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RESEARCH PAPER

A robust sensor platform for label-free detection of anti-*Salmonella* antibodies using undiluted animal sera

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Abstract We present a portable and easy-to-use biosensor platform, allowing for label-free detection of diagnostic markers in undiluted animal serum. Exemplarily, this is shown for the detection of anti-*Salmonella* antibodies. 1-lambda-Reflectometry was used as detection method, making the new biosensor platform portable, cheap, and robust. As recognition elements, lipopolysaccharides (LPSs) from *Salmonella typhimurium* bacteria were immobilized as sensitive layer on the transducer to carry out serological tests via a direct assay format. For this purpose, a new surface preparation protocol has been worked out allowing for immobilization of the LPS via hydrophobic interactions. It has been shown that results obtained by 1-lambda-Reflectometry are equivalent to those obtained by the non-portable Reflectometric Interference Spectroscopy setup. The new sensor platform was calibrated in both matrices, buffer and undiluted serum. Good sensitivity, selectivity and intra chip reproducibility have been observed. Furthermore, inter chip reproducibility was examined and recovery rates were found to be between 99 and 117 % in undiluted serum.

Keywords 1-lambda-Reflectometry · RIfS · Label-free · *Salmonella* infection · Optical Sensors · Biosensor

Introduction

Detection of diagnostic markers in animals is not only important due to health care but also due to economic issues. Especially within large animal farms, costs per test are a matter of importance. Instead of sending samples to centralized laboratories, on-site measurements allow for continuous herd screening, quick countermeasures, and therefore avoiding subsequent contamination of a production site by an infected animal. For this reason, cheap consumables, time-efficient methods, reliable results, and portable devices are desirable.

A standard test used in veterinary diagnostics is the Enzyme Linked Immunosorbent Assay (ELISA) that is known to be a highly sensitive method [1]. As this procedure includes several working, incubation and washing steps, it is rather time consuming and therefore does not allow for immediate countermeasures in case of an infection. Furthermore, additional reagents like secondary antibodies, enzymes, and substrates are necessary causing additional costs per test [2].

The method we present within this work is a new type of Reflectometric Interference Spectroscopy (RIfS) [3]. For better understanding, the common RIfS setup is described first.

A glass transducer coated with several thin layers [4] is illuminated from its backside with a halogen light source not passing the sample solution. Light is reflected at each interface and the reflected light beams interfere with each other. 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 read out by a shift of the interference spectrum. As RIfS is a direct optical method, there is no need for expensive labeling reagents. Contrary to ELISA, neither binding inhibition assay nor sandwich assay format is necessary, as RIfS allows for a direct assay format. No additional reagents and only one working step is needed to obtain a result: the analyte is flushed over a sensitive layer and a time resolved binding signal is received within several minutes. All transparent

This paper is dedicated to Professor Franz Dickert on the occasion of his 70th birthday.

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materials like plastics or glass can be chosen as transducer material, allowing for cheap consumables. Furthermore, the transducer does not need a gold coating or other optical structures like evanescent field methods, e.g., surface plasmon resonance (SPR) [5] or grating couplers [6]. This is not only advantageous concerning costs per tests, but also favorable for the immobilization of biological recognition elements as gold may denature proteins due to protein sulfur binding. Another main advantage of RIfS is the robustness against temperature fluctuations, as refractive index and physical thickness change in the opposite ways concerning temperature effects [7]. Therefore, the optical thickness is nearly temperature independent allowing RIfS to provide a robust measuring setup.

The new type of RIfS, 1-lambda-Reflectometry, combines all advantages of RIfS with a much more simpler and portable measuring setup. Using 1-lambda-Reflectometry, a light-emitting diode illuminates the transducer from its backside by using confocal optics. In case of a binding event, a change in the optical thickness occurs in a change of the reflection coefficient. This can be read out by a photodiode at a certain wavelength as a change of reflected light intensity. Therefore, the white light source, the waveguide and the spectrometer are negligible, making the 1-lambda-Reflectometry setup simple and portable. The 1-lambda-Reflectometry setup and its detection principle are illustrated in Fig. 1.

