Self-Assembled Plasmonic Sensor Platforms: a Promising Approach for Monitoring Enzymatic Degradation of Thin Gelatin Layers

Markus Weiler and Stefan B. Quint
Department of Biosystems and New Materials,
Max Planck Institute of Intelligent Systems
Stuttgart, Germany

Supratim Basak and Claudia Pacholski
Institute of Chemistry, University of Potsdam
Potsdam, Germany
e-mail: cpachols@uni-potsdam.de

***Abstract* - Plasmonic sensors based on periodic hole arrays in metallic films are investigated for monitoring enzymatic degradation of extracellular matrix. Sensors are fabricated using a bottom-up strategy in which soft colloidal lithography is combined with chemical gold film deposition. The resulting plasmonic sensors show extraordinary transmission of light supported by surface plasmon polaritons. These are highly sensitive to refractive index changes in close proximity to the sensor surface and are utilized for the quantitative detection of extracellular matrix degradation.**

Keywords - Optical sensor; bottom-up; enzymes; surface plasmon resonance; real-time monitoring.

I. INTRODUCTION

Qualitative and quantitative detection of biomolecular interactions, such as DNA hybridization, antigen/antibody recognition, as well as enzymatic reactions is nowadays routinely achieved by optical biosensors (overview in [1]). These sensor platforms are composed of a recognition layer providing specificity for the target analyte and a transducer converting the recognition events into a measurable optical signal.

A prominent transduction method exploited in these sensors is Surface Plasmon Resonance (SPR) – a resonant oscillation of conduction band electrons occurring at a dielectric/metal interface (reviewed in [2]). The resonance wavelength of SPR depends on the refractive index of the environment in proximity to the metal surface. SPR can be subdivided in Propagating Surface Plasmon Resonance (PSPR) and Localized Surface Plasmon Resonance (LSPR). The latter, LSPR, occurs in metallic nanostructures – especially nanoparticles - and can be directly excited by light. A vital difference between LSPR and PSPR is the field decay length of the SPR. Whereas LSPR in gold nanoparticles can detect refractive index changes only several nanometers (maximal 10 nm – 20 nm) above the gold surface, PSPR are sensitive to refractive index changes several 100 nm above the gold film. However, PSPR cannot be directly excited. In order to match the momentum of incoming light to that of the plasmon, commercial available SPR biosensors employ the so-called Kretschmann configuration in which a thin gold film on a prism is used. Another way to generate SPPs is to introduce a period structure (grating) into the gold film. It has been reported that periodic hole arrays in metallic films show an extraordinary transmission of light (EOT), meaning that more light is transmitted through the sub-wavelength holes than expected from Bethe's law. EOT has been traced back to the excitation of PSPR by the grating [3].

Sensors based on nanohole arrays could offer several advantages in comparison to SPR sensor based on thin metal films on prisms, such as more convenient experimental geometries, higher spatial resolution and greater reproducibility [4]. For example, the presented plasmonic sensor can be operated in transmission mode and therefore could be incorporated in multi-well plates. Thus, multiplexing in combination with the advantages of PSPR sensors could be exploited for detecting target analytes, determining equilibrium binding constants or enzymatic activities. Due to these tremendous advantages over commercial PSPR sensors, optical sensors based on this technology have already been used for the detection of biomolecular interactions in a proof-of-concept study [5]. However, the full potential of these sensor platforms has not been tapped.

Two challenges can be identified for this. On the one hand plasmonic sensors based on hole arrays in metallic films have a lower sensitivity than thin gold films investigated in Kretschman configuration. In order to increase the sensitivity of the hole array sensors, different approaches have been suggested including the deposition of fluorescent material [6] or metallic nanoparticles in the holes [7] of the perforated metal film. On the other hand - on the background of the numerous promising applications - it seems evident that simple, fast and inexpensive techniques to fabricate hole arrays are needed to make them accessible to the many research groups who could benefit from such devices. However, periodic arrays of nanoholes in opaque metal films are commonly fabricated by focused ion beam milling or electron beam lithography. These techniques are expensive and provide only small nanostructured areas at low throughput. An alternative approach is photolithographic fabrication but it suffers from the complicated production of a master and/or the requirement to work in a clean-room.

