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Multiplex surface plasmon resonance biosensing and its transferability towards imaging nanoplasmonics for detection of mycotoxins in barley†

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A 6-plex competitive inhibition immunoassay for mycotoxins in barley was developed on a prototype portable nanostructured imaging surface plasmon resonance (iSPR) instrument, also referred to as imaging nanoplasmonics. As a benchmark for the prototype nanoplasmonics instrument, first a double 3-plex assay was developed for the detection of deoxynivalenol (DON), zearalenone (ZEA), T-2 toxin (T-2), ochratoxin A (OTA), fumonisin B_1 (FB_1) and aflatoxin B_1 (AFB_1) using a well-established benchtop SPR instrument and two biosensor chips. To this end, ovalbumin (OVA) conjugates of mycotoxins were immobilized on the chip *via* amine coupling. The SPR response was then recorded upon injection of a mixture of antibodies at a fixed concentration and the sample (or matrix-matched standard) over a chip with immobilized mycotoxin–OVA conjugates. The chips were regenerated after each sample using 10 mM HCl and 20 mM NaOH and could be used for at least 60 cycles. The limits of detection in barley (in μg kg^{-1}) were determined to be 26 for DON, 6 for ZEA, 0.6 for T-2, 3 for OTA, 2 for FB_1 and 0.6 for AFB_1. Preliminary in-house validation showed that DON, T-2, ZEA and FB_1 can be detected at the European Union regulatory limits, while for OTA and AFB_1 sensitivities should be improved. Furthermore, measurement of naturally contaminated barley showed that the assay can be used as a semi-quantitative screening method for mycotoxins prior to liquid chromatography tandem mass spectrometry (LC-MS/MS). Finally, using the same bio-reagents the assay was transferred to a 6-plex format in the nanoplasmonics instrument and subsequently the two assays were compared. Although less sensitive, the 6-plex portable iSPR assay still allowed detection of DON, T-2, ZEA and FB_1 at relevant levels. Therefore, the prototype iSPR shows potential for future applications in rapid in-field and at-line screening of multiple mycotoxins.

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Introduction

Mycotoxins are secondary metabolites of fungi commonly found in several foods and ingredients such as nuts, cereals (*e.g.*, barley), coffee, oil seeds, fruits and, as a result, also occur in beer, wine as well as in feed.[1] Barley is used in unprocessed forms as well as processed forms in food commodities and is known to be occasionally infected with several fungal species. The most relevant mycotoxins for barley are deoxynivalenol (DON), zearalenone (ZEA or ZEN, referred to as ZEA throughout this article), T-2 and HT-2 toxin, ochratoxin A (OTA), fumonisin B_1 (FB_1), and aflatoxin B_1 (AFB_1).[2] Several of these mycotoxins are known for their teratogenicity, mutagenicity and/or carcinogenicity and could be a threat to both human and animal health. According to EU legislation, the maximum levels (MLs) in μg kg^{-1} for unprocessed barley are 1250 for DON, 100 for ZEA, 200 for T-2 (recommendation for sum of T-2 and HT-2), 5 for OTA, 2 for AFB_1 and 2000 for FB_1 (sum of FB_1 and FB_2 in unprocessed maize).[3,4]

Several methods for mycotoxin detection have been developed in recent years each with their own merits and limitations.[5–8] Chromatography-based techniques for mycotoxin detection, such as high-pressure liquid chromatography (HPLC) and gas chromatography (GC) in combination with mass spectrometry (MS), are very sensitive and allow quantitative analysis of multiple mycotoxins.[9] However, they are rather expensive, require skilled personnel, and sometimes involve

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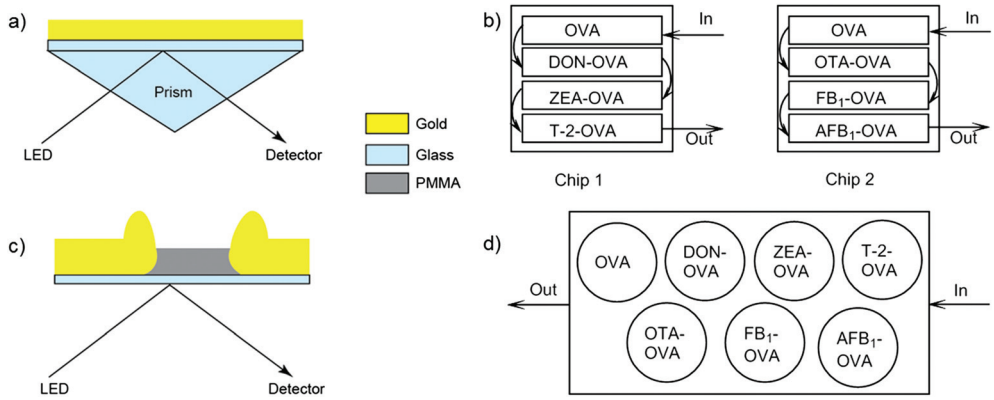

Fig. 1 Schematic representation of (a) a benchtop state-of-the-art SPR setup with flat gold as sensor surface and a prism to couple the light into the gold film (b) chip microfluidic array for double 3-plex assay (two chips each with an OVA channel as reference and three toxin conjugates) (c) portable imaging SPR (iSPR) setup with nanostructured gold as sensor surface thus omitting the need for any prism and (d) chip microfluidic array for 6-plex assay (single chip with OVA as reference and six toxin conjugates).

extensive sample preparation. One or a few mycotoxins have been measured using metal-enhanced fluorescence,[10] chemiluminescent immunoassay,[11,12] immuno-polymerase chain reaction,[13] real-time electrochemical profiling,[14] fluorescent immunosorbent assay,[15] microsphere based assay[16,17] and direct analysis in real time coupled with high-resolution mass spectrometry (DART-HRMS).[18] Amongst the immunochemical methods, enzyme-linked immunosorbent assay (ELISA) and lateral flow immunoassays (LFIA) are the most popular approach as they allow, respectively, semi-quantitative or qualitative detection of multiple mycotoxin samples in parallel.[19,20] Surface plasmon resonance (SPR) based biosensors have emerged as a reliable, label-free and sensitive alternative immunochemical method for the semi-quantitative detection of mycotoxins.[21–23] SPR, in addition, benefits from real-time monitoring of the interaction kinetics and reusability of the biosensor chip. Within the SPR field, imaging SPR (iSPR)[24,25] is an interesting development as in principle it allows the simultaneous detection of all mycotoxins of interest using a single sensor chip. The multiplexing capability of iSPR saves both time and cost in terms of bioreagents as a single injection gives information about multiple mycotoxins. But this potential has not been realized until now as only two mycotoxins have been detected simultaneously using iSPR.[26] In addition to multiplexing, there is an increasing demand for miniaturized instruments in order to bring the lab to the sample. Recently, a new prism-free prototype portable nanostructured iSPR instrument, also referred to as imaging nanoplasmonics (iNPx), was introduced.[27] However, the merits of this instrument remain to be demonstrated. In a 2015 critical review about nanoplasmonic biosensing[28] it is observed that no studies in literature perform a fair benchmark test against state-of-the-art SPR. Unfortunately most earlier SPR studies focused on only one or two mycotoxins,[29–32] while the only report[33] on multiplex SPR assays neither includes all experimental details nor an application to real sample matrices.

