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FULL ARTICLE

Surface functionalization allowing repetitive use of optical sensors for real-time detection of antibody-bacteria interaction

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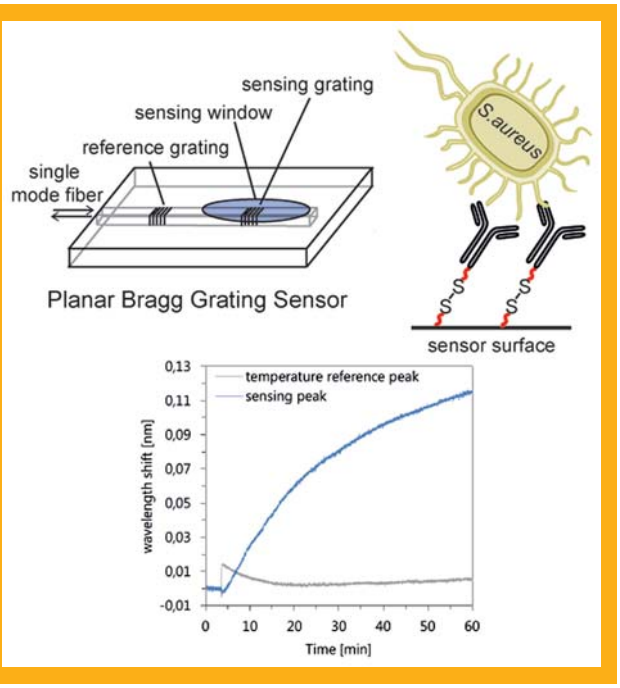
In this study, sensor surface functionalization allowing the repetitive use of a sensing device was evaluated for antibody-based detection of living bacteria using an optical planar Bragg grating sensor. To achieve regenerable immobilization of bacteria specific antibodies, the heterobifunctional cross-linker N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) was linked to an aminosilanized sensor surface and subsequently reduced to expose sulfhydryl groups enabling the covalent conjugation of SPDP-activated antibodies via disulfide bonds. The immobilization of a capture antibody specific for *Staphylococcus aureus* on the sensor surface as well as specific binding of *S. aureus* could be monitored, highlighting the applicability of optical sensors for the specific detection of large biological structures. Reusability of bacteria saturated sensors was successfully demonstrated by cleaving the antibody along with bound bacteria through reduction of disulfide bonds and subsequent re-functionalization with activated antibody, resulting in comparable sensitivity towards *S. aureus*.



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1. Introduction

Detection methods for pathogens, microorganism and other biological specimens are in great demand in the field of life sciences and clinical diagnostics, in particular in the context of infectious diseases. Especially for ubiquitous and often persistent pathogens such as *Staphylococcus aureus*, immediate detection and identification is crucial for the proper choice of medical treatment allowing to avoid systemic spreading and persistence of the infection as well as development of antibiotic resistance [1]. Biosensors promise to improve pathogen detection, providing a wide field of application and permitting sensitive, specific, and rapid analysis of diverse specimens [2–4]. Specifically, label-free optical biosensors such as planar Bragg grating sensors (PBGs), among others, emerged as alternatives to traditional clinical analysis methods, allowing to reduce costs, improve handling and avoid labeling [5–9].

Highly selective detection of pathogens may be achieved by immobilizing target-specific antibodies on the surface of the sensing region of PBGs [3]. Binding of complementary antigens to the antibody-modified sensor surface changes the refractive index in proximity of the sensing grating and leads to a detectable spectral shift of the reflected Bragg wavelength [9].

