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ORIGINAL PAPER

Development of an enzymatic fiber-optic biosensor for detection of halogenated hydrocarbons

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Abstract An enzyme-based biosensor was developed by co-immobilization of purified enzyme haloalkane dehalogenase (EC 3.8.1.5) and a fluorescence pH indicator on the tip of an optical fiber. Haloalkane dehalogenase catalyzes hydrolytic dehalogenation of halogenated aliphatic hydrocarbons, which is accompanied by a pH change influencing the fluorescence of the indicator. The pH sensitivity of several fluorescent dyes was evaluated. The selected indicator 5(6)-carboxyfluorescein was conjugated with bovine serum albumin and its reaction was tested under different immobilization conditions. The biosensor was prepared by cross-linking of the conjugate in tandem with haloalkane dehalogenase using glutaraldehyde vapor. The biosensor, stored for 24 h in 50 mM phosphate buffer (pH 7.5) prior to measurement, was used after 15 min of equilibration, the halogenated compound was added, and the response was monitored for 30 min. Calibration of the biosensor with 1,2-dibromoethane and 3-chloro-2-(chloromethyl)-1-propene showed an excellent linear dependence, with detection limits of 0.133 and 0.014 mM, respectively. This biosensor provides a new tool for continuous *in situ* monitoring of halogenated environmental pollutants.

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Abbreviations

BSA	bovine serum albumin
CF	5(6)-carboxyfluorescein
CF-BSA	conjugates of 5(6)-carboxyfluorescein and albumin
CLEA	cross-linked enzyme aggregate
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
ISE	ion-selective electrode
NHS	*N*-hydroxysuccinimide
PVA	poly(vinyl alcohol)

Introduction

Halogenated aliphatic hydrocarbons make up a large group of environmental pollutants owing to their extensive use in industry and agriculture. In addition, some of them are produced by naturally occurring processes [1, 2]. Many of these hazardous compounds are chlorinated or brominated alkanes and alkenes, e.g., 1,2-dibromoethane, 1,2-dichloroethane, 1,1,2-trichloroethylene, and 1,2,3-trichloropropane. These compounds belong to prevalent groundwater contaminants and are significant components of hazardous wastes and landfill leachates. Since halogenated aliphatic hydrocarbons have adverse health effects, their accurate monitoring in the environment is necessary.

Traditional analytical techniques for determination of these pollutants are gas and liquid chromatography, which

require a preliminary extraction step to concentrate the analyte [3–7]. Chromatographic methods are highly selective and sensitive, but are not generally suited for continuous and online measurements. In addition, they are time-consuming and expensive [8]. Therefore, there is a growing need to develop simple systems that provide fast and automatic direct analysis of pollutants. Application of biosensors is very promising in environmental monitoring owing to their sensitivity, low costs, and adaptability for on-site field measurements [9–13].

Several authors reported detection of halogenated aliphatic hydrocarbons using both biosensors and bioassays with the potential for adaption to a biosensor format. Most of these biosensors utilized an ion-selective electrode (ISE) for detection of released halide ions. Henrysson and Mattiasson [14] constructed a biosensor system utilizing *Hyphomicrobium* DM2 cells immobilized in alginate. A combination of a flow-calorimeter and an ISE was used for detection of dichloromethane. Hutter et al. [15] described an assay based on an ISE and alginate beads with cells of *Rhodococcus* sp. DSM 6344 expressing haloalkane dehalogenase. Peter et al. [16] adapted this sensing design to produce a compact biosensor for detection of ethylenebromide and 1-chlorobutane. Han et al. reported a whole-cell biosensor for determination of trichloroethylene. This device utilized *Pseudomonas aeruginosa* JI104 immobilized on a polytetrafluoroethylene filter which was mounted onto an ISE in a batch-type [17] or a flow-type [18] configuration. The lifetime of the trichloroethylene biosensor was extended using *Pseudomonas* cells adsorbed on glass beads in a reactor with a dual-electrode system [19]. Maliszewska and Wilk [20] developed a microbial assay for quantification of 2,2-dichloropropionic and 2-chloropropionic acid. Cells of *Pseudomonas* sp. MB58 producing 2-haloacid dehalogenase were entrapped in alginate beads and an ISE was used for evaluation of released chlorides. Recently, Bachas-Daunert et al. [21] reported a system for detection of 2-haloacids based on haloacid dehalogenase from the thermophile *Sulfolobus tokodaii*. The enzyme was covalently linked to Sepharose in a disposable column and released halogenide ions were detected using an ISE. Also two examples of whole-cell biosensors based on optical fiber as a transducer were reported. Campbell et al. [22] immobilized cells of *Xanthobacter autotrophicus* GJ10 expressing haloalkane dehalogenase in alginate on a fluoresceinamine-based pH optode. The protons released from the dehalogenation reaction were detected as a fluorescence change of the pH indicator. This biosensor was used for quantification of 1,2-dichloroethane. The demonstrated two-layer sensing concept was further used for immobilization of cells of *Rhodococcus* sp. GJ70 for detection of 1,2-dibromoethane [23].