Therefore, the aims of the present study were twofold, the first aim being the development of a double 3-plex benchmark assay (including a reference channel in each chip) for the detection of the six most relevant mycotoxins (DON, ZEA, T-2, OTA, FB_1 and AFB_1) in barley using state-of-the-art SPR with flat gold biosensor chips as commonly used in Biacore instruments (Fig. 1a and b). A detailed study of the cross-reactivity and matrix effects has been performed, together with a preliminary in-house validation and the analysis of naturally contaminated samples. Finally, using the developed benchtop SPR assay as a benchmark, our second aim was to demonstrate the transferability of the state-of-the-art double 3-plex SPR immunoassay to a 6-plex iSPR format with a single nanostructured gold biosensor chip in a portable iSPR instrument (Fig. 1c and d) using the same bio-reagents and assay conditions and to compare the nanoplasmonics iSPR assay with the benchmark SPR assay.

Experimental

Chemicals, biosensor chips and barley samples

Flat gold chips (modified with carboxymethylated dextran hydrogel, CMD) were purchased from Xantec (Düsseldorf, Germany). The nanostructured gold chips for iSPR were produced using colloidal lithography and plasma-enhanced chemical vapor deposition (PE-CVD).[34] In short: poly(methyl methacrylate) (PMMA) was deposited on the glass substrate by PE-CVD using methyl methacrylate as liquid precursor. Spin coating was then used to cover the film with polystyrene (PS) beads (500 nm with a nominal size dispersion of 10%). Next, the sample was exposed to oxygen plasma etching to remove the PMMA from the areas not covered by the beads and to reduce the size of the PS beads to provide the required periodicity. A gold layer of 100–200 nm was then deposited on the surface by physical vapor deposition. Finally, the polystyrene beads were removed resulting in a surface with a gold film

perforated by PMMA (Fig. 1c). The combined control of deposition and etching parameters allowed fine tuning of film thickness and well diameters. The PMMA regions with diameters of 200–250 nm and periodicity (distance between the centers of the two PMMA wells) of 500–600 nm make up around 20% of the total surface area. Detailed characterization of the nanostructured iSPR chips can be found in an earlier publication.[35]

16-Mercaptohexadecanoic acid (MHDA), pentafluorophenol (PFP), *N*,*N*′-dicyclohexylcarbodiimide (DCC), poly(ethylene glycol) 2-aminoethyl ether acetic acid (average MW 3500) (NH_2–PEG–COOH), ovalbumin (OVA), *N*-hydroxysuccinimide (NHS), ethanolamine hydrochloride, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), sodium dodecyl sulfate (SDS), Tween20, anhydrous sodium acetate, glacial acetic acid and absolute ethanol were purchased from Sigma Aldrich (Zwijndrecht, The Netherlands). All chemicals were used as obtained, except for dichloromethane (Sigma-Aldrich, >99.8%), which was further purified using a Pure Solv 400 solvent purification system (Innovative Technology, Amesbury, USA). HBS-EP buffer (0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% v/v Surfactant P20) was purchased from GE Healthcare (Uppsala, Sweden). MilliQ water (18.3 MΩ cm resistivity) was obtained using a Merck Millipore (Billerica, USA) water purification system.

The OVA conjugates of mycotoxins (DON–OVA, ZEA–OVA, T-2–OVA, OTA–OVA, FB_1–OVA and AFB_1–OVA) and monoclonal antibodies (mAbs) against DON (AB-02-22), ZEA (AB-01-01) and T-2 (AB-07-02) were bought from Aokin AG (Berlin, Germany). The mAbs against OTA (SFH-OTA-005) were from Soft Flow Biotechnology (Pecs, Hungary), while the mAbs against FB_1 were produced and kindly provided by RIKILT (Wageningen, the Netherlands). The mAbs against AFB1 (12E6) were bought from Europroxima (Arnhem, the Netherlands). Mycotoxin reference solutions of DON, ZEA, T-2, OTA, FB_1, AFB_1 and nivalenol (NIV) were purchased from Biopure *via* RomerLabs (Tulln, Austria). The other mycotoxins deoxynivalenol-3-glucoside (D3G), 3-acetyl-DON (3ADON), α-zearalanol (α-ZEL), HT-2, fumonisin B_2 and B_3 (FB_2 and FB_3) were kindly provided by RIKILT.

Barley reference material surpassing the maximum level for ZEA (RMM-01-363a, 729 ± 244 μg kg^{-1}) was purchased from Aokin AG. The contaminated samples for T-2/HT-2 sample (60 μg kg^{-1} as measured by LC-MS/MS), for DON (289 μg kg^{-1} as measured by ELISA, <50 μg kg^{-1} as measured by LC-MS/MS) and for OTA (25 μg kg^{-1} as measured by ELISA, 210 μg kg^{-1} as measured by LC-MS/MS) were obtained from project partners. Three different blank samples (containing no mycotoxins based on ELISA or LC-MS/MS pre-screening) were also obtained from project partners. An extraction container and a filtering device were obtained from Abraxis (Warmister, USA) as part of a soil extraction kit.

Surface modification of nanostructured biosensor chips

The nanostructured iSPR chips were modified with PEG3500 as described previously.[35] In short: the chips were rinsed with ethanol and water followed by drying with nitrogen. They were then immersed in a 1 mM solution of 16-mercaptohexadecanoic acid (MHDA) in ethanol for 24 h. The MHDA-modified chips were removed, rinsed with ethanol and water, and dried with nitrogen. The acid-terminated chips were activated by immersing overnight in a 1 : 1 mixture of 0.4 M PFP and 0.4 M DCC in dichloromethane.[36] The PFP-terminated chips were washed with DCM and then immersed in a 1 mg mL^{-1} solution of NH_2–PEG–COOH (PEG3500) in DCM for 24 h. The PEG3500-modified chips were removed and rinsed with DCM, sonicated in the same solvent and dried with nitrogen. All the surface modifications were confirmed by comparison with static water contact angle values and XPS results from a previous publication.[35]

Immobilization of mycotoxin–OVA conjugates

Conventional SPR chips. Ovalbumin (OVA) and mycotoxin–OVA conjugates were immobilized on the carboxymethylated dextran chips using a Biacore 3000 SPR instrument (GE Healthcare, Uppsala, Sweden) as follows: HBS-EP was used as running buffer and the entire immobilization was performed at a constant flow rate of 10 μL min^{-1}. The microfluidics of the Biacore allows all the channels to be connected in series (Fig. 1b) or used separately with their own inlet and outlet. Each channel was individually activated using a 1 : 1 mixture of 0.4 M EDC and 0.1 M NHS for 7 min. This was followed by a 7 min injection of 50 μg mL^{-1} of OVA or mycotoxin–OVA conjugate (diluted in 10 mM acetate buffer pH 4 except for ZEA–OVA in 10 mM acetate buffer pH 3.5) to achieve a response between 3000–5000 (relative to the HBS-EP baseline). For OVA conjugates of DON, T-2 and OTA, a second injection of 50 μg mL^{-1} had to be performed to achieve the required response. Following the immobilization, the chips were blocked with 1 M ethanolamine, pH 8.5 for 7 min. The chips were then stored at 4–8 °C until use.

