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Research Paper

Detection of diclofenac molecules by planar and nanostructured plasmonic sensor substrates

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ABSTRACT

Surface plasmon resonance (SPR) sensors are well-established and widely used in the field of environmental and life sciences. To overcome the limitation of SPR sensors to applications in the laboratory environment initial studies on a low-cost nanostructured sensor substrate fabricated by nanoimprint lithography for the development of a SPR-based on-site biosensor system were conducted. For this purpose, diclofenac molecules were successfully immobilized both on planar and nanostructured gold sensor substrates, which was proved by XPS and SPR measurements. For the latter substrates, a specific binding between an anti-diclofenac antibody and immobilized diclofenac can be observed in form of a localized surface plasmon resonance shift in the optical transmission spectrum. The results show that our low-cost sensor substrate is wellsuited as transducer element for future SPR-based biosensors.

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1. Introduction

Nowadays, many products of daily use as well as biocides, industrial chemicals and other micropollutants are continuously released into the environment through human activities [1–3]. Beyond these classical pollutants, the presence of pharmaceuticals is increasingly attracting attention as they have been detected in remarkable concentrations (in the range of ng/L to low μg/L levels) in surface waters, waste water treatment plant (WWTP) effluents, as well as in lower concentrations in ground and drinking water [1,3–10]. Problematic about this development is that more and more parent compounds and metabolites are insufficiently eliminated during conventional waste water treatment processes [11]. Consequently, these residues are introduced into surface waters serving as prefiooders or are adsorbed by sewage sludge and reach agriculture land or landfills. Veterinary pharmaceuticals on the other hand enter the aquatic environment mostly directly via excretion or fertilizers [12–14]. In Germany alone 8120 t of 1200 different pharmacologically active substances were deployed in 2012 [15].

With an annual consumption of about 80 t the nonsteroidal anti-inflammatory drug (NSAID) diclofenac is one of the most prescribed and likewise most frequently detected compounds in Germany [10,16–18]. In usual WWTPs diclofenac and its metabolites are barely removed which leads to concentrations in the range of 1–10 μg/L [19] in effluents [6,20]. The amounts found are not acute toxic to humans yet and health risks arising from acute burden are unlikely. However, possible effects of long-term exposure on human health and wildlife are still lingering questions [4,12,13,21,22]. For example, ecotoxicological effects on the aquatic wildlife at an exposure of low concentrations (1 μg/L) of diclofenac are reported to lead to organ damage and general impairment in animals [8,11,23–25]. To point out the importance, in 2013 the European Union added three pharmaceuticals with diclofenac amongst them to the first watch list for emerging water pollutants [26–28]. Hence, diclofenac may be considered as an indicator substance for monitoring the presence of a broader range of pharmaceutical species, e.g. in the effluents of WWTP.

In this context, the fast and reliable measurement of the diclofenac concentration is of utmost importance but yet not entirely solved. Current analytical methods for diclofenac, e.g. liquid chromatography-tandem mass spectrometry (LC–MS/MS) [29,30], high-resolution mass spectrometry (HRMS) with liquid chromatography (LC) [31], gas chromatography-mass spectrometry (GC–MS) [18], or ultra performance liquid chromatography

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(UPLC) [32,33] are carried out by time-consuming and costly sampling and subsequent analysis in laboratory environment [31,34–36]. To enable fast, automated and continuous on-site monitoring of pharmaceuticals in complex water matrices, small and sensitive biosensors are highly in need [28,37–40]. For on-site operation, e.g. at the effluent of a WWTP, such sensors have to be especially rigid and robust. This is one of the reasons why established biosensor systems cannot easily be used for field applications.

This holds true especially for surface plasmon resonance (SPR) spectroscopy which is known to be one of the most sensitive label-free detection methods for molecular interactions [41,42]. In the classical case, SPR sensors are based on the excitation of propagating surface plasmon polaritons (SPPs) in a gold film on the surface of a glass prism (known as the Kretschmann-Raether arrangement) [43]. By reflection of incident light focused on the gold coating, changes in the near-surface refractive index of the metal film can be observed as a dip shift in the angular intensity of the reflected light. By monitoring the angular shift in time, molecular binding events at the functionalized gold surface can be quantified which represent changes in the refractive index [41,42,44]. However, this measurement configuration is limited to laboratory operations due to both the bulkiness and the elaborate adjustments of the optics necessary for excitation of propagating SPPs.

To circumvent these drawbacks but anyway exploit the benefits of SPR spectroscopy, we have developed a robust and miniaturized interrogation unit for optical sensors based on a wavelength-sensitive photodiode which was described elsewhere [45]. The system is suited to interrogate the spectral properties of nanostructured gold surfaces which are known to support localized surface plasmon polaritons (LSPPs) in addition to propagating SPPs. LSPPs show a similar sensitivity to refractive index changes induced by molecular binding processes, but can be excited in simple transmission geometry without the need of optical alignment, bulky optics and their angular dependency [44,46–50]. The combination of our robust interrogation unit and the simple excitation of the nanostructured gold surface of the sensor substrate may pave the way to on-site sensor systems.