All reported systems were based on the microbial cells containing dehalogenating enzymes, because fabrication of the whole-cell biosensors is easy and cheap and no protein purification steps are required. In addition, cells can be simply entrapped on the transducer with no effect on enzymatic activity. However, these biosensors can suffer from lower sensitivity, poor selectivity, and limited stability, owing to the leakage of the cells out of the matrix and the activity of proteases.

To overcome these limitations, we constructed a novel biosensor based on purified enzyme haloalkane dehalogenase LinB from *Sphingobium japonicum* UT26. The unique features of this biosensor are (1) the use of purified enzyme haloalkane dehalogenase and (2) immobilization of both the enzyme and the pH indicator in one sensitive layer on a fiber tip using conjugation of a fluorescent dye with bovine serum albumin (BSA).

Experimental

Chemicals

All chemicals were of analytical grade and used without further purification. Ammonium hydroxide, 2′,7′-*bis*(2-carboxyethyl)-5(6)-carboxyfluorescein, 5-(4,6-dichloro-*s*-triazin-2-ylamino)fluorescein hydrochloride, fluorescein, 6-[fluorescein-5(6)-carboxamido]hexanoic acid, hydroxylamine hydrochloride, 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt, 2-mercaptoethanol, and potassium dihydrogen phosphate were purchased from Fluka (Switzerland). Ampicillin sodium and isopropyl β-D-thiogalactopyranoside were obtained from Duchefa (The Netherlands). 2-(*N*-morpholino)ethanesulfonic acid was purchased from Carl Roth (Germany). All other chemicals were purchased from Sigma-Aldrich (USA). Dimethyl sulfoxide and methanol of high performance liquid chromatography grade were purchased from Chromservis (Czech Republic). Aqueous solutions were prepared with Milli-Q water obtained using a Simplicity 185 water purification system (Millipore, USA).

Enzyme and indicator preparation

Haloalkane dehalogenase LinB from *Sphingobium japonicum* UT26 [24] was overexpressed in *Escherichia coli* BL21(DE3) containing plasmid pAQN::*linB-UT* [25]. The His-tagged LinB was purified using an HR 16/10 chromatographic column (GE Healthcare, Sweden) with nickel nitrilotriacetic acid Sepharose (Qiagen, Germany) attached to an ÄKTA fast protein liquid chromatography system (GE Healthcare, Sweden) as described earlier [25]. The prepared LinB was lyophilized using an ALPHA 1-2 LD freeze dryer (Martin Christ, Germany). The enzyme activity was assayed by the Iwasaki method [26].

The fluorescent pH indicator 5(6)-carboxyfluorescein (CF) was covalently coupled to BSA using a procedure

described earlier [27, 28] with the following modifications. CF (5 mg) was dissolved in 1 ml of 0.1 M 2-(morpholino) ethanesulfonic acid with 0.5 M sodium chloride (pH 6.0) and added slowly to the cross-linker 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC; 25.5 mg) and *N*-hydroxysuccinimide (NHS; 3.8 mg) in a glass vial. The reaction mixture was incubated for 1 h at 25 °C. The cross-linker was inactivated by addition of 1.4 μl of 2-mercaptoethanol. BSA (15 mg) was dissolved in 1.5 ml of 0.1 M sodium bicarbonate buffer (pH 8.3). The reactive dye solution (0.9 ml) was slowly added to the stirred BSA solution and the mixture was incubated for 1 h at 25 °C. To stop the reaction, 0.15 ml of 1.5 M hydroxylamine (pH 8.5) was added to the mixture and the mixture was incubated for 1 h at 25 °C. The mixture was filtered through a Millipore Millex syringe filter with a pore size of 0.45 μm (Millipore, USA). The conjugates (CF–BSA) were separated from unreacted dye by size-exclusion chromatography using Sephadex G-25 Superfine (GE Healthcare, Sweden). The CF–BSA conjugates were stored in lyophilized form at 4 °C.