Nanostructured iSPR chips. For the PEG3500-modified nanostructured iSPR chips, the immobilization was performed manually on the bench: the entire chip was activated using a 1 : 1 mixture of 0.1 M NHS and 0.4 M EDC for 20 min followed by washing with water and drying with nitrogen. Two μL of OVA (1 mg mL^{-1} in 10 mM acetate buffer pH 3.5) and 2 μL of each of the six mycotoxin–OVA conjugates (1 mg mL^{-1} of ZEA–OVA, 1 mg mL^{-1} of AFB_1–OVA, 2.8 mg mL^{-1} of T-2–OVA, 2.8 mg mL^{-1} of FB_1–OVA, 5 mg mL^{-1} of DON–OVA and 3 mg mL^{-1} of OTA–OVA, all in 10 mM acetate buffer pH 3.5) were each spotted (manually using a micropipette) on different areas (regions of interest, ROI) of the activated chip. The chip was kept in an air-tight container for 2 h to avoid evaporation and drying out of the spots. Following the spotting, the chips were washed with water and dried with nitrogen. They were then blocked with ethanolamine for 20 min, rinsed with MilliQ water and dried with nitrogen. The spotted chips were stored at 4–8 °C.

Measurement protocols for double 3-plex assay (SPR) and 6-plex assay (iSPR)

SPR measurements on carboxymethylated gold chips were performed using a Biacore 3000 and iSPR measurements on

PEG3500-modified nanostructured gold chips using a prototype portable imaging nanoplasmonics instrument (Plasmore Srl., Italy).[27] The channels in the Biacore were connected in series for all measurements (Fig. 1b). Typical sensorgrams generated by both instruments can be found in the ESI (Fig. S1†). In both formats (double 3-plex with SPR and 6-plex with iSPR), chips were flushed for 1 min (t = 0–60 s) with running buffer (HBS-EP). The samples were mixed with the antibodies and were injected at a constant flow rate of 20 μL min^{-1} for 2 min (t = 60–180 s). The chip was then flushed with running buffer (HBS-EP) for 2 min (t = 180–240 s). The response obtained after 2 min of buffer flow, relative to the starting baseline, was used in calculations. The chip was regenerated by injecting 10 mM HCl (100 μL min^{-1}) for 30 s (t = 250–280 s) followed by 20 mM NaOH (100 μL min^{-1}) for 30 s (t = 350–380 s). The regeneration was followed by washing with HBS-EP buffer (2 min, t = 400–460 s) to remove any remaining regeneration solution. The entire cycle took approximately 15 min. In both SPR and iSPR, OVA was used as a reference, *i.e.*, the response of the OVA channel was subtracted from all the other responses, to correct for any non-specific interactions. The sensorgrams were plotted using Origin 8.5 and Excel 2010. Sensorgrams (for iSPR) were smoothened using the Savitzky-Golay function in Origin 8.5 (10 points of window, polynomial order 2).

Calibration curves, limit of detection and cross-reactivity

For the calibration curves, a fixed concentration of single or multiple antibodies in HBS-EP (without methanol) was mixed with an equal volume of the standard solution containing single or multiple toxins. The toxin standards were diluted in HBS-EP containing 20% methanol (for singleplex and multiplex assays in buffer) or in barley extract (see below about extraction of barley) in HBS-EP containing 20% methanol (for multiplex assays in barley). The (i)SPR response obtained for the solution containing both toxins and antibodies (B) relative to the maximum response obtained for the solution containing antibodies only (B_0) was used to calculate the relative response as (B/B_0) × 100%. The relative binding was plotted against the concentration of the toxin to obtain the calibration curves. The calibration curves were fitted in GraphPad Prism (Version 6.0, GraphPad Software Inc., USA) using a non-linear four-parameter model to obtain the IC_{50} values (concentration at which 50% inhibition of binding occurs). The IC_{10} values were used as a measure of the limit of detection (LOD) as explained in the results section. Additionally, the IC_{80} and IC_{20} values (concentrations at which 80% and 20% inhibition of binding occurs, respectively) allowed determination of the working range of the assay. The specificity of the antibodies was tested by determining the cross-reactivity of the following modified mycotoxins (masked myctoxins and metabolites) using the calibration curves for the target mycotoxin and modified mycotoxin in buffer as (IC_{50} target/IC_{50} modified mycotoxin) × 100%: D3G, 3ADON, NIV, α-ZEL, FB_2, FB_3, HT-2, AFB_2, AFG_1, AFG_2, AFM_1.

Extraction of barley samples

The extraction protocol for blank and naturally contaminated barley samples was based on a previous study.[17] In short: 2.5 g of ground barley was weighed in a 50 mL centrifuge tube. After adding 10 mL of 80% methanol, the sample was vortexed vigorously in a Lab dancer (IKA, Staufen, Germany), and shaken for 30 min using an Labquake (Fischer Scientific, Landsmeer, the Netherlands) tube shaker/rotator. Following the extraction, the sample tubes were kept in the fridge for 30 min. Next, the tubes were centrifuged for 10 min at 4000*g*. The supernatant was then collected and diluted four times using HBS-EP buffer solution to obtain an extract in HBS-EP containing 20% methanol. Finally, the solution was centrifuged for 10 min at 12 000*g* to remove any insoluble components formed as a result of the decrease in organic solvent content. This extract in HBS-EP containing 20% methanol is referred to as "barley extract" throughout the manuscript and was used for matrix matched calibration curves as well as blank barley for validation experiments.

