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Development of Immunological Methods for the Detection of Micropollutants in Fresh Water Samples

The aim of this work was to elaborate immunological methods for the detection of micropollutants in water samples. These pollutants are highly variable regarding their origin and molecular structures. By using monoclonal antibodies (mAbs), highly specific and sensitive immunoassays can be provided as recently shown for benzo[a]pyrene and microcystin-LR. Another class of emerging pollutants are pharmaceuticals like the non-steroidal anti-inflammatory drug (NSAID) diclofenac. It is found worldwide in the aqueous environment and therefore, has raised increased public concern on potential long-term impact on human health and wildlife. The importance of DCF has been emphasized by the European Union by including this pharmaceutical in the first watch list of priority hazardous substances in order to gather EU-wide monitoring data recently. Rapid and cheap methods of analysis are therefore required for fresh and wastewater monitoring with high sample load. In this work, for the first time, well-characterized mAbs against DCF were generated and a highly sensitive ELISA developed. The best antibody (mAb 12G5) was screened by testing 4 promising mAbs regarding affinity, specificity towards similar pharmaceuticals and DCF metabolites as well as matrix effects in fresh water samples.

Mab 12G5 has turned out to be highly affine (K_D = 1.5 x 10^{-10} M), stable to potential matrix interferences such as pH value (pH range 5.2-9.2), calcium ion concentration (up to 75 mg/L) and humic acid content (up to 20 mg/L). The LOD (S/N = 3) and IC_{50} of the ELISA calibration curve were 7.8 ng/L and 44 ng/L, respectively. The working range was defined between 11-180 ng/L. On average, an about 5% cross reactivity was found for DCF metabolites 5-OH-DCF, 4'-OH-DCF and DCF-acyl glucuronide by using an optimized ELISA protocol. Other structurally related NSAIDs showed binding ≤ 5% compared to the parent compound. While DCF concentrations at the low ppt-range were measured in river and lake water, higher values of 2.9 µg/L and 2.1 µg/L were found in wastewater influents and effluents, respectively. The results could be confirmed by SPE combined with HPLC-MS.

In order to allow the parallel detection of micropollutants in water samples, the mAbs were used to develop a microarray-based competitive ELISA with chemiluminescence (CL) detection in a flow-through device called MCR 3. This device was previously developed at

the TU München (Kloth, 2009 a). The microarray chips are prepared on glass carriers, which are modified with a PEG layer that carries terminal amino groups. These glass carriers are glued to a PMMA carrier. On the PEGylated glass slides, an immobilization strategy had to be developed for the different analytes BaP, DCF and MC-LR as model analytes. The immobilization should not require laborious derivatization of the immobilized hapten and should allow sensitive mAb detection by a high coupling density of the ligand on the microarray surface. In this work, a dioxane based microspotting method was developed that allows the immobilization of hydrophobic 1-pyrenebutanoic acid – a suitable surrogate for BaP – next to DCF and MC-LR. In comparison to previously developed methods (Oswald, 2013), signal intensities were higher and small amounts of hapten (≤ 1 mM in 100 µL) were needed for the microspotting method based on dioxane. The high yield of immobilzation is caused by the absence of salts and the basic character of dioxane to activate the terminal amino groups on the DAPEG modified glass carrier. Additionally, the water/dioxane mixture used to the microspotting method (v/v = 1/1) is coating the PEG surface with a lower contact angle than pure water (15° in contrast to 40°). For MC-LR, the same microspotting method could be applied after using Traut's reagent for generating terminal thiol groups on the surface. The developed method allowed the regenerable detection of anti-target analyte antibodies on the microarray chip with low signal fluctuations (< 10%).

