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Development and adaptation of a multiprobe biosensor for the use in a semi-automated device for the detection of toxic algae

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Abstract

Worldwide monitoring programs have been launched for the observation of phytoplankton composition and especially for harmful and toxic microalgae. Several molecular methods are currently used for the identification of phytoplankton but usually require transportation of samples to specialised laboratories. For the purpose of the monitoring of toxic algae, a multiprobe chip and a semi-automated rRNA biosensor for the *in-situ* detection of toxic algae were developed. Different materials for the electrodes and the carrier material were tested using single-electrode sensors and sandwich hybridisation that is based on species-specific rRNA probes. Phytoplankton communities consist of different species and therefore a biosensor consisting of a multiprobe chip with an array of 16 gold electrodes for the simultaneous detection of up to 14 target species was developed. The detection of the toxic algae is based on a sandwich hybridisation and an electrochemical detection method.

Keywords: Disposable multiprobe chip; Electrochemical detection; Toxic algae; Sandwich hybridisation; rRNA probes

1. Introduction

Rapid identification of aquatic microorganisms as well as physical and chemical measurements of the environment are important to understand coastal dynamics and processes that can impact marine ecosystems, such as the initiation of harmful algal blooms (LaGier et al., 2005). Worldwide monitoring programs have been introduced to observe phytoplankton composition and especially harmful microalgae, the latter of which includes two types of causative organisms: the toxin producers and the high-biomass producers. Approximately 4000 marine planktonic microalgae have been described so far and of these, around 97 species are toxic (mainly dinoflagellates) and about 200 can be noxious (Zingone and Enevoldsen, 2000, Moestrup, 2004). Many toxic algae species are cosmopolitan, and many areas are threatened by multiple species of toxic algae (Metfies et al., 2006). The genus *Alexandrium* includes a number of species producing saxitoxins, which are potent neurotoxins responsible for paralytic shellfish poisoning (Penna, 1999), e.g., *Alexandrium tamarense* and *A. ostenfeldii*. A traditional method for the monitoring of toxic algae is the use of light microscopy, but this can be time consuming when many samples have to be routinely analysed. Unicellular algae are taxonomically challenging and some of them have few morphological markers; thus reliable species identification requires trained personnel and expensive equipment (Tyrrell et al., 2002; Ayers et al., 2005). Diverse molecular methods are currently used for the identification of phytoplankton, such as whole cell fluorescent *in-situ* hybridisation (Anderson et al., 2005; Hosoi-Tanabe and Sako, 2005; Kim and Sako, 2005), PCR-based assays (Penna, 1999; Guillou et al., 2002) and sandwich hybridisation assays (Tyrrell et al., 2002; Ayers et al., 2005). Metfies et al. (2005) introduced a biosensor in combination with a hand held device for the detection and identification of the toxic dinoflagellate *A. ostenfeldii* (Metfies et al., 2005) using sandwich hybridisation and molecular DNA probes that specifically targeted the rRNA of toxic algae. This method simplifies the detection of toxic algae by reducing the analysis time of a sample, although manual RNA isolation and manual manipulation of the hybridisation steps are necessary.

A simultaneous detection of multiple species is important for detection of harmful algae because phytoplankton communities consist of different species that vary greatly temporally and spatially. Various sectors, such as clinical diagnostics, environmental monitoring, biothreat detection and forensics, apply

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single-electrode sensors as well as arrays for the detection of their targets (Berganza et al., 2006; Lermo et al., 2006; Taylor et al., 2006). Arrays of electrodes enable a simultaneous detection of multiple species with different molecular DNA probes (Dock et al., 2005; Farabullini et al., 2007). Still most of the molecular techniques and the conventional methods for the monitoring of harmful algae usually require transportation of samples to specialised laboratories. This becomes increasingly difficult to manage as the number of samples and frequency of their collection increases, reaching a point where analysis of a given sample can take days (Metfies et al., 2006). Rapid identification of aquatic microorganisms is important to understand coastal dynamics and processes that can impact marine ecosystems, such as the introduction and spreading of microbial pollutants and the initiation of harmful algal blooms (LaGier et al., 2005). Biosensors can be used on-site and therefore circumvent the need to return samples into the laboratory.

