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Technical Note

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Versatile Electrochemical Sensing Platform for Bacteria

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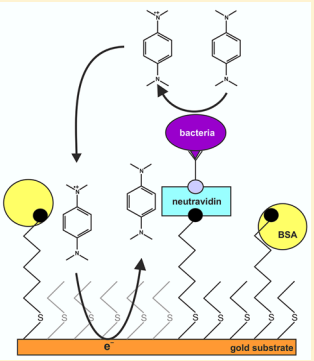
Supporting Information

ABSTRACT: Bacterial infections present one of the leading causes of mortality worldwide, resulting in an urgent need for sensitive, selective, cost-efficient, and easy-to-handle technologies to rapidly detect contaminations and infections with pathogens. The presented research reports a fully functional chemical-detection principle, addressing all of the above-mentioned requirements for a successful biosensing device. With the examples of *Escherichia coli* and *Neisseria gonorrheae*, we present an electrochemical biosensor based on the bacterial expression of cytochrome c oxidase for the selective detection of clinically relevant concentrations within seconds after pathogen immobilization. The generality of the biochemical reaction, as well as the easy substitution of target-specific antibodies make this concept applicable to a large number of different pathogenic bacteria. The successful transfer of this semidirect detection principle onto inexpensive, screen-printed electrodes for portable devices represents a potential major advance in the field of biosensor development.



Bacterial infections represent one of the leading causes of mortality worldwide,[1] whereby most infections occur through contaminated water, food, and bodily fluids. According to the World Health Organization, outbreaks and epidemics related to bacterial infections, such as *Escherichia coli*, meningococcal diseases, or sexually transmitted infections (STIs), are frequently reported on all continents.[2−4] Although numerous reports for the electrochemical detection of bacteria have been published,[5−8] no point-of-care device has entered the global market yet, because of the challenge of combining a rapid detection mechanism with sensitivity, selectivity, cost-efficiency, and a facile handling procedure.

Over the past two decades, it has become evident that electrochemical sensing methods can not only overcome the limitations of both culture and biochemical methods in terms of assay time, sensitivity, and specificity but furthermore offer more convenience of handling, as this technology can be implemented into a hand-held device for which technical training becomes minimal. One of the key advantages of electrochemical sensing for bacteria is its sensitivity, and detection limits of one to only a few colony-forming units (CFU) for different bacteria have been achieved in the literature.[9−13] In general, an electrochemical-current on-signal,[14−16] based on the direct measurement of bacterial metabolites, compares favorably with an off-signal,[17] which can be obtained by impedance spectroscopy and is often prone to false-positive results. The aspect of selectivity is commonly addressed through the use of nanomaterials,[18−21] wherein the potential risk of nanomaterial release into the environment should not be neglected.[22] The development of paper- or plastic-based substrates for the electrochemical detection of pathogens presents an inexpensive and easy-to-handle alternative to standard electrodes.[10,11,23]

Herein, we report a fully functional electrochemical detection assay for the recognition of pathogenic bacteria that addresses all of the above-mentioned criteria. Using the examples of *E. coli* and *Neisseria gonorrheae* bacteria, we demonstrate the versatility of the proposed recognition strategy across cell lines, and it is suggested that the outlined principle can be adapted to any organism expressing cytochrome c oxidase. Because of its significant impact on the food industry in correlation with food contamination and food-related illnesses,[24] *E. coli* represents an ideal target for the proof of concept of our electrochemical sensing principle. Furthermore, we address the health issue of STIs, which account for about 340 million new infections annually worldwide,[4] and the number of infected individuals is increasing every year, particularly in North America[25] and

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Europe.[3] People of all ages can be affected, and because STIs are highly contagious even at early stages, and their reoccurrence rates are high if not thoroughly cured,[26] an early, reliable, and accurate method of diagnosis is crucial. One of the current analysis techniques for urine or blood samples is the long, old, established method of cell culture, which is easy to handle but involves a delay of 3 to 4 days before a conclusive diagnosis can be reached.[27] Modern methods, such as polymerase chain reaction (PCR) or ligase chain reaction, reduce the analysis time but involve difficult and expensive equipment and material.[28] Because of these limitations in STI detection and diagnostics, an accessible and cost-effective technology to detect STIs is an urgent need in public-health monitoring.