Only a few attempts have been made to use simpler approaches, like colloidal nanolithography, to fabricate hole arrays in metallic films. However, even in these approaches

 ISBN: 978-1-61208-660-6

expensive equipment, such as reactive ion etching machines were used. Recently, our group reported a novel strategy to produce sub-wavelength hole arrays in metallic films, which is solely based on simple chemical techniques [7]. The procedure, which is based on soft colloidal nanolithography and electroless plating, has great advantages over standard fabrication procedures as it allows high throughput, can be easily applied to areas of square centimeters, and requires only standard lab equipment. Furthermore, this method might enable the deposition of periodic hole arrays in metallic films in multiwell plates.

The sensors fabricated by utilizing this 'pure' bottom-up approach showed comparable sensitivity to refractive index changes as nanohole arrays in metal films prepared by more sophisticated and expensive methods. Furthermore, the adsorption of bovine serum albumin to the sensor surface and protein A/IgG interactions could be monitored in real-time using these self-assembled plasmonic sensors. Highly sensitive detection of biomolecular interactions has also been reported for nanohole arrays in metallic films produced by electron beam lithography [5].

However, sensors based on periodic hole arrays in metallic films have not been investigated for determining enzymatic activity – to the best of our knowledge. This fact is quite surprising as biosensors for detecting enzymatic activity were and still are in the focus of biosensor research [8]–[10]. The high interest in this topic is based on the crucial role that certain enzymes (proteases) play in physiological processes, e.g., matrix metalloproteases in cancer development – degradation of extracellular matrix (ECM) [11]–[13]. Consequently, detection of enzymatic activity using biosensors which exploit surface plasmon resonance as transduction method has already been reported. Here, either thin gold films deposited on prisms (Kretschmann configuration) [14] or gold nanoparticles on solid substrates were utilized [15].

In this contribution, we report on the bottom-up fabrication of an optical sensor composed of a periodic hole array in a metallic film, which allows for monitoring enzymatic activity in real-time. The paper is structured in the following way: In Section I an introduction to the topic is given. Afterwards, experimental details on the preparation and characterization of the presented plasmonic sensor platforms are described in Section II. Obtained scientific results on the fabrication and optical behavior of the plasmonic sensors, as well as their sensing performance are presented and discussed in Section III. Finally, the content of the paper is summarized in Section IV (conclusions).

II. EXPERIMENTAL SECTION

A. Materials & Methods

All chemicals were used as received without any further purification except of *N*-isopropylacrylamide (NIPAM), which was purchased together with (3-amino-propyl)triethoxysilane (APTES) from Acros Organics. NIPAM was recrystallized from hexane before synthesizing microgels. Tetrachloroauric(III) acid trihydrate, hydroxyl-amine hydrochloride, and sodium citrate were obtained from Sigma Aldrich. Ethanol, *N,N'*-methylene(bisacrylamide) (BIS), hydrochloric acid (fuming), sulfuric acid (96%), calcium chloride dihydrate, glycerol, N-2-HydroxyEthyl Piperazine-N'-2-Ethane Sulphonic acid (HEPES) and glass cover slips (20 × 20 mm or 24 × 24 mm) were received from Carl Roth. Nitric acid (65%) and hydrogen peroxide (30%) were supplied by Merck. Potassium peroxodisulfate (KPS) was obtained from Fluka. Methanol was purchased from BASF. Collagenase, Type III from *Clostridium histolyticum* was purchased from Merck. Water was deionized to a resistance of at least 18.2 MΩ using an Ultra-Pure Water System (TKA, Germany).

Transmission spectra were recorded with a Cary 5000 UV-VIS-NIR spectrometer (Varian, USA) at normal incidence using unpolarized light.

Scanning electron micrographs were taken with a Zeiss Ultra 55 "Gemini" scanning electron microscope.