Several authors have evaluated antibody modified sensors for the detection of biological entities including bacteria [2–5, 7, 10–13]. However, in most studies antibodies were covalently attached to the sensor surface, permitting only single use or requiring laborious and/or harsh cleaning procedures prior to subsequent analysis. In contrast, reusable sensors would not only reduce installation time, costs and preparatory work but would also minimize waste and consumption of resources. Towards this end, antibody conjugation through interaction of the Fc part of antibodies with protein A or G would represent a straightforward approach, allowing regeneration of the sensor through cleavage of protein A/G-antibody interaction, e.g. by incubation in acidic guanidine solution [3–5, 14, 15]. But, both costs and high molecular weight of the linker molecules represent significant drawbacks of this strategy. To alleviate these disadvantages and to improve sensor reusability we developed antibody modified PBGs using low molecular weight cleavable linkers and tested their sensing capabilities towards different bacterial strains. This paper describes the synthetic strategy used for conjugation of antibodies to the sensor surface by cleavable linkers, provides evidence for successful linker and antibody conjugation based on sensor response and presents the results of an initial proof-of-concept study employing antibody modified PBGs for repetitive specific detection of *S. aureus*.

2. Materials and methods

2.1 Materials

All applied chemicals were at least of analytical grade unless stated otherwise. The cross-linker N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) was sourced from Thermo Fisher Scientific Inc. (Rockford, IL, USA). DMSO (Dimethyl sulfoxid, 99.9%, Bio Reagent), APTES ((3-Aminopropyl) triethoxysilane) as well as DTT (DL-Dithiothreitol, ≥98% (TLC), ≥99.0% (titration)) were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). UK-66, a monoclonal antibody (mAb) targeting the immunodominant staphylococcal antigen A (IsaA), was provided by the Institute for Molecular Infection Biology (University of Wurzburg, Germany), where also the bacterial strains *Staphylococcus aureus* Newman wildtype and *Escherichia coli* 536 were cultivated [16]. The cell densities were adjusted to $3 \cdot 10^{10}$ cfu/ml in 3 ml PBS before use.

2.2 Planar Bragg grating sensor

The sensors are based on a periodic refractive index perturbation in an optical waveguide which reflects a particular wavelength. The spectral position of the reflected Bragg wavelength (λ_B) is determined by the period of the refractive index perturbation Λ and the effective refractive index n_{eff}. In this study, a PBGs was used which was fabricated with the direct writing technique [17, 18]. Initially, the integrated waveguide and the Bragg gratings had been isolated from the environment by a silica layer. To allow interaction of the evanescent field with the surrounding medium, which is essential for the detection of changes of the refractive index, the cladding layer above the sensing grating was partially removed by hydrofluoric etching. Subsequently, the sensor surface was coated with a high refractive index titanium dioxide layer. Since the covering layer above the other grating remained unchanged, preventing the evanescent field of this buried grating to interact with the environment, its reflected Bragg wavelength could be used as a reference signal to detect temperature fluctuations during measurements. By connecting a single mode (SM) fiber to the PBGs using UV curable glue, both gratings could be simultaneously monitored during the experiments. A detailed description of the fabrication process of the PBGs can be found elsewhere [19–22]. Figure 1 illustrates the structure of the planar sensor chip, including a cross-section indicating the interaction of the evanescent field with a surrounding medium.

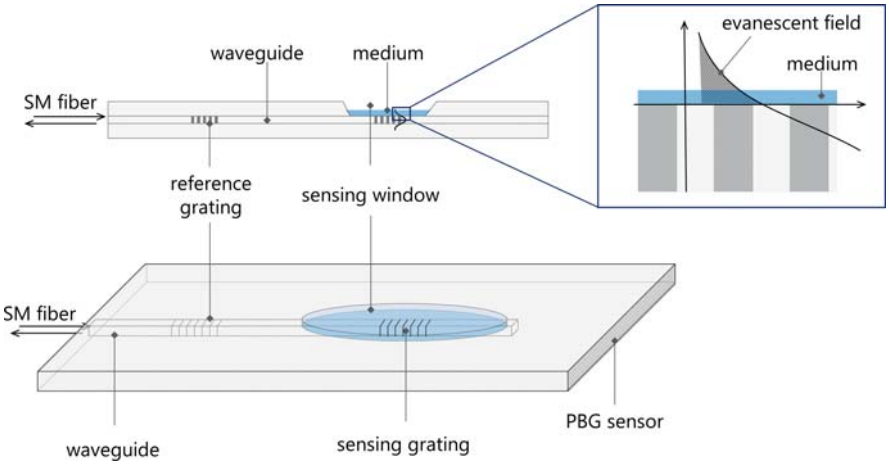

Figure 1 Illustration of the composition and the operation principle of a silica based optical planar Bragg grating sensor.