To demonstrate the performance of 1-lambda-Reflectometry in veterinary diagnostics, an assay for detection of anti-*Salmonella* antibodies has been setup. Salmonellosis is a zoonosis that can cause serious illness in humans [8]. Typically, *Salmonella* infections in humans occur due to the consumption of insufficiently cooked pork or poultry products. For this reason, the detection of *Salmonella* infections in farm animals plays an important role in public health [9]. In case of an infection with *Salmonella*, the animal's immune system recognizes surface molecules of *Salmonella* bacteria as antigen and reacts by producing antibodies that bind in a highly specific way to the antigens. One of those molecules are lipopolysaccharides (LPSs) [10] that are located on the outer membrane of *Salmonella* bacteria. The serovar most frequently isolated from infected pigs is *Salmonella typhimurium* [11]. For this reason, LPSs from *Salmonella typhimurium* were immobilized as recognition elements of the sensitive layer for quantitative determination of anti-*Salmonella* antibodies.

The immobilization of recognition elements can be realized by covalent bonding [12], by their electrostatic adsorption to the surface [13, 14] or via hydrophobic interactions between the recognition element and the sensor surface. The latter was used for the immobilization of LPSs from *Salmonella typhimurium*.

Recently, several examples were shown that demonstrate the good performance of RIfS in complex matrices like diluted serum [15] or milk [16]. With 1-lambda-Reflectometry, we have shown for the first time that label-free sensor calibration in whole serum is possible.

Calibration of the sensor in both, buffer and serum together with good reproducibility demonstrate the capability of the new sensor platform for fast, cheap, and reliable detection of diagnostic markers in animals.