Atomic force microscopy (AFM) was carried out with a device from Asylum research (MFP-3D-BIO). Commercially available silicon cantilever (Olympus, spring constant 0.02 N /m, resonance frequency 11 kHz) were used for the measurements.

B. Sensor fabrication

Plasmonic sensors were prepared according to Quint and Pacholski [7]. In this fabrication method, poly-*N*-isopropylacrylamide (polyNIPAM) beads were employed for colloidal lithography, previously synthesized by aqueous dispersion copolymerization of NIPAM with BIS in the presence of sodium dodecyl sulfate as described elsewhere [16]. Prior to use, the bead dispersion was purified by centrifugation and filtration.

Microscope cover slips were utilized as substrates. To clean them and provide a hydrophilic surface, the glass cover slips were immersed in piranha solution (concentrated sulfuric acid : hydrogen peroxide 3 : 1, v : v) for 1.5 h followed by thorough rinsing with ultra-pure water. The cleaned cover slips were stored in deionized water from 1 to 24 h and were blown dry with nitrogen just before the deposition of polyNIPAM beads.

Deposition of polyNIPAM bead arrays onto glass substrates was achieved by spin coating. Spin coating was carried out using a Laurell WS-400A-6NPP Lite spin coater (Laurell Technologies Corporation, North Wales). Cleaned glass substrates were mounted in the spin coater and a 40 µl droplet of diluted polyNIPAM bead dispersion was placed in the center of the substrate. Subsequently, 5 µl of ethanol were added to facilitate self-assembly of polyNIPAM beads in hexagonal patterns and to obtain a complete substrate wetting. Subsequently, the sample was rotated for 6 min at 500 rpm followed by 1 min at 6000 rpm using an acceleration of 100 rpm/s in both cases. This procedure yielded high ordered arrays of polyNIPAM beads on the glass substrate surface.

In order to allow a binding of gold colloids to the substrate, the glass surface was functionalized with amino groups. To do this, previously spin coated samples were encased in a standard exsiccator together with a small dish containing 40 µl of APTES. The exsiccator was then

 ISBN: 978-1-61208-660-6

evacuated until a pressure of 0.3 mbar was reached. The samples were kept under these conditions for 1 h to allow a dense silanization of the glass surface. Afterwards the samples were incubated for 1 h in a drying oven at 80 °C. Subsequently, the sensor surface was wetted with a ~ 12 nM colloidal gold solution [17] and incubated at 40 °C for 30 min to allow gold nanoparticle binding to the free amino groups. Afterwards the samples were rinsed with ultra-pure water.

In the next step, a gold film was formed by electroless deposition. For this purpose, the samples were immersed in an aqueous solution of 0.4mM hydroxylamine hydrochloride and 0.5% $HAuCl_4 \cdot 3H_2O$ [18]. The solution was agitated on a vibrating table to ensure the formation of a homogenous gold film. After 1 h the samples were rinsed with water and blown dry under a nitrogen stream. Finally, the colloidal mask was removed by flame annealing in a propane/butane flame which leads to pyrolysis of the polyNIPAM beads. In an alternative approach, the colloidal mask was removed by ultrasonication after the deposition of gold colloids.

C. Sensor performance

1) Deposition of gelatin layer

Spin-coating was employed for obtaining thin films of gelatin. Here, 100 µL of a warm solution of gelatin in ultra-pure water (50 °C) was dropped on a 20 × 20 mm glass substrate, which was rotated at 6000 rpm at this moment and for a further minute after applying the gelatin solution. Thereby, complete drying of the gelatin layer was ensured. In order to control the thickness of the deposited gelatin layer, different concentrations of gelatin in solution were investigated. Moreover, glutardialdehyde was added to the gelatin solution prior to spin-coating in order to cross-link the gelatin. For example, warm gelatin solution (2.5 wt% gelatin) was mixed with 0.5 wt% glutardialdehyde (final concentration) and then immediately spin-coated onto cleaned glass substrates.

