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Plasmonic sensor for the on-site detection of diclofenac molecules

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Editor's Preface

The proportion of residues of environmentally harmful pharmaceuticals in waste water has been rising steadily for years. In Germany alone, this concerns a quantity of more than 8000 t of about 1200 different substances, a whole series of which, unfavourably, cannot be adequately treated by waste water treatment in sewage plants. Even if the concentration is often only in the µg/L or ng/L range, significant ecotoxicological effects can still occur.
For this reason, inline monitoring of trace concentrations of such pharmaceuticals on-site at waste water treatment plants is of great importance. However, great demands are placed on the corresponding process sensors in terms of sensitivity, accuracy, cross-sensitivities, long-term stability, and process suitability. Up to now, there have been no satisfactory sensor solutions available for measuring the concentrations of such relevant substances directly in the process on site. The main reasons for this are that the sensor principles used are not sufficiently robust and reliable.

This work now addresses this issue by proposing the use of plasmonic sensor substrates to which appropriate recognition molecules for the pharmaceuticals of interest are attached. Plasmonic sensors are very robust and well-suited for industrial inline processes. Furthermore, the shift of the plasmonic resonance frequency can be evaluated with little effort by simple electronic evaluation circuits.

On this basis, this work impressively demonstrates that it is possible to detect residues of environmentally harmful pharmaceuticals on site in wastewater treatment plants. As an example of such non-degradable or poorly degradable substances, the anti-inflammatory diclofenac (DCF) was chosen, which is used in many painkilling drugs and, thus, enters the wastewater almost unchanged. Typical concentrations are in the range of 1...10 µg/L.

The results of this work clearly show that, using such a concept, inline-capable sensors can be realized that are robust and reliable as required for industrial applications. In other words, this means that the work presented here has both great economic and environmental potential in perspective. For this reason, I feel confident that this volume of the book series "Dresden Contributions to Sensor Technology" will earn that amount of attention it fully deserves.

Dresden, October 2021
Gerald Gerlach

Abstract

Every day, residues of polluting pharmaceuticals enter rivers, streams or lakes passing through waste water treatment plants (WWTPs). Monitoring these molecules in WWTPs is, thus, a pressing issue. However, due to the lack of suitable measurement systems especially the on-site monitoring of such molecules present in the range of ng/L to µg/L is currently not possible.
To address this problem, a biosensor being perspectively deployed directly at WWTPs is presented using diclofenac (DCF) as a guiding substance for water quality assessment. To begin with, a protocol developed for the detection of DCF molecules is evaluated using a commercial surface plasmon resonance (SPR) device and x-ray photoelectron spectroscopy. Commercial SPR sensors, however, are limited to applications in the laboratory environment due to their bulky nature and alignment-sensitive optics needed. Thus, a cost-effective and robust sensor approach is set up based on a nanostructured metal surface serving as an optical transducer. The plasmonically active nanostructure is fabricated by nanoimprint lithography and embedded in a simple transmittance setup and microfluidic system. For molecular detection, the transducer is surface-functionalised with DCF molecules. Using an indirect immunoassay, binding events between anti-DCF antibodies (preincubated with DCF molecules) and the sensor surface are detected in form of localised surface plasmon resonance (LSPR) shifts in the optical transmittance spectrum.
In initial experiments, a working range between 3 and 14 µg/L concentration of DCF and at least 75-fold regeneration of the sensor surface has been demonstrated. For further sensor miniaturisation, a photocurrent based interrogation unit is additionally employed for data acquisition. The results confirm that, using the LSPR sensor, DCF concentrations in environmentally relevant ranges can be detected and it, thus, may pave the way for the on-site detection of (polluting) molecules.

Kurzfassung

Tagtäglich gelangen Rückstände umweltschädlicher Pharmazeutika in Flüsse, Bäche oder Seen, indem sie Kläranlagen passieren. Die Überwachung dieser Moleküle in Kläranlagen ist daher ein sehr aktuelles Thema. Mangels geeigneter Messsysteme ist aber insbesondere die Vor-Ort-Überwachung solcher Moleküle, die im ng/L- bis µg/L-Bereich vorliegen, derzeit nicht möglich. Um dieses Problem anzugehen, wird ein Biosensor vorgestellt, der zukünftig direkt an Kläranlagen eingesetzt werden soll und Diclofenac (DCF) als Leitsubstanz zur Beurteilung der Wasserqualität verwendet.