Preliminary in-house validation

In-house validation was performed using 21 blank and 21 spiked barley samples. The 42 samples (blank and spiked) were composed of three different barley batches, one containing 0 μg kg^{-1} DON according to ELISA (B1), one with 0 μg kg^{-1} OTA according to ELISA (B2) and one mixed barley (B3) selected as blank for all six toxins after LC-MS/MS analysis. For fortification of the samples, the following levels of toxins were used: DON (1/2 × ML, 625 μg kg^{-1}), ZEA (1/2 × ML, 50 μg kg^{-1}), T-2 (1/8 × ML, 25 μg kg^{-1}), OTA (2 × ML, 10 μg kg^{-1}), FB_1 (1/5 × ML, 400 μg kg^{-1}) and AFB_1 (4 × ML, 8 μg kg^{-1}). B1, B2 and B3 (blank and spiked) were each extracted in triplicate and measured on the same day to obtain the intra-day variation. The same experiment was then repeated on two additional days to obtain the inter-day variation. Due to the limited quantity, sample B3 could only be analyzed in triplicate on day 1 and could not be used for inter-day variation (see Scheme S1†). All the responses shown are relative to the response of a mixed barley (MB) analyzed in triplicate at the beginning of each day. The mixed barley was composed of equal volumes of all the 21 blank barley extracts. For spiked samples, the required volumes of each toxin standard for 2.5 g of barley were mixed and the toxin mixture (volume not exceeding a total of 50 μL) was spiked on the barley by gently pipetting the mixed solution on the inner side of a 50 mL centrifuge tube. The solution was mixed with the barley by vigorous vortexing followed by air-drying (left with cap open in the fume hood) for 30 min. The barley samples were then extracted as explained in the extraction section. The obtained barley extracts were mixed with the same volume of an antibody solution. Assuming a 100% recovery, the mycotoxin concentration in the final solution (in ng mL^{-1}) is 1/32 of the original concentration in the barley (in μg kg^{-1}). Data evaluation was done based on regulations for validation of screening methods.[37] Validation of a semi-quantitative screening method requires

identification of a cut-off level (Fm) and threshold value (T). When the SPR response if at or below the cut-off level, the sample is categorized as 'screen positive' and liable to confirmatory analysis. As described in Annex II of the guidelines,[37] the threshold value (T) was defined as $T = B - 1.725 \times \mathrm{SDb}$, where B is the average response of the blank samples, and SDb is the standard deviation of these responses. The cut-off factor (Fm) was defined as $\mathrm{Fm} = M + 1.725 \times \mathrm{SD}$, where M is the average responses of spiked samples and SD is the standard deviation of these responses. For a successful validation, the Fm value has to be lower than the T value and only 5% samples (one of 21) are allowed to be false negative.

Naturally contaminated samples

Naturally contaminated barley samples containing DON (289 μg kg^{-1} as measured by ELISA, <50 μg kg^{-1} as measured by LC-MS/MS), ZEA (CS2, 729 ± 244 μg kg^{-1}), T-2/HT-2 (CS3, 60 μg kg^{-1}), and OTA (25 μg kg^{-1} as measured by ELISA, 210 μg kg^{-1} as measured by LC-MS/MS) were tested. Three samples were prepared for each contaminated sample measurement: blank barley (B−), spiked barley (B+) and contaminated sample (CS). Each sample (B−, B+ and CS) was extracted in triplicate and each extract was measured in triplicate. The following levels of toxins were present in the spiked barley: DON (1/2 × ML, 625 μg kg^{-1}), ZEA (1/2 × ML, 50 μg kg^{-1}), T-2 (1/8 × ML, 25 μg kg^{-1}), OTA (2 × ML, 10 μg kg^{-1}), FB_1 (1/5 × ML, 400 μg kg^{-1}) and AFB_1 (4 × ML, 8 μg kg^{-1}). All the barley extracts were prepared as described in the Experimental section except for ZEA where the contaminated barley extract had to be diluted an additional ten times in 20% methanol (final concentration of 2.2 ng mL^{-1} assuming 100% recovery) before 1 : 1 dilution with antibody mixture, to be able to measure within the detection range of the double 3-plex assay. The responses were relative to the response of a mixed blank barley (MB, see section on validation above) analyzed in triplicate prior to each contaminated sample.

Simplified sample preparation for field application

For possible in-field applications, a simpler extraction protocol was tested with one of the contaminated sample (CS2). A kit (see Fig. S2†) consisting of a plastic container and a stainless steel ball (d = 1.5 cm) for extraction and a filter device was used. All components of the kit were rinsed several times with 80% methanol before use. In short: 1.25 g of ground barley was weighed in the plastic container. After adding 5 mL of 80% methanol, the ball was added and the sample was vigorously shaken manually for 1 min. The extract was allowed to stand on the bench for 5 min and then transferred to the bottom container of the filtration device. A plunger containing the actual filter was attached to the bottom container of the device. Upon applying gentle pressure to the plunger, the extract is filtered into the plunger. The filtrate was collected and diluted four times using HBS-EP buffer solution to obtain an extract in HBS-EP containing 20% methanol.

Results and discussion

Double 3-plex benchtop SPR immunoassay development

Carboxymethylated dextran (CMD)[38,39] is the most commonly used surface in SPR and was used in all the Biacore experiments. OVA and OVA conjugates of mycotoxins were immobilized on the CMD surface *via* amine coupling after one step NHS/EDC activation of the carboxylic acid groups. The covalent attachment of the conjugates is important for re-use and stability of the chips. The six mycotoxins had to be divided over two chips as the Biacore 3000 has four channels, thus allowing the detection of a maximum of three toxins per biosensor chip when using a reference channel. OVA conjugates of DON, ZEA and T-2 were immobilized on one chip as the simultaneous detection of these toxins is relevant from an application point of view. On the other chip, AFB_1 and OTA were paired together as they both have very low legal limits and might require additional sample treatment. FB_1 was the only one remaining and was thus included on the second chip. Once the chips were stable (no significant loss of SPR signal from conjugates after multiple injections of 20 mM NaOH), the concentration and buffer composition were optimized for the individual antibodies. HBS-EP containing 20% methanol was chosen as the dilution buffer for the antibodies during assay development because for most of the antibodies at a fixed concentration, the binding with the immobilized mycotoxin–OVA conjugates was similar (anti-T-2, anti-ZEA, anti-FB_1, anti-OTA) or higher (for anti-DON and anti-AFB_1) in the methanolic buffer than in pure HBS-EP (Fig. S3†). Such higher affinity of antibodies in buffer containing methanol has been reported previously in the literature and is not uncommon.[32,40] Furthermore, for matrix-matched calibration curves in barley, 10% methanol is present after the extraction and 1 : 1 mixing with antibodies. Thus, measuring the antibody binding and constructing the calibration curve in HBS-EP containing 10% methanol is the most appropriate approach. The optimal concentration of the antibodies (corresponding to an SPR response of 100–200 units) was 0.1 μg mL^{-1} for anti-FB_1, 1 μg mL^{-1} for anti-OTA and anti-ZEA, and 2 μg mL^{-1} for anti-T-2, anti-DON and anti-AFB_1. After optimization of the binding conditions, the regeneration step was optimized. Regeneration is one of the advantages of SPR as it allows reuse of the biosensor chips, unlike most other immunoassay formats. However, for multiplexing applications this is quite challenging since both the binding and regeneration steps have to be optimized for all the different antibodies and the toxins involved. In this study, the most critical antibody-conjugate pairs with respect to regeneration were those for DON, T-2 and AFB_1. OVA conjugates of DON and T-2 were sensitive to regeneration and the conjugates on the surface were partly lost according to the SPR response. On the other hand, the antibodies against AFB_1 were very strongly bound to the chip surface and could not be removed completely upon regeneration. However, after testing a large range of regeneration conditions as recently suggested in literature,[41] we found that the combination of 10 mM HCl (30 s at 100 μL min^{-1}) followed by 20 mM NaOH (30 s at

100 μL min^{-1}) provided the best compromise for the critical toxins (Fig. S4†) and the other three toxins (data not shown). Cross-talk of these antibodies with the untargeted mycotoxin conjugates was tested by injecting single antibodies over all the biosensor channels. Some non-specific interaction of anti-T-2 was seen with DON–OVA and ZEA-OVA but turned out to be mainly caused by OVA. This could thus be corrected by using the OVA reference channel (see Experimental section about measurement protocols), underlining the importance of a suitable reference channel in SPR biosensor immunoassays to avoid misinterpretation of results.