2) Monitoring enzymatic reactions

Gelatin coated sensors were mounted in a custom-made flow cell and exposed to buffer solution (50mM HEPES, 10 mM $CaCl_2$, 5% glycerol, pH 7.4). After establishing a constant baseline, the buffer solution was replaced by a solution of collagenase in buffer. Different concentrations of collagenase were tested. Transmission spectra were recorded every 30 s and the position of the transmission minimum in each spectrum was determined by a fitting routine. Briefly, to extract the positions of the transmission minimum, we have used a Matlab script which first determined the absolute minimum. This minimum was taken as starting point. Then, we chose 40 data points, which surrounded this minimum, and fitted these points with the polyfit Matlab function with a degree of n = 2 ($f(x) = ax^2 + bx + c$).

III. RESULTS & DISCUSSION

A. Sensor fabrication and characterizaion

Three major steps are involved in the presented fabrication method for periodic hole arrays in metallic films (Figure 1). In the first step, a lithographic mask is prepared on a glass cover slip. In contrast to previous colloidal lithographic approaches, polyNIPAM beads are used for the mask production. This allows for directly creating loosely packed, hexagonally ordered arrays of microspheres as polyNIPAM undergoes a reversible phase transition from a swollen to shrunken state upon drying. This property is very advantageous as it supersedes any further treatment of the mask, which is necessary in conventional colloidal nanolithography approaches. The obtained two dimensional hexagonal array can be readily used as a lithographic mask for the fabrication of hole arrays.

In the following step, a homogenous gold film is deposited by electroless plating. The reaction requires Au(0) as nucleation seeds for the chemical reduction of dissolved Au(III) [18]. Thus, the glass surface is functionalized with 3-(aminotriethoxy)silane and subsequently decorated with gold colloids, which serve as seeds for the growth of a homogenous gold-layer by electroless plating. Removal of the colloidal mask was achieved by thermal treatment or ultrasonication. This fabrication process has already been published [7].

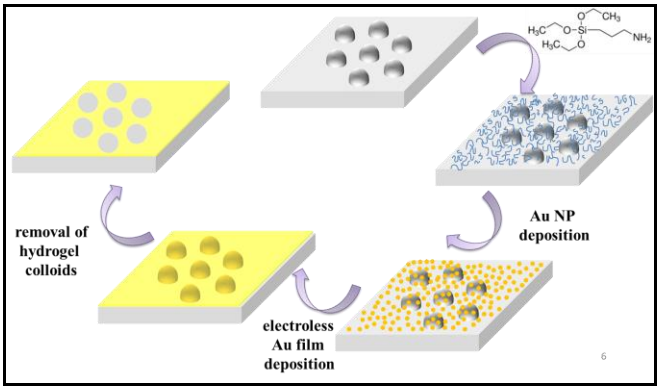

Figure 1. Major steps involved in the fabrication of sub-wavelength holes in gold films.

In Figure 2, a representative scanning electron microscopy (SEM) image of a periodic hole arrays in a gold film, prepared by the previously presented method, is displayed. On the first glance a quasi-hexagonal order of the holes can be noticed. To provide a quantitative measure for the lattice constant of the hole array, we have calculated the radial distribution function by analyzing several SEM images [19].

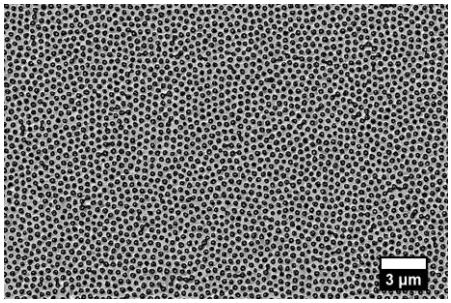

Figure 2. Representative SEM image of a prepared periodic hole array in a metallic film.

