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RESEARCH PAPER

A multi-analyte biosensor for the simultaneous label-free detection of pathogens and biomarkers in point-of-need animal testing

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Abstract For the first time, a multi-analyte biosensor platform has been developed using the label-free 1-lambda-reflectometry technique. This platform is the first, which does not use imaging techniques, but is able to perform multi-analyte measurements. It is designed to be portable and cost-effective and therefore allows for point-of-need testing or on-site field-testing with possible applications in diagnostics. This work highlights the application possibilities of this platform in the field of animal testing, but is also relevant and transferable to human diagnostics. The performance of the platform has been evaluated using relevant reference systems like biomarker (C-reactive protein) and serology (anti-*Salmonella* antibodies) as well as a panel of real samples (animal sera). The comparison of the working range and limit of detection shows no loss of performance transferring the separate assays to the multi-analyte setup. Moreover, the new multi-analyte platform allows for discrimination between sera of animals infected with different *Salmonella* subtypes.

Keywords 1-lambda-Reflectometry · Multi-analyte · Label-free · *Salmonella* infection · CRP · Optical biosensors · Point-of-need (PON)

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Introduction

In the field of biomolecular interaction analysis, parallel detection of multiple analytes gained in importance during the last years [1–3]. Especially in the areas of food safety [4, 5], environmental analysis [6], and medical diagnostics [7], multiplex assays that are integrated on portable devices are desired to obtain results of high scientific quality within a short time and at low costs.

To fulfill this demand, the use of biosensors is particularly interesting. Due to their small size, they are portable and can be used for on-site measurements. Immunosensors that rely on the specific interaction between an antigen and an antibody allow for highly specific and highly sensitive analyte detection and therefore offer the opportunity for a qualitative and quantitative determination of multiple analytes from complex matrices [8]. In combination with label-free optical detection technologies, they can form a powerful, low-priced complement to standard analytical methods.

For these reasons, we developed a label-free portable biosensor platform, allowing for the detection of multiple analytes in one sample in a time efficient and cost efficient manner. The direct optical method named 1-lambda-reflectometry [9] is a type of reflectometric interference spectroscopy (RIfS) [10]. As RIfS it is based on the interference of reflected light at thin layers. When the analyte is captured by recognition elements at the sensitive layer, a change of refractive index and physical thickness occurs resulting in a change of reflected light intensity. Monitoring this change via a

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photodiode at a certain wavelength allows for a simple and portable measuring setup.

Compared to other optical, label-free techniques, 1-lambda-reflectometry has some main advantages: First, transducers do not need any gold coating or other optical surface structures like it is the case within evanescent field methods, e.g., SPR [11], grating couplers [12] or resonant mirrors [13]. Second, 1-lambda-reflectometry is very robust in regard to temperature fluctuations, as refractive index and physical thickness change in opposite ways concerning temperature effects [14]. Third, 1-lambda-reflectometry allows for a simple and portable measuring setup, as the optical components, the light source and the photodiode are integrated within a confocal working detection module that nearly has the size of a smartphone [15].

The feasibility of 1-lambda-reflectometry to quantify small amounts of analyte in complex matrices within a short time already has been demonstrated in detail for the detection of *Salmonella* antibodies out of undiluted animal sera [8]. Recently, a parallelized, imaging type of 1-lambda-reflectometry has been realized as a laboratory setup using a CCD camera for the detection of multiple antigen–antibody interactions within a single measurement [16]. This paper also focuses on multi-analyte detection within one sample, but in this work a portable and lower-priced setup is aimed to be used instead. For this reason, the transducer is situated on an *x*-*y*-movable table allowing the 1-lambda-reflectometry detection module to monitor different spots on the transducer one after another. By running several cycles over all spots, a time-resolved signal for each spot is obtained offering the opportunity to calculate thermodynamic and kinetic constants for each receptor-analyte interaction [17].

To demonstrate the principal feasibility of the new multi-analyte platform, two assays have been developed. To give an example for the detection of antibodies in the field of veterinary diagnostics, the first assay has been developed for monitoring *Salmonella* infections. To exemplarily show the applicability of the new platform for the detection of relevant parameters in the field of human diagnostics, the second assay has been developed for monitoring the inflammation marker C-reactive protein (CRP). However, CRP is also a relevant biomarker in animal diagnostics. As the concentration of CRP in blood for both humans and animals increases in case of bacterial infections, the two parameters are complementary towards each other. Therefore, measuring both parameters out of one sample allows for detecting *Salmonella* infections and for determining the status of the infection by quantifying CRP. Both assays have been fully characterized using a single-spot 1-lambda-reflectometry setup first. To find the best working assay conditions for the detection of both analytes in parallel, single-assay conditions like sensitive layer components, working buffer and regeneration solution had to be harmonized. After assay harmonization, both assays have been transferred to the new multi-analyte platform by combining two recognition spots on one transducer. Due to the repeated movement of the transducer within this setup, robustness of the new platform concerning variations in the distance of the transducer to the detection module has been investigated. Furthermore, a new multi-analyte flow cell has been characterized concerning depletion effects along the flow channel. By measuring both *Salmonella* antibodies and CRP out of one sample, calibration curves and working ranges, as well as recovery rates, have been determined and have been compared to those obtained by single-spot measurements.