We have recently shown that the use of *N,N,N′,N′*-tetramethyl-para-phenylene-diamine (TMPD) in chronoamperometry measurements of pathogenic bacteria led to the quantification of cytochrome c oxidase activity in *E. coli* and *Bacillus subtilis*, which could be seen for the first time in aerobically grown *E. coli* bacteria.[16] This sensing principle is based on the expression of cytochrome c oxidase in target bacteria, thus oxidizing TMPD, which is then regenerated at an electrode. An enhanced electrochemical current thereby indicates the presence of bacteria. However, bacteria could not be detected in solution and had to be dropcasted onto the electrode surface in order to achieve measurable electrochemical signals. The present manuscript overcomes these limitations, by combining immunohistochemistry and electrochemical sensing to provide the concept for a potential electrochemical sensing device. Herein we develop the previous nonselective chemical principle[16] into a fully applicable selective-detection method for medically pertinent organisms at clinically relevant concentrations. We show the selective immobilization of target bacteria to gold macroelectrodes by employing thiol chemistry and antibody binding, allowing the subsequent sensitive quantification of cytochrome c oxidase activity within seconds. This allows the specific detection of organisms in solution, which is crucial for a biosensing device. The presented detection of bacteria based on an electrochemical on-signal thereby reduces the risk of false-positive results, compared with that of off-signals, which are observed because of blockage of the electroactive surface of the electrode by bacteria or any kind of impurity. Furthermore, the direct interaction of the redox species with the bacteria greatly reduces the chances of interferences and reduces the cost of the experimental equipment, as expensive instrumentation, such as that needed for impedance spectroscopy, is not required. The presented analytical detection method was transferred onto screen-printed electrodes (SPEs) after evaluation of several commercially available sensing platforms, allowing the possibility of point-of-care applications. In addition, a limit of detection for *E. coli* is presented, and the applicability to the detection of *N. gonorrheae* is illustrated.

EXPERIMENTAL SECTION

Chemical Reagents. All chemicals were purchased from Sigma-Aldrich, if not indicated otherwise. Phosphate-buffered saline (PBS, 0.17 M) solution was prepared by mixing 8 g of sodium chloride (≥99%), 0.2 g of potassium chloride (≥99%), 1.44 g of sodium phosphate dibasic (≥99%), and 0.24 g of potassium phosphate dibasic (≥99%). Nanopure water with resistivity not less than 18.2 MΩ cm at 25 °C (Millipore water-purification system) was added to 1 L. *N,N,N′,N′*-Tetramethyl-para-phenylene-diamine tetrafluoroborate (TMPD-BF_4) was prepared following the method of Yamauchi et al.[29] Crystals were characterized by their brownish-purple appearance and melting point of 125 to 127 °C.

Immobilization of Bacteria. The number of bacteria in solution was determined by the optical density at a wavelength of 600 nm (*E. coli*[30] OD_{600} of 1.0 indicates 8.0×10^8 cells mL^{-1}, *A. faecalis*[31] OD_{600} of 0.14 indicates 3.0×10^7 cells mL^{-1}). The capture of bacteria at the gold electrodes, following the procedure by Maalouf et al.,[32] was carried out by the formation of self-assembled monolayers (SAMs) on the gold substrates. $HSCH_2[CH_2]_9CH_2NHCOBiotin$ (ProChimia Surfaces) was used as a biotinylated thiol, and 1-octanethiol functioned as a spacer. In a 1:1 mix of ethanol and chloroform, 0.2 mM biotin−thiol and 0.8 mM spacer were mixed and exposed to the gold electrodes for about 24 h at room temperature. The electrodes were washed thoroughly with PBS by careful immersion of the electrodes multiple times in solution. To block any nonspecific binding sites, the electrodes were exposed for 30 min to 1 μM bovine-serum albumin (BSA) in nanopore H_2O and again washed with PBS. Incubation in 10 μM neutravidin in nanopure H_2O for 45 min (ThermoFisher) resulted in the replacement of BSA at the biotinylated thiols because of the high affinity of avidin for biotin, with a disassociation constant of 10^{-15} M.[33] A 1:200 dilution of a commercially available biotin-tagged polyclonal anti-*E. coli* antibody (GeneTex, GTX40765) was prepared in PBS and exposed to the electrodes for 45 min to achieve binding to neutravidin. Incubation with a cell suspension for 45 min at 37 °C while the vial was occasionally gently tapped resulted in the immobilization of target bacteria on the surfaces of the electrodes.