For this purpose, we have read out the positions of the holes in Cartesian coordinates with the help of the software ImageJ. In a second step, the pairwise distance r_{ij} of all colloids and then the radial distribution density g (r) was calculated as follows:

$$g(r)=\frac{1}{2\pi r \Delta r \rho}\sum_{i=1}^{N}\sum_{j>i}^{N}\chi(r_{ij}-r) \quad (1)$$

where N is the total number of colloids in the image, ρ the density per µm^2, Δr is the step size and r the considered colloid distance. χ is defined as:

$$\chi(r_{ij}-r)=\begin{cases}1, if\ |r_{ij}-r|\le\Delta r\\ 0, if\ |r_{ij}-r|>\Delta r\end{cases} \quad (2)$$

The function g (r) thus describes the mean number of colloids in the distance r + Δr, which is found from any colloid. The first maximum in the radial distribution function corresponds to the lattice constant, which was determined to be (470 ± 100) nm in the shown SEM image.

A transmission spectrum of this hole array in a gold film is presented in Figure 3. The spectrum is characterized by two transmission maxima, which can be assigned to the (1,0) gold,glass resonance at ~1200 nm and to the (1,0) gold,air resonance at ~800 nm using the following equation [20]:

$$\lambda_{SPP}=\frac{a_0}{\sqrt{\frac{4}{3}(n^2+nm+m^2)}}\sqrt{\frac{\varepsilon_m\varepsilon_d}{\varepsilon_m+\varepsilon_d}} \quad (3)$$

Where λ_{SPP} is the resonance wavelength, a_0 is the lattice constant, n as well as m are integer numbers, and ε_m and ε_d are the dielectric constants of the metal and the dielectric medium, respectively.

The horizontal line in the spectrum indicates the open area fraction of the particular hole array, determined by the software ImageJ. As the open area fraction is lower than the transmission of the (1,0) Au,glass resonance of the hole arrays, extraordinary transmission of light is observed.

We have determined the sensitivity of the fabricated hole arrays by immersion of the sensors in solutions with different refractive indices. The position of the transmission minimum in the spectrum was evaluated, which can be found at ~ 600 nm in air and is particularly sensitive. The position of this minimum shifted linearly to longer wavelengths with increasing refractive indices of the immersion medium. It shows a sensitivity of 540nm / RIU and is in the same sensitivity range as published for hole arrays fabricated by conventional lithography. Hence, the presented method for preparing periodic hole arrays in metallic films is highly attractive for mass production, as it is simpler as well as more cost- and time-efficient in comparison to approaches using electron beam lithography or focused ion beam milling. Moreover, the presented bottom-up strategy is capable to nanostructure large areas in the range of cm^2.

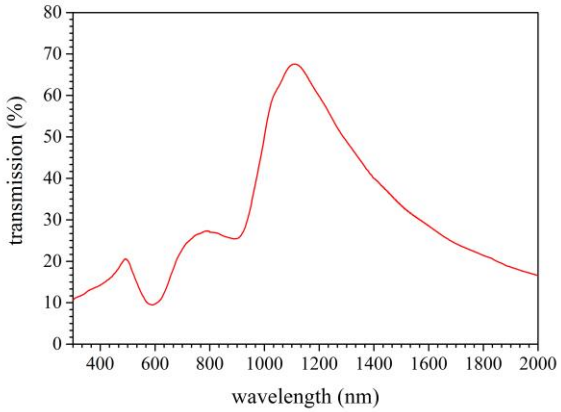

Figure 3. Transmission spectrum of a fabricated optical sensor.

B. *Deposition of ECM layer*

In order to obtain thin films of gelatin (= ECM), we applied spin-coating. The thickness of gelatin films can be adjusted by varying the gelatin concentration of the gel. As it can be seen from Figure 4, film thickness is an exponential function of the gelatin concentration, when temperature, spinning speed, and suspension volume are kept constant.

To determine the gelatin film thickness we scratched a groove into the film and measured the step height using a white light interferometer. Each data point in Figure 4 coressponds to 6 measured thickness values.

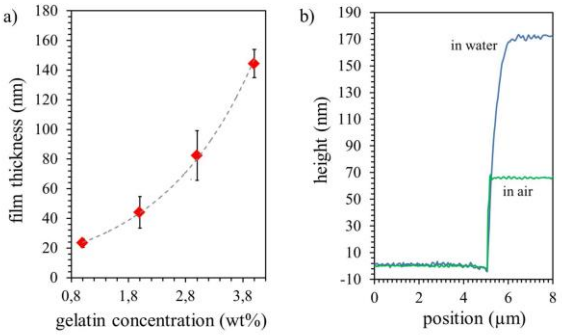

Figure 4. Gelatin films on sensor surface. a) gelatin film thickness in dependence of the gelatin concentration in the soultion used for spin-coating. b) Gelatin film thickness in air and in water determined using atomic force microscopy (AFM).