Zu diesem Zweck wird zunächst ein Protokoll zur Detektion von DCF-Molekülen entwickelt, das mit einem kommerziellen Oberflächenplasmonenresonanz- (SPR-) Gerät und Röntgen-Photoelektronenspektroskopie evaluiert wird. Kommerzielle SPR-Sensoren sind allerdings aufgrund ihrer Massivität und der erforderlichen justagesensitiven Optiken auf Anwendungen in der Laborumgebung beschränkt. Daher wird in dieser Arbeit ein kostengünstiges und robustes Sensorsystem entwickelt, das auf einer nanostrukturierten Metalloberfläche basiert und gleichzeitig als optischer Transducer dient. Die plasmonisch aktive Nanostruktur wird mittels Nanoimprint-Lithographie hergestellt und in einen einfachen Transmissionsaufbau und ein mikrofluidisches System eingebettet. Für die molekulare Erkennung wird der Transducer mit DCF-Molekülen oberflächenfunktionalisiert. Mit einem indirekten Immunassay werden Bindungsereignisse zwischen Anti-DCF-Antikörpern (vorinkubiert mit DCF-Molekülen) und der Sensoroberfläche in Form von lokalisierten Oberflächenplasmonenresonanz- (LSPR-) Verschiebungen im optischen Transmissionsspektrum nachgewiesen.

Erste Versuche zeigen einen Arbeitsbereich des Sensors zwischen 3 und 14 µg/L DCF und eine mindestens 75-fache Regenerierbarkeit der Sensoroberfläche. Für eine weitere Miniaturisierung des Sensors wird zusätzlich eine photostrombasierte Abfrageeinheit zur Datenerfassung eingesetzt. Die Ergebnisse bestätigen, dass mit dem LSPR-Sensor DCF-Konzentrationen in umweltrelevanten Bereichen detek-

tiert werden können und damit möglicherweise der Weg für die Vor-Ort-Detektion (umweltbelastender) Moleküle geebnet werden kann.

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Part I

Fundamentals and goals of this work

1 Introduction

1.1 Motivation

Industrial and household chemicals, pesticides and personal care products are only a few examples of organic micropollutants being nowadays continuously released into the aquatic environment through human activities [1]. Beyond these, the presence of pharmaceuticals in trace concentrations in surface waters (in the range of ng/L to low µg/L levels) has attracted much attention in recent years as potential effects on nature and wildlife are still lingering questions [2–5].
Major entry paths are related to municipal waste water in addition to veterinary medicine [6–8]. The reasons are obvious: In Germany alone 8120 t of 1200 different pharmacologically active substances are deployed annually, many of which are excreted unchanged or as metabolised degradation products by the human body [9]. Reaching the waste water treatment plant (WWTP) with the waste water, pharmaceutical residues are insufficiently eliminated [10]. Consequently, they are released, although still active, into surface waters and are thus present in constantly increasing concentrations in the water cycle [11].
One of the most frequently detected compound is the non-steroidal anti-inflammatory drug diclofenac (DCF) being ubiquitous to surface waters around the world [3, 5, 12]. In WWTP effluents DCF concentrations in the range of 1-10 µg/L have been detected [2]. At these trace concentrations, ecotoxicological effects on the aquatic wildlife have already been observed as studies on organ damage in fish, for instance, have shown [7, 12, 13]. To point out the importance, in 2013 the European Union added three pharmaceuticals with DCF amongst them to the first watch list for emerging water pollutants [14–16][1]. Hence, following the discussion above, DCF

[1] A legally regulated environmental quality standard (EQS) does not yet exist for the pharmaceuticals on the EU watch list, but concentration-related proposals for their occurrence in surface waters. At the European level, EQS for DCF between 0.1 and 0.01 µg/L have been drafted [17]. In comparison, the average value of the DCF concentration in WWTP effluent is 3 µg/L.

may be considered as an indicator substance signifying the presence of a broader range of pharmaceutical species, e.g. in the effluents of WWTPs.
In this context, targeted expansions of WWTPs to include an additional, so-called fourth treatment stage (as an end-of-pipe approach) are becoming increasingly important to effectively reduce pharmaceuticals reaching surface waters. In Switzerland, for instance, a new Water Protection Act came into force at the beginning of 2016, stipulating the fourth treatment stage in certain WWTPs [18][2]. Current process engineering technologies for the fourth treatment stage essentially comprise:

- chemical (advanced) oxidation processes, e.g. ozone addition, to oxidize contaminants directly or through the formation of hydroxyl radicals,
- sorption on special adsorbents, e.g. activated carbon,
- dense membranes allowing size exclusion of low molecular weight pollutants, e.g. nanofiltration.