A detection system with two major parts was developed during the EU-project ALGADEC: a multiprobe biosensor and a semi-automated device. We present here the development of a multiprobe biosensor for the simultaneous detection of 16 different target molecules and thus for the detection of specific compositions of toxic algae. The design and adaptation of the semi-automated device for the *in-situ* analysis of toxic algae using the multiprobe biosensor is described in another publication (Diercks et al., unpublished). The detection of the target algae involves a sandwich hybridisation using two different DNA probes, a capture probe and a signal probe. Oligonucleotide DNA probes have a length of 18–25 base pairs and are usually designed to bind to complementary sequences of the small and the large subunit ribosomal RNA algal genes, as a result of their high target number in cells and their varying target specificity based on more or less conserved regions (Groben et al., 2004). The capture probe is immobilised on the working electrode surface of a biosensor and binds to rRNA isolated from the target organism. A second digoxigenin-labelled probe also binds to the rRNA and carries the signal moiety. An antibody–enzyme complex directed against digoxigenin is added and incubated and a redox-reaction takes place after substrate addition and the resulting electrical current can be measured with a potentiostat.

This study describes the determination of the most efficient and cost-effective materials for working electrodes as well as the development and adaptation of a multiprobe chip for the detection of toxic algae.

2. Materials and methods

2.1. DNA probe sets

One set of capture and signal 18S-DNA probes (AOST1: 5′-CAACCCTTCCCAATAAGGT-3′ and AOST2: 5′-GAATCACCAAGGTTCCAAGCAG-3′, (Metfies et al., 2005)), specific for the 18S-rRNA of *A. ostenfeldii*, was used to compare the performance of carbon sensors and gold sensors (Gwent Electronic Materials (GEM), UK) and for the design of a multiprobe chip. These DNA probes have at least one mismatch against all non-target organisms listed in the database. DNA probes and positive control were synthesized from Thermo Electron Corporation (Ulm, Germany). Oligonucleotide synthesis is a chemical process performed on automated synthesising machines (for further information please see: http://www.thermo.com/com/cda/product/detail/1,22383,00.html).

2.2. Single-electrode chips

2.2.1. Immobilisation of DNA probes on carbon sensors

The immobilisation of the biotinylated capture DNA probe (AOST2) on single-electrode carbon sensors used in this study was done according to a previous protocol (Metfies et al., 2005). All incubation steps were carried out in a moisture chamber to prevent evaporation. The surface of the carbon-working electrode was pretreated with carbonate buffer (50 mM $NaHCO_3$, pH 9.6) that was followed by an incubation with NeutrAvidin in a concentration of 0.5 mg/mL (Pierce Biotechnology, Rockford, USA) at 4 °C for at least 4.5 h. Subsequently, the sensor was washed with PBS (BupH phosphate saline pack, Pierce Biotechnology, USA) to remove excessive NeutrAvidin. For blocking, the working electrode was incubated with 3% (w/v) casein in PBS at room temperature and afterwards the sensors were washed in PBS. Prior to the application on the electrodes, the DNA probes were diluted in bead buffer (0.3 M NaCl/0.1 M Trizma base, pH 7.6) to achieve a concentration of 10 μM. For the immobilisation of the DNA probes on the electrodes, the sensors were incubated at room temperature for 30 min. The unbound DNA probe was removed from the electrode by a washing step with hybridisation buffer (75 mM NaCl/20 mM Trizma base, pH 8.0 /0.04% SDS).