A Bragg meter, consisting of a tunable laser diode, emitting a wavelength spectrum in the telecommunication range around 1550 ± 40 nm, a circulator and a photodiode were used for the interrogation of the planar Bragg grating sensors. The sensing system exhibited a sampling rate of 2 Hz and a resolution of 1 pm. Prior to each experiment, the sensor was equilibrated at the respective starting conditions until a stable baseline was observed for at least 5 minutes.

2.3 Modification of the sensor surface with S. aureus specific antibody

To prepare the sensor surface for subsequent conjugation steps, the PBGs were first functionalized by aminosilanization. After cleaning the sensor surface with PBS and acetone, the PBGs were immersed in 3 ml 2% (V/V) APTES in acetone for approximately 60 min and rinsed with acetone to remove excess APTES solution. The formed aminosilane layer was cured at 60 °C for 105 min. For immobilization of the monoclonal antibody on the sensor surface, the heterobifunctional cross-linker SPDP was used (Figure 2) [23–25]. Both, mAb and sensor surface were separately modified with SPDP (Figure 2,Ia and Ib). SPDP solution was freshly prepared (5.2 mg/ml in DMSO) and mixed with mAb solution (10 mg/ml in PBS, pH 7.4) at a molar ratio of approximately 12.5 : 1. The mixture was allowed to react at room temperature for 30 min and the modified mAb was purified from reaction byproducts and excess SPDP by gel filtration into PBS containing 10 mM EDTA (PBS-EDTA) using Illustra NAP-25 columns (GE healthcare, Buckinghamshire, UK).