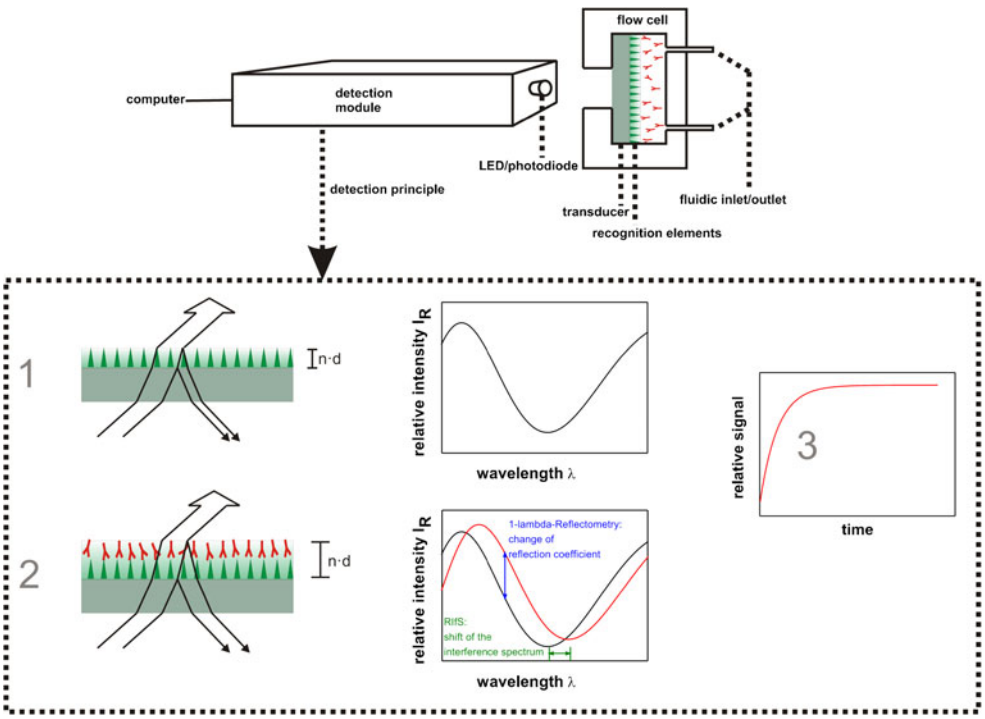

Fig. 1 1-lambda-Reflectometry setup, including detection module, transducer with flow cell and computer. Within the detection module, the LED and photodiode are sitting close to each other and a confocal filter allows for a free jet setup. Detection principle: (*1*) The transducer is illuminated with a light source from its backside. The reflected beams interfere and form a characteristic interference spectrum. (*2*) The binding of the analyte to the biosensor surface causes a change in optical thickness, resulting in a shift of the interference spectrum. (*3*) Timely resolved binding signal is obtained in RIfS via observation of the shift of the turning points in the interference spectrum and in 1-lambda-Reflectometry via detection of the change of the reflection coefficient

Materials and methods

Materials

RIfS transducer chips made of a 1-mm-thick D263-glass substrate with a layer of 10 nm Ta_2O_5 and 330 nm SiO_2 on top were purchased from Schott AG, Mainz, Germany.

1-lambda-Reflectometry transducer chips made of a 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.

Common organic compounds were obtained from Fluka, Neu-Ulm, Germany; Sigma-Aldrich, Deisenhofen, Germany and Merck, Darmstadt, Germany. 3-Glycidyloxypropyltrimethoxysilane (GOPTS) was purchased from Fluka Neu-Ulm, Germany. Amino functionalized dextran (AMD; M_w= 100 kDa, 50 % amino functions) was purchased from Innovent e.V. Jena, Germany. Lipopolysaccharides *Salmonella typhimurium* was purchased from Sigma-Aldrich, Deisenhofen, Germany. Polyclonal antibody "rabbit anti Salmonella group antigen" was obtained from AbD Serotec, Oxford, UK. Bovine serum albumin (BSA), fetal calf serum (FCS) and Pepsin 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 buffer solution.

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 (*ELGA LabWater*, Celle, Germany) and dried in a nitrogen stream.

Silanization

A 7.5-μL volume 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

A 10-μL volume 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 decanoic acid

A 1.3-μL volume of diisopropylcarbodiimide 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.

Immobilization of lipopolysaccharides

A 15-μL volume of a 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.

Antigen/antibody treatment and storage

The antibodies were dissolved in PBS at pH 7.4 yielding to a stock solution of 4 mg/mL. The stock solution was stored in aliquots of 100 μL at −20 °C. LPS was also dissolved in PBS yielding to a stock solution of 1 mg/mL. Aliquots of 15 μL were stored at −20 °C.

Assay setup

Direct assay

A defined concentration of anti-*Salmonella* antibody was injected into the flow cell as described in detail under *RIfS setup/1-lambda-Reflectometry setup*. The flow cell contained the sensor chip with its surface processed as described above. Antibodies binding to the immobilized LPS were detected via 1-lambda-Reflectometry. Resulting binding signals were proportional to the antibody concentration in the sample.

Binding inhibition assay

To make sure that measuring signals obtained via 1-lambda-Reflectometry are caused by specific interaction between the analyte and the recognition element, a binding inhibition assay was carried out as a control experiment before sensor calibration.

Therefore, an excess of LPS was pipetted to a defined LPS antibody solution and the mixture was flushed over the sensitive layer. Due to blocking of the paratopes, specific binding to the surface is thereby inhibited, proving specificity of the observed interaction.

Contact angle measurement setup

Left and right contact angles were measured with a CAM 200 (KSV, Helsinki), using fresh ultrapure water in laboratory atmosphere. Three measurements of the contact angles were done per sample at different positions on the sensor chips. Results from left and right contact angle were averaged, respectively. Mean values and standard deviation of the threefold measurements were calculated for each chip.