2.2.2. Immobilisation of DNA probes on gold sensors

The immobilisation of thiolated DNA probes on single-electrode gold sensors was done according to a modified protocol that was first introduced by Carpini et al. (2004). Prior to the immobilisation of the DNA probes onto the gold-working electrode, the thiolated DNA probes were dissolved at a concentration of 10 μM in 0.5 mol/L phosphate buffer. The gold-working electrode surface was incubated with the DNA probe at room temperature for at least 16 h. During all incubation steps, the sensors were stored in a moisture chamber to protect the solutions from evaporation. In order to minimize the non-specific interaction between the gold surface and the DNA probes, a post-treatment with 6-mercapto-1-hexanol (MCH; 1 mmol/L aqueous solution) was carried out for 1 h. Excessive DNA probe and MCH were removed by washing the sensor with 2× saline sodium citrate buffer.

2.2.3. Storage of coated sensors

The sensors were coated with 2% (w/v) Trehalose in PBS and dried for approximately 30 min at 37 °C. Afterwards coated sensors can be stored at 4 °C. The long-term stability of coated carbon and gold sensors was tested after 4, 6 and 12 months by hybridisation with test-DNA (positive control) and the detection DNA probe (AOST1).

2.2.4. Hybridisation of test-DNA on single-electrode sensors

The hybridisation mixture for the detection of test-DNA contained 1× hybridisation buffer (75 mM NaCl/20 mM Trizma base, pH 8.0/0.04% SDS), 0.25 μg/μL herring sperm DNA, 0.1 pmol/μL digoxigenin-labelled DNA probe AOST1 and 0.1 pmol/μL test-DNA (positive control) as target for the DNA probes. The negative control contains no test-DNA. The test-DNA is a synthetic oligonucleotide that matches exactly the combined region of the capture and signal DNA probe. Denaturation of the target nucleic acid was carried out by incubating the hybridisation mixtures at 94 °C for 4 min. Two microlitre of the mixture was applied to the working electrode and the sensor was incubated at 46 °C for 30 min. The biosensors were stored in a moisture chamber during hybridisation to prevent evaporation. Subsequently, the sensors were washed with POP buffer (50 mM $NaH_2PO4 \times H_2O$, pH 7.6/100 mM NaCl).

2.2.5. Electrochemical detection with single-electrode sensors

An antibody–enzyme complex directed against the digoxigenin coupled to horseradish-peroxidase (anti-DIG-POD, 7.5 U/mL in PBS, pH 7.6/0.1% BSA (w/v)/0.05% Tween 20 (v/v)) was applied onto the single-electrode sensor and incubated at room temperature for 30 min. Unbound antibody–enzyme complex was removed by washing the sensor with POP buffer and the sensor was inserted into the measurement device, PalmSens (Palm Instruments BV, Houten, Netherlands). Twenty microlitre of substrate solution (4-aminophenylamine hydrochloride, ADPA (44 μg/mL)/0.44% ethanol (v/v)/0.048% H_2O_2 (v/v)/50 mM $NaH_2PO_4 \times H_2O$/100 mM NaCl) was added to the working electrode and the resulting electrochemical signal was directly measured for 10 s at a potential of −147 mV (versus Ag/AgCl) after 8 s of equilibration. All experiments were carried out in triplicate, the mean value of the signals was calculated and the standard derivation was determined with the following formula (x is the measured value, n is the number of measurements):

$$\text{Standard deviation} = \frac{\sqrt{n\sum x^2 - (\sum x)^2/n(n-1)}}{\sqrt{n}}$$

2.3. Multiprobe chips

2.3.1. Design of the multiprobe chip

A disposable multiprobe chip was designed from iSiTEC GmbH (Bremerhaven, Germany) with the size of a conventional glass slide and produced by GEM (Pontypool, UK). The multiprobe chip consisted of a carrier material that contained 16 gold-working electrodes, each with the size of 1.5 mm and a combined counter/reference electrode above the electrode array (Fig. 1). Working and counter/reference electrodes were encircled with a dielectric layer. The stems of the electrodes were fitted to a typical connecting strip.