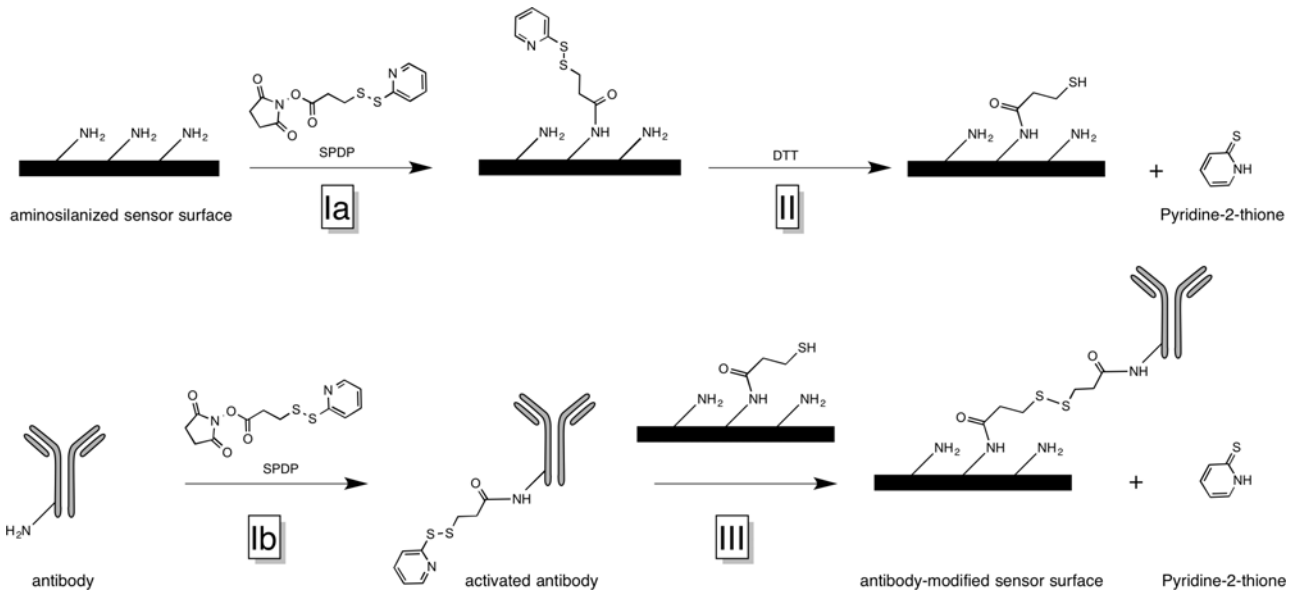

Figure 2 Schematic illustration of the strategy for coupling anti-IsaA antibodies to the functionalized sensor surface using SPDP as a cross-linker.

The sensor was incubated in a 1:21 mixture of SPDP (10 mM in DMSO) and PBS-EDTA, pH 7.4 for 30 min at room temperature and subsequently immersed in PBS-EDTA to remove reaction byproducts and excess SPDP. The SPDP-modified sensor surface was incubated in 50 mM DTT for 45 min to generate free sulfhydryl groups (Figure 2,II) and immersed in PBS-EDTA to remove excess DDT as well as reaction byproducts. Finally, the sulfhydryl-activated sensor surface was incubated with SPDP-activated mAb solution for at least 18 h at 4 °C (Figure 2,III) and non-conjugated mAb was removed by immersion in PBS-EDTA. The mAb modified sensor was stored in PBS-EDTA until use. The process of the mAb conjugation onto the sensor surface was monitored by recording the sensor response during each functionalization step. Prior to each modification, the sensor was equilibrated in PBS-EDTA until a stable baseline signal was obtained. The binding of the single reagents onto the sensor surface was analyzed by observation of absolute Bragg wavelength shift.

2.4 Real-time detection of antibody-bacteria interaction

To evaluate the response of antibody modified sensors in the presence of *S. aureus* or *E. coli,* absolute wavelengths shifts were recorded. After equilibration in PBS-EDTA, an antibody-functionalized sensor was exposed to an *E. coli* cell suspension ($3 \cdot 10^{10}$ cfu/ml) for 5 min and subsequently re-immersed into PBS-EDTA. After equilibration of the monitored signal, the same sensor was incubated in a *S. aureus* bacteria suspension ($3 \cdot 10^{10}$ cfu/ml) for 90 min to analyze the selective binding of *S. aureus* to the mAb-modified sensor surface. Subsequently, the sensor was rinsed with PBS-EDTA to remove any unbound bacteria. A second mAb-modified sensor was immersed directly in *S. aureus* bacteria suspension ($3 \cdot 10^{10}$ cfu/ml) for 60 min to investigate the absolute wavelength shift caused by the binding of *S. aureus* without the influence of unspecifically adsorbed *E. coli*. As a control for unspecific binding to the aminosilanized sensor surface, the response of a sensor without attached antibody toward both bacteria strains was investigated. The control sensor was initially exposed to *E. coli* for 20 min and afterwards regenerated using 5 M NaCl for 10 min before incubation with *S. aureus* for 60 min (both at $3 \cdot 10^{10}$ cfu/ml).

2.5 Regeneration of the sensor

For practical application of the sensing device, reusability of the SPDP-modified sensor is a crucial requirement and was investigated in detail. The bacteria saturated sensor was incubated in 50 mM DTT for 30 min (Figure S1) while the shift of the sensor signal was recorded [26]. After regeneration of sulfhydryl groups, the sensor was incubated with SPDP-modified mAb overnight (about 20 h) at 4 °C to re-functionalize the sensor surface with fresh mAb (Figure S1). To demonstrate the functionality of the regenerated sensor, the response to the *S. aureus* bacteria suspension ($3 \cdot 10^{10}$ bacteria/ml) was analyzed.