After optimization of the binding and regeneration conditions, singleplex calibration curves were prepared for all the individual toxins to determine the detection range. These calibration curves (Fig. S5†) were then compared to the multiplex calibration curves in buffer (Fig. S5† and Fig. 2a). The minor differences between the singleplex and multiplex formats show that interference upon mixing different antibodies and toxins was insignificant. Finally, multiplex calibration curves were prepared in barley extract (Fig. S5† and Fig. 2b) and compared to the multiplex calibration curves in buffer to test for a possible matrix effect. The IC_{80} values (Table S1†) of all toxins (except AFB_1) in barley extract were only slightly (about two times) higher than in buffer; the most significant difference was seen for DON where the value in barley (100 ng mL^{-1}) was four times higher than the value in buffer (25 ng mL^{-1}). This is expected for calibration curves in sample matrices where several other components contribute to a higher signal than in buffer. In the case of ZEA and OTA, the IC_{20} and IC_{50} were not or hardly affected by the sample matrix (barley). For T-2 and AFB_1, both the IC_{20} and IC_{50} values were affected by the sample matrix. For T-2, the IC_{20} value for multiplex in barley (0.02 ng mL^{-1}) was five times lower than for multiplex in buffer (0.1 ng mL^{-1}) whereas the IC_{50} for multiplex in barley (0.5 ng mL^{-1}) was more than two times lower than for multiplex in buffer (1.2 ng mL^{-1}). Similarly, for AFB_1, the IC_{20} value for multiplex in barley (0.02 ng mL^{-1}) was ten times lower than for multiplex in buffer (0.2 ng mL^{-1}) and the IC_{50} for multiplex in barley (0.8 ng mL^{-1}) was two times lower than for multiplex in buffer (1.7 ng mL^{-1}). Such shifts of the calibration curve in barley towards higher sensitivity, as seen here for T-2 and AFB_1, have also been observed in a multiplex microsphere immunoassay format.[17] A significant difference in the IC_{50} value was observed for DON where the value in barley (15 ng mL^{-1}) was almost four times higher than the value in buffer (3.9 ng mL^{-1}). For FB_1, the only significant difference was in the IC_{10} values that was almost three times higher for multiplex in buffer (0.2 ng ml^{-1}) compared to multiplex in barley (0.07 ng ml^{-1}). Since all the assays were influenced by the sample matrix (barley) and the effect is not the same for all toxins, matrix-matched calibration curves are recommended prior to measurement of contaminated samples. As seen from the LODs (Table 1), the double 3-plex assay allows detection of all six toxins within the legal requirements of the EU. However, in case of OTA and AFB_1, the working range does not allow analysis at ML levels. The performance of the assay, especially for AFB_1 and OTA, may be improved by the production of more sensitive antibodies. Alternatively, less dilution of the sample may be considered but at the risk of increased matrix effects and higher methanol concentrations.

Direct comparison of the sensitivity of the double 3-plex SPR assay with literature values is complicated due to differences in assay types, antibodies, sample matrices, sample preparation as well as the calculation method for determination of the limit of detection (LOD). Nevertheless, the most relevant literature values reported for different mycotoxins have been compiled in Table 2. The reported LODs are often calculated by subtracting three times the standard deviation from the average blank response.[30,31] Due to the very low noise of the Biacore instrument, the signal corresponding to such an LOD is at 1–5% relative inhibition. The standard deviations of the blank sample of these reported SPR assays are already higher than the noise of the Biacore, making such LOD calculations less preferable.[30,31] Therefore, IC_{10} values (10% inhibition of binding) were used in the present work as a reasonable estimate of sensitivity (LOD). In case of DON and T-2, IC_{20} values were used as LOD because the fitting of the calibration curve did not allow determination of IC_{10} values. Compared to another SPR study on detection of DON and T-2/HT-2, our assay is comparable for DON while being more sensitive for T-2/HT-2.[30,31] Compared to the reported values of a previous multiplex SPR study, our assay may be judged less

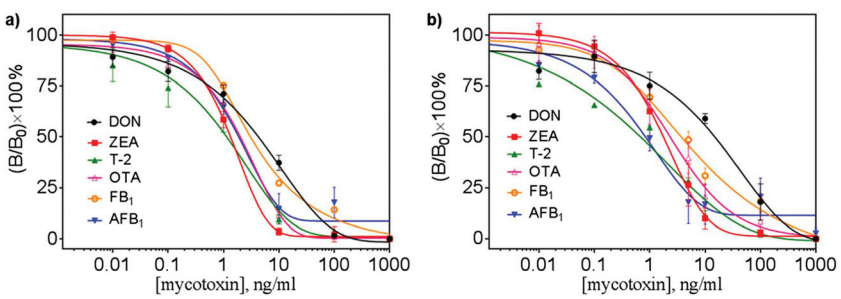

Fig. 2 Calibration curves in (a) buffer and (b) barley extract for the six mycotoxins measured in a double 3-plex format using SPR biosensing (Biacore) (n = 3). Two carboxymethylated dextran modified flat gold chips were used: one for DON, T-2 and ZEA and another one for AFB_1, OTA and FB_1.

Table 1 The maximum levels (MLs in μg kg^{-1}) for the six toxins as specified by the EU,[3,4] limit of detection (LOD), IC_{50} and the working range obtained from calibration curves in barley extract measured using the double 3-plex SPR assay and 6-plex nanostructured iSPR assay (n = 3). All values are expressed in μg of toxin per kg of barley

		Double 3-plex SPR			6-Plex nanostructured iSPR		
Toxins	MLs	LOD[a]	IC_{50}	Working range[b]	LOD[a]	IC_{50}	Working range[b]
DON	1250	26[e]	480	26–3200	64	1220	192–4800
ZEA	100	6	51	16–160	96	960	224–8000
T-2	200[c]	0.6[e]	16	0.6–290	26	580	80–3800
OTA	5	3	67	13–320	160	1280	320–5700
FB_1	2000[d]	2	112	10–1200	13	480	48–3800
AFB_1	2	0.6	26	3–260	10	640	48–8000

[a] LOD is defined as IC_{10} (concentration at which 10% inhibition of binding occurs) unless marked with an asterisk. [b] Working range is defined as IC_{20}–IC_{80} (concentration at which 20%–80% inhibition of binding occurs). [c] Recommendation for sum of T-2 and HT-2. [d] Sum of FB_1 and FB_2 in unprocessed maize. [e] IC_{20} is used as LOD, as the fitting does not allow determination of IC_{10} for this toxin.