RIfS setup

The details of the setup have been described elsewhere [3]. White light is guided to the backside of a transducer via a Y-optical fiber and is partially reflected at each interface. The reflected beams superimpose and are guided to a diode array spectrometer (Spekol 1100, Analytik Jena, Germany) forming interference spectra. Liquid handling consists of a flow cell and an automated sample injection analyzer (Ismatec, Wertheim-Mondfeld, Germany) with an autosampler, two independent pumps, a load/inject- and a six-way valve.

1-lambda-Reflectometry setup

The 1-lambda-Reflectometry setup consists of a modified detection module (ESE-LOG USB, Qiagen Lake Constance GmbH, Germany) [17] including a light-emitting diode with a wavelength of 470 nm. The detection module focuses the transducer from its backside by integrated confocal optics. Light is partially reflected at each interface of the transducer. The reflected beams superimpose and reflected light intensity is detected via an integrated photodiode at the certain wavelength. 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) was used allowing for fully automated measurements.

Calibration

To calibrate the sensor in buffer and serum, each antibody concentration was measured threefold. During the calibration in buffer, measurements were carried out in random order, while they were repeated from lower to higher concentration during the calibration in FCS.

The different steps of a measurement cycle with RIfS can be divided into a baseline with buffer, followed by the injection of a 1-mL sample. After observing dissociation by flushing the flow cell for about 1 min with buffer, the sensitive layer is regenerated with guanidine hydrochloride solution (6 M; pH 1.5) and again flushed with buffer.

For measurements in serum, a typical measurement cycle was carried out in the same way like in buffer but with an additional FCS injection to block nonspecific binding before sample injection. Furthermore, an additional regeneration of the surface with 50 μM pepsin in acidified PBS (pH 1.9) was introduced avoiding the complete blocking of the recognition elements with serum and therefore a successive decrease in the monitored signal.

The different steps of a measurement cycle in 1-lambda-Reflectometry have been the same as described for RIfS but only a volume of 0.5 ml sample was injected with a flow rate of 0.5 μl/s.

Data evaluation

Contrary to the RIfS setup, 1-lambda-Reflectometry causes negative binding signals due to the different reflectivity behavior of 1-lambda-Reflectometry transducers in dependence of thickness changes in the sensitive layer.

For data evaluation in buffer, a linear fit with $y=z$, where y is the measured signal and z a constant value, was carried out over a defined region of the baseline before as well as after sample injection and the difference of the z values was calculated. In serum, matrix related drifts in the baseline after sample injection were observed and therefore, the difference of the measured signals at one defined position of the baseline before as well as after sample injection was calculated.

By plotting the mean values of the absolute signal change over the antibody concentration on semi logarithmic scale, a dataset is obtained that can be fitted by following four-parameter function (1).

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

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 [18]. 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 [19]. 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):

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

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

Results and discussion

To fulfill the demands on the immunoassay to show a good performance concerning sensitivity, selectivity and reliability, it is of crucial importance to setup an accordingly working assay. First, the recognition element has to be immobilized to the transducer surface. The aim is to obtain a high surface loading with low nonspecific binding that allows for high sensor signals. A high surface loading means that more binding sites on the surface are available in comparison to the amount of analyte in solution and a mass transport limited signal is obtained [20]. Several immobilization techniques were tested and the chemistry as described in detail above turned out to be the best suitable. Dextrane provides a large number of binding sites on the surface [21] and reduces nonspecific binding considerably [22]. On the other hand, a high surface loading with decanoic acid should result in a high hydrophobicity of the surface allowing for compact LPS adsorption via its hydrophobic Lipid A residue [23].

To control whether dextrane modification with decanoic acid results in a hydrophobic surface, contact angle measurements before and after modification with decanoic acid were carried out. Contact angles with water on three transducers with AMD and three transducers with decanoic acid were measured threefold at different positions on each surface. Results are shown in Fig. 2. The definite higher hydrophobicity obtained for AMD transducers modified with decanoic acid indicates a successful immobilization of decanoic acid on AMD, as decane residues are more hydrophobic than polar amine groups of AMD. Those hydrophobic residues build the basis for hydrophobic interaction to the Lipid A of the LPS.