For this alternative SPP spectroscopy approach, a reliable biofunctionalization and a sufficient sensitivity of the plasmonic sensor substrate for the detection of target molecules are crucial. In this study, experiments for the detection of diclofenac using a nanostructured plasmonic sensor substrate were conducted. The two overall aims were a) to provide a reliable biofunctionalization for the nanostructured gold sensor surface which allows to investigate the interaction between diclofenac and its antibody, and b) to prove a sufficient sensitivity of the LSPP spectral resonance position to detect the refractive index change induced by the chosen diclofenac-antibody binding reaction.

2. Experimental section

2.1. Materials

Standard chemicals were purchased from Sigma-Aldrich (Taufkirchen, Germany) and Carl Roth (Karlsruhe, Germany) in analytical grade. HS-PEG-NH_2 x HCl (Mw = 3230 Da) was obtained from Rapp Polymere (Tübingen, Germany), HS-PEG-COOH (Mw = 2100 Da) and diclofenac sodium salt from Sigma-Aldrich (Taufkirchen, Germany). SPR sensor chips for the ^{li}SPR system (capitalis technology, Berlin, Germany) were purchased from Fraunhofer IOF (Jena, Germany). The monoclonal mouse anti-diclofenac antibody 12G5 was prepared as described by Hübner et al. [28].

2.2. Fabrication of sensor substrates

Plasmonic sensor substrates consisting of an array of nanopillars were fabricated by nanoimprint lithography (NIL) according to the process reported by Yang et al. [44] in cooperation with GeSiM mbH (Großerkmannsdorf, Germany). Briefly, a transparent mold of PDMS is formed from a silicon master into which the nanostructure was written by means of electron beam lithography. The PDMS mold now carrying a negative of the nanostructure is imprinted into a UV-curable photoresist spin-coated on a glass wafer by means of the μContactPrinter 4.1 (GeSiM mbH). After filling all the cavities of the soft mold, the polymer is exposed to UV radiation in order to cure. Subsequently, the soft mold is removed resulting in a $3 \times 3\,mm^2$ area of polymer nanopillars arranged in a hexagonal lattice with a diameter of 230 nm and a center-to-center distance of 450 nm. The height of the pillars is 150 nm. As UV radiation solely takes place locally, the imprint process can be repeated on the same glass wafer with lateral displacement. Finally, for LSPPs, 2 nm titanium and 25 nm gold were deposited on the nanopillar array as adhesive and active layer, respectively. The glass wafer was cut into squares each having a size of $10 \times 10\,mm^2$ with a sensitive nanopillar area of $3 \times 3\,mm^2$ in the center. In addition to these nanostructured sensor substrates also standard SPR spectroscopy substrates (planar gold films) for the ^{li}SPR system (capitalis technology, see section 2.6) were used in reference experiments.

2.3. Preparation and functionalization of sensor substrates

Prior to functionalization all sensor substrates were rinsed with pure ethanol, dried under a stream of nitrogen and subsequently treated with UV/ozone (UV/ozone ProCleaner, NanoAndMore GmbH, Wetzlar, Germany) for 30 min. Next, 50 μL of HS-PEG-NH_2 x HCl dissolved in dimethyl sulfoxide (DMSO) were diluted to a concentration of 3 mg/mL in 20 mM phosphate buffer saline (PBS, pH 7.4) and incubated on the cleaned sensor substrate for 2 h at room temperature (RT). Finally the sensor substrate was thoroughly rinsed with PBS.

2.4. Immobilization of diclofenac

For the attachment of diclofenac to the amino-functionalized sensor substrates diclofenac is initially activated using EDC/NHS (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide/N-hydroxysuccinimide) as follows. A volume of 250 μL of 20 mg/mL diclofenac in 70% (v/v) ethanol and 5 mM of EDC and NHS was filled up with 5 mL 0.1 M 2-(*N*-morpholino) ethanesulfonic acid buffer (MES, pH 6,0) in a reaction tube and incubated for 30 min at RT. Next, sensor substrates were covered by 50 μL of the activated diclofenac solution diluted 1:1 in PBS buffer and once again incubated overnight at RT. To quench remaining active groups 50 μL of 50 mM hydroxylamine was incubated for 10 min followed by rinsing with PBS. A graphic representation of this protocol is presented in Fig. 1.

2.5. Interrogation of functionalized sensor substrates

Transmission spectra of the functionalized nanostructured sensor substrates were acquired in a simple home-built setup. The substrates were placed horizontally on a transparent holder and illuminated with a LED source in the spectral window between 750 and 1000 nm. The transmitted light was collected below the transparent holder by means of an optical fiber and directed to a spectrometer (iHR550, Horiba Jobin Yvon GmbH, Unterhaching, Germany). With this setup, an optical transmission spectrum of diclofenac-functionalized sensor substrates covered with PBS was measured. Then, 20 μL of 27 μg/mL anti-diclofenac antibody was

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