However, these technologies are energy-intensive and involve thus an additional financial effort. In this respect, a fast sensory monitoring, i.e. to register concentration breakthroughs of filters or to determine the energy required for ozonation on the basis of the actual contamination present, is crucial but yet not entirely solved. Figure 1.1 schematically depicts what such a sensory solution for monitoring (DCF) concentrations might look like.
Analytical methods currently being carried out in the laboratory, e.g. chromatographic separation (gas or liquid) and mass spectrometric detection, however, are still based on representative sampling [19–21]. This is only one reason, why those very sensitive and highly specific detection methods cannot be integrated on-site. Additionally, they need a high preparation effort and are, beyond that, time-consuming and costly.
Enabling automated and continuous on-site monitoring of pharmaceuticals in complex water matrices, small and sensitive biosensors are highly in need. For on-site operation, e.g. at the effluent of a WWTP, such sensors have to be especially rigid and robust. That is one of the reasons why biosensors established in the laboratory in the fields of environmental and life sciences, for example surface

[2] In Germany, there are already several pilot plants, however, a fourth treatment stage is not legally required at present.

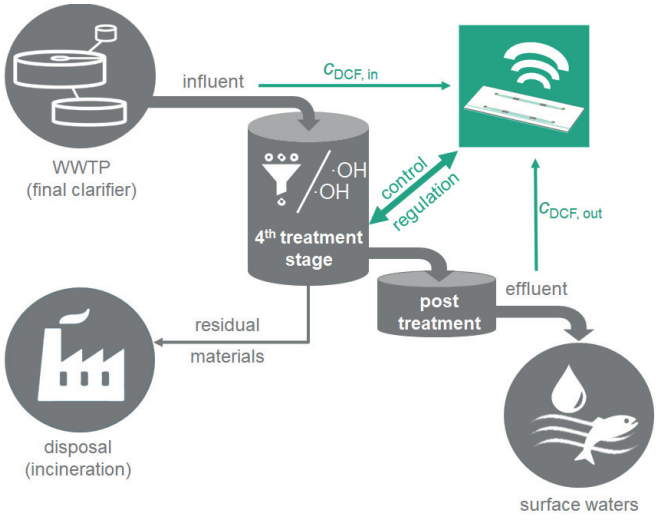


Fig. 1.1: Schematic concept for the perspective sensory monitoring of diclofenac (DCF) concentrations at waste water treatment plant (WWTP) effluents.

plasmon resonance (SPR) sensors, cannot easily be used for on-site applications. Therefore, this thesis aims at demonstrating a novel optical, plasmonically active biosensor concept for water quality monitoring, allowing the detection of DCF in the low µg/L range for the perspective on-site monitoring of WWTP effluents.

1.2 State of the art

1.2.1 Measurement of sum parameters

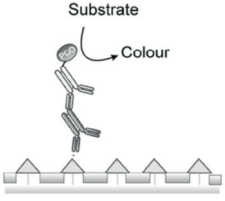
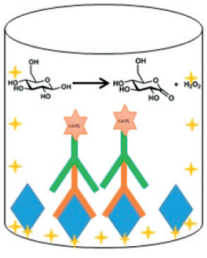
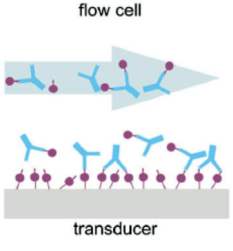
For the regulation of the fourth treatment stage, spectral sensors are currently used to record sum parameters, e.g. DOC (dissolved organic carbon), COD (chemical oxygen demand) or SAC_{254} (spectral absorption coefficient at 254 nm). By far the most frequently used parameter is the SAC_{254}. This parameter relies on the ability of various dissolved organic compounds to absorb UV light, e.g. aromatic molecules, and is thus a measure of the dissolved organic load in water. Several studies have