Two different carrier materials, valox (a polyester blended material) and ceramic (alumina), were chosen for comparison of spotting properties and signal intensities. Additionally two variations of the ceramic were tested, a plain ceramic material and ceramic with a hydrophobic polymer (formulation D2061107D4, for further information please contact GEM).

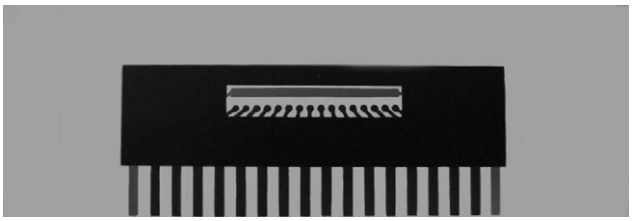

Fig. 1. Multiprobe chip with 16 gold-working electrodes.

2.3.2. Spotting of multiprobe chips

Multiprobe chips were either hand-spotted or spotted with a non-contact dispenser (Biodot Ltd., UK) from GEM. Hand-spotted chips were covered with 10 μL of thiolated capture DNA probe (10 μM in 0.5 mol/L phosphate buffer) and incubated as described above. Ten microlitres of MCH solution were added and incubated for 1 h, subsequently, unbound DNA probe and MCH were removed by washing the sensor with 2× saline sodium citrate buffer. The multiprobe chips were blocked with 10 μL of 5% (w/v) BSA and washed again with 2× saline sodium citrate buffer. Multiprobe chips were spotted by immobilising 0.05 μL thiolated capture DNA probe per electrode and adding of 0.05 μL of MCH after incubation. Wash steps and blocking of the surface was carried out as previously described. The multiprobe chips were subsequently coated with 10 μL 2% (w/v) Trehalose in PBS buffer and dried for storage and shipment.

2.3.3. Hybridisation mixture and electrochemical detection

The hybridisation mixture using test-DNA (positive control), antibody solution and substrate solution for the multiprobe chip were prepared as described above. A volume of 10 μL hybridisation mixture and antibody solution was applied each time onto the chip to cover the whole electrode array. Electrochemical detection was carried out by placing the multiprobe chip into a substrate reservoir that harboured the substrate solution. The electrochemical signals were measured using a multiplexer, which can measure eight electrodes simultaneously, and the PalmSens detector (Palm Instruments BV, Houten, Netherlands). Electrodes 1–8 were measured first and then electrodes 9–16.

3. Results

The signals of the electrochemical detection are measured with negative values, but for simplification of analysis and presentation, the signals are multiplied by −1 unless otherwise noted.

3.1. Sensor design using single-electrode sensors

3.1.1. Comparison of electrochemical signals of carbon and gold sensors

In order to determine the most efficient and cost-effective material for the working electrodes on the sensors, two differ-

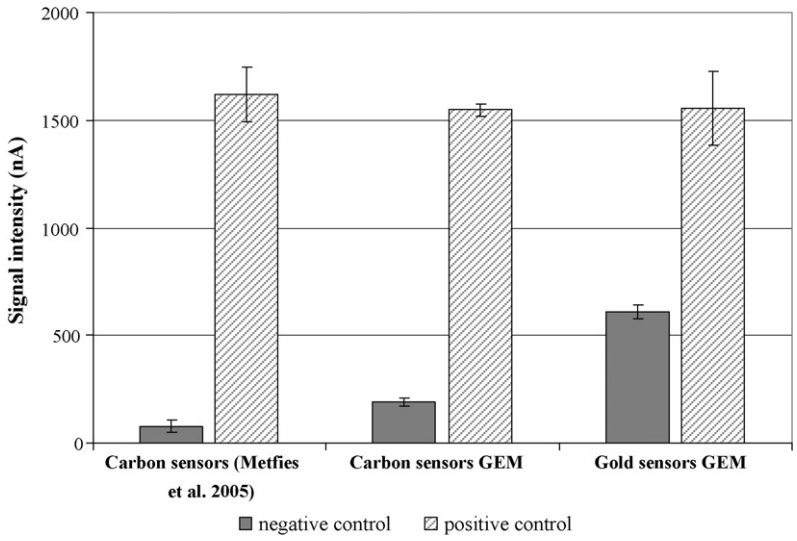

Fig. 2. Comparison of signal intensity of carbon and gold sensors was done using DNA probes AOST2 and AOST1 and test-DNA.