Gelatin is a network of collagen filaments comprising hydrophilic protein chains. Therefore, gelatin is a highly absorbent polymer matrix, which swells upon immersion in water. The swelling of gelatin depends on the crosslinking density of the gel. Not crosslinked gelatin films can increase roughly tenfold in film weight due to water uptake when

 ISBN: 978-1-61208-660-6

stored in a buffer solution. The swelling ratio is important for us because of two reasons. First of all, we would like to tune the thickness of the film in such a way that it matches the penetration depths of the surface plasmons of our sensor. The surface plasmons are only sensitive to changes in the refractive index which are within their penetration depth, which is roughly 100 nm at the resonance minimum we consider for following refractive index changes. If we know the swelling ratio, we can easily estimate the thickness of the film in water after measuring the thickness in air. A measurement in air is much easier to perform than in water, but can give us only relevant information when we know the swelling ratio. Second, the swelling ratio will also have an effect on the refractive index of the film. The more water the film takes up the more the refractive index will be shifted towards that of water. Also, this has an effect on the sensitivity of our sensor.

A way to affect the swelling ratio of gelatin films is cross linking by GluTardiAldehyde (GTA). Therefore, we measured the Swelling Ratio (SR) for different contents of GTA, which were added prior to spin-coating. We used AFM to measure the height profiles of gelatin films in air and in water, and to determine the swelling ratio of thin gelatin films, which is simply the thickness in water divided by the thickness in air. Figure 4 b) shows two typical height profiles for a gelatin film in air and water. A SR of ~2 was determined by using this method in accordance with published values [21].

C. Monitoring ECM degradation

For the degradation experiments the sensor surface was coated with a gelatin layer, which has a thickness of (57 ± 8) nm in the dry state (determined by white light interferometry). Figure 5 shows transmission spectra of a sensor, before and after coating the sensor surface with gelatin, recorded in air.

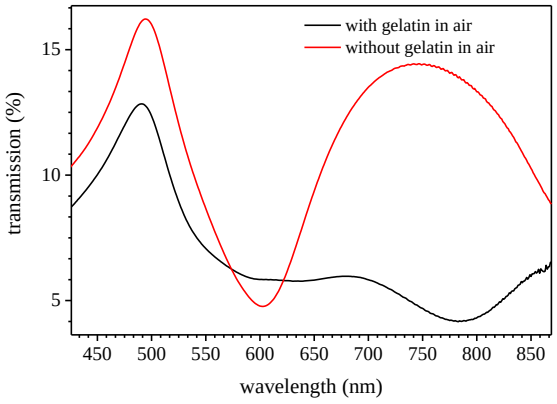

Figure 5. Transmission spectra of a sensor before and after coating with gelatin.

The spectrum of the bare sensor is characterized by a transmission maximum at ~760 nm which can be assigned to the (1,0) gold,air resonance. After coating the sensor with gelatin this maximum shifts to longer wavelengths due to the increase of the refractive index at the sensor surface. In this case, the maximum is close to the wavelength region in which water absorption interferes with measuring reliable spectra for determining the sensor response to refractive index changes. Therefore, we have chosen to monitor the position of the transmission minimum located at ~800 nm after coating the sensor with gelatin.

To carry out the degradation experiments the sensor was mounted in a custom-made flow cell and rinsed with HEPES buffer at 37 °C. Transmission spectra of the sensor were recorded throughout the whole degradation experiment every 30 s. In Figure 6, the position of the transmission minimum on the wavelength scale is plotted versus time. First, a stable baseline in buffer was established for each sensor. Then, at 0 min, a solution composed of collagenase in HEPES buffer was added. An enzyme concentration of 1 units/mL led to a quasi-linear decrease in the peak position until after about 180 min the signal remains almost unchanged. In this case, the same results were obtained under flow and static conditions (red circles and black squares, respectively).