Electrochemical Measurements. Gold macroelectrodes (Basi, 3 mm diameter) were employed as working electrodes, and an in-house-fabricated gold microelectrode with a diameter of 6.9 mm was used to determine the concentration of TMPD-BF_4 in solution by cyclic voltammetry.[16] Working electrodes were polished prior to experiments using a water−alumina mix (1.0, 0.3, and 0.05 mm; 30 s for each grade) on microcloth polishing pads (Buehler).[34] After polishing, macroelectrodes were sonicated for approximately 2 min to remove alumina powder from the electroactive surface. A platinum mesh and a saturated calomel electrode were used as the counter and reference electrodes, respectively. All bioelectrochemical experiments were carried out in a thermostated (37 °C) electrochemical cell inside a Faraday cage, using a modular potentiostat (PGSTAT302N, Autolab).

Chronoamperometry was carried out, respecting a delay time of 45 s after the electrode was brought in contact with the TMPD-BF_4 solution to maximize the electrochemical response of the target bacteria.[16] Hence, following an open-circuit potential for 45 s, an oxidative potential of 250 mV was applied for 5 s. This was followed by a potential step to −150 mV for 5 s. All potentials are indicated versus a saturated calomel reference electrode (SCE).

RESULTS AND DISCUSSION

Immobilization of Target Bacteria on Gold Substrates and Electrodes. To enable the electrochemical detection of specific bacteria, gold substrates, macroelectrodes, and screen-printed electrodes (SPEs) were modified by thiol chemistry, following the method of Maalouf et al.[32] As shown in Figure 1A, a biotin−thiol and spacer−thiol self-assembled

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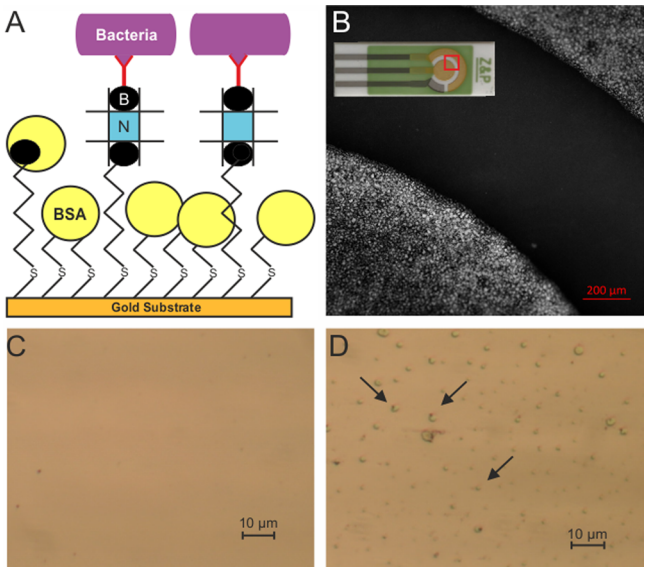

Figure 1. Surface modification of gold substrates and electrodes. (A) Schematic representation of surface modifications. The self-assembled monolayers of biotinylated thiols, capped with BSA, allow the binding of neutravidin (N) with high affinity to a biotinylated antibody (red), which captures target bacteria. (B) Fluorescence imaging of different substrates on an SPE containing immobilized, biotinylated fluorescent beads instead of antibody, illustrating the precision of the presented electrode-modification method. (C) Optical micrograph of a bare gold substrate and (D) optical micrograph of a fully functionalized gold surface with immobilized *E. coli* bacteria. Arrows point to three immobilized bacteria as examples.