To demonstrate the performance of the new platform in real matrices, measurements with reference sera of *Salmonella* infected chicken have been carried out. Discrimination between positive sera and negative sera was possible and reproducible. Moreover, the classification of *Salmonella*-infected serum to the appropriate *Salmonella* serovar was successful.

Materials and methods

Materials

1-lambda-Reflectometry transducer chips (10×10 mm sized for one-spot measurements, 37×12 mm sized for multi-spot measurements) made of 1-mm-thick BK7-glass substrate with a layer of 45 nm Ta_2O_5 and 20 nm SiO_2 on top were purchased from Berliner Glas, Berlin, Germany.

Ultrapure water was taken from an in-house PURELAB Classic device from ELGA LabWater, Celle, Germany.

Common organic compounds were obtained from Fluka, Neu-Ulm, Germany; Sigma-Aldrich, Deisenhofen, Germany and Merck, Darmstadt, Germany.

3-Glycidyloxypropyl-trimethoxysilane (GOPTS) was purchased from Fluka Neu-Ulm, Germany.

Amino functionalized dextran (AMD; M_w=100 kDa, 50 % amino functions for *Salmonella* assay and 10 % amino functions for CRP and multi-analyte assay) was purchased from Innovent e.V. Jena, Germany.

Lipopolysaccharides *Salmonella enterica* serovar Typhimurium and *Salmonella enterica* serovar Enteritidis were purchased from Sigma-Aldrich, Deisenhofen, Germany.

Polyclonal antibody “rabbit anti *Salmonella* group antigen” was obtained from AbD Serotec, Oxford, UK.

Antibody-positive and antibody-negative reference sera from *Salmonella*-infected chicken were purchased from GD Animal Health Service, Deventer, Netherlands.

The protein CRP and the monoclonal anti-CRP antibody (C5-clone, IgG_{2a}) were purchased from Meridian Life Science, Inc., Memphis, USA.

Bovine serum albumin (BSA) and fetal bovine serum (FBS) were purchased from Sigma-Aldrich, Deisenhofen, Germany.

Phosphate-buffered saline (PBS), pH 7.4, comprising 150 mM sodium chloride and 10 mM potassium phosphate monobasic was used as running buffer for single-spot *Salmonella* assay.

HEPES, pH 7.4, comprising 10 mM HEPES, 150 mM sodium chloride and 5 mM calcium chloride was used as running buffer for single-spot CRP assay and multi-spot measurements.

For regeneration of the biosensor surface, a solution of 6 M guanidine hydrochloride at pH 1.5 was used for *Salmonella* assay and multi-analyte assay. For CRP single-spot assay, a solution of 0.5 % sodium dodecyl sulfate (SDS) at a pH of 2 was used.

Surface chemistry

Cleaning and activation of the transducer surface

The transducers were cleaned in a 6 M potassium hydroxide solution for 1 min, rinsed with water and then activated by ultrasonication in a freshly prepared solution of hydrogen peroxide and concentrated sulfuric acid (2:3 ratio) for 15 min. After that, the transducers were rinsed with ultrapure water and dried in a nitrogen stream.

Silanization

For modification of transducers, 7.5 μL of GOPTS was pipetted on a freshly activated transducer and then covered with a second transducer. After 1 h, the single slides were rinsed with dry acetone and dried in a nitrogen stream.

Immobilization of the biopolymer

For modification of transducers, 10 μL of AMD solution of 0.2 mg/μL in ultrapure water was pipetted on a silanized transducer and covered with a second one. After reaction for up to 24 h within a humidity chamber, the single transducers were rinsed with ultrapure water and dried in a nitrogen stream.

Immobilization of lipopolysaccharides

A volume of 1.3 μL diisopropylcarbodiimide (DIC) was added to a solution of 0.5 mg decanoic acid in 8 μL *N*,*N*′-dimethylformamide (DMF). The mixture was pipetted on an AMD-modified transducer and a second AMD transducer was used to cover it. After reaction for at least 6 h within a DMF saturated chamber, the single transducers were rinsed with DMF, ultrapure water and then dried in a nitrogen stream. For immobilization of lipopolysaccharides (LPS), 15 μL of an LPS solution of 1 mg/mL in PBS was pipetted on the middle of a transducer with decane residue and left to react for at least 24 h within a humidity chamber. Prior to the measurement, the transducers were rinsed with ultrapure water and dried in a nitrogen stream.