Table 2 SPR detection limits of mycotoxins in buffer (in ng mL^{-1}) and in different matrices (in μg kg^{-1}) reported in the literature. Sensitivity of the present work can be found in Table 1 and S1

	Buffer (ng mL^{-1})		Matrix (μg kg^{-1})	
Toxins	Singleplex	Multiplex	Singleplex	Multiplex
DON	—	—	—	0.5, 1–29, 68–84[26,31,33]
ZEA	—	—	—	0.01, 40–64[26,33]
T-2	—	—	26[30]	31–47[31]
OTA	—	—	0.06–0.5[32]	0.1[33]
FB_1	—	—	—	50[33]
AFB_1	3.0[29]	—	—	0.2[33]

Table 3 IC_{50} values and corresponding cross-reactivities of mycotoxin metabolites measured in singleplex format using SPR (Biacore) (n = 3)

Metabolite	IC_{50} (ng mL^{-1})	Cross-reactivity (%)	Metabolite	IC_{50} (ng mL^{-1})	Cross-reactivity (%)
DON	5.6	100 ± 7	FB_1	2.7	100 ± 9
D3G	10	56 ± 5	FB_2	2.6	104 ± 2
3ADON	5.0	112 ± 12	FB_3	2.0	135 ± 4
NIV	ND	<1			
ZEA	1.5	100 ± 8	T-2	1.9	100 ± 8
α-ZEL	1.9	79 ± 5	HT-2	2.5	76 ± 5
AFB_1[a]	0.1	100			
AFB_2[a]	0.6	17			
AFG_1[a]	0.7	14			
AFG_2[a]	1.1	9			
AFM_1[a]	1.1	9			

ND = not detectable. [a] Determined by ELISA.

sensitive for all toxins except FB_1 and AFB_1.[33] However, such a comparison is unjustified as the method for calculating the LOD is not described in ref. 33 and the calibration curves are not shown in matrix (nevertheless the LODs are reported in ng g^{-1} for "sample" without specification of the sample[33]). In case of AFB_1, the reported sensitivity[33] is comparable to our assay. The most significant difference can be seen in our FB_1 assay, which is 25 times more sensitive than the previous SPR assay.[33] For AFB_1, our assay in buffer is almost ten times more sensitive than another SPR assay reported in literature.[29] For OTA in cereals, our assay is ten times less sensitive than the assay in a previous report.[32] This can be easily explained by the gold nanoparticles used in ref. 32 for SPR signal enhancement as the authors claim a 17-fold increase in sensitivity due to the nanoparticles. Compared to benchtop iSPR,[26] our benchtop SPR assay is about 2–3 times more sensitive for DON and 6–10 times more sensitive for ZEA.

Cross-reactivity with modified mycotoxins

The double 3-plex immunoassay was tested with modified mycotoxins that commonly occur in food and feed (Table 3). The antibodies against DON showed significant cross-reactivity towards D3G and 3ADON as seen in previous reports.[31] However, in contrast with literature, no cross-reactivity was seen with NIV in our case. This could be due to the use of polyclonal antibodies in the earlier studies compared to monoclonal antibodies used here. Such lack of cross-reactivity with NIV has been seen in other reports where antibodies similar to ours were used.[17,26] Antibodies against ZEA (anti-ZEA) were cross-reactive towards α-ZEL and the anti-FB_1 towards FB_2 and FB_3. The measured cross-reactivity of anti-T-2 towards HT-2 has been reported earlier.[30,31] According to ELISA studies performed by the supplier and the data provided, antibodies against AFB_1 show cross-reactivities towards AFB_2, AFG_1, AFG_2 and AFM_1. Such cross-reactivities from ELISA have also been reported in earlier literature.[29] The observed cross-reactivity might lead to overestimation of the targeted mycotoxin concentration in real samples. Note that in some cases, such as T-2 and HT-2, FB_1 and FB_2, and the aflatoxins (data obtained using ELISA from Europroxima), the cross-reactivity is desirable as the regulations specify the summed toxin concentration. In other cases such as DON, ZEA and AFB_1 where the cross-reactivity leads to overestimation of target mycotoxin, the "suspect" samples can be subjected to further quantitative LC-MS/MS analysis.

Durability of SPR chips following multiple analyses and regenerations

All three calibration curves for the double 3-plex SPR assay (singleplex in buffer, multiplex in buffer, multiplex in barley extract) were measured in triplicate using the same concentration of antibodies on the same chip. The minor differences between the first (singleplex in buffer) and last calibration curve (multiplex in barley extract) showed that the chips can be re-used at least 60 times.

Preliminary in-house validation of the double 3-plex SPR assay

For validation, 21 blank barley and 21 spiked barley samples were measured using the double 3-plex SPR assay. The following levels of toxins were used for spiking of barley: DON (1/2 × ML, 625 μg kg^{-1}), ZEA (1/2 × ML, 50 μg kg^{-1}), T-2 (1/8 × ML, 25 μg kg^{-1}), OTA (2 × ML, 10 μg kg^{-1}), FB_1 (1/5 × ML, 400 μg kg^{-1}) and AFB_1 (4 × ML, 8 μg kg^{-1}). For four toxins (DON, ZEA, T-2 and FB_1), the cut-off factor (Fm) was smaller than the threshold value (T) (Fig. S6†) as required for a successful validation (see Experimental section).[37] The concentrations corresponding to the average measured response were 22% lower for DON, 24% lower for ZEA, 28% lower for T-2 and 26% lower for FB_1, when compared to the spiked levels probably due to incomplete extraction. Despite this, inhibition was seen for all four toxins in 19 out of 21 samples. These samples would be considered "suspect" in our screening test and would be subjected to further quantitative analysis by LC-MS/MS. For OTA and AFB_1, based on the calibration curve, validation at the relevant MLs is not possible as discussed earlier. Based on the results obtained at 2 × ML for OTA and 4 × ML for AFB_1 (data not shown), the current double 3-plex assay would require levels of approximately 5 × ML for OTA and 20 × ML for AFB_1 for a successful validation (Fm < T).

The inter-day and intra-day precision (RSD%) of the assay were calculated as the standard deviation of the data discussed above and are depicted in Table 4. Since the 21 samples were divided over three different days, the average values of the different inter- and intra-day RSDs are reported. In all cases, as expected the inter-day RSDs are higher than the intra-day. The RSDs of our assay (comparable to previous SPR assays[30,31]) demonstrate that the assay is fit-for-purpose.