3. Results and discussion

3.1 Sensor functionalization

The sensor surface was functionalized by formation of a self-assembled aminosilane monolayer using APTES [27, 28] enabling subsequent reaction with SPDP (Figure 2,Ia). SPDP is a heterobifunctional cross-linker featuring an amine-reactive part represented by the N-hydroxysuccinimide (NHS) ester as well as a sulfhydryl-reactive part represented by a 2-pyridyldithio group [29–31]. In this study, SPDP was used to cross-link primary amines of the antibody to the aminated sensor surface. For this, both, amino groups of the antibody and of the sensor surface were modified separately with SPDP (Figure 2, Ia and Ib). The binding of SPDP altered the refractive index on the sensor surface resulting in a positive shift of the reflected Bragg wavelength by approximately 800 pm over 50 min (Figure 3A). Concurrently, the reference peak wavelength remained constant indicating a constant temperature during the binding process. Subsequently, the sensor surface was treated with DTT to generate free sulfhydryl groups by releasing pyridine-2-thione (Figure 2,II). Cleaving of pyridine-2-thione caused a reduction of the refractive index, resulting in a negative wavelength shift of 40 pm over 25 min (Figure 3B). Finally, a SPDP-modified antibody, which specifically recognizes the IsaA epitope on *S. aureus*, was covalently attached to free sulfhydryl groups on the sensor surface forming disulfide bonds (Figure 2,III). The immobilization of antibodies could be monitored in real-time through a steady and significant shift of the reflected Bragg wavelength of 300 pm over 50 min (Figure 3C).

3.2 Specific detection of S. aureus

The antibody-functionalized sensors were exposed to different bacteria suspensions in PBS to test their ability to specifically detect *S. aureus*. The real-time

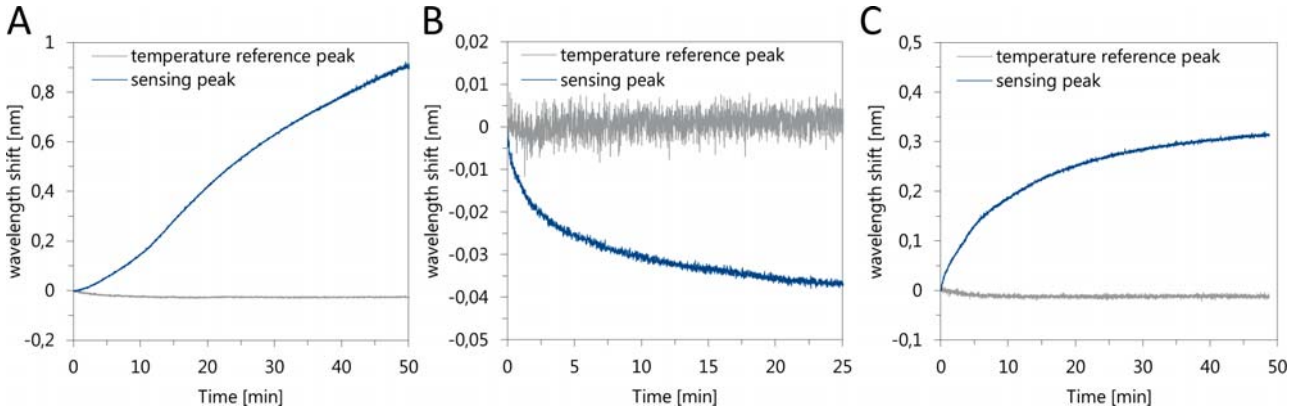

Figure 3 Monitoring of sensor surface functionalization: (**A**) binding of SPDP to the aminosilanized sensor surface, (**B**) cleavage of disulfide bonds using DTT exposing sulfhydryl-groups and (**C**) conjugation of SPDP modified antibody onto the surface.