shown a correlation between the elimination of different organic trace substances and the relative reduction of SAC_{254} [22]. The parameter is measured using a UV/VIS probe.
However, other undesirable substances also absorb light in this spectral range, e.g. humic acids. For this reason, the measured values must be elaborately calculated with complex algorithms in order to achieve sufficient accuracy. Besides, substances that cannot be detected and thus falsify the measured value, or substances that are of importance, are not recorded. This is a general problem of sum parameter analysis. In addition, certain organic or anorganic components of surface waters may cause measurement drifts, which is why regular maintenance, cleaning and laboratory comparisons are necessary [23]. Generally speaking, regarding UV/VIS probes, representative sample cross-sections might be recorded, but no specific concentration statements of individual environmentally relevant trace substances can be made.

1.2.2 Measurement of individual parameters

A real-time and individual molecule measurement for monitoring the fourth treatment stage (or DCF concentrations) is not yet possible. Nonetheless, more recently promising optical biosensor concepts for the detection of DCF in water based on immunoassays have been published [24–28]. Their basic detection principle is summarised in Table 1.1. Biochemical immunosensors offer many advantages compared with laboratory methods due to their simplicity, potential for miniaturisation, fast response time, and reusability reducing time and cost of analysis.

Tab. 1.1: Immunosensor concepts for the detection of DCF in water. Enzyme-linked immunosorbent assay (ELISA), charged-coupled device (CCD).

System	Description	Reference
	• ELISA-based DCF detection in an automated flow-through device • functionalised silicon oxide chip as transducer • indirect, competitive assay format using an enzyme-labeled secondary antibody • signal imaging by a CCD camera	Hübner et al. 2015 [25, 26]
	• flow-through platform for DCF detection via a competitive, plasmonic ELISA • hydrogen peroxide yielded from a glucose oxidase labeled secondary antibody and Au(III)/citrate act as measurand • in-line generation of gold nanoparticles and monitoring of their growth rates	Kaewwonglom et al. 2019 [28]
	• label-free immunosensor based on reflectometric interference spectroscopy • liquid handling by a microfluidic system • functionalised silicon oxide chip as transducer • binding inhibition assay (using an anti-DCF antibody) • signal detection by a diode array spectrometer	Rau et al. 2015 [24, 27]

All biosensor concepts listed in the table have in common that DCF detection in the range from ng/L to low µg/L in water is demonstrated using an anti-DCF antibody as recognition element.
The ELISA-based flow-through platforms from Hübner et al. [25, 26] and Kaewwonglom et al. [28] require, moreover, an enzyme-labeled secondary antibody for chemiluminescene response or monitoring the growth rates of in-line generated gold nanoparticles, respectively. A label-free immunosensor is, on the other hand, presented by Rau et al. [24, 27] based on reflectometric interference spectroscopy. The intensity of the reflected light upon binding of anti-DCF antibodies to prefunctionalised silicon oxide chips is detected by a spectrometer.
However, all these immunosensors are not designed for the on-site use, i.e directly at WWTP effluents. First and foremost, the sensor setups are neither robust nor rigidly constructed due to the elaborate adjustments of the optics needed for signal generation and detection (e.g. spectrometer). Furthermore, ELISA based on end-point measurements lack ruggedness in that the results presented are highly dependent on most experimental conditions [28].

1.3 Objectives and structure of the work

On that account, a biochemical immunosensor concept allowing the detection of DCF as a guidance substance in the low µg/L range for the perspective on-site monitoring of WWTPs is demonstrated in this thesis. A basic principle of the sensor set-up is elucidated in Figure 1.2. At this, a nanostructured, plasmonically active metal surface serves as optical transducer. The plasmonic sensor chips allow a simple, compact and, hence, inline-capable sensor setup without the need of optical alignment of angular-dependent optics. In this sensor system, plasmonic oscillations are excited in a simple transmittance configuration. The plasmonic oscillations sensitively respond to changes in the refractive index on the metal surface [29], which in turn show up in spectral changes, e.g. in the transmittance spectrum of the metal nanostructure.
Making the transducer surface specific for the detection of DCF molecules, a stable and functional biological recognition element and its surface functionalisation is of utmost importance and plays a key role in the present thesis. In addition to the chemically functionalised transducer with DCF, an anti-DCF antibody is applied in