monolayer (SAM) was formed on the gold surface, with $HSCH_2[CH_2]_9CH_2NHCOBiotin$ and octanethiol as the biotinylated thiol and spacer, respectively. Following the blockage of unspecific binding sites with bovine-serum albumin (BSA), incubation with neutravidin resulted in the replacement of BSA at the biotinylated thiols. A commercially available polyclonal anti-*E. coli* antibody was added, binding to neutravidin and immobilizing target bacteria on the substrate during incubation with a cell suspension. The optical micrographs shown in Figure 1D demonstrate the successful immobilization of *E. coli* bacteria on a fully functionalized gold substrate compared with on a control surface lacking the SAM (Figure 1C). The accuracy of the presented electrode-modification method and its applicability to screen-printed electrodes (SEPs) was demonstrated by replacing the antibody with biotinylated fluorescent beads (Figure 1B). Fluorescence microscopy revealed the successful binding of beads on the gold surface of a fully functionalized SPE. The rapid and sensitive detection of pathogens by electrochemistry on the basis of the binding of specific target bacteria to gold electrodes is described below.

Electrochemical Recognition of Bacteria and Control Measurements. Our previous studies reported the recognition of bacterial cytochrome c oxidase using the compound *N,N,N′,N′*-tetramethyl-para-phenylene-diamine (TMPD) by electrochemistry.[16] The oxidation of TMPD consists of two electron-transfer reactions (Supplementary Figure 1). In the first oxidation step, the radical cation $TMPD^{+\cdot}$ is formed, which has a characteristic blue color,[35] as seen in the oxidase test in cell-culture applications. This step is highly reversible, both chemically and electrochemically, whereas the product of the second electron transfer is unstable and decomposes quickly. Because of the rapid oxidation of TMPD by atmospheric oxygen, the radical cation salt *N,N,N′,N′*-tetramethyl-para-phenylene-diamine tetrafluoroborate ($TMPD\text{-}BF_4$) was synthesized according to the procedure of Yamauchi et al.[29] and exposed to bacteria dropcasted onto gold macroelectrodes. Because of the expression of cytochrome c oxidase, the immobilized bacteria were capable of oxidizing TMPD to its radical cation, $TMPD^{+\cdot}$, which was then regenerated to TMPD at the macroelectrode, resulting in a statistically significant increase in electrochemical current in the presence of the bacteria.[16] The electrochemical detection of specific pathogens was not addressed in our previous publication and is presented in the following.

As a proof-of-concept experiment, *E. coli* bacteria were immobilized onto a gold macroelectrode by exposing a functionalized macroelectrode to a bacterial suspension at a concentration of 10^9 cells per milliliter for 45 min at 37 °C. It should be noted that the fractions of living and dead bacteria in solution was not determined in order to mimic the conditions of a real sample, such as urine. Hence, we purposely use the term cells per milliliter instead of colony-forming units (CFU). In this study, six bare gold macroelectrodes functioned as control sensors. As shown in Figure 2A, in the presence of *E. coli* bacteria, a significant increase in electrochemical current was recorded. This increase is attributable to the oxidation of TMPD by the bacteria, as no significant change in current was observed at a functionalized electrode in the absence of *E. coli* (Supplementary Figure 2), excluding potential influences on the electrochemical-current signal by the SAM. Furthermore, all individual chemical compounds of the SAM were tested for their electroactivity and found not to result in a faradaic current, as shown in Supplementary Figure 3.

To assess the specificity of the antibody for *E. coli* detection, *Alcaligenis faecalis* bacteria were employed as control organisms. *A. faecalis* are Gram-negative rod-shaped bacteria that can be found in soil, water, and the general environment of humans.[36] These bacteria express cytochrome c oxidase, which can be electrochemically detected when organisms are dropcasted onto a macroelectrode, as shown in Figure 2B. Exposing a macroelectrode containing *A. faecalis* to a 2 mM $TMPD\text{-}BF_4$ solution while applying a potential at the electrode exceeding the formal potential for the reduction of $TMPD^{+\cdot}$, an increase in the electrochemical current was observed. A turnover number of 6.4×10^8 L mol^{-1} s^{-1} was determined, representing a 70% increase in cytochrome c oxidase activity compared with that of aerobic *E. coli*, as reported previously.[16] Please see the Supporting Information for detailed calculations. However, when incubating a modified electrode containing an anti-*E. coli* antibody with *A. faecalis*, no significant current increase was observed, demonstrating the specificity of the method for the detection of *E. coli*, as no *A. faecalis* could bind to the surface of the macroelectrode (Figure 2C). These control studies demonstrate that the electrode modifications do not compromise the electrochemical concept behind the recognition of specific target bacteria at macroelectrodes, and the specific detection of *E. coli* can be achieved within seconds after bacteria immobilization.