By using label-free detection methods such as SPR, RIfS or 1-lambda-Reflectometry, nonspecific binding of the analyte or matrix components to the sensitive layer can cause artifacts in binding signals. Anything that adsorbs on the sensitive layer results in a signal and it is not distinguishable whether this is derived from a specific interaction between the analyte and the recognition element or a nonspecific adsorption. Therefore, it is necessary to block nonspecific binding of the analyte and its matrix to the sensitive layer.

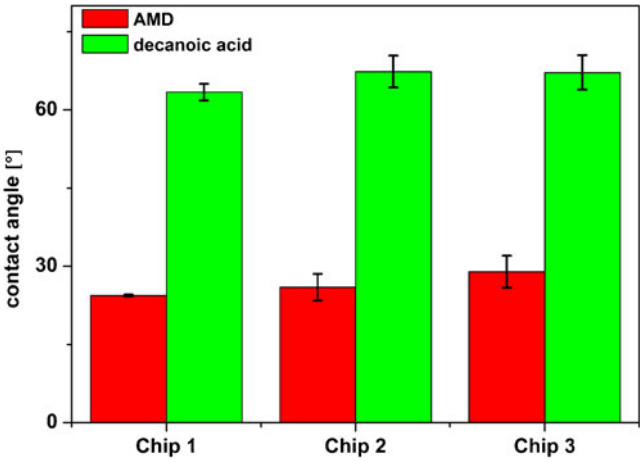

Fig. 2 Contact angle measurements on three different transducers before and after modification with decanoic acid

Before measurements were carried out with anti-*Salmonella* antibody, 1 g/L BSA was injected as sample and flushed over the surface. In spite of the 100-fold higher protein concentration of the BSA compared to the antibody solution, no binding signal has been observed (data not shown).

By obtaining an antibody binding curve, it is not proven that the antibody binds specifically with its paratope to the epitope on the immobilized LPS. Changes in reflection coefficient would also occur in a comparable way if the antibody is adsorbed in a nonspecific manner to the sensitive layer. For this reason, a binding inhibition test was carried out. To guarantee fully occupied antibody binding sites, 10 mg/L anti-*Salmonella* antibody was inhibited with a 12-fold excess of LPS antigen in an incubation step and subsequently this solution was injected over the sensor surface. The lack of a binding signal due to inhibition in Fig. 3 is a proof for specific interaction between the antibody and the LPS.

For assay development as well as for reduction of time and costs, it is desirable to carry out several measurements on one transducer. A solution containing 6 M guanidine hydrochloride at a pH of 1.5 was used to remove bound antibody from the sensitive layer without damaging the LPS binding sites at the surface.

Based on these results, we first calibrated a single chip with the RIfS setup in PBS buffer. After successful calibration, the assay was transferred to the new 1-lambda-Reflectometry platform.

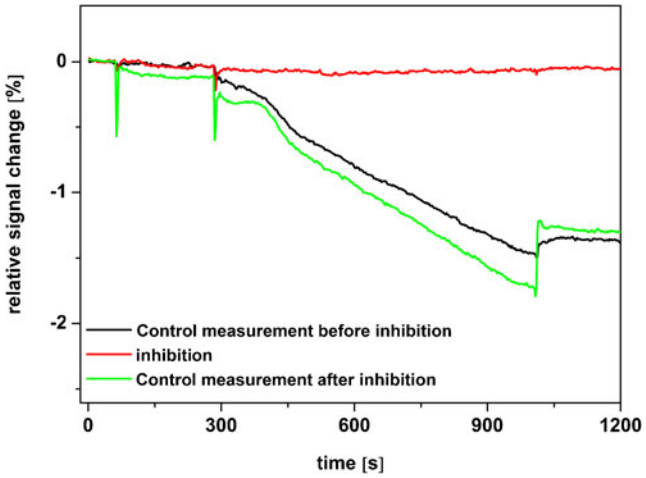

Fig. 3 Binding inhibition test using a solution of 8 mg/L LPS and 10 mg/L anti-*Salmonella* antibody. Control measurements before and after the inhibition were carried out with 10 mg/L anti-*Salmonella* antibody

Figure 4 shows the calibration curves obtained via RIfS and 1-lambda-Reflectometry. MDC and RDL values, the slope *p* as well as the dynamic signal range of the calibration curves are compared in Table 1.