ent materials, carbon and gold were tested to compare signal intensity and the effectiveness of DNA probe immobilisation (AOST2). Additionally, the signals were compared to the signals shown by Metfies et al. (2005) with carbon sensors from a different manufacturer. The achieved signals for the positive controls detected on electrodes with different materials and sensors from different manufacturers are comparable being in the range of ∼1500 nano ampere (nA) (Fig. 2). However, the signal intensity of the negative control for the different surface materials varied considerably. The carbon sensor from Metfies et al. (2005) showed the lowest signal with 78 nA, whereas for the carbon sensor from GEM a signal of 190 nA was achieved. The gold sensor showed a very high signal of 611 nA. Therefore, the immobilisation protocol for gold sensors was optimised to reduce the background noise of the gold sensors.

3.1.2. Optimisation of immobilisation protocol for gold sensors

The optimisation of the immobilisation protocol was carried out by adding a surface-blocking step to the protocol subsequent to the immobilisation of the DNA probe (AOST2) and the treatment with MCH. Two different blocking reagents, casein and bovine serum albumin (BSA), known from the literature for their blocking properties (Bhatia et al., 1989; Che et al., 2001) were examined for their attributes to reduce the background noise of the gold surface. As a control, gold electrodes with no blocking reagents were hybridised. The blocking with 3% casein in PBS was accomplished at room temperature for 1 h, and could reduce the signal of the negative control to 281 nA but also reduced the signal of the positive control to 1168 nA (Fig. 3). Different concentrations of BSA, 3%, 5% and 10% in 4× hybridisation buffer, were applied to the gold

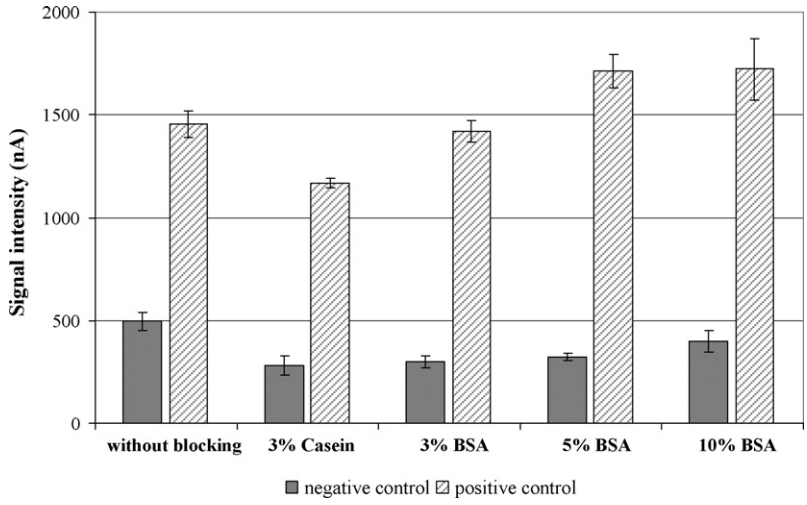

Fig. 3. Reduction of background signal by using casein and bovine serum albumin as blocking solutions on gold sensors coated with the thiolated DNA probe AOST2.