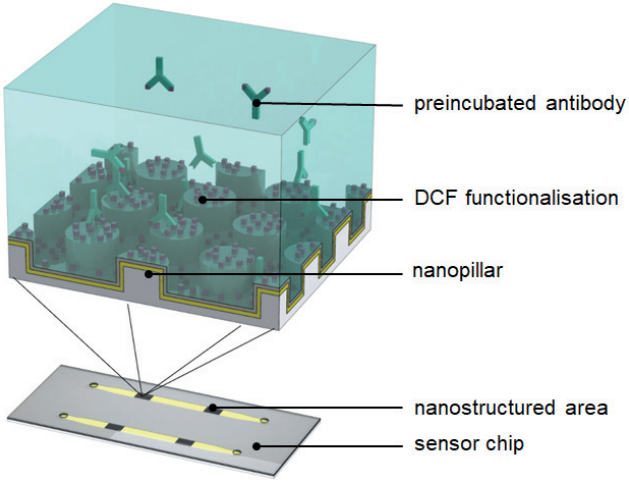


Fig. 1.2: The basic principle of the sensor set-up for detection of DCF molecules. A plasmonically active and DCF-functionalised sensor chip sensitively responds to antibody binding.

an indirect assay format. The decisive, measurable signal changes of the biosensor described originate from the binding event between anti-DCF antibodies and the sensor surface. The signal changes are detected using a spectrometer as well as in a photocurrent-based read-out approach [30].
The thesis therefore describes, in Part I, the background of SPR immunosensors and the topics encompassed in the development of a flow-through sensor chip. Providing an understanding of why the use of a plasmonic nanostructure is favorable for the set-up of an on-site biosensor, basic physical principles of plasmonics are introduced. Furthermore, enabling specific molecular detection, the biosensor's transducer is biofunctionalised. On that account, important aspects on molecular binding to surfaces are pointed out.
In Part II, the development of an immunoassay protocol on gold sensor chips for the detection of DCF is presented and successfully applied on a commercially available SPR-sensor. Following this, the knowledge is transferred for developing a novel plasmonic flow-through sensor for the perspective on-site monitoring of DCF molecules described in detail in Part III. Here, the key results of this work are presented and discussed. Furthermore, initial experimental results using a photocurrent-based read-out approach [30] instead of a spectrometer are presented.

To conclude, the last Part IV summarises the most important findings of this work and presents an outlook on the future work in this field.

2 Fundamentals

2.1 Biosensors

2.1.1 Basic setup of a biosensor

In the following some relevant parts of a biosensor as well as commonly used terms within biosensor technology are introduced. According to the International Union of Pure and Applied Chemistry a biosensor is generally understood to be a measuring system, in which a biochemical recognition element is closely coupled with a suitable physicochemical signal converter (transducer) [31]. This basic principle is depicted schematically in Figure 2.1.

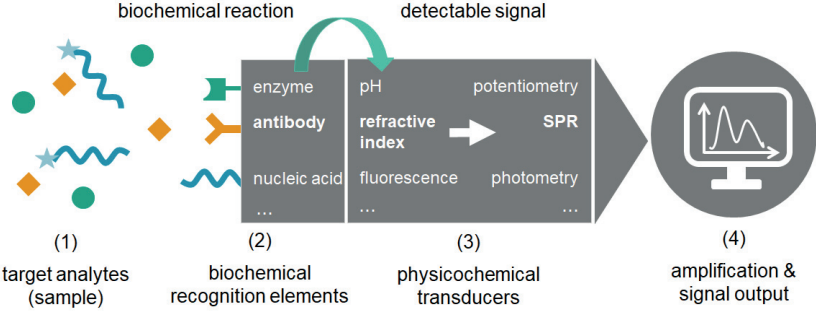


Fig. 2.1: Schematic representation of the principle of a biosensor. Bold: elements used in this work.

If now a molecular interaction between the selective recognition structure and the target molecule (analyte) takes place, a physicochemical change is generated being converted into a measured value after electronic signal processing [32, 33].