Immobilization of anti-CRP antibodies

To change terminal amino groups of biopolymer coated transducers into carboxyl functions, 7.5 μL of a glutaric anhydride solution of 2 mg/μL in DMF was pipetted on an AMD-coated transducer and covered with a second one (sandwich). After reaction for at least 6 h within a DMF saturated chamber, the single transducers were rinsed with DMF, ultrapure water and then dried in a nitrogen stream. The transducers were activated as sandwich with 7.5 μL of a solution of 1 M NHS/1.5 M DIC in DMF for 4 h. Afterwards, the single transducers were rinsed with DMF and acetone and dried in a nitrogen stream. For immobilization of anti-CRP antibodies, 10 μL of an anti-CRP antibody solution of 0.1 g/L in PBS was pipetted on a freshly activated transducer and a second activated transducer was used to cover it. After reaction for 2 h within a humidity chamber at room temperature, the modified transducers were further incubated at 4 °C overnight.

Immobilization of lipopolysaccharides and anti-CRP antibodies on one transducer

For each modification step carried out on transducers for multi-spot measurements, the same reagent concentration, incubation time, and incubation chamber was used as within modification steps on single-spot transducers, respectively. Due to their larger size, modification of transducers for multi-spot measurements was carried out with a 4.5-fold larger volume of GOPTS and AMD, respectively. For parallel modification of AMD modified multi-analyte transducers using decanoic acid for one spot and glutaric anhydride for another spot, 5 μL of the appropriate solution was pipetted on the designated position and covered with a 18×18 mm coverslip, respectively.

For activating the glutaric acid position, 5 μL of the appropriate solution was pipetted on the glutaric acid spot and covered with a coverslip. At the same time, no modification was carried out at the decane residue spot.

For immobilization of the appropriate recognition elements, LPS was pipetted on the decane spot while 5 μL of anti-CRP antibody solution was pipetted on the activated spot and covered with a coverslip.

Single- and multi-analyte 1-lambda-reflectometry setup

The 1-lambda-reflectometry setup consists of a modified detection module (ESE-LOG USB, Qiagen Lake Constance GmbH, Germany) [18] including a light-emitting diode with a wavelength of 470 nm. The detection module focuses a

transparent glass transducer coated with several thin layers [19] from its backside by integrated confocal optics. Light is partially reflected at each interface of the transducer and the reflected beams superimpose. In case of a binding event on top of the transducer due to recognition, the upper layer changes in refractive index and physical thickness. When light passes this layer and is reflected, changes in the optical thickness (product of refractive index and physical thickness) can be monitored by a change of the reflection coefficient. This can be read out via an integrated photodiode at the certain wavelength as a change of reflected light intensity.

The multi-analyte 1-lambda-reflectometry setup consists of a detection module as it is used for single-analyte measurements and a movable *x*-*y*-table including a carrier for a multi-analyte transducer with flow cell. By moving the *x*-*y*-table, single measuring positions on the transducer can be read out by the detection module that is situated underneath. A schematic illustration of the multi-analyte 1-lambda-reflectometry setup can be found in Fig. 1.

The liquid handling system consists of a flow cell that connects the transducer with a Hamilton dilutor Microlab (Hamilton, Switzerland) with two syringe pumps and an eight-way valve. For sample transfer to the flow cell, a CETAC ASX-130 autosampler (CETAC Technologies, USA) is used allowing for fully automated measurements.

Calibration

For calibration, each antibody concentration was measured threefold. Single-spot measurements were carried out in random order to exclude any carry-over effects in the liquid handling system. During calibration of the multi-analyte sensor, measurements were repeated from lower to higher concentration due to reasons of convenience.

The different steps of a measurement cycle can be divided into a baseline with buffer, followed by the injection of a defined volume of sample. After observing dissociation by flushing the flow cell for about 1 min with buffer, the sensitive layer is regenerated with acidified regeneration solution and again flushed with buffer. All sample injections were carried out at a flow rate of 0.5 μL/s.

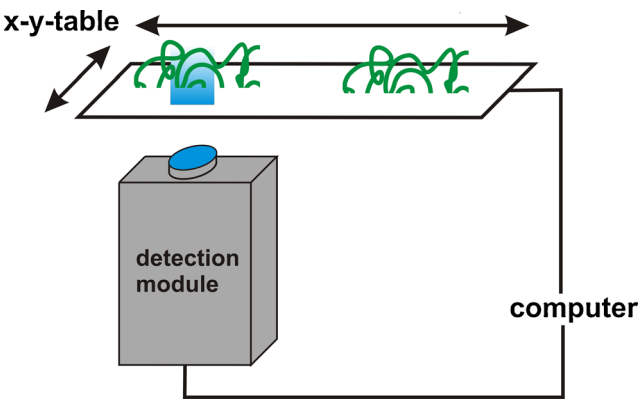

Fig. 1 Scheme of the multi-analyte 1-lambda-reflectometry setup with an *x*-*y*-movable table and a detection module

For detection of anti-*Salmonella* antibodies, sample volume was 500 μl. As binding kinetics of CRP were very fast, signals for different CRP concentrations stopped to change after some seconds of injection as they reached equilibrium. Therefore, CRP sample volume was reduced to 250 μl. For detection of both, anti-*Salmonella* antibodies and CRP using the multi-analyte platform, sample volume was set to 500 μl.

