Biosensor platform based on surface plasmon-enhanced fluorescence spectroscopy and responsive hydrogel binding matrix

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ABSTRACT

We report a novel biosensor platform based on surface plasmon-enhanced fluorescence spectroscopy (SPFS) and a responsive N-isopropylacrylamide (NIPAAm) hydrogel binding matrix. This binding matrix highly swells in aqueous environment and it can be modified with receptor biomolecules by using active ester coupling chemistry. After the binding of target analyte molecules contained in a sample by receptor biomolecules immobilized in the hydrogel matrix, the captured analyte molecules can be compacted on the surface through the collapse of the gel triggered by an external stimulus. A thin hydrogel NIPAAm–based film was attached to a gold sensor surface and modified with mouse IgG receptor molecules. The affinity binding of antibodies against mouse IgG that were labeled with Alexa Fluor chromophores was observed by surface plasmon-enhanced fluorescence spectroscopy. We demonstrate that the collapse of the hydrogel matrix results in the enhancement of measured fluorescence intensity owing to the increase in the concentration of captured molecules within the evanescent field of surface plasmons.

Keywords: SPR, surface plasmon, sensor, biosensor, stimuli-responsive hydrogel, fluorescence spectroscopy

1. INTRODUCTION

Optical biosensors based on surface plasmon resonance (SPR) hold potential for rapid and sensitive detection of chemical and biological species in numerous important fields such as medical diagnostics, food control and security [1]. In these devices, biomolecular recognition elements (e.g. antibodies) are anchored to a metallic sensor surface that is brought in contact with an analyzed liquid sample. The capture of target molecules contained in a liquid sample by the biomolecular recognition elements on the metallic sensor surface is probed by surface plasmons. The SPR-based measurement of refractive index changes induced by the molecular binding events was proved to allow for sensitive and label-free detection of wide range of analytes including proteins [2], nucleic acids [3] and carbohydrates [4]. However, the analysis of samples with extremely low analyte concentration or the detection of small molecules which do not produce sufficient refractive index changes remains a challenge for SPR biosensors. Therefore, SPR biosensors were combined with fluroscence spectroscopy in a method referred as to surface plasmon-enhanced fluorescence spectroscopy (SPFS) [5]. In this method, the surface plasmon-enhanced intensity of electromagnetic field allows for highly efficient excitation of captured fluorophore-labeled molecules which is directly translated to an increased fluorescence signal. By using SPFS, only fluorophores adhered to sensor surface are excited by the evanescent field of surface plasmons while those contained in the whole sample are not. In SPFS biosensors, a spacer between fuorophores and metal surface need to be used in order to avoid fluorescence quenching through Förster energy transfer. In order to suppress the fluorescence quenching and to increase the binding capacity within the evanescent field of surface plasmons, Yu et al. used a thin dextran brush with the thickness of about 100 nm as a three-dimensional binding matrix [6]. They showed that the SPFS readout of molecular binding events in such matrix allowed for the ultra-sensitive detection of chromophore-labeled molecules at concentrations as low as 0.5 fM. In this communication, we demonstrate that the responsive hydrogel based on poly(N-isopropylacrylamide) (NIPAAm) [7] can be similarly used as a binding matrix to support an SPFS biosensor and that it offers additional features enabling increasing its sensitivity. The used NIPAAm-based hydrogel matrix can be prepared with higher thickness (up to several micrometers) and it can be functionalized by

Optical Sensors 2009, edited by Francesco Baldini, Jiri Homola, Robert A. Lieberman, Proc. of SPIE Vol. 7356, 735625 · © 2009 SPIE · CCC code: 0277-786X/09/$18 · doi: 10.1117/12.820988

receptor molecules with high surface density [8]. We observe the affinity binding of antibodies against mouse IgG antibodies covalently anchored in the NIPAAm-based hydrogel via detection of the induced refractive index variations (SPR) and fluorescence light emission (SPFS). After the capture of target molecules, target molecules within the matrix can be compacted on the sensor surface by the collapse of the NIPAAM-hydrogel triggered by external stimulus. We show that by this means more efficient excitation of captured molecules is achieved resulting in additional enhancement of SPFS signal.