response of the sensor during incubation with *S. aureus* is shown in Figure 4A. The immediate and steady shift of the reflected Bragg wavelength of over 110 pm during 55 min was associated with the binding interaction between *S. aureus*, containing the surface antigen IsaA, and the anti-IsaA antibody immobilized on the sensor surface [16]. In contrast, immersing anti-IsaA antibody-functionalized sensor in an *E. coli* suspension of the same concentration resulted in a constant sensor signal (Figure 4B). From the shift of the temperature reference peak when transferring the sensor from PBS to the bacteria suspension, it was apparent that the temperature of the bacteria suspensions was higher than pure PBS but decreased slightly during incubation. Taking into account the negative wavelength shift of 5 pm induced by the temperature fluctuations, a slight positive net shift of the sensing signal in the presence of *E. coli* resulted. However, when returning the sensor into PBS solution, an instantaneous minimal negative shift of the sensing signal was observed indicating desorption of unspecifically bound *E. coli*. In summary,

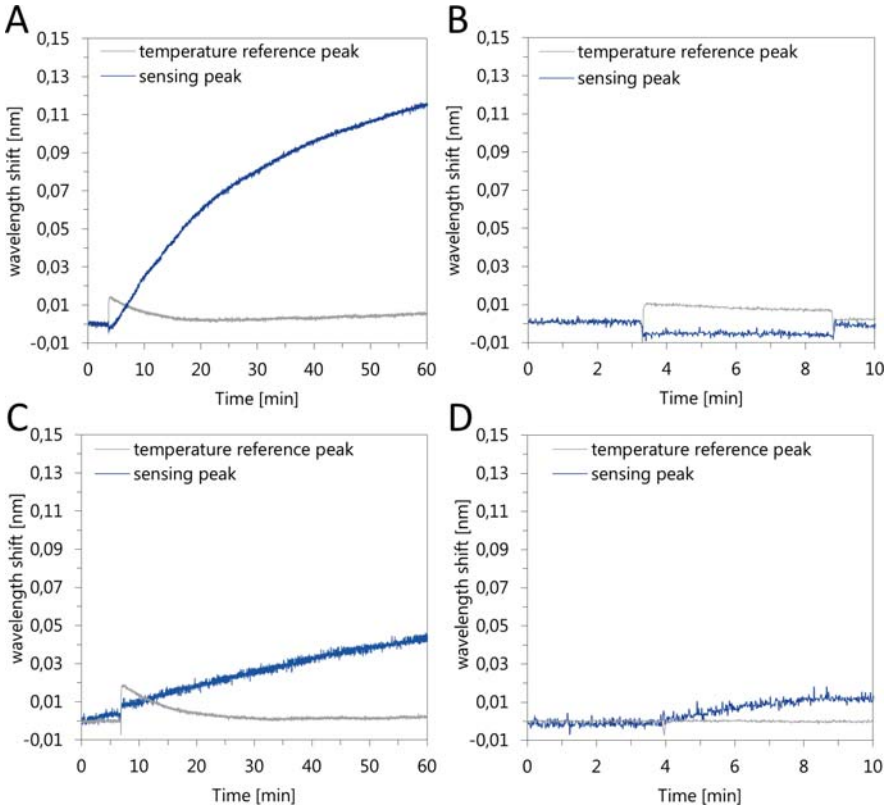

Figure 4 Bragg wavelength shifts of a mAb modified sensor resulting from exposure to (**A**) *S. aureus*, (**B**) *E. coli* and of an aminosilanized sensor to (**C**) *S. aureus* and (**D**) *E. coli*.

incubation of mAb-modified sensors with *E. coli* led to minimal, unspecific and reversible adsorption of bacteria to the sensor surface. When the same sensor was subsequently exposed to *S. aureus,* a significant shift of the reflected wavelength was observed, indicating specific binding of *S. aureus* to the antibody-modified sensor surface even after exposure to *E. coli* (Figure S2).