For multi-analyte measurements using reference sera, samples were diluted 1:10 with buffer. Furthermore, a blocking step using FBS in a dilution of 1:2 with buffer was introduced before sample injection to avoid nonspecific binding. For single spot-measurements using reference sera, sample volume was 250 μl.

Data evaluation

For a comparison of data, absolute measuring signals given by the detection module always were standardized on the first detected data point of a measurement. The level of the first baseline before sample injection was always set to 0 %.

For the evaluation of concentration dependent single- and two-spot measurements with anti-*Salmonella* antibodies, the relative signal change was determined through the level at a defined position of the second baseline.

For the evaluation of concentration-dependent measurements with CRP, relative signal change was determined after 307 s (two-spot measurements, 330 s) of sample injection at the point where equilibrium of the signal for the highest CRP concentration began, respectively.

Due to the reflectivity behavior of 1-lambda-reflectometry transducers in dependence of thickness changes in the sensitive layer, 1-lambda-reflectometry causes negative binding signals. As the optical thickness actually increases, we preferred to change the algebraic sign of mean values before we illustrated evaluated data graphically.

By plotting all mean values of the relative signal changes over the antibody concentration on semilogarithmic scale, a dataset is obtained that can be fitted by following four-parameter function (1).

$$y = A_2 + \frac{(A_1 - A_2)}{1 + \left(\frac{x}{x_0}\right)^p} \quad (1)$$

A_1 is the lower asymptote and A_2 the higher asymptote. The inflection point is given by the variable x_0, and the slope of the tangent in this point equates to the parameter p. This function describes a sigmoid curve progression which is a well-known approach to quantitatively evaluate immunoassay data [20]. Additionally, the 95 % confidence belt is plotted. To compare

the range in which the calibration curves obtained can be used to quantify the analyte, the minimum detectable concentration (MDC) and the reliable detection limit (RDL) values were determined [21]. These values are calculated by applying a tangent to the upper confidence curve at the lowest concentration. The intersection with the calibration curve marks the MDC while the intersection with the lower confidence curve marks the RDL. The working range is defined as concentration area of the 10–90 % dynamic signal range.

Recovery rates

For determination of recovery rates (RR), threefold measurements were carried out. The mean value of the absolute signal change is set as y in the four-parameter logistic function used for calibration. After solving the logistic function for x (2), the ratio of the calculated concentration and the real concentration is given as recovery rate (3):

$$x_{\mathrm{calc}} = \sqrt[p]{\left(\frac{A_1 - A_2}{y - A_2} - 1\right)} \cdot x_0 \tag{2}$$

$$\mathrm{RR} = \frac{x_{\mathrm{calc}}}{x_{\mathrm{real}}} \cdot 100\% \tag{3}$$

Results and discussion

To obtain a successful working biosensor, the preparation of its sensitive layer is of crucial importance and some demands should be fulfilled. Recognition elements should be immobilized in a stable way without any loss of their ability to recognize the analyte. Furthermore, when measurements are carried out in complex matrices, the sensitive layer should avoid nonspecific binding to matrix components. For the detection of *Salmonella* antibodies, immobilization of lipopolysaccharides of *Salmonella* was realized via hydrophobic interaction between the Lipid A of LPS [22] and decane residue on the sensor surface. For the detection of CRP, anti-CRP antibodies were immobilized covalently via amine coupling reaction. Resulting binding curves are shown in Fig. 2.

Before 1-lambda-reflectometry single-spot measurements were carried out with anti-*Salmonella* antibody and CRP, 1 g/L BSA was injected as sample and flushed over the surface. In spite of the 50–100-fold higher protein concentration of the BSA compared to the analyte solution, no binding signal has been observed indicating that the constructed sensitive layers avoid nonspecific binding to non-analyte molecules. The resulting binding curves for *Salmonella* antibodies and CRP, respectively, indicate that the recognition elements on