Specific Detection of Pathogenic Bacteria at SPEs. To transfer the presented concept to substrates suitable for operation within a portable, hand-held device, the applicability of the proposed methodology was tested on commercial disposable SPEs purchased from Zimmer&Peacock. Prior to the formation of SAMs, redox chemistry of TMPD was carried

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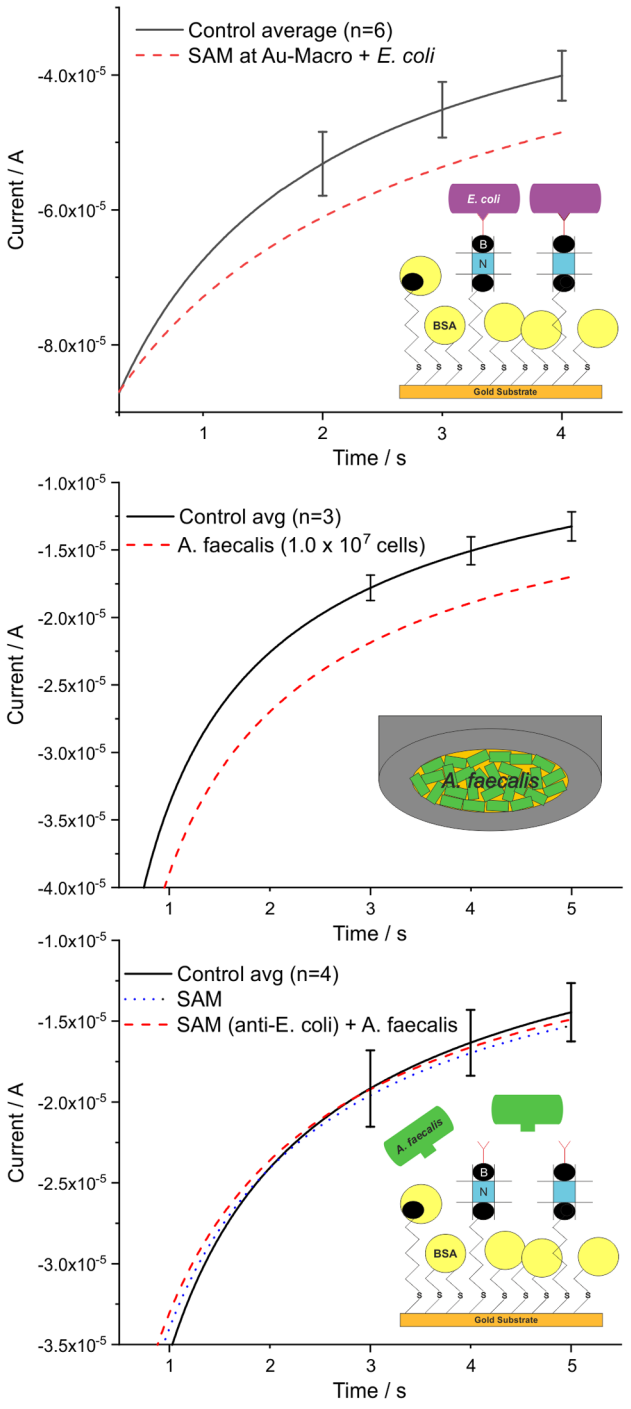

Figure 2. *E. coli* recognition and control studies. (A) Electrochemical-current increase observed following the binding of *E. coli* to a fully functionalized macroelectrode (red, dashed line), compared with that of a bare electrode (black, solid line). (B) Dropcasted *A. faecalis* bacteria detected at a macroelectrode, shown as an increase in electrochemical current (red, dashed line). (C) Exposure of *A. faecalis* to a functionalized electrode containing an anti-*E. coli* antibody. This does not result in an enhanced electrochemical-current signal (red, dashed line) compared with those of the control electrodes (black, solid line: bare electrode; blue, dotted line: modified electrode in the absence of bacteria). All error bars represent three times the standard deviations.

out. It should be noted that all SPEs were used as received without the need for polishing or pretreatment prior to the electrochemical studies. Sensors provided by Zimmer&Peacock revealed stable electrochemical behavior during voltammetry. Along with their clean and stable electrochemistry, SPEs by Zimmer&Peacock were easy to handle and withstood organic solvents easily. Other SPEs were evaluated but did not compare favorably with SPEs from Zimmer&Peacock, which were selected for further studies.

