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Plasmonically amplified bioassay – Total internal reflection fluorescence *vs.* epifluorescence geometry

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ABSTRACT

This paper investigates plasmonic amplification in two commonly used optical configurations for fluorescence readout of bioassays – epifluorescence (EPF) and total internal reflection fluorescence (TIRF). The plasmonic amplification in the EPF configuration was implemented by using crossed gold diffraction grating and Kretschmann geometry of attenuated total reflection method (ATR) was employed in the TIRF configuration. Identical assay, surface architecture for analyte capture, and optics for the excitation, collection and detection of emitted fluorescence light intensity were used in both TIRF and EPF configurations. Simulations predict that the crossed gold diffraction grating (EPF) can amplify the fluorescence signal by a factor of 10^2 by the combination of surface plasmon-enhanced excitation and directional surface plasmon-coupled emission in the red part of spectrum. This factor is about order of magnitude higher than that predicted for the Kretschmann geometry (TIRF) which only took advantage of the surface plasmon-enhanced excitation. When applied for the readout of sandwich interleukin 6 (IL-6) immunoassay, the plasmonically amplified EPF geometry designed for Alexa Fluor 647 labels offered 4-times higher fluorescence signal intensity compared to TIRF. Interestingly, both geometries allowed reaching the same detection limit of 0.4 pM despite of the difference in the fluorescence signal enhancement. This is attributed to inherently lower background of fluorescence signal for TIRF geometry compared to that for EPF which compensates for the weaker fluorescence signal enhancement. The analysis of the inflammation biomarker IL-6 in serum at medically relevant concentrations and the utilization of plasmonic amplification for the fluorescence measurement of kinetics of surface affinity reactions are demonstrated for both EPF and TIRF readout.

1. Introduction

Fluorescence spectroscopy represents an established method for detection of chemical and biological species and it supports a wide range of optical readers for protein and oligonucleotide assays [1,2]. In order to advance the sensitivity of such assays and to use them for rapid analysis of trace amounts of biomolecules, we witnessed increasing research efforts in near-field optics and particularly plasmonics [3]. Plasmonics concerns the manipulating of light at sub-wavelength scale by its coupling to surface plasmons (SPs) originating from collective oscillations of charge density at surface of metallic films and metallic nanoparticles. Plasmonic nanostructures have found their applications in amplification of signal in optical spectroscopy including surface enhanced Raman spectroscopy (SERS), surface enhanced infrared spectroscopy (SEIRA), and plasmon-enhanced fluorescence (PEF) spectroscopy [4–6]. In fluorescence spectroscopy-based detection of molecular analytes, a sandwich assay format with *e.g.* secondary antibody labeled with a fluorophore is typically used. These fluorophores are coupled with the confined field of SPs to increase the excitation rate at their absorption wavelength and improve extraction of fluorescence light at their emission wavelength from the sensor surface. In addition, the coupling with SPs mediates the emitter radiation rate which can improve its quantum yield η (particularly for fluorophores with intrinsically low η). Due to the strongly confined field profile of SPs, the coupling occurs in close vicinity to the sensor surface which allows selectively amplifying the signal originating from the specific binding without increasing background.

Up to now, numerous implementations of PEF that utilize the excitation of SPs propagating along continuous metallic films, as well as localized SPs supported by metallic nanoparticles, were pursued [7–9]. Either the attenuated total reflection (ATR) method with Kretschmann configuration or diffraction on periodically corrugated metallic surface was employed for the coupling of propagating SPs with far field optical waves. Kretschmann

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geometry was utilized for fluorescence immunoassays with SP-enhanced excitation and collection of fluorescence light in the beginning of nineties of the last century [10]. This approach was later re-introduced and served for the combined SP-enhanced fluorescence with refractometric surface plasmon resonance (SPR) sensing [11]. The directional SP-coupled emission was exploited based on the reverse Kretschmann configuration by using prism elements [8] or concentric diffractive structures [12]. The Kretschmann geometry allows for the implementation of plasmonic amplification into fluorescence readers that resemble total internal reflection fluorescence (TIRF) configuration. Diffraction gratings offer an alternative means for the coupling of far field with SPs and can be used in epifluorescence (EPF) devices. The coupling with propagating SPs was demonstrated to amplify the fluorescence signal by a factor up to 10^2 with respect to configurations where the fluorophores are present in homogenous environment and emit isotropically into the far field [13,14].

This paper concerns the implementation of plasmonic amplification for *in situ* readout of fluorescence assays. It describes TIRF and EPF configurations for the detection of fluorescence signal through a microfluidic device which requires using low numerical aperture lens optics for its collection. We compare the readout of a realistic assay by using TIRF and EPF geometries that utilize the SP-assisted excitation and extraction of emitted fluorescence light. For each readout configuration key characteristics of the assay are determined and improvements and limitations associated with the coupling with SPs are discussed. As a target analyte cytokine interleukin 6 (IL-6) is selected as it plays important role in regulating inflammatory and immune responses and is aberrantly expressed in diseases including cancer [15,16]. Therefore we witness a need for the timely and accurate monitoring of this protein in clinical research, disease diagnosis as well as in therapy. Enzyme-linked immunosorbent assay (ELISA) is arguably a golden standard method for the highly specific analysis of IL-6 at concentrations below pg/mL [17]. Research is pursued in areas such as point-of-care diagnostic sensors in order to simplify the IL-6 analysis which currently requires laboratory conditions and can only be used by trained personnel. In this field, a range of techniques is investigated including electro- or electrochemical sensors [18,19] as well as surface acoustic wave sensors or fluorescence biosensors [20–22].