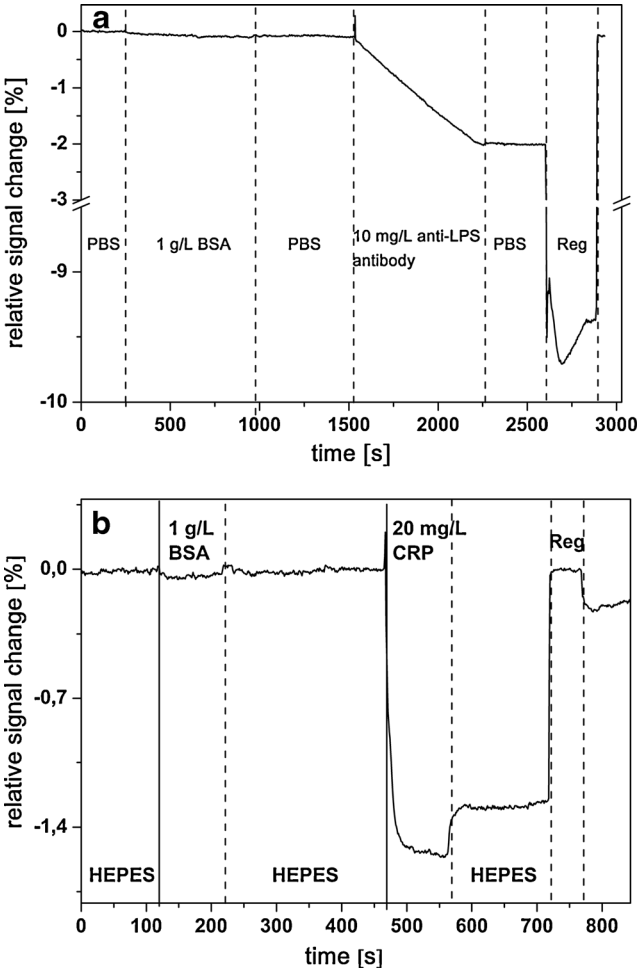

Fig. 2 1-lambda-Reflectometry binding signal obtained for 1 g/L bovine serum albumin (BSA) and 10 mg/L anti-*Salmonella* antibody (**a**), as well as for 1 g/L bovine serum albumin (BSA) and 20 mg/L CRP (**b**)

the surface kept their ability to still recognize their binding partner after the immobilization process.

Using regeneration solutions, multiple measurements on one chip were possible and concentration dependent signals within 1-lambda-reflectometry single-spot measurements could be obtained for both analytes (Fig. 3). Values of calibration curves obtained by single-spot measurements are given later in this paper, when they are compared to those obtained by two-spot measurements (see Table 2). Recovery rates obtained via measurements on chips of different preparation batches are given in Table 1.

By transferring both assays on one transducer, any cross reactivity or nonspecific binding between an analyte and a non-complementary recognition element has to be excluded. For this reason, we proved whether CRP molecules bind to immobilized LPS of *Salmonella* and whether anti-*Salmonella* antibodies bind to immobilized anti-CRP antibodies. In Table S1 in the Electronic Supplementary Material (ESM), results were compared to values obtained out of specific binding signals. Relative binding signals for the interaction of

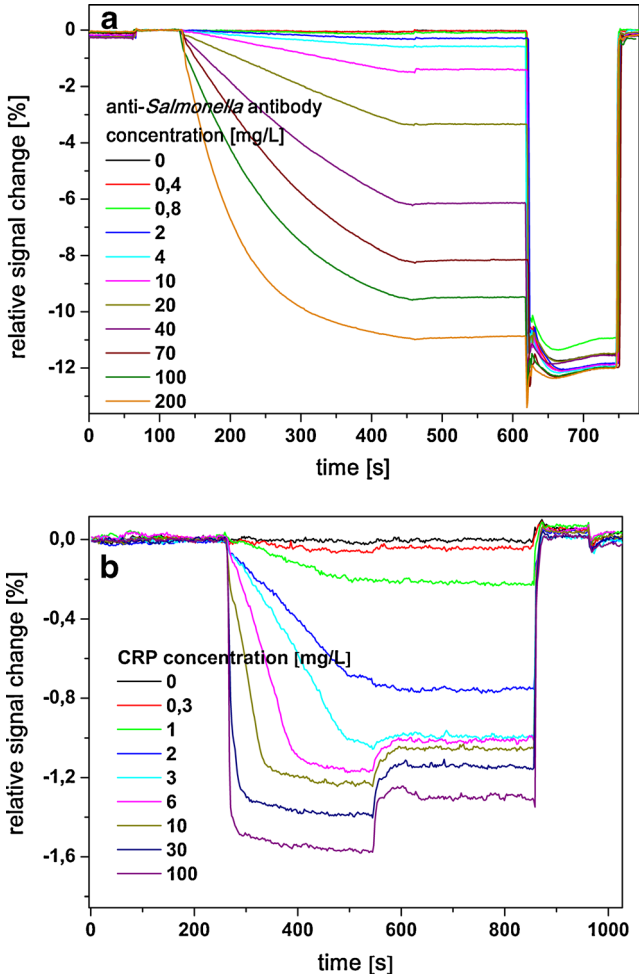

Fig. 3 Concentration dependent signals within 1-lambda-Reflectometry single-spot measurements for anti-*Salmonella* antibodies (**a**) and CRP (**b**). Different shapes of the binding curves can be traced back to different surface loading with the recognition elements LPS and anti-CRP antibody, respectively

analytes with non-complementary recognition elements are insignificantly small compared to respective signals for specific interaction of the appropriate analyte.