The results obtained by the new 1-lambda-Reflectometry method provide a calibration curve covering a working range between 7.74 mg/L and 113.23 mg/L for the quantification of anti-*Salmonella* antibody. Calculated MDC and RDL values as well as the working range and the slope p of the calibration curve at the inflection point fit well to the results obtained via RIfS. Despite 1-lambda-Reflectometry allows for a simpler measuring setup by detecting one wavelength, has a small size comparable to a mobile phone and is affordable due to its low priced commercially available components, good capability of 1-lambda-Reflectometry for quantification of anti-*Salmonella* antibodies could be proven.

Table 1 Slope p, MDC, and RDL values with the resulting working range obtained by calibration via RIfS and 1-lambda-Reflectometry in PBS buffer

Method type	Slope *p* [%L/mg]	MDC [mg/L]	RDL [mg/L]	Working range [mg/L]
RIfS	1.34	1.74	2.59	5.66–102.15
1-lambda-Reflectometry	1.39	1.64	2.48	7.74–113.23

To demonstrate the high chip to chip reproducibility, recovery rates for different concentrations of anti-*Salmonella* antibody were determined using chips of three different batches. Results are given in Fig. 5. Mean values of recovery rates were found to be between 81 and 103 %.

For the determination of veterinary diagnostic markers, more complex matrices like meat juice, serum, or even whole blood have to be analyzed [24]. As matrix components can interact with the sensor surface or the analyte itself, this can cause problems due to nonspecific binding signals or disturbed antigen/antibody interactions. For this reason, we wanted to show that the 1-lambda-Reflectometry platform works also well in serum. For sensor calibration, FCS was chosen as model matrix for real sera and a blocking step with FCS before sample injection was introduced into the assay procedure.

The calibration curve obtained by serum measurements with 1-lambda-Reflectometry is shown in Fig. 6. Calculated MDC and RDL values as well as the working range and the slope p of the calibration curve at the inflection point are compared in Table 2 with those obtained for calibration in buffer.

In comparison to the calibration of the sensor in buffer, even lower MDC and RDL values were obtained in FCS,