To demonstrate that the immobilization of anti-IsaA antibody on the sensor surface was a necessary prerequisite for specific detection of *S. aureus*, both, *E. coli* and *S. aureus* suspensions were introduced to an aminosilane coated sensor without conjugated antibody (Figure 4C and 4D). A slow increase of the reflected wavelength of approximately 40 pm over 60 min was observed in the case of *S. aureus* suspension (Figure 4C), while immersion in *E. coli* suspension resulted in a shift of approximately 13 pm in the first 5 minutes and remained constant thereafter (Figure 4D). The positive Bragg wavelength shift observed for both bacteria suspensions may be explained by attractive electrostatic interaction between negatively charged surface of bacteria and the positively charged aminosilanized sensor surface [28, 32]. It is interesting to note that *E. coli* was shown to exhibit a lower adsorption rate to positively charged surfaces than *S. aureus* in the study by Gottenbos et al., potentially explaining the less distinct amplitude shift of the sensor signal in the case of *E. coli* [32]. Furthermore, *E. coli* showed faster desorption than *S. aureus* in the same study [32]. Hence, the constant sensor signal observed after only 5 minutes may be interpreted as a result of the balance between adsorption and desorption of *E. coli* on the aminosilanized sensor surface. These control experiments suggest that wavelength shifts observed for antibody-modified sensors were not due to unspecific adsorption of bacteria to the sensor surface but resulted from specific antibody-antigen interaction with *S. aureus*.

We would like to emphasize that the sensitivity of modified PBGs described herein is not yet up to par with alternative optical sensing principles and requires further optimization. A similar PBGs was described by Bhatta et al., where mAbs were immobilized via interaction with protein A/G and used for the detection of *E. coli* and other biological agents [3]. In this report, *E. coli* were successfully detected at $4.6 \cdot 10^8$ cfu/ml, suggesting higher sensitivity of this sensor. However, the intention of the study of Bhatta et al. and ours was to show the feasibility of bacteria detection with mAb-modified PBGs and both studies did not determine the limit of quantification or limit of detection of sensors. Further improvement of the sensitivity of mAb-modified PBGs for bacteria detection might be achieved through optimization of several sensor-related parameters in future studies. The oriented immobilization of mAbs on the sensor surface was shown to be conducive for sensitivity since random conjugation may result in steric hindrance and hence loss of binding capacity [12, 33, 34]. Among other strategies, oriented antibody immobilization may be achieved through oxidation of carbohydrates in the Fc part and subsequent reductive amination [35]. Antibody surface density is another critical parameter closely related to mAb orientation. It has been shown that an optimum mAb density exists, above which steric hindrance results in reduced sensitivity. On the other hand, too low mAb density limits sensitivity due to sub-optimal binding capacity [36, 37]. Sensitivity improvements could also be achieved through direct conjugation of Fab' fragments, allowing to reduce molecular weight of mAbs while achieving oriented conjugation via disulfide bridges between the Fab fragment and SPDP-modified sensor surface [38, 39].

It should also be noted that antibody selection critically affects performance and reliability of the sensing principle. On the one hand, high selectivity for the target microorganism is required and on the other hand different strains of target bacteria should be detected with comparable efficiency. Lastly, mutation of surface features of the target microorganism may reduce detection efficiency. *S. aureus* specific anti-IsaA antibody used herein to our current knowledge fulfills these requirements. It has been shown that the targeted IsaA antigen is involved in the cell wall metabolism of *S. aureus*, and is regarded as standard housekeeping protein, resulting in lower likelihood of mutation compared to virulence associated antigens [40, 41]. Furthermore, IsaA is found conserved in all sequenced *S. aureus* strains and efficient binding of anti-IsaA antibody to major clinical lineages was shown [16, 42].

3.3 Regeneration of the sensor

Finally, the feasibility of PBGs regeneration after cleavage of bacteria-saturated antibody was studied. The disulfide-containing cross-linker between the antibody and the sensor could be cleaved by incubation with DTT resulting in an instantaneous distinct shift of the reflected wavelength by approximately 250 pm over 30 min (Figure 5A). DTT exhibited a higher refractive index compared to PBS-ETDA leading to a steep increase of the wavelength shift after immersion into DTT solution and a comparable decrease during the transition back to PBS-EDTA (Figure 5A). For regeneration of the *S. aureus* specific sensor, SPDP-modified antibody was conjugated to the sensor surface via free sulfhydryl groups as described above, resulting in a positive shift of the sensor signal by approximately 350 pm over 30 min (Figure 5B). The functionality of the regenerated sensor was verified by real-time monitoring of cap-