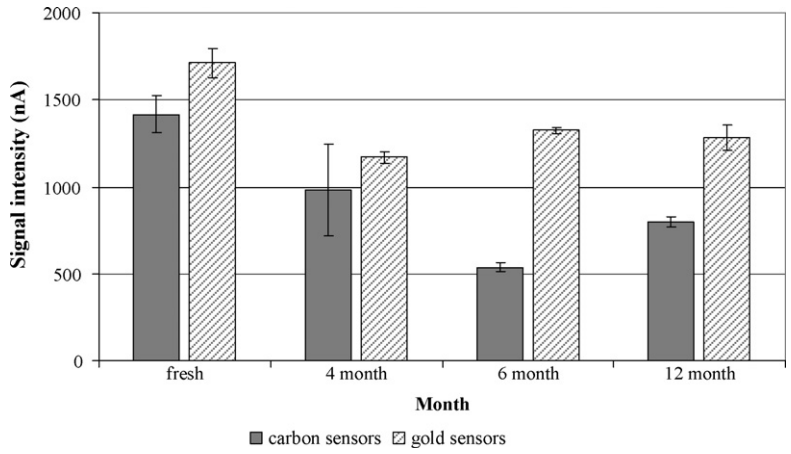

Fig. 4. Long-term stability of carbon and gold sensors after coating with 2% Trehalose and storage at 4 °C over indicated intervals.

sensors and incubated at 46 °C for 1 h. All treatments caused a decrease in signal of the negative control regardless which concentration of BSA was used, but 3% and 5% BSA showed the best improvement. Additionally, the signals of the positive control of the gold sensors blocked with 5% and 10% BSA increased about 200 nA. In consideration of these results, the 5% BSA blocking solution was chosen for further experiments.

3.1.3. Long-term stability of sensors

Long-term stability of carbon and gold sensors was tested by coating the sensors with Trehalose after immobilisation of the DNA probes (AOST2) onto the working electrode (Fig. 4). The sensors were stored at 4 °C and hybridised with target-DNA and the detection DNA probe (AOST1) after 4, 6 and 12 months. Signal intensity decreased from freshly prepared carbon sensors with 1416–798 nA for carbon sensors stored over 12 months at 4 °C. Also the signals for gold sensors decreased from 1711to 1282 nA.

3.1.4. Optimisation of the substrate concentrations

The enhancement of signals intensity was examined using carbon sensors (GEM) and different concentrations of substrate (POD) by varying the concentration of the mediator 4-aminophenylamine hydrochloride (ADPA) and of the hydrogen peroxide (H_2O_2). Fig. 5 shows that an increase of signal was achieved from 1530 nA of normal POD substrate to 3971 nA of 6.6 mg ADPA and 600 mM of H_2O_2 by increasing concentrations of ADPA and H_2O_2, simultaneously. The highest signal was obtained with 6.6 mg of ADPA and 600 mM of H_2O_2, but the signal of the negative control also increased from 38 to 203 nA.

3.2. Development of a multiprobe chip

3.2.1. Signal transmission between working electrodes

Every second working electrode (e.g. WE 2, 4, 6) of a multiprobe chip with valox carrier material was spotted by hand with thiolated DNA probe. Signals were detected only for the spotted working electrodes (data not shown); non-coated electrodes