Table 4 Inter- and intraday precision of the mycotoxin assay for blank and spiked barley samples measured with double 3-plex SPR assay

		RSD for each assay in %					
Sample	Precision	DON	ZEA	T-2	OTA	FB_1	AFB_1
Blank	Intra-day (n = 21)	5.4	3.6	3.1	1.8	4.4	1.5
	Inter-day (n = 18)	11.7	9.4	8.9	4.6	7.1	5.5
Spiked	Intra-day (n = 21)	0.9	4.3	1.2	3.4	4.3	5.2
	Inter-day (n = 18)	9.4	9.6	9.9	6.2	7.2	11.4

Measurement of naturally contaminated barley samples using the double 3-plex SPR assay

To demonstrate the application of the double 3-plex assay, naturally contaminated barley samples containing DON (CS1, 289 μg kg^{-1} as measured by ELISA, <50 μg kg^{-1} as measured by LC-MS/MS), ZEA (CS2, 729 ± 244 μg kg^{-1}), T-2/HT-2 (CS3, 60 μg kg^{-1}) and OTA (CS4, 25 μg kg^{-1} as measured by ELISA, 210 μg kg^{-1} as measured by LC-MS/MS) were tested. Compared to the mixed blank used for normalization and the blank samples (B−), all the contaminated samples (CS) showed significant inhibition (Fig. 3), thus proving the applicability of the double 3-plex assay for screening of naturally contaminated samples. For naturally contaminated barley samples containing DON, the measured concentration is 27% lower than the value obtained by ELISA. Although the sample was considered as blank by LC-MS/MS, the higher concentration measured in our assay could be due to the presence of other modified forms of DON (<100 μg kg^{-1} DON3G, <40 μg kg^{-1} 3ADON and <40 μg kg^{-1} 15ADON according to LC-MS/MS analysis) and their cross-reactivity with anti-DON used in the study. The measured concentration for naturally contaminated samples containing OTA is only 36% lower than values obtained by ELISA. But the LC-MS/MS value for the sample is 800% higher than measured by ELISA. Although the reason for such a variation is not completely clear, such differences between values measured by ELISA and LC-MS/MS have been reported earlier in the literature[42] and may be caused by sample inhomogeneity or other discrepancies between the sample matrix and the blank matrix used for calibration. Compared to the known values, the measured concentrations are about 21% lower for ZEA and 46% lower for T-2. The lower measured concentrations in naturally contaminated samples of ZEA, T-2 and OTA are probably partially due to incomplete extraction recovery (see validation results). In contrast to LC-MS/MS analysis where a stable isotope internal standard can be used, no correction for incomplete recovery is possible in our case. Therefore, a matrix matched calibration curve would be required before analysis of contaminated samples. Naturally contaminated samples for the other two mycotoxins (FB_1 and AFB_1) were not available and therefore not included in the present study.

Transferability of the double 3-plex SPR assay to a 6-plex assay on a prototype portable iSPR instrument

One of the hurdles for SPR to be used in field applications is the size and weight of the instrument. Therefore, a prototype portable iSPR instrument with nanostructured iSPR chips, previously used to study the antifouling properties of biosensor chips[35] and demonstrated in a medical application,[27] was tested for the transferability of the developed assay. OVA and the same six mycotoxin–OVA conjugates as used in the benchtop SPR assay were covalently immobilized on a PEG3500-modified nanostructured iSPR chip. PEG3500 was chosen because of its well-characterized antifouling properties.[35] Although higher concentrations (50–200 times) of conjugates

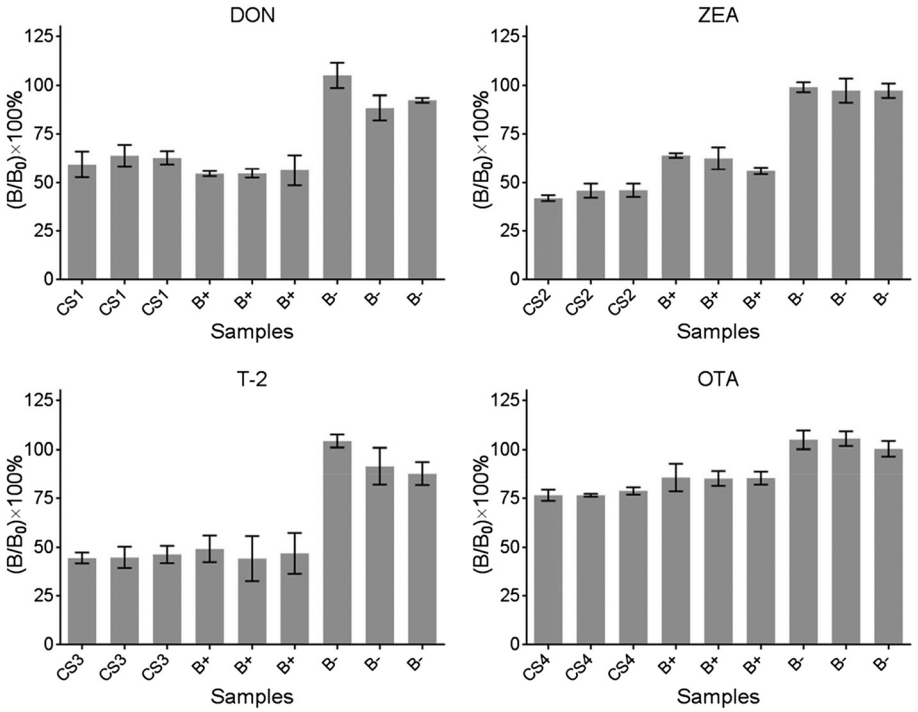

Fig. 3 Relative responses measured for naturally contaminated barley samples for presence of DON, ZEA, T-2 and OTA using double 3-plex benchtop SPR (Biacore). Three types of barley extract were used for each contaminated sample measurement: blank barley (B–), spiked barley (B+) and contaminated sample (CS). Each sample (B–, B+ and CS) were extracted in triplicate and each extract was measured in triplicate. The responses are relative to the response of a mixed blank barley. The error bars are the standard deviation of triplicate responses of each extract.

were used during the immobilization on the iSPR chips, manual spotting required much lower sample volumes (2 μL) compared to the Biacore (100 μL). The optimal concentrations of the antibodies (corresponding to an SPR response of 0.04–0.05 units) in the 6-plex assay were 5 μg mL^{-1} for FB_1 and 50 μg mL^{-1} for OTA and ZEA, T-2, DON, and AFB_1. Note that the nanoplasmonics iSPR assay required higher concentrations of antibodies (50 times for FB_1, OTA and ZEA and 25 times for DON, T-2 and AFB_1) relative to the double 3-plex SPR (see above), which is due to two main factors: difference in surface chemistry of the two surfaces and hardware sensitivity. The iSPR chips are coated with a 2D polymer (PEG3500) having fewer binding groups (COOH) compared to the 3D hydrogel (CMD) in the double 3-plex SPR set-up. This limits the amount of mycotoxin conjugates that can be immobilized on the surface, thus requiring higher concentrations of antibodies. Furthermore, as seen in our previous study, the sensitivity of the prototype iSPR instrument is approximately ten times lower than that of the Biacore,[35] thus more antibodies are required to give a detectable response in the inhibition assay.