To determine a detection limit for the recognition of *E. coli* at SPEs, functionalized SPEs were exposed to cell suspensions ranging from 5×10^3 to 1×10^7 cells per milliliter (Figure 3A)

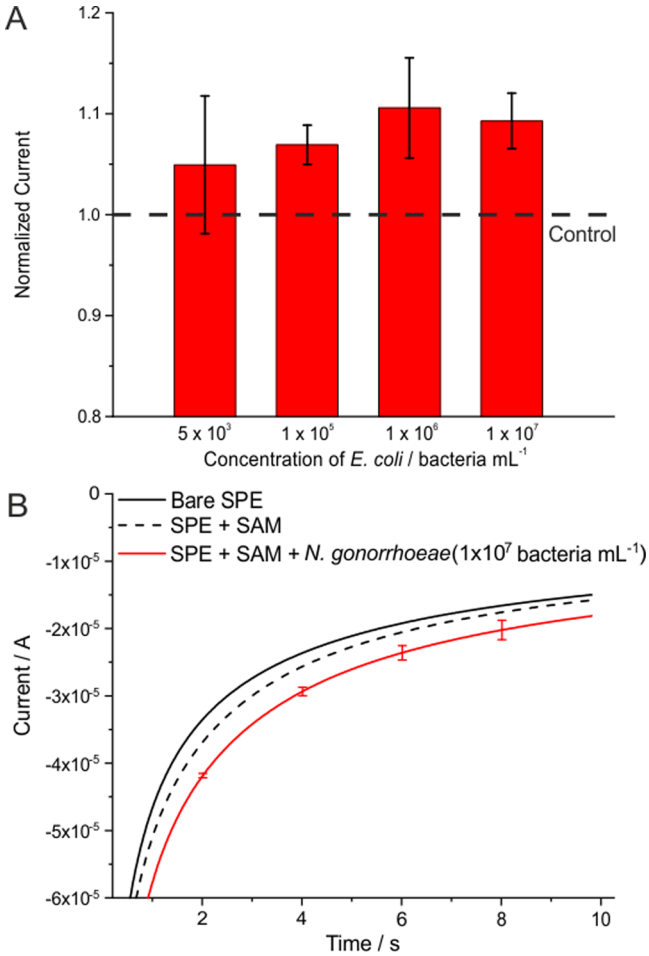

Figure 3. Detection of bacteria on SPEs. (A) Detection of various *E. coli* concentrations, revealing a detection limit of 1×10^5 bacteria per milliliter. (B) Detection of *N. gonorrheae* on functionalized SPEs. Immobilized bacteria result in a significantly enhanced electrochemical current (red), in contrast to a blank electrode (black, solid line) and a functionalized sensor in the absence of bacteria (black, dotted line). Error bars represent three times the standard deviations.

and incubated for 45 min at 37 °C. Following incubation, chronoamperometric measurements revealed a detection limit of 10^5 cells per milliliter (Supplementary Figure 4). The electrochemical measurements at all concentrations were performed in triplicate and are shown as averages with error bars representing three times the standard deviations. Three functionalized SPEs in the absence of bacteria functioned as controls, and their average is represented as a dotted line in Figure 3A.