2. MATERIALS AND METHODS

2.1 Chemicals

Sodium acetate, acetic acid, sodium chloride (NaCl), phosphate buffered saline (PBS, 137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, and a pH of 7.4) and ethanol amine hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO). Mouse immunoglobulin G (IgG) and goat anti-mouse IgG (a-IgG) labeled with Alexa 633 were obtained from Abcam (Cambridge, UK). 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) was from Pierce (Rockford, IL). 10 mM acetate buffer (ACT), pH 4, was used. Sodium para-tetrafluorophenol-sulfonate (TFPS) was synthesized in house as described in the literature [9].

2.2 Optical setup

In this study, we used an optical setup described previously in detail [10]. Briefly, a monochromatic light beam from He-Ne laser (PL610P, Polytec, Germany, power 2 mW, wavelength λ=632.8 nm) was linearly polarized using a polarizer, passed through a chopper (Princeton Applied Research, USA) and then was coupled to a LASFN9 prism, see Fig.1. A glass slide with 50 nm gold thin layer was optically matched to the prism base by refractive index matching oil (Cargille, USA). The intensity of the light beam reflected at the prism base was measured using a photo-diode and a lock-in amplifier (Model 5210, Princeton Applied Research, USA). The angle of incidence of the light beam was controlled by a motorized rotation stage (Hans Huber AG, Germany). A flow-cell consisting of a poly(dimethylsiloxane) gasket and a transparent glass substrate was attached to the sensor surface to contain liquid samples (volume approximately 10 µL). The input port of the flow-cell was connected to a peristaltic pump (Reglo, Ismatec, Switzerland) using rubber tubing (Tygoon R3607, from Ismatec, Switzerland) in order to flow the liquid sample across the sensor surface. The flow rate of 50 µL/min was used. The fluorescence light emitted through the flow cell was collected using a lens, passed through a bandpass filter (670FS10-25, L.O.T.-Oriel, Germany) and was projected into the input of a photomultiplier (H6240-01, Hamamatsu, Japan) that was connected to a counter (53131A, Agilent, USA). The optical setup and the data collection were controlled by WasPlas software developed at Max Planck Institute for Polymer Research in Mainz, Germany. As seen in Fig.2a, the excitation of surface plasmons is manifested as a resonant dip in the reflectivity spectrum. The optical properties of the hydrogel and the changes in surface mass density on the gold sensor surface were determined by the fitting of angular reflectivity spectra with a transfer matrix model as described in elsewhere [8]. Simultaneous determining of thickness d_h and the refractive index n_h of the gel was possible by fitting the angle of incidence at which SPR occurs and the arising hydrogel waveguide mode (HW) in vicinity to the critical angle.

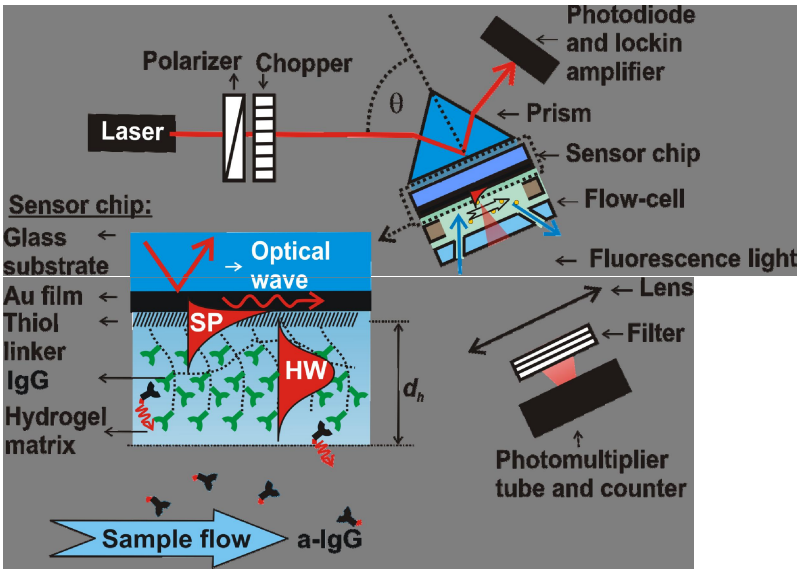


Fig. 1 Scheme of the optical setup of SPFS sensor with a hydrogel binding matrix.

