Ang-1 (product number SRP3007) and with Vascular Endothelial Growth Factor (VEGF-A—product number SRP4364) antigen, as negative control. Before the experiment starts all the microfluidic system is passivated with a bovine serum albumin (BSA) solution, to avoid non specific adsorption of analyte prior reaching the sensor surface. Real time experiments on the Anti-Ang-1-modified hydrogel is also carried out with a VEGF-A antigen, non-specific for the employed antibody (A0604), by using exactly the same incubation conditions employed for Ang-1 binding reaction. All the solutions are made using double deionized water (dd-H_2O). The predicted isoelectric point for both the antigen Ang-1 and VEGF-A is pI = 6.3, therefore during the experiment the species are neutral or at least positively charged, while the dextran hydrogel is negatively charged.

In order to set the incubation time for Ang-1, an ELISA-like assay has been performed. The Anti-Ang-1 modified hydrogel has been exposed to Ang-1 at 0.5 ng/ml concentration, then a biotinylated 2nd Anti-Ang-1 has been incubated in order to specifically bind to the Ang-1 previously immobilized. Finally, a Streptavidin-HRP adsorption and a Luminol activation for Chemiluminescence have been performed. Upon analysis of the ElectroChemiLuminescent (ECL) images (not shown here), an incubation time of around 60 minutes have been defined as the best compromise between Ang-1 surface coverage and homogeneity.

As the measurement starts, the spectral position of the BSW-assisted scattering peak is continuously monitored (integration time 10 s) through the spectrometer. We operated at an incidence angle θ=63 deg, corresponding to a resonance peak at about λ=570 nm. After a stable baseline is recorded, a solution containing the analyte is injected into the microfluidic cell and made recirculating for 30 min. Then, a rinsing step is performed. A tubing and valve system allows to directly switch between Ang-1 injection (and recirculation) and rising without contacting the hydrogel to air at any moment. In Fig. 3, an illustrative sensorgram for the sequential injection of three concentrations of Ang-1 is presented (7 pM, 14 pM, 70 pM), corresponding to 0.5 ng/ml, 1 ng/ml, 5 ng/ml, inducing a gradual increase of refractive index and associated spectral red-shift of the BSW resonance (Δλ) related to the Ang-1 sample concentrations. Firstly, water is flowed over the 1DPC surface in order to establish a stable baseline. Afterwards, 250 μl aqueous solution of Ang-1 is injected for 30 min. At the injection, it can be observed that the initial part of the curve shows a steep increase, due to the refractive index change of the liquid (bulk effect). The bulk shift is small and proportional to the antigen concentration. In the second part the curve shows a kinetics, which indicates an association interaction between the antigen and the surface. This gradual increase in the BSW resonance position (spectral redshift), is

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due to a refractive index change induced by the antigen binding within the hydrogel. After 60 minutes, according to the incubation time set in the ELISA-like assay, the buffer is flowed again on the sensor surface to rinse any excess of Ang-1. Rinsing is performed for at least 40 minutes. This step is characterized by a decrease of the refractive index due to the removal of non-specifically bound Ang-1 and requires more time as the antigen concentration increases. After this washing step the spectral shift stabilizes.

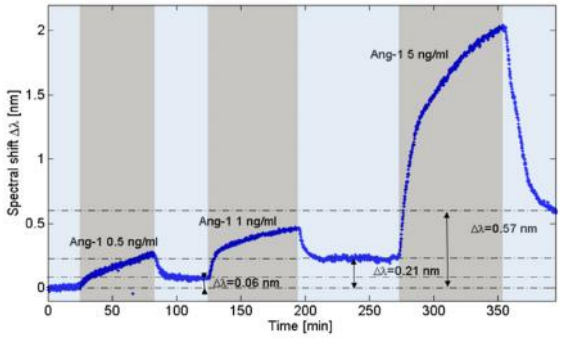

Fig. 3. Illustrative time-resolved sensorgram related to the incubation and binding of Ang-1 at sequential concentrations of 0.5 ng/ml, 1 ng/ml, 5 ng/ml. Darker bands correspond to antigen incubation periods.

In order to demonstrate the specificity of the proposed biosensing approach, a negative control has been set up. VEGF-A and Ang-1 are both potent pro-angiogenic factors. VEGF-A could represents an interferent of Ang-1 in standard ELISA assays. VEGF-A and Ang-1 possess a very similar molecular mass, 40 and 60 KDa, respectively [21,22]. Here, we inject a VEGF-A solution at 1 ng/ml concentration followed by an injection of Ang-1 by the very same amount. As the exemplary sensorgram in Fig. 4 demonstrates, no residual BSW spectral shift due to VEGF-A presence is observed, while Ang-1 produces a shift comparable with previous observations (see Fig. 3), due to specific binding with the immobilized antibody. We may argue that the difference in the observed spectral shifts is probably due to some lack of reproducibility of the hydrogel binding matrix on the multilayer.

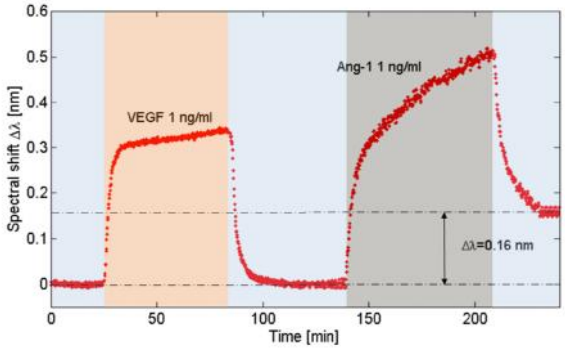

Fig. 4. Illustrative time-resolved sensorgram related to the incubation of a Vascular Endothelial Growth Factor (VEGF)-A (negative control) and Ang-1 at a concentration of 1 ng/ml. Contrary to Ang-1, VEGF-A results in a rather negligible spectral shift, meaning that no specific binding is observed.

IV. Conclusion

In conclusion, a refractometric biosensor based on surface waves (BSW) coupled on hydrogel-modified dielectric multilayers (1DPC) is demonstrated for real-time, label-free detection of the Ang-1 tumoral biomarker. The photonic structure is constituted by a stack of dielectric layers tailored with a functional dextran hydrogel layer, carrying specific receptors for the analyte. Because of the hydrogel swelling in water, a large number of receptors are efficiently exposed to the target molecules diffusing in the matrix. In accordance with an SPR-based scheme, the BSW resonance shift is tracked to measure refractive index variations occurring within the hydrogel matrix. The proposed working principle is successfully demonstrated in real-time detection of picomolar concentration of Ang-1. As the use of hybrid organic/inorganic materials is largely exploited in high-sensitivity label-free bio-sensing [23,24], our approach based on three-dimensional functional hydrogel can be applied to a number of different geometries including optical fibers and waveguides.

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