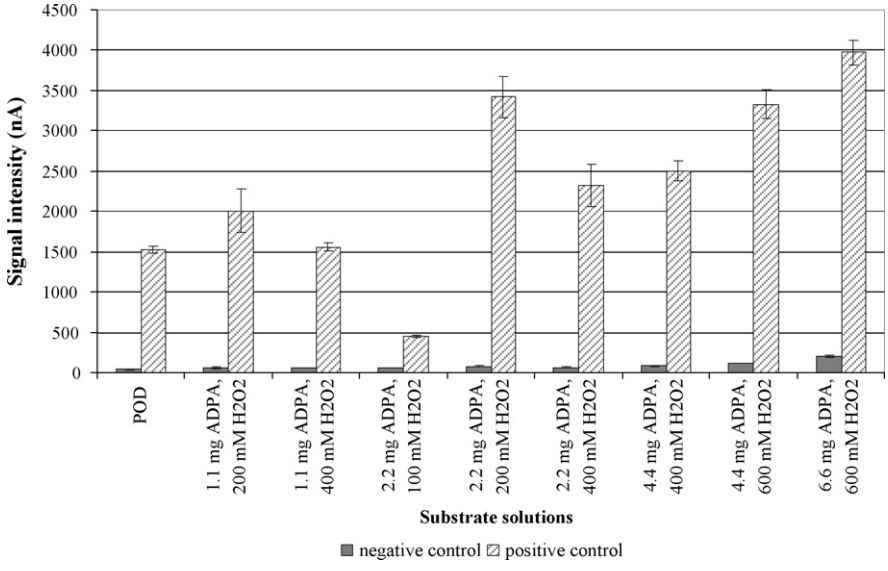

Fig. 5. Signal enhancement by varying substrate concentrations.

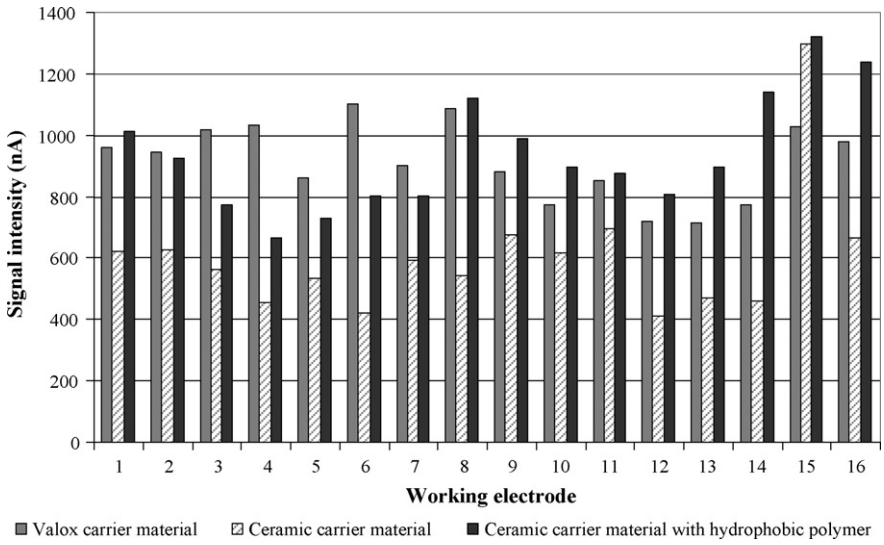

Fig. 6. Comparison of hand-spotted multiprobe chips with different carrier materials.

gave signals from 62 to 129 nA. There was no signal transmission between the electrodes. The signals were on average 3× lower than the signals for the single-electrode sensors because the diameter of the electrodes was smaller.

3.2.2. Selection of carrier material for multiprobe chips

Fig. 6 shows the signal intensity for two different carrier materials, valox and ceramic, and additionally a variation of ceramic with a hydrophobic polymer underneath the working electrodes. The valox material showed signals from 716 up to 1099 nA with a mean signal of 913 nA, the ceramic material with a hydrophobic polymer had signals from 728 to 1324 nA with a mean of 937 nA, whereas the uncoated ceramic showed the lowest signals from 421 to 1296 nA with a mean of 602 nA. The valox material showed a higher stability of DNA probe drops during spotting, whereas the uncoated ceramic could not be spotted with a biodot because of the hydrophilic properties of the ceramic (data not shown). Ceramic with a hydrophobic polymer coat underneath the working electrodes showed good stability of DNA probe drops (data not shown). During the experiments, the valox material was found to be difficult to cut into the correct size and spotted with DNA probes because the material bent too easily and all cutting was done by hand.