2.3 Surface architecture

The synthesis of the NIPAAm-based hydrogel composed of the terpolymer with N-isoproprylacrylamide, methacrylic acid, and 4-methacryloyl benzophenone was performed as described elsewhere [7]. As shown by Beines et al., this hydrogel film highly swells in water and its collapse can be triggered by changing temperature, pH or ionic strength [7]. A thin hydrogel film was deposited on a gold surface modified by a benzophenon-terminated thiol layer by spin-coating from ethanol solution (4% wt. of the polymer) and dried overnight in vacuum at 50 ºC. The polymer chains were cross-linked and anchored to the gold surface via benzophenone units by UV irradiation (λ=365 nm, irradiation energy density of 2 J×cm^{-2}). The thickness of the dry crosslinked polymer layer was 68 nm as measured with a surface profiler. In order to couple protein molecules into the NIPAAM-based hydrogel matrix, the hydrogel layer was swollen in ACT buffer at pH 4 followed by the activation of the carboxylic groups within the gel with a EDC/TFPS solution for 90 min. TFPS and EDC dissolved at the concentration of 21 mg mL^{-1} and 75 mg mL^{-1}, respectively, in water. After, the surface was washed out with ACT buffer for 3 minutes and the solution with mouse-IgG, 100 µg/mL, was flowed through the flow-cell. After the reaction of mouse-IgG with activated carboxylic groups, the gel was washed with the ACT buffer for 10 min, incubated in ethanolamine for 10 min in order to block the un-reacted binding sites and rinsed again with ACT buffer for 15 min. The ethanolamine was dissolved in water at the 1 M concentration and the pH of the solution was adjusted to 8.5 with sodium hydroxide.

3. RESULTS AND DISCUSSION

3.1. Immobilization of ligands and affinity binding of target molecules

The in situ immobilization of mouse IgG molecules was probed with surface plasmons excited at the interface between the gold and the hydrogel film, see Fig.1. The time kinetics of the reflectivity measured at the angle of incidence of θ=58.2° upon the immobilization of IgG molecules can be found in Fig.2b. Firstly, the ACT buffer was flowed through the flow-cell for 30 min in order to stabilize the sensor response. The fitting of the angular reflectivity spectrum measured upon the flow of ACT buffer revealed that the swollen hydrogel film exhibited the thickness of d_h=607 nm and its refractive index was n_h=1.349, see Fig.2a. In order to covalently conjugate mouse IgG with the hydrogel film, the carboxyl groups within the gel were activated by the incubation in a solution with EDC/TFPS followed by the flow of IgG molecules dissolved in ACT buffer, incubation in ethanolamine and rinsing with ACT in order to wash out unbound molecules. The analysis of reflectivity spectrum measured after the ACT rinsing showed that the thickness of hydrogel decreased to d_h=454 nm and its refractive index increased to n_h=1.370. Afterwards, the PBS at pH 7.4 was injected to the

flow-cell resulting in the increase of hydrogel thickness to d_h=684 nm and a decrease in the refractive index to n_h=1.353. The variations in the thickness d_h upon the modification of the hydrogel matrix by IgG molecules were probably due to the changes in its net charge. As summarized in Tab.1, the measured optical parameters of the gel film corresponds to its surface mass of Γ=42 ng/mm^2 and Γ=67 ng/mm^2 prior and after the binding of mouse IgG molecules, respectively. These data indicate that the surface mass density of covalently coupled mouse-IgG was of 25 ng/mm^2.

The affinity binding of anti-mouse IgG antibodies (a-IgG) tagged with Alexa 633 chromophore was observed from the variations in surface plasmon resonance as well as by surface plasmon-enhanced fluorescence spectroscopy. In this experiment, we changed the angle of incidence to θ=58.7° and measured time evolution of the reflectivity and fluorescence intensity upon 100 minute flow of a-IgG molecules dissolved in PBS at the concentration of 40 µg/mL followed by rinsing PBS. As seen in Fig.2, the affinity binding of a-IgG was manifested as a gradual increase in the fluorescence signal in the angular regions where the hydrogel waveguide mode and surface plasmons were resonantly excited. This figure shows a negligible change in the SPR reflectivity. This effect can be explained by the analysis of the angular reflectivity spectrum measured after the affinity binding of a-IgG. As seen in Fig.2a, the binding of a-IgG did not induce a pronounced shift of the SPR dip however the excitation of hydrogel waveguide mode was altered significantly. The analysis of the spectrum revealed that the refractive index was n_h=1.353 was not changed however the thickness of the hydrogel matrix increased to d_h=750 nm. Based on these results, the surface mass density Γ after the affinity binding of IgG was determined as 75 ng/mm^2. Therefore, the resulting mass density of captured a-IgG of Γ=8 ng/mm^2 indicates that approximately one third of IgG molecules was capable to react with affinity partners in the sample. The reason for this observation is probably due to the steric hindrance of the molecular binding within the hydrogel matrix.