Instrumentation

Fluorescence spectra were measured using a FluoroMax-4P spectrofluorometer (HORIBA Jobin Yvon, USA) with a 150-W xenon arc lamp as a light source. A quartz cuvette with a 1-cm pathlength was used. The excitation and emission spectra were recorded at the maximum emission and excitation wavelengths (Table 1). All measurements were carried out in 10 mM phosphate buffer at 21 °C. An Orion Star 2 pH meter (Thermo Scientific, USA) with a Double Pore pH electrode (Hamilton, USA) calibrated using three pH reference buffers (Hamilton, USA) was used for pH measurements. The pH of the solutions was adjusted by addition of 3 M hydrochloric acid or 3 M sodium hydroxide.

The biosensor system (Fig. 1) consisting of a fluorescence photometer (University of Hanover, Germany), an optical fiber, and a biosensor tip was similar to that described by Campbell et al. [22]. The optical fiber of 60-cm length and with a 1-mm poly(methyl methacrylate) core with a polyethylene jacket (Toray Industries, Japan) was coupled to the fluorescence photometer with a light source, a light detector, and signal processing. The light from blue-light-emitting diode with a guaranteed stability for 1,000 h at 20 °C (Fig. S1) was filtered to 480 nm and guided to an optical fiber. The emitted light from the biosensor tip was filtered to 520 nm and focused into an H5784-20 photomultiplier tube (Hamamatsu Photonics, Japan). The signal was recorded using Advanced Serial Data Logger 2.5.0.39 (AGG Software, Russia).

The substrate concentration for biosensor calibration was determined by a Trace GC gas chromatograph (Thermo-Quest Scientific, UK) equipped with a DB-FFAP capillary column (J&W Scientific, USA) and a flame-ionization detector.

Preparation of the biosensor tip

The biosensor tip consists of one sensitive layer of LinB and CF–BSA immobilized on the poly(methyl methacrylate) optical fiber (Fig. 1). The surface of the fiber was polished with very fine grit paper (Softflex, Germany) before immobilization. Different techniques of immobilization were applied for preparation of the sensitive layer.

Cross-linking with glutaraldehyde

Lyophilized LinB (5 mg) and CF–BSA (10 mg) were dissolved in 80 μl of 25% (v/v) glycerol and 2 μl of this mixture was applied on the fiber tip with a pipette. Fibers were exposed to 70% (v/v) glutaraldehyde vapor for 30 min at 21 °C.

Cross-linking with EDC

A solution of the cross-linker was prepared by dissolution of 7 mg of EDC in 250 μl of 50 mM pyridine (pH 7.0). Adipic acid dihydrazide (8 mg) was dissolved in 250 μl of 50 mM pyridine (pH 7.0). Lyophilized LinB (5 mg) and CF–BSA (10 mg) were dissolved in 25 μl of EDC solution, then 8 μl of adipic acid dihydrazide solution was added. Immobilization mixture (2 μl) was applied on the fiber tip. The mixture was allowed to polymerize for 30 min at 21 °C.

Cross-linking with glutaraldehyde and entrapment in poly(vinyl alcohol)

LinB and CF-BSA were cross-linked with glutaraldehyde as described previously. Poly(vinyl alcohol) (PVA; 1 g) and poly(ethylene glycol) with M_r ~ 1,000 (0.6 g) were heated in 6.6 ml of water to 98 °C. Then the mixture was slowly cooled to 35 °C. Optical fibers with an cross-linked layer were immersed in the mixture and then left in the air for 15 min at 30 °C to complete gelation. Then the fibers were incubated in 0.1 M sodium sulfate for 40 min. The prepared fibers were rinsed with 50 mM phosphate buffer, pH 7.5 [29].

Cross-linked enzyme aggregates and entrapment in PVA

Lyophilized enzyme LinB (38.4 mg) and CF–BSA (38.4 mg) dissolved in 9.6 ml of water were precipitated by 28.8 ml of saturated ammonium sulfate (pH 8.0) for 45 min under stirring. Precipitated proteins were cross-linked with 3.1 ml of dextran polyaldehyde [30] for 45 min. Precipitation and cross-linking steps were performed in an ice bath. When the cross-linking was complete, the