Before both assays were transferred to the multi-analyte platform, some preliminary tests characterizing the new setup were run. As the two-spot transducer is shifted multiple times within a measurement, changes in the distance between the transducer and the detection module could occur due to a shift in the position. Therefore, we first tested whether such changes influence the detected binding signal. For this reason, we carried out the same measurement one after another while changing the distance between detection module and transducer from measurement to measurement. Three different measuring positions were investigated, by starting with a position resulting in a maximal detector signal and shifting the transducer gradually closer to the detector.

A variation in the distance between transducer and detector is resulting in a change of the absolute signal detected by the photodiode. This is resulting in an appropriate change in the binding signal (see ESM Fig. S1A). After standardization of the absolute signals given by the detection module, relative binding signals all have the same slope (see ESM Fig. S1B). Thus, evaluated binding signals are independent from the distance chosen between transducer and detector and allow for a robust measuring setup that delivers reproducible results.

By monitoring binding events at multiple spots of a transducer, it is important to make sure that there are no depletion effects along the flow channel. For this reason, we completely covered a transducer surface with antigen and detected analyte binding signals at a position next to the inlet as well as next to the outlet of the flow channel. Results in ESM Table S2 show that in spite of a completely covered surface with recognition elements, no depletion effects along the flow channel could be observed, allowing for reproducible results at each measuring position on the transducer.

Based on these results, we transferred both single assays to the multi-analyte platform and carried out calibration by

Table 1 Recovery rates for anti-*Salmonella* antibody and CRP, respectively obtained via measurements on chips of three different preparation batches

Analyte	Concentration [mg/L]	Recovery rate [%]	Coefficient of variation [%]
Anti-*Salmonella* antibody	3	100	11
Anti-*Salmonella* antibody	30	94	9
Anti-*Salmonella* antibody	50	83	10
CRP	0,6	103	18
CRP	4	80	20
CRP	20	80	6

Table 2 Comparison of slope *p*, MDC, and RDL values with the resulting working range obtained by calibration via single-spot and two-spot 1-lambda-reflectometry measuring setup for both analytes, anti-*Salmonella* antibody and CRP, respectively

Analyte	Total no. of spots	Slope *p* [% · L/mg]	MDC [mg/L]	RDL [mg/L]	Working range [mg/L]
Anti-*Salmonella* antibody	1	1.43	1.62	2.43	8.26–179.34
	2	1.44	1.56	2.21	5.74 – 122.52
CRP	1	1.30	1.02	1.59	1.81 – 44.66
	2	1.39	0.51	0.72	1.26–29.56

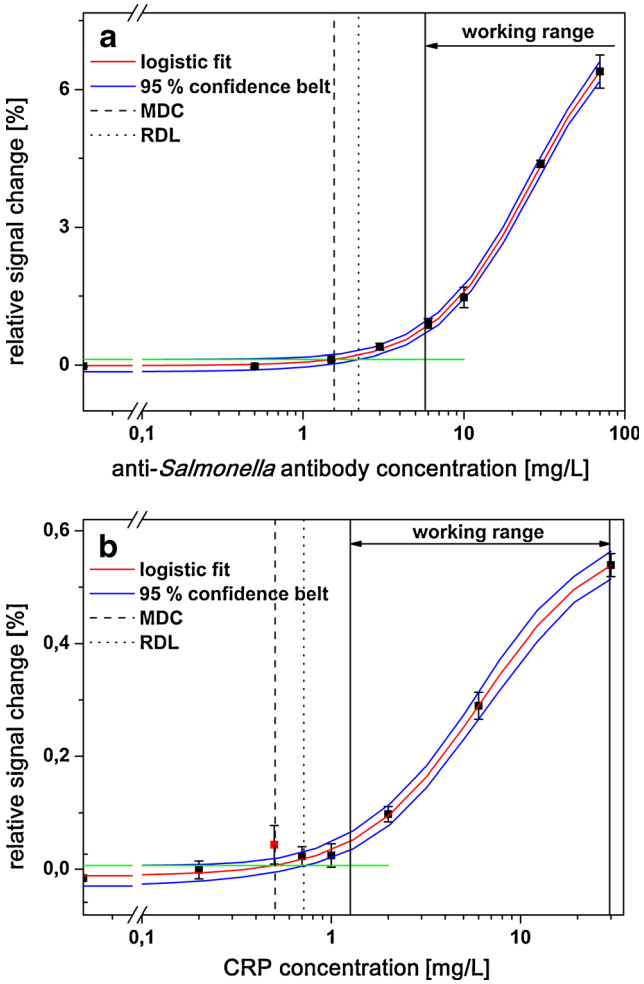

Fig. 4 Calibration curves obtained via 1-lambda-reflectometry two-spot measurements for anti-*Salmonella* antibodies (**a**) and CRP (**b**). Different concentrations of anti-*Salmonella* antibody and CRP were determined threefold

detecting both, anti-*Salmonella* antibodies and CRP out of one sample.