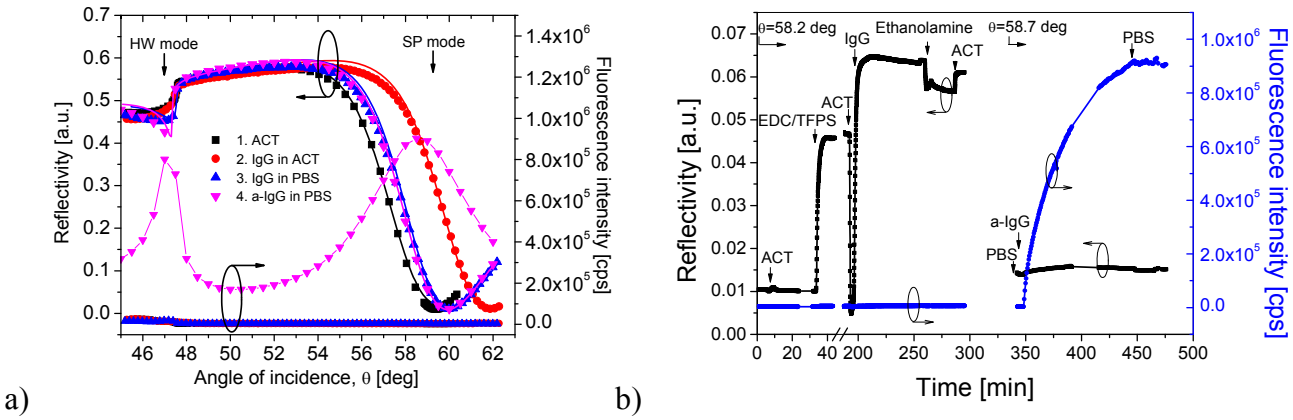


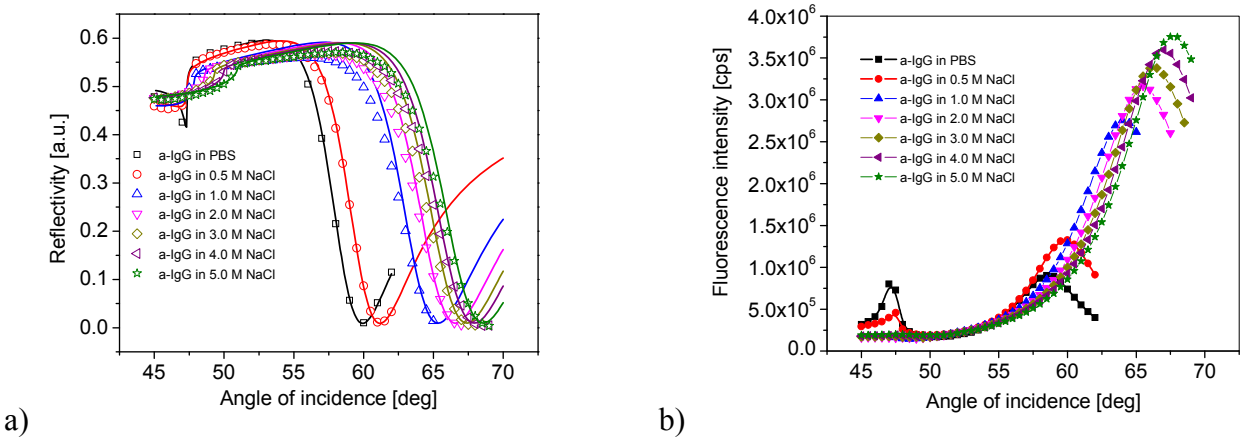
Fig. 2 a) The angular reflectivity and fluorescence intensity spectra and b) time evolution of the reflectivity and fluorescence signal measured at a fixed angle of incidence upon the covalent coupling of mouse-IgG and affinity binding of anti-mouse IgG labeled with a chromophore in NIPPAm-based hydrogel.