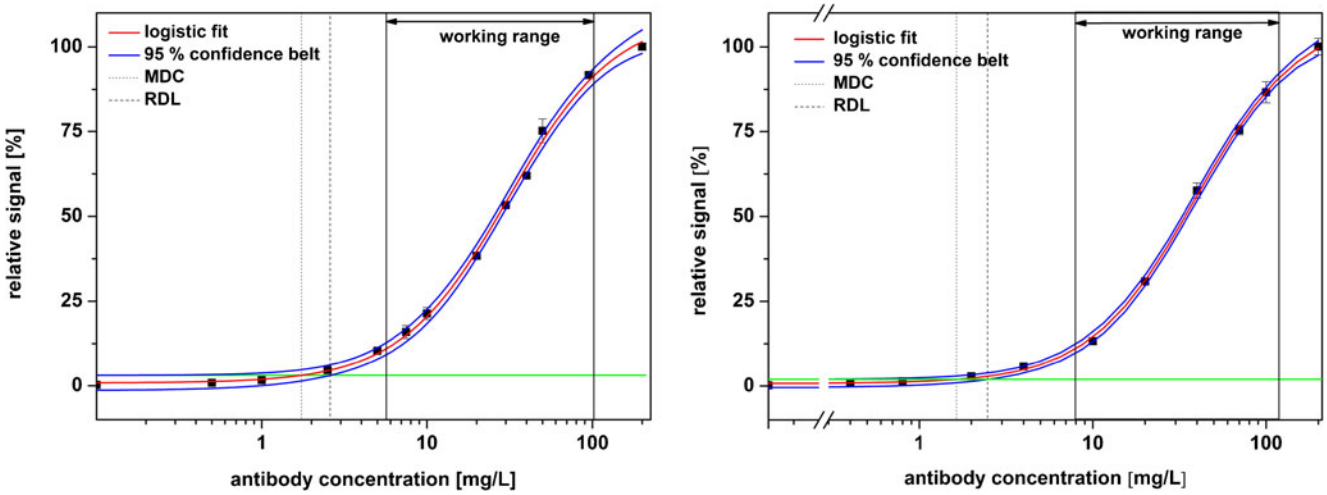

Fig. 4 Calibration curves in buffer obtained via RIfS (*left*) and via 1-lambda-Reflectometry (*right*). Different concentrations of anti-*Salmonella* antibody ranging from 0 to 200 mg/L were determined threefold

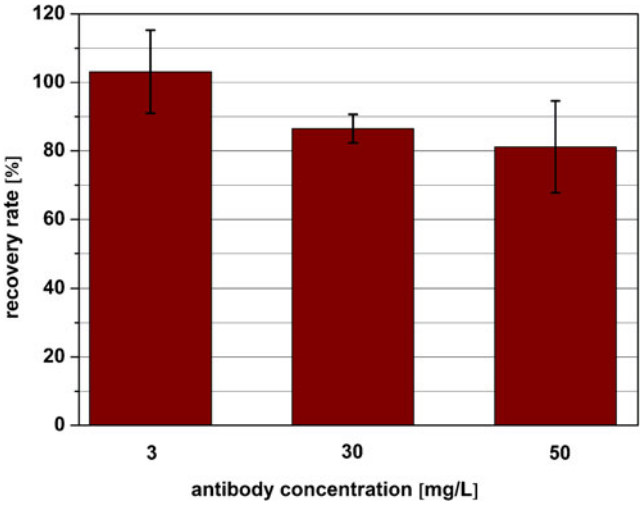

Fig. 5 Recovery rates obtained for anti-*Salmonella* antibody concentrations 3, 30, and 50 mg/L in PBS by threefold measurements on chips of three different batches

whereas the working range is a bit narrower. This might be caused on the one hand by a supporting effect of serum to the antigen/antibody interaction that results in a higher sensitivity (slope of the calibration curve). On the other hand, serum might block some of the surface binding sites and thus hinder binding of the antibodies. In case of low antibody concentration, a lot of binding sites on the surface are available and a mass transport limited linear signal is obtained. Serum that blocks a part of the surface binding sites will not influence the binding signal due to the excess of binding sites on the surface in comparison to the amount of analyte. In case of higher antibody concentration, comparatively fewer free binding sites are available at the sensor surface and the kinetics of the binding event is the limiting process that is monitored. In that case, the signal changes

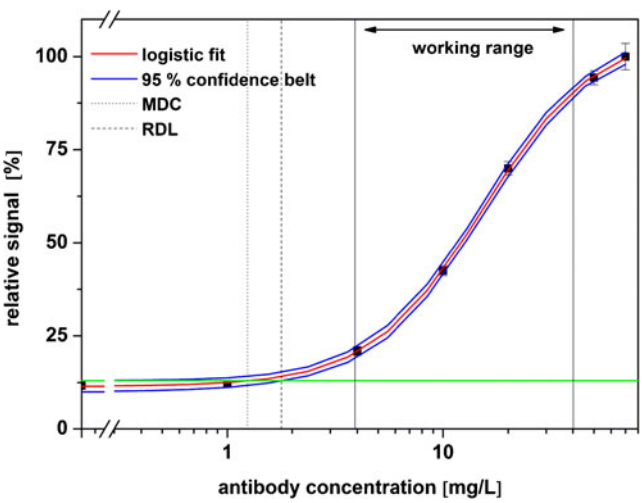

Fig. 6 Calibration curve in FCS obtained via 1-lambda-Reflectometry. Different concentrations of anti-*Salmonella* antibody ranging from 0 to 70 mg/L were determined threefold