2. Materials and methods

2.1. Materials

Polydimethylsiloxane elastomer (PDMS) Sylgard 184 was obtained from Dow Corning (USA) and the UV-curable polymer Amonil MMS 10 was from AMO GmbH (Germany). The carboxylate (SPT 0014A6) and PEG-dithiols (SPT 0013) were purchased from SensoPath Technologies (USA). EDC [1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide] and NHS (N-hydroxysuccinimide) were obtained from Thermo Scientific (Germany). Ethanolamine (1 M in water) was purchased from Sigma Aldrich (Austria) and adjusted to pH 8.5 with HCl. 10 mM acetate buffer (ACT) with pH 4.0 was prepared from sodium acetate and acetic acid and the pH was adjusted by HCl and NaOH. The used antibody pair of purified monoclonal rat anti-IL-6 (clone MQ2-13A5; product number 13-7068) and biotinylated monoclonal rat anti-human IL-6 (clone MQ2-39C3; product number 14-7069), as well as human IL-6 (product number 34-8069) were obtained from eBioscience (Austria). A reference capture antibody against p53 from Sigma Aldrich was used. Streptavidin Alexa Fluor 647 conjugate (product number S32357; in average 3 fluorophores per molecule) was purchased from Life Technologies (USA). Phosphate buffered saline (PBS) and Tween 20 were obtained from Sigma Aldrich (Austria) and biotin-free albumin fraction V was from Roth, Germany. Standard serum samples with concentrations of human IL-6 of 8.4, 131 and 437 pg/mL were acquired from Randox (UK).

2.2. Sensor chip preparation

For plasmonically amplified measurements relying on TIRF geometry, 2 nm thick chromium adhesion promoting layer and 45–50 nm thick gold film were subsequently deposited on a glass substrate by using vacuum thermal evaporation (HHV AUTO 306 from HHV LTD). For the EPF experiments, a 4 nm thick chromium adhesion promoting layer and a 100 nm thick gold film were prepared by the same means on a substrate with periodically corrugated surface. These substrates were structured by soft lithography as we described before [13]. Briefly, a master crossed-grating structure was fabricated by using laser interference lithography and transferred to a PDMS working stamp. This stamp was used for the preparation of multiple replicas to an UV-curable polymer Amonil MMS 10 that was spin-coated on a BK7 glass substrate. The corrugation profile $h(x,y)$ of copied rectangular crossed grating surface with deposited gold film can be described by using Cartesian coordinates (x and y axis are in the plane of the substrate) as the function $h(x,y)=\alpha(\sin[(2\pi/\Lambda)x]+\sin[(2\pi/\Lambda)y])+\beta(\sin[(2\pi/\Lambda)x]+\sin[(2\pi/\Lambda)y])^2$, where Λ is the period and α and β are modulation amplitudes. α describes the linear and β the nonlinear response of the UV sensitive photoresist used in interference lithography for master fabrication. The parameters of the crossed grating profile used further are $\Lambda=434$ nm, $\alpha=4$ nm and $\beta=2.5$ nm as determined by atomic force microscopy (AFM). The formula of the surface profile allows for implementation of the crossed grating geometry in the simulation method, as later mentioned. It is worth of noting that corrugations with low values of the non-linear parameter β exhibit weak Bragg scattering of propagating SPs which simplifies design of the grating for efficient amplification of fluorescence signal.

2.3. Optical setup

The used optical setup for TIRF and EPF readout of a sandwich IL-6 immunoassay is presented in Fig. 1. Identical light-source, sensor chip biointerface architecture, flow cell, and a module for

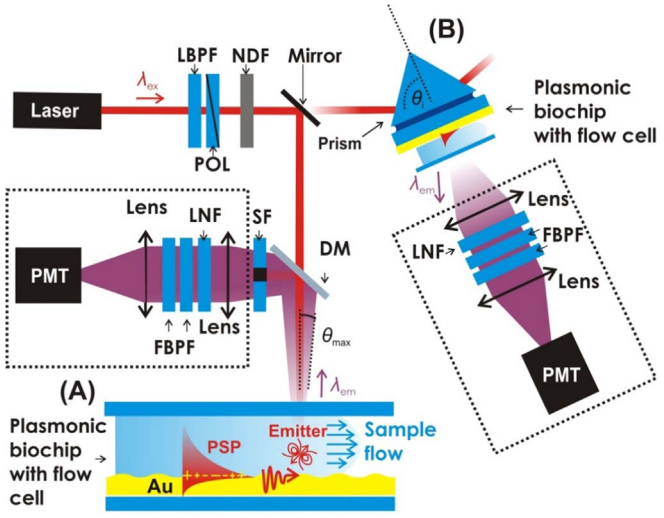

Fig. 1. Optical setup of used for assay with *in situ* plasmonically enhanced fluorescence readout relying on (A) epifluorescence geometry and (B) total internal reflection fluorescence.