Figure 4 shows the calibration curves obtained via 1-lambda-reflectometry two-spot measurements. MDC and RDL values, the slope *p* as well as the working range of the calibration curves are compared to those obtained via single-spot measurements in Table 2.

For both analytes, the mean values of threefold measurements for different concentrations fit well to the four-parameter logistic function, respectively.

Calibration curve for anti-*Salmonella* antibodies obtained via two-spot measurements is resulting in a working range between 5.74 and 122.52 mg/L that is a little narrower than within the calibration curve obtained via one-spot measurements. As assay harmonization led to the use of a biopolymer with 10 % amine functions (instead of 50 % used for single-spot measurements), this could be an explanation why the calibration curve obtained via two-spot measurements reaches signal saturation earlier. Values for slope *p*, MDC, and RDL obtained via two-spot measurements are very close to those obtained within the single-spot 1-lambda-reflectometry setup for anti-*Salmonella* antibody detection.

Calibration curve for CRP obtained by two-spot measurements is slightly shifted to smaller values for MDC and RDL as well as for the working range, while the slope *p* of the calibration curve is little higher than within the one-spot calibration curve. Thus, sensitivity (slope *p*) and detection limit (MDC) determined via two-analyte CRP calibration are even better than those obtained via 1-spot CRP calibration.

To demonstrate that calibration of the two-analyte sensor is valid, recovery rates for different samples containing both anti-*Salmonella* antibodies and CRP have been determined threefold using transducers of different batches. Results are given in Table 3.

In general, in spite of another measuring setup used for two-spot measurements as well as slightly different assay conditions due to assay harmonization, values obtained via the new multi-analyte setup conform well to values obtained via single-spot 1-lambda-reflectometry setup. Comparison of those values for both parameters, anti-*Salmonella* antibodies and CRP, allow concluding that the new multi-analyte platform can be used for multi-analyte assays.

Real sample single-spot measurements

To demonstrate the applicability of the multi-analyte platform for complex matrices, anti-*Salmonella* antibody negative serum and two sera with anti-*Salmonella* antibodies against the serovar Typhimurium and Enteritidis have been examined concerning their interaction with LPS out of *Salmonella* Typhimurium and *Salmonella* Enteritidis, respectively.

First, single-spot measurements have been carried out and specificity of binding signals was proven by a binding inhibition assay. Results obtained at two different transducers, one covered with LPS Typhimurium and the other covered with LPS Enteritidis are shown in Fig. 5.

At the transducer covered with LPS Typhimurium, a large binding signal is obtained for serum of *Salmonella*

Table 3 Recovery rates for different samples containing both, anti-*Salmonella* antibodies and CRP

	Concentration [mg/L]	Recovery rate [%]	Coefficient of variation [%]
Anti-*Salmonella* antibody	4	102	26
	10	110	15
	20	82	15
CRP	3	84	21
	6	96	13
	10	81	29