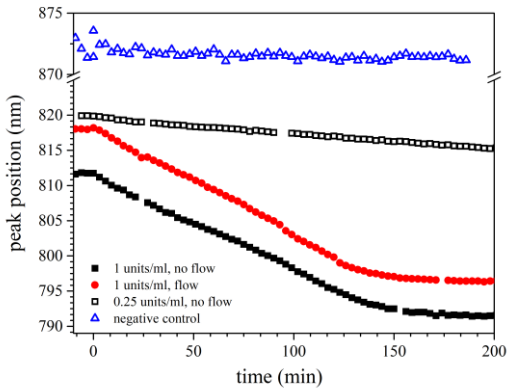

Figure 6. Real-time monitoring of enzymatic activity. At 0 min the enzyme was added to the buffer solution.

If the concentration of the enzyme in the solution was lower (0.25 units/mL) the degradation reaction was much slower – as expected. A negative control experiment was carried out by injecting only HEPES buffer at 0 min into the flow cell. In this case, no degradation of the gelatin layer could be detected by monitoring the transmission minimum position (open blue triangles). Hence, a first proof-of-concept for utilizing periodic hole arrays in metallic films was demonstrated. The enzymatic activity was reliably determined, but the sensitivity of the sensor should be improved for being capable of detecting smaller amounts of enzymes. For this purpose, it is planned to replace the gelatin layer with a more sophisticated layer mimicking ECM.

IV. CONCLUSIONS

In summary, periodic hole arrays in metallic films were prepared using a bottom-up strategy and successfully tested

 ISBN: 978-1-61208-660-6

as optical sensors for monitoring enzymatic degradation of gelatin layers in real-time.

Fabrication of sensor platforms was achieved by combining soft colloidal lithography with subsequent chemical deposition of a gold film. The resulting periodic hole array in a gold film showed extraordinary transmission of light facilitated by the excitation of surface plasmon resonance by the grating and leading to distinct transmission maxima and minima in their optical spectrum. Surface plasmon resonance is confined to the sensor surface and probes only refractive index changes occurring with a maximum distance of approximately 100-200 nm from the gold film (for the reported sensor). These characteristics of the prepared sensors were successfully exploited for detecting enzymatic activity by following the position of a transmission minimum on the wavelength scale in the optical spectrum.

ACKNOWLEDGMENT

This project was funded by the VolkswagenStiftung, the Heidelberg Academy of Sciences and Humanities, and the Max Planck Society. In addition, C. Pacholski is grateful for funding by the DFG (Heisenberg fellowship / project number: 286735196).

REFERENCES

[1] X. Fan et al., "Sensitive optical biosensors for unlabeled targets: A review," *Anal. Chim. Acta*, vol. 620, no. 1–2, pp. 8–26, Jul. 2008.

[2] J. Homola, S. S. Yee, and G. Gauglitz, "Surface plasmon resonance sensors: review," *Sens. Actuators B Chem.*, vol. 54, no. 1–2, pp. 3–15, Jan. 1999.

[3] T. W. Ebbesen, H. J. Lezec, H. F. Ghaemi, T. Thio, and P. A. Wolff, "Extraordinary optical transmission through sub-wavelength hole arrays," *Nature*, vol. 391, no. 6668, pp. 667–669, Feb. 1998.

[4] A. De Leebeeck et al., "On-Chip Surface-Based Detection with Nanohole Arrays," *Anal. Chem.*, vol. 79, no. 11, pp. 4094–4100, Jun. 2007.

[5] A. G. Brolo, R. Gordon, B. Leathem, and K. L. Kavanagh, "Surface Plasmon Sensor Based on the Enhanced Light Transmission through Arrays of Nanoholes in Gold Films," *Langmuir*, vol. 20, no. 12, pp. 4813–4815, Jun. 2004.