The detection limit is recorded as the bacterial concentration that results in an electrochemical current exceeding

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three times the standard deviation for each condition. Interestingly, recent reports suggest urinary-tract infections diagnosed with CFU values less than 10^5 cells per milliliter result in the inappropriate use of antibiotics,[37] as the human immune system usually copes well with exposure to pathogens below this concentration. For the development of biosensors, it is recommended to focus less on reaching detection limits below 10^5 CFU and rather focus on selective, rapid, easy-to-handle, portable, and compact devices.[8] The sensibility of the presented methodology therefore sits at the immediate threshold for clinically significant bacterial concentrations and directly addresses current sensing needs. To illustrate the convenience of the proposed method, the reusability of single sensors for the recording of both baseline and bacterial signals were assessed. A freshly prepared functionalized SPE was exposed to TMPD-BF_4 during chronoamperometry. The same sensor was reused after 1 h of incubation in PBS (Supplementary Figure 5A). No degradation in the electrochemical current was observed, demonstrating the stability of the electroactive surface area. Furthermore, two individual functionalized SPEs were employed to record the electrochemical signal before the incubation with *E. coli*, and the same sensors were reused to detect immobilized *E. coli* bacteria after a 45 min exposure to a cell suspension of 10^6 cells per milliliter at 37 °C. As shown in Supplementary Figure 5B for two individual SPEs, the presence of *E. coli* bacteria resulted in a significant current increase. These results demonstrate that a single SPE is sufficient for recording both the initial baseline current as well as the electrochemical-current signal as a result of bacteria immobilization at the surface of the SPE.

Our findings using the example of the pathogen *E. coli* illustrate the usefulness of the proposed procedure for the detection of contaminants in the food industry and for clinical applications in connection with *E. coli*. To widen the impact of the technology within the medical sector, the *E. coli* antibody was replaced with a polyclonal anti-*N. gonorrheae* antibody. Figure 3B shows chronoamperometry in 2.0 mM TMPD-BF_4 carried out on a bare SPE, an SPE containing the SAM layer including the anti-*N. gonorrheae* antibody, and a fully functionalized SPE after exposure to 10^7 cells per milliliter *N. gonorrheae* in solution for 45 min at 37 °C. A significantly enhanced electrochemical current was recorded in the presence of *N. gonorrheae*. A turnover number of 1.2×10^9 L mol^{-1} s^{-1} was determined, which corresponds to about 200% of the value obtained for *A. faecalis*. The straightforward substitution of antibodies and successful recognition of specific target bacteria demonstrates the versatility of the proposed method. Given the generality of the biochemical reaction, by exchanging the target specific antibody, such biosensors could be envisioned for a large number of different pathogenic bacteria that exhibit cytochrome c oxidase, including species related to food contamination, sexually transmitted infections, and other diseases. Furthermore, the use of disposable SPEs in combination with an antibody reduces the current costs of diagnosis to under $5.00 per test, as SPEs are produced at high volumes for less than $1.00 and a volume of 500 μL of antibody is sufficient to produce about 500 to 600 functionalized SPE sensors.

CONCLUSION

Herein, we report an electrochemical method for the sensitive, rapid, selective, cost-efficient, and easy-to-perform detection of specific bacteria. Using the examples of *E. coli* and *N. gonorrheae*, the detection of pathogens was achieved at clinically relevant concentrations. The immobilization of target bacteria on SPEs by thiol chemistry and antibody binding can be applied to a large range of bacteria that exhibit cytochrome c oxidase, enabling the specific detection of pathogens within seconds by electrochemistry. As pathogens are successfully detected with inexpensive SPEs, which are commonly used for portable potentiostatic devices, results can in principle be obtained with the push of a button. Furthermore, the proposed use of polyclonal antibodies is ideal for providing a first alert for contamination or infection with a suspected bacterial species, which can then initiate the analysis of samples by more rigorous techniques. This approach will find broad application, such as in the food industry, as well as in veterinary and human medical health services.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.9b00326.

Electrochemistry of TMPD; electrochemical current of a bare electrode and the functionalized electrode in the absence of *E. coli* bacteria; electrochemistry of SAM components; detection of *E. coli* on functionalized SPEs containing anti-*E. coli* antibodies; reusability of the SPE sensors; and additional methods: bacterial culture, determination of species concentration, and determination of turnover numbers (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. S.K. designed the research, conducted experiments, analyzed data, and wrote the manuscript. R.A.S.C. conducted experiments and prepared figures. R.M.E. contributed *E. coli* and *A. faecalis* cultures, and H.L. provided *N. gonorrheae* bacteria. C.C.T. provided funding, materials, and facilities for *N. gonorrheae* cultures. R.G.C. directed the research, is the corresponding author, and wrote the manuscript. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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