This promising surface preparation was further investigated by using the surface analysis methods provided by the French project partners. The strategy for preparing PEG coatings for subsequent hapten immobilization on glass-type silica surfaces was transferred to QCM-D chips and Si wafer. In this way, it could be characterized in detail. The substrates - glass slide and silicon wafers - are bearing terminal silanol groups. For the functionalization, surfaces were first thoroughly cleaned and pretreated to generate additional silanol groups. Then, a silane layer with terminal epoxy groups was created using 3-glycidyloxypropyltrimethoxysilane (GOPTS). Epoxy groups were used to bind a layer of diamino-poly(ethylene glycol) (DAPEG) with terminal amino groups. Finally, the low molecular weight compound diclofenac was bound to the surface to be used as model ligand for competitive biosensing of haptens. The elementary steps were characterized using Atomic Force Microscopy (AFM), Water Contact Angle measurements, Grazing-Angle Attenuated Total Reflection (GA-ATR) FT-IR spectroscopy, and X-Ray Photoelectron

Spectroscopy (XPS). The data collected using these techniques have confirmed the successive grafting of the molecular species, evidencing, that homogeneous monolayers were created on the silica surfaces and validated the proposed mechanism of functionalization. The resulting surfaces were used to investigate polyclonal anti-diclofenac antibodies recognition and reversibility using QCM-D measurements or on the MCR 3. For both techniques, recognition and reversibility of the antibody binding were observed. MAb 12G5 was identified as reliable for surface interaction by using QCM-D, too. The stability of sensors over time was also assessed and no decrease in CL response was observed over 14 days in aqueous solution. The herein presented strategy for surface functionalization can be used in the future as reproducible and reusable universal biosensor platform for small molecules.

In order to further raise the sensitivity of the CL read-out immunoassay on the MCR 3, different ways to incorporate AuNPs or Au enhancement into the flow cells were tested. AuNPs were grafted on the surface or coupled to the anti-mouse-HRP secondary antibody. Moreover, the PMMA carrier was covered with gold. However, none of these methods allowed to further increase the CL signal. Therefore, no sensitivity enhancement of the flow-through assay could be achieved.

For multi-analyte detection of emerging pollutants, the assay protocol was optimized to allow reproducible calibration experiments. For the detection of BaP, 50% of MeOH had to be added to the sample in order to avoid non-specific adsorption to the flow-through capillaries and channels of the MCR 3. This increase of organic solvent was also tolerated by the anti-DCF and anti-MC-LR mAbs 12G5 and 10E7. The maximum CL signal intensity was achieved by using a flow rate of 2 µL/min for the mAb binding step to the microarray surface. The measurements based on this optimized assay program were also reproducible in fresh water and wastewater samples with RSD < 10% for blank measurements in the different matrices. In calibration experiments, the LOD was 34 ng/L for DCF, 220 ng/L for BaP and 580 ng/L for MC-LR. The inter-assay RSD was in general below 20% for the three analytes on three different measurement days on three different microarray chips. Subsequently, the developed assay allows sensitive and reproducible calibration curves for the three different emerging pollutants. Finally, the recoveries in fresh water were tested and they were on average between 93 and 122 % for the three different analytes in 5

different fresh water samples. False positive results were not found in different fresh water samples. These results show the general applicability of the method for fresh water analysis. In comparison to chromatography-based methods, this analysis requires less sample pre-treatment and less laboratory equipment. The monoclonal antibodies are robust and reliable detection reagents for the three different target analytes from different chemical classes.

In the future, additional priority pollutants can be added to the microarray like E2 and EE2 or antibiotics for screening applications. The presented coupling strategy can be transferred to other haptens that were coupled to proteins *via* amino groups for immunization. For the reliable detection of these haptens, however, it is crucial to have access to well characterized and highly reproducible antibodies like mAbs. Therefore, a standardized antibody preparation and characterization will be required to raise the acceptance of the immunological methods for analytical chemistry in general.

For the high-throughput analysis of fresh water analysis, another important development will be the implementation of standardized reference materials for this matrix including PAHs as well as pharmaceuticals, pesticides and toxins. (Elordui-Zapatarietxe, 2015)