For the double 3-plex SPR assay (Biacore), the singleplex calibration curves in buffer were rather similar to the multiplex calibration curves in buffer (Fig. S5†). Upon measuring FB_1 calibration curves using the prototype nanostructured iSPR instrument, the two calibration curves were again similar. Extrapolating on this, for the other five toxins only multiplex calibration curves in buffer and barley extract were measured in the prototype iSPR (Fig. S7† and Fig. 4). As expected, the calibration curves indicate a decrease in sensitivity compared to the double 3-plex SPR assay. For most toxins, the sensitivity (Table 1) is about 3–20 times less than for the Biacore and similar to a benchtop iSPR instrument (for DON and ZEA),[26] except for OTA and T-2 where the iSPR assay is almost 40 and 50 times less sensitive than the benchtop SPR assay, respectively. The difference could be due to the amount of OVA conjugates immobilized on the surface. Although the spots can be visualized in the imaging instrument, the actual amount of immobilized conjugates cannot be as easily checked as in the Biacore. Note that even in the benchtop SPR assay (Biacore), a second injection of OTA–OVA and T-2–OVA was required to achieve a good response. For four toxins (DON, ZEA, T-2 and FB_1), the 6-plex assay is sensitive enough to detect the toxins at MLs as defined by EU legislation.[3,4] In case of OTA and AFB_1, better binding antibodies will be required to obtain the required sensitivity. One of the contaminated samples containing 729 ± 244 μg kg^{-1} of ZEA (22.7 ng ml^{-1} assuming 100% recovery) was also measured using the prototype nanoplasmonics iSPR instrument. Although, the measured concentration was 31% lower than stated, inhibition was clearly seen relative to the mixed blank barley sample (Fig. S8†). To demonstrate the applicability of the assay as a screening method for in-field appli-

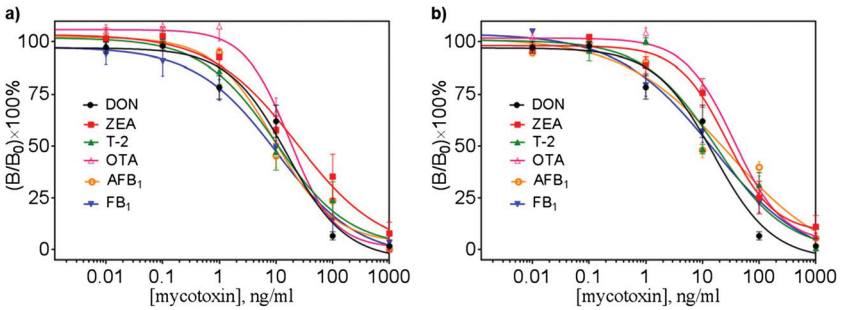

Fig. 4 Calibration curves in (a) buffer and (b) barley extract for the six mycotoxins measured in a 6-plex format measured using a prototype nanostructured iSPR instrument. A single PEG3500 chip was used for detection of DON, ZEA, T-2, OTA, FB_1 and AFB_1.

cations, the same contaminated sample was extracted using a simpler extraction protocol (see Experimental section on naturally contaminated samples). Both extraction protocols gave similar results (Fig. S8†) showing the potential of such an extraction protocol for future field applications. The simplified sample preparation is both time and cost effective as it required less equipment and expert personnel. The performance of the portable iSPR assay may be further improved by using a 3D hydrogel chip surface comparable to CMD or by functionalized zwitterionic polymer brushes. Another approach for sensitivity improvement could be based on confinement-induced signal enhancement.[43,44] In a nanostructured chip, in addition to the bulk SPR due to the propagation of surface plasmons, localized SPR (LSPR) occurs. The LSPR mode has been reported to be eight times more sensitive than the bulk SPR mode.[27] The maximum of the evanescent wave from LSPR is at the top of the PMMA wells as described in ref. 44. Therefore, controlled and precise filling of these wells in such a way that the OVA–toxin conjugates are exactly near the top of the well after immobilization, may help to boost sensitivity. The performance of the prototype instrument itself can be improved by upgrading the hardware.

The benchtop SPR and prototype nanostructured iSPR each have their own advantages and disadvantages. While nanostructured iSPR is in this application less sensitive than benchtop SPR, it allows simultaneous detection of six toxins using one chip. This would be beneficial for rapidly detecting both targeted and other less frequently occurring mycotoxins in real samples. Additionally, if the sensitivity could be improved, nanostructured iSPR would save costs in terms of the chip, reagents as well as assay time. However, it should be kept in mind that optimization of regeneration conditions can be difficult in a one-chip format. In a double 3-plex format with benchtop SPR, regeneration-sensitive conjugates (such as DON–OVA and T-2–OVA) can be separated from the conjugates that are more resistant to regeneration (such as AFB_1–OVA and FB_1–OVA). The toxins can also be separated based on their required detection levels set by EU. For some toxins, such as DON and FB_1, diluted sample extracts would be desirable, whereas for others (OTA and AFB_1) concentrated extracts would be desirable.

Conclusions

Mycotoxin contamination of food and feed is a major concern for producers and quality controllers. Most published mycotoxin detection studies have focused on the detection of one or a few mycotoxins. In addition to multiplex detection, there is also a demand for a miniaturized instruments to bring the lab to the sample. However, a benchmark to properly evaluate such prototype miniaturized instruments is missing from the literature. This work provides such a comprehensive study for the multiplex detection of six of the most relevant mycotoxins. The developed benchmark method allows detection of deoxynivalenol (DON), zearalenone (ZEA), T-2 toxin, ochratoxin A (OTA), fumonisin B_1 (FB_1) and aflatoxin B_1 (AFB_1) in a double 3-plex format using a benchtop SPR (Biacore) with two separate SPR biosensor chips. The detection range of the assays allows the detection of four mycotoxins (DON, ZEA, T-2 and FB_1) in barley at the regulatory limits. For OTA and AFB_1, further improvement in sensitivity, either by using better antibodies or using less diluted samples, is still required for screening at the regulatory limits. The sensitivity of our assay is comparable to the reported literature values for singleplex SPR assays, but offers the benefit of multiplexing. In case of FB_1, our assay is 25 times more sensitive than earlier SPR assays. The validation and measurement of contaminated samples show that the recovery of the toxins is about 75%. Thus, the developed double 3-plex SPR assay could serve well as a semi-quantitative screening method, to be followed up by further analysis of any "suspected" samples using LC-MS/MS. Finally, the transfer to a prototype portable iSPR instrument showed the simultaneous detection of all six mycotoxins in a 6-plex format using a single nanostructured iSPR chip. The prototype iSPR sensitivity, although less than that of the benchtop instrument (Biacore), could be improved by different strategies including change of surface chemistry, selection of better antibodies and hardware upgrades. The demonstrated transferability provides a step forward towards use of a miniaturized iSPR for at-line and in-field biosensing screening of mycotoxins in barley.

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