[6] S. Wu, P. Guo, W. Huang, S. Xiao, and Y. Zhu, "Dielectric Thickness Detection Sensor Based on Metallic Nanohole Arrays," *J. Phys. Chem. C*, vol. 115, no. 31, pp. 15205–15209, Aug. 2011.

[7] S. B. Quint and C. Pacholski, "A chemical route to sub-wavelength hole arrays in metallic films," *J. Mater. Chem.*, vol. 19, no. 33, pp. 5906–5908, 2009.

[8] Y. Zhou, S. Jiang, S. Krause, and J.-N. Chazalviel, "Biosensor Arrays Based on the Degradation of Thin Polymer Films Interrogated by Scanning Photoinduced Impedance Microscopy," *Anal. Chem.*, vol. 79, no. 23, pp. 8974–8978, Dec. 2007.

[9] C. Sumner, A. Sabot, K. Turner, and S. Krause, "A Transducer Based on Enzyme-Induced Degradation of Thin Polymer Films Monitored by Surface Plasmon Resonance," *Anal. Chem.*, vol. 72, no. 21, pp. 5225–5232, Nov. 2000.

[10] B. Schyrr, S. Boder-Pasche, R. Ischer, R. Smajda, and G. Voirin, "Fiber-optic protease sensor based on the degradation of thin gelatin films," *Sens. Bio-Sens. Res.*, vol. 3, pp. 65–73, Mar. 2015.

[11] L. A. Liotta et al., "Metastatic potential correlates with enzymatic degradation of basement membrane collagen," *Nature*, vol. 284, no. 5751, pp. 67–68, Mar. 1980.

[12] M. A. Moses et al., "Increased incidence of matrix metalloproteinases in urine of cancer patients," *Cancer Res.*, vol. 58, no. 7, pp. 1395–1399, Apr. 1998.

[13] R. A. Cairns, R. Khokha, and R. P. Hill, "Molecular mechanisms of tumor invasion and metastasis: an integrated view," *Curr. Mol. Med.*, vol. 3, no. 7, pp. 659–671, Nov. 2003.

[14] A. Shoji, M. Kabeya, and M. Sugawara, "Real-time monitoring of matrix metalloproteinase-9 collagenolytic activity with a surface plasmon resonance biosensor," *Anal. Biochem.*, vol. 419, no. 1, pp. 53–60, Dec. 2011.

[15] S.-H. Jung et al., "Rapid analysis of matrix metalloproteinase-3 activity by gelatin arrays using a spectral surface plasmon resonance biosensor," *The Analyst*, vol. 135, no. 5, p. 1050, 2010.

[16] R. H. Pelton and P. Chibante, "Preparation of aqueous latices with N-isopropylacrylamide," *Colloids Surf.*, vol. 20, no. 3, pp. 247–256, Oct. 1986.

[17] J. Turkevich, P. C. Stevenson, and J. Hillier, "A study of the nucleation and growth processes in the synthesis of colloidal gold," *Discuss. Faraday Soc.*, vol. 11, no. 0, pp. 55–75, 1951.

[18] K. R. Brown and M. J. Natan, "Hydroxylamine Seeding of Colloidal Au Nanoparticles in Solution and on Surfaces," *Langmuir*, vol. 14, no. 4, pp. 726–728, Feb. 1998.

[19] S. B. Quint and C. Pacholski, "Getting real: influence of structural disorder on the performance of plasmonic hole array sensors fabricated by a bottom-up approach," *J. Mater. Chem. C*, vol. 2, no. 36, pp. 7632–7638, 2014.

[20] T. Thio, H. F. Ghaemi, H. J. Lezec, P. A. Wolff, and T. W. Ebbesen, "Surface-plasmon-enhanced transmission through hole arrays in Cr films," *J. Opt. Soc. Am. B*, vol. 16, no. 10, p. 1743, Oct. 1999.

[21] C. M. Ofner and H. Schott, "Swelling studies of gelatin. I: Gelatin without additives," *J. Pharm. Sci.*, vol. 75, no. 8, pp. 790–796, Aug. 1986.

 ISBN: 978-1-61208-660-6