Table 1 Summary of the characteristics of hydrogel binding matrix prior and after protein conjugation

Spectrum	buffer	Thickness d_h [nm]	n_h	Surface mass density Γ [ng/mm^2]
1.	ACT	607	1.349	42
3.	PBS	684	1.353	67
4.	PBS	750	1.353	75

3.2 Amplification of the fluorescence signal through hydrogel collapse

Further, we demonstrate the increase of the fluorescence signal due to molecular binding events by externally triggered collapse of the NIPAAm-based hydrogel binding matrix. In this experiment, the concentration of NaCl in buffer solution flowed over the hydrogel was sequentially increased from 0.5 M to 5 M. As seen in Fig. 3a, the SPR dip and critical angle in the reflectivity spectrum shift towards high angles when increasing the concentrations of NaCl. From these spectra, the changes of refractive index of the buffer and the refractive index and thickness of hydrogel were determined. As shown in Fig. 4, the hydrogel collapses (thickness d_h decreases and the refractive index n_h increases) when increasing the ionic strength of the buffer due to the osmotic and electrostatic screening effects [11-13]. By the collapse of the gel, the hydrogel binding matrix is compressed leading to an increase in the concentration of captured analyte within the evanescent field of SPs and subsequent enhancement of the fluorescence signal, see Fig.3b. For the 5M concentration of NaCl in the buffer, the thickness of the gel of d_h=240 closely matches the penetration depth of surface plasmons into the hydrogel (approximately 180 nm). As Fig.4 shows, the dependence of the measured fluorescence signal is inversely proportional to the thickness d_h. The enhancement of the fluorescence signal in the 5M NaCl solution was increased by the factor of 3.2 with respect to those measured in PBS buffer. In addition the 3.1 fold decrease in the hydrogel film thickness d_h was observed.



a) b)

Fig. 3 Angular spectrum of a) reflectivity and b) fluorescence intensity measured for the incubation of a hydrogel matrix with affinity bound a-IgG in buffer solutions with ionic strengths between 0.1 and 5M.

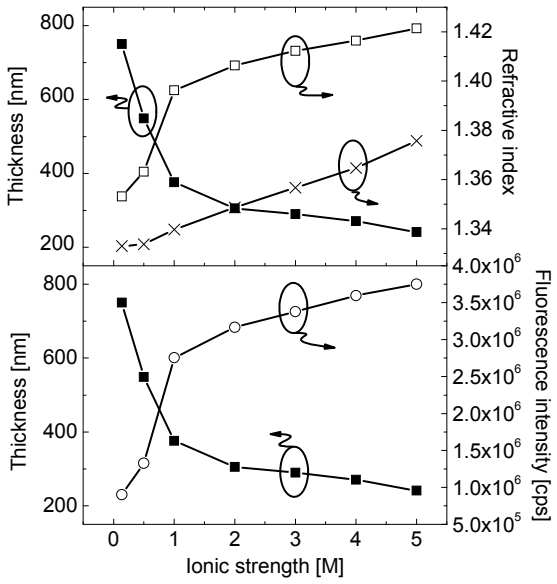


Fig. 4 The upper panel: The changes of the hydrogel thickness (■) and refractive index of hydrogel (□) and buffer (×) as a function of ionic strength. Lower panel: The dependence of hydrogel thickness (■) and maximum fluorescence signal (○) as a function of ionic strength.

CONCLUSIONS

We demonstrated that a highly swollen responsive NIPAAm-based hydrogel film can serve as an efficient binding matrix in surface plasmon-enhanced fluorescence spectroscopy (SPFS) biosensors. In a model experiment, we functionalized the gel with IgG antibodies and observed the affinity binding of antibodies against IgG by SPR and SPFS. Through the measurement of induced refractive index and thickness changes, we determined the ligand surface mass density of 25 ng/mm^2 when IgG molecules were covalently immobilized into a gel with the thickness of approximately 700 nm. In this binding matrix, around 32% of covalently bound IgG molecules reacted with their affinity partners contained in a liquid sample. We showed the enhancement of the SPFS signal by compacting the captured analyte molecules on the sensor surface through a externally triggered collapse of NIPAAm-based hydrogel. In this study, a 3.1 fold decrease in the thickness of the gel resulted in the enhancement of fluorescence intensity with a similar factor.

ACKNOWLEDGEMENTS

Alena Aulasevich, Basit Yameen and Martina Knecht from Max Planck Institute for Polymer research in Mainz (Germany) for the synthesis of NIPPAm polymer and benzophenon-terminated thiol are gratefully acknowledged.

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