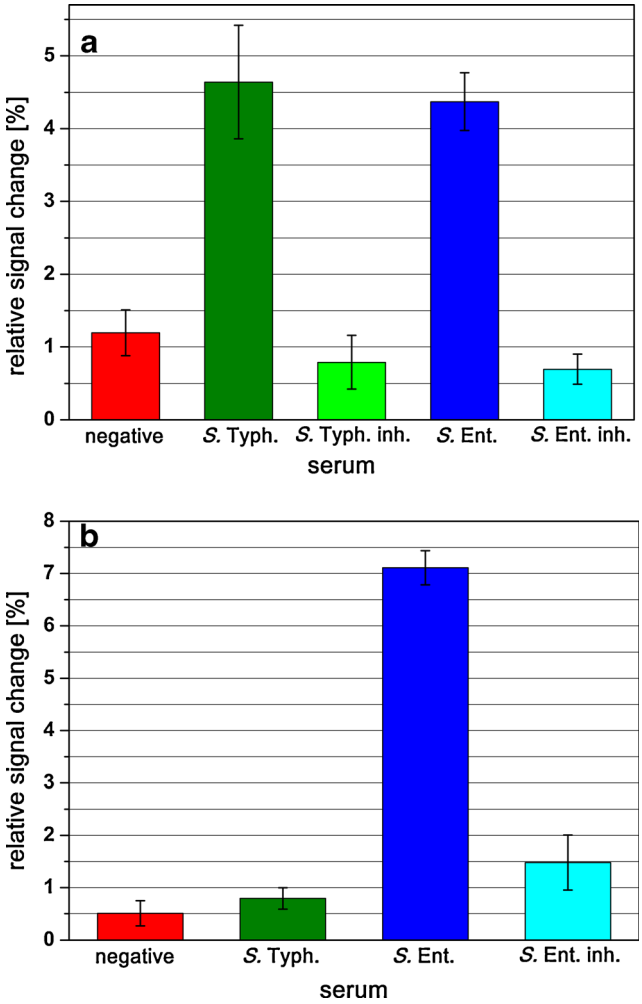

Fig. 5 Single-spot measurements with antibody negative, antibody-positive (*S. Typh./S. Ent.*) and antibody-inhibited (*S. Typh inh./ S. Ent. inh.*) sera at two different transducers, one covered with LPS Typhimurium (**a**) and the other covered with LPS Enteritidis (**b**)

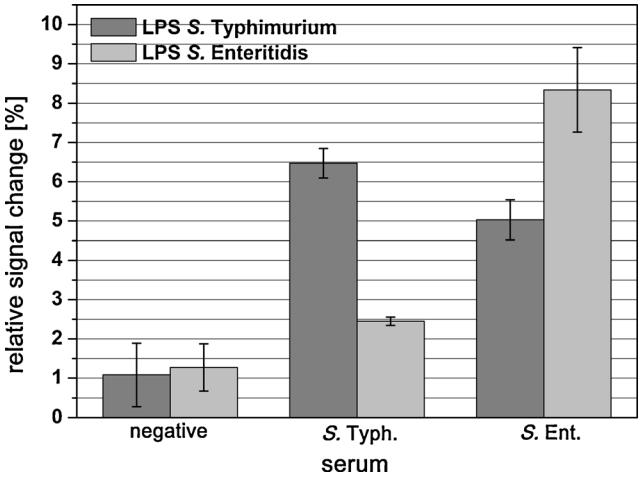

Fig. 6 Results obtained on one transducer detecting different reference sera at two measuring spots, one covered with LPS of *Salmonella* Typhimurium and the other covered with LPS of *Salmonella* Enteritidis

Typhimurium-infected chicken as well as for serum of *Salmonella* Enteritidis-infected chicken. As LPS out of *Salmonella* can be very similar, cross reactivity between antibody-positive sera and different LPS is possible. To prove whether monitored binding signals can be traced back to specific recognition, sera have been inhibited with an excess of LPS Typhimurium. In this case, all paratopes of anti-*Salmonella* antibodies should be blocked and should therefore no longer be able to bind to LPS on the sensor surface. Thus, no binding signal should occur. For both antibody-positive sera, inhibition is successful and binding signals for inhibited sera are in the range of signals obtained for negative serum.

At the transducer covered with LPS Enteritidis, no cross reactivity with serum of *Salmonella* Typhimurium-infected chicken can be observed as the binding signal is in the range of negative serum. For serum of *Salmonella* Enteritidis-infected chicken, a large binding signal is obtained and the signal is inhibited by giving an excess of LPS Enteritidis to the appropriate serum. Therefore, the monitored binding signal can be traced back to specific recognition.

Real sample two-spot measurements

By combining both LPS of *Salmonella* Typhimurium and LPS of *Salmonella* Enteritidis on one transducer and carrying out measurements with reference sera, we could show that the new multi-analyte platform is also applicable for measurements in real matrices. Results obtained for different sera at the appropriate spots are shown in Fig. 6 and include the same information obtained via single-spot measurements while the number of measurements was reduced from 6 to 3.

Conclusion

The aim was to demonstrate the good applicability of 1-lambda-reflectometry for multiplex testing within a low-priced and portable measuring setup. We therefore exemplarily established two label-free assays to quantify anti-*Salmonella* antibodies and CRP. Both sensitive layers allowed for several measurements on one chip with high intra- and interchip reproducibility. After assay harmonization, no cross reactivity between non-complementary analytes could be observed, proving a well-working immobilization protocol for both recognition spots. The new multi-analyte setup was very robust concerning mechanical movement of the transducer and no depletion effects could be observed using a new flow cell. Comparison between calibration of single- and multi-analyte measurements for *Salmonella* antibodies and CRP, respectively allow concluding that the new multi-analyte platform is able to detect two analytes out of one sample simultaneously, while sensitivity and MDC were similar or even

better than values that could be observed within single-spot measurements.

By measuring reference sera out of chicken, we could demonstrate the good performance of the new platform in complex matrices and the applicability of the whole setup to discriminate between *Salmonella* antibody-positive sera and negative sera as well as between the different *Salmonella* serovars Typhimurium and Enteritidis in parallel.

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