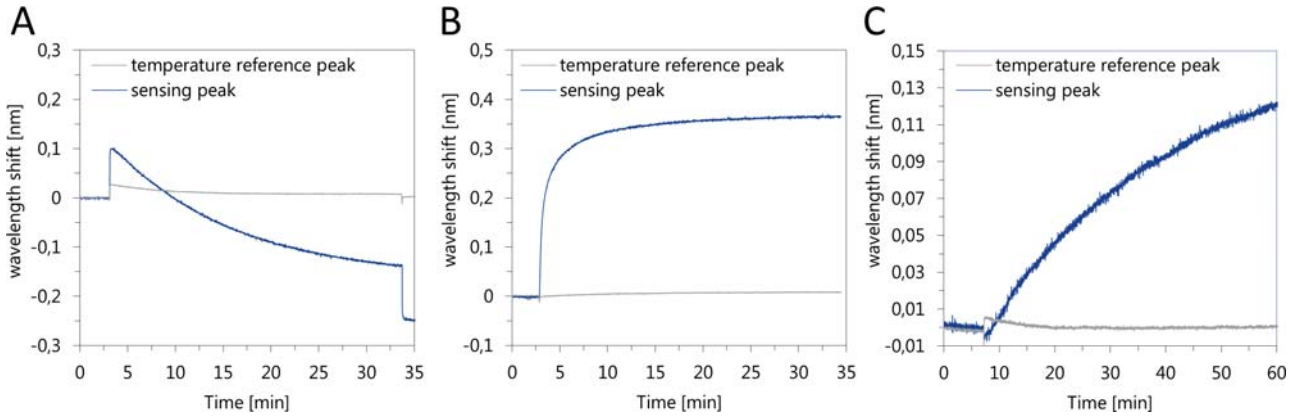

Figure 5 Regeneration of a bacteria saturated sensor by (**A**) cleaving of the disulfide bond between the cross-linker and the antibody along with bound bacteria using DTT, (**B**) coupling of fresh activated antibody to the sensor surface and (**C**) detection of *S. aureus* using the regenerated planar Bragg grating sensor.

ture of *S. aureus*. Incubation of the regenerated sensor with *S. aureus* suspension resulted in a positive reflected wavelength shift of approximately 120 pm within 60 min confirming comparable binding of *S. aureus* before and after sensor regeneration (Figure 5C).

4. Conclusion

Anti-IsaA antibody modification of aminosilanized PBGs can be used to specifically and selectively detect *S. aureus*. Furthermore, the individual steps during antibody conjugation to the sensor surface are associated with distinct changes of the sensor signal, allowing real-time monitoring of surface modifications. The disulfide-containing cross-linker employed herein allows cleavage of bacteria-saturated antibodies from the sensor surface and simple regeneration of the sensor through incubation with SPDP-activated antibody, resulting in similar sensitivity towards *S. aureus* after regeneration.

These results confirm the applicability of established bioconjugation techniques in the context of bacteria-specific PBGs, allowing for straightforward, inexpensive sensor surface modification. Compared to alternative conjugation strategies based on avidin-streptavidin or protein A/G interaction, the conjugation technique employed herein not only reduces costs and improves sensor ruggedness but also allows regeneration of the sensor and reduces cross-linker molecular weight, potentially promoting broad application and future sensitivity improvements, respectively. While proof-of-concept has been confirmed, the low sensitivity of the current sensor limits its applicability. We anticipate that the combination of established methods for sensitivity enhancement of optical sensors with the inexpensive, regenerable surface functionalization established herein will ultimately lead to reusable antibody-based sensors with high sensitivity.

Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's website.

Figure S1. Schematic illustration of the sensor regeneration with DTT induced cleavage of the disulfide bond between the sensor surface and bacteria-saturated antibodies as well as renewed immobilization of activated mAb onto the sensor surface.

Figure S2. Sensor response to incubation with *S. aureus* following incubation with *E. coli*.

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