4. Discussion

4.1. Design of sensors and comparison of immobilisation protocols

Two materials for sensors, carbon and gold, were tested and an immobilisation protocol for gold sensors was developed and tested. Immobilisation of DNA probes to gold surfaces had already been established (Carpini et al., 2004; Mannelli et al., 2005) and our previously published methods (Metfies et al., 2005) were adapted with some modifications to immobilise the DNA probes to the gold sensors. Because of the considerable variations of signal intensity of the negative controls (background noise) of the different sensors, the immobilisation protocol for gold sensors was optimised by adding a surface-blocking step. Thus, the signal formation of a gold or carbon-covered surface with capture probe was similar and efficient. Signal comparison of long-term stability tests showed that the signals of carbon and gold sensors decreased over several months of storage about 45% and 26%, respectively, but stored gold sensors performed better and achieved higher signals. Long-term storage enables the production and coating of sensors in advance of use and consequently mass production and introduction to the market would be possible. Experiments with higher substrate concentrations revealed the potential of enhanced signals. A substrate concentration of 2.2 mg ADPA and 200 mM of H_2O_2 would be sufficient for a twofold signal increase. However, the immobilisation protocols for the different sensors have advantages and disadvantages concerning costs. One advantage of the carbon sensors is the lower price of the carbon paste in comparison to the gold paste. Gold sensors have the benefit that the coating with expensive NeutrAvidin can be omitted given that thiolated DNA probes bind directly to the surface of the gold. Because the gold sensors require fewer immobilisation steps in the protocol, which reduced manufacturing costs and also produced higher values after long-term storage, the gold sensors were chosen over the carbon sensors for further development of a biosensor.

4.2. Development of multiprobe chip

A multiprobe chip was designed from iSiTEC GmbH with 16 gold electrodes that can detect 16 different targets. The chip was developed with the size of a conventional glass slide, which offers the possibility to use automated dispensing systems for the spotting of DNA probes, e.g., piezo spotter. Furthermore, the chips are easy to handle because of their size and can be stored in standard boxes. The size of the working electrodes was reduced in comparison to the single-electrode sensors to decrease the electrode area and consequently the volume of reagents needed

for analysis. Signal transmission between the electrodes was assessed by coating only every second electrode with DNA probe and only background noise was determined. This is an important attribute because the 16 working electrodes will be coated with different species probes and false positive signals must be avoided. Different carrier materials for the electrodes were investigated for signal formation and DNA probe spotting properties. Valox material showed high signals and can be spotted with DNA probes by hand; however, the signals from electrodes spotted with DNA probes by hand were irregular and unacceptable. Automated spotting with a dispenser requires accurately cut chips because the recognition of the electrodes is not processed by image recognition but with position recognition. The valox material was easily bent, making this material unsuitable for automated spotting. To overcome this problem, the ceramic carrier material was chosen for the multiprobe chips. It could be accurately cut using a laser and then by snapping off pieces manually, and thus be spotted with an automated dispenser. The experiments with the ceramic chips showed lower signals than the valox material because the hydrophilic surface hampered the spotting. We observed that the liquid accumulated onto the hydrophilic surface and was drawn from the working electrodes resulting in non-coated electrodes. Finally, the addition of a hydrophobic polymer coat underneath the working electrode area overcame this last problem and the liquid stayed on the working electrodes. However, during the manufacturing of these chips, difficulties using the automated dispenser for DNA probe spotting occurred. We anticipate that in the production of these chips for commercial use that all spotting difficulties encountered here will be overcome.

5. Conclusion

A multiprobe chip with 16 gold electrodes was designed and adapted for the use in a sandwich hybridisation assay. This multiprobe biosensor can be used for the simultaneous detection of 14 different targets plus two controls and thus for the detection of species compositions in harmful algal blooms. The spotting of the multiprobe chips with DNA probes has to be automated to achieve a regular signal formation and to increase the sensitivity of the system. Different DNA probes, i.e., species will be spotted onto a chip thus chips specific for different geographic areas can be developed. Several specific DNA probe sets for toxic algae have been developed (Diercks et al., 2008) and need to be adapted to the chips. Furthermore, for the conversion of the electronic signal with the help of custom-made software into the concentration of toxic cells the sensors must be calibrated for each DNA probe set. The multiprobe chip can be inserted in a portable semi-automated device for the detection of toxic algae in less than 2 h (Diercks et al., unpublished).

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