Table 2 Slope p, MDC, and RDL values with the resulting working range obtained by calibration via 1-lambda-Reflectometry in PBS buffer and FCS serum

Matrix	Slope *p* [%L/mg]	MDC [mg/L]	RDL [mg/L]	Working range [mg/L]
PBS buffer	1.39	1.64	2.48	7.74–113.23
FCS serum	1.67	1.24	1.78	3.91–40.15

from a linear to an exponential one that comes to a saturation [25, 26]. When serum binds to free binding sites, this condition will be achieved earlier than without serum and a narrower working range is obtained in comparison to measurements carried out in buffer.

To show that high inter chip reproducibility is also given in the complex matrix serum, recovery measurements with different antibody concentrations were performed threefold on chips from different batches and the results evaluated using the calibration curve shown in Fig. 6. The recovery rates are given in Fig. 7.

Good inter chip reproducibility has also been achieved in the complex matrix serum with mean recovery rates between 99 and 117 %. The results demonstrate the feasibility to use one calibration for the quantification of analyte with different chips. This is important when sensor chips are used as disposables due to hygienic reasons and cross contamination.

Conclusion

The aim was to demonstrate the good capability of 1-lambda-Reflectometry for serology testing in complex matrices. We therefore exemplarily established a label-free

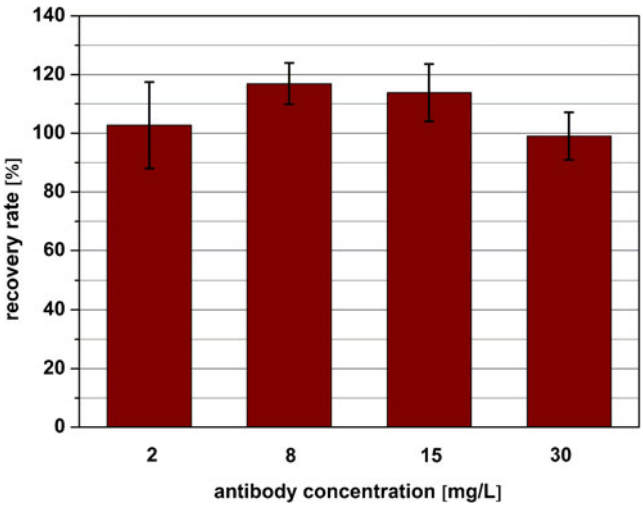

Fig. 7 Recovery rates obtained by threefold measurements of anti-*Salmonella* antibody concentrations 2, 8, 15, and 30 mg/L in FCS on chips of different batches

assay to quantify anti-*Salmonella* antibodies in undiluted serum. To perform a direct assay format, a new surface chemistry protocol was developed to attach the antigen to the transducer. Hydrophobic interaction between decanoic acid and Lipid A offered a stable sensitive layer that allowed for several measurements on one chip with high intra and inter chip reproducibility. We were able to show that the LPS has been immobilized with accessible epitopes by the proof of a binding inhibition test.

We have demonstrated that results of 1-lambda-Reflectometry are equivalent to results of RIfS by comparison of the sensitivity and the working range. With 1-lambda-Reflectometry, calibration of the sensor in undiluted serum has been possible and good sensitivity and inter chip reproducibility have been achieved.

As the 1-lambda-Reflectometry setup is robust and portable, it is a useful tool for on-site measurements. Together with the simple measuring setup, reliable results can be obtained within several minutes even by untrained personal. The obtained results highlight the new platform being applicable for the surveillance of livestock. For its use in herd screening, further work will be performed to discriminate between the statuses of *Salmonella* infection in sera of different pigs. Thereby, not only the veterinary sector but also food industry could benefit from these results.

Due to the good performance in the complex matrix serum, the application of 1-lambda-Reflectometry in other fields, e.g., human diagnostics and food safety, is also thinkable in the near future.

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