Table 1 Fluorescence properties of the fluorescent pH indicators tested

pH indicator	λ_{ex} (nm)	λ_{em} (nm)	pH range	pH range[a]	Fluorescence intensity (%)
5(6)-Aminofluorescein	491	511	<2.8 5.1–7.4 8.2–10.2	3.0–6.6	24.5
2′,7′-*Bis*(2-carboxyethyl)-5(6)-carboxyfluorescein	492	520	5.6–8.5	3.0–6.6	8.4
5(6)-Carboxyfluorescein	491	513	5.0–7.8	5.0–7.7	62.5
5-(4,6-Dichloro-*s*-triazin-2-ylamino)fluorescein	492	514	5.2–7.9 >9.3	5.3–7.7	5.7
Fluorescein	490	514	5.4–8.0	3.0–6.6	100.0
6-[Fluorescein-5(6)-carboxamido]hexanoic acid	491	515	5.2–7.9	3.9–7.5	63.3
8-Hydroxypyrene-1,3,6-trisulfonic acid	450	511	5.9 – 8.6	6.1–8.5	23.0

[a] The pH-dependent response of the fluorescent dyes measured with the fluorescence photometer at 520 nm (excitation at 480 nm). Other data were acquired by using a spectrofluorometer at the excitation and emission maxima

suspension was centrifuged at 4,000*g* for 20 min at 4 °C. The supernatant was withdrawn and the resulting cross-linked enzyme aggregates (CLEAs) were resuspended in 30.7 ml of saturated sodium hydrogen carbonate. Sodium borohydride (61.4 mg) was added to the solution and was allowed to react for 30 min at 4 °C under stirring. CLEAs were washed three times with 50 mM phosphate buffer (pH 7.5) and separated by centrifugation at 4,000*g* for 20 min at 4 °C. The mixture of PVA and poly(ethylene glycol) was prepared as described previously. A suspension of CLEAs (1.8 g) was slowly added to the cooled PVA mixture and carefully mixed. Optical fibers were coated with the mixture.

The fibers with immobilized LinB and CF–BSA were stored for 24 h in 50 mM phosphate buffer (pH 7.5) at 4 °C unless specified otherwise.

Measurement procedure

The biosensor tip was immersed in 1 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 8.2) in a well-stirred glass vial shielded from ambient light at 21 °C. The optical fiber was connected to the fluorescence photometer. The photomultiplier control voltage was set to 0.8 V and the biosensor signal was recorded every 5 s. An aliquot of halogenated substrate was added to the measurement solution after a steady signal had been obtained from the biosensor. The response of the biosensor was calculated as the difference between the initial and the final fluorescence intensity, recorded in units of millivolts from the photomultiplier in the fluorescence photometer. The calibration data were fitted using Origin 6.1 (OriginLab, USA).

Results and discussion

Selection of fluorescent pH indicator

The selection of the pH-sensitive dye was the first decision in the construction of the dehalogenase biosensor. The appropriate fluorescent pH indicator has to fulfill several requirements [31]: excitation and emission wavelengths must be compatible with the fluorescence photometer, pH

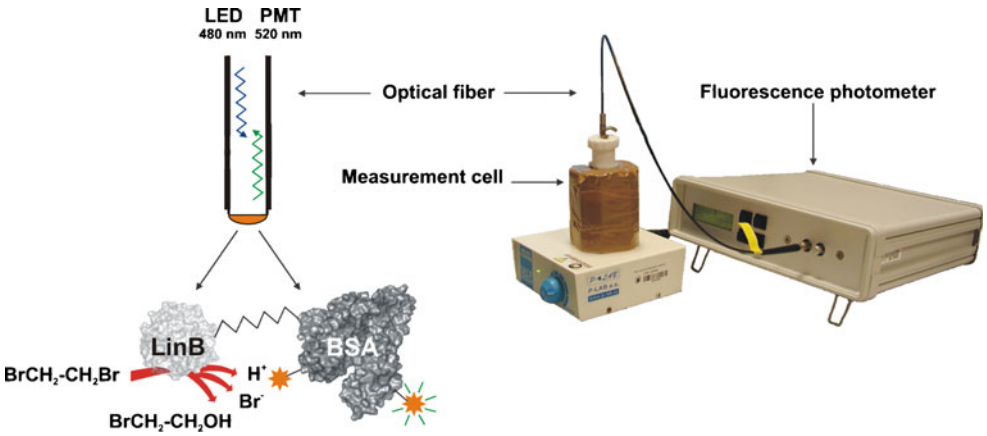

Fig. 1 Fiber-optic biosensing system composed of a fluorescence photometer, an optical fiber, and a biosensor tip. The tip consists of the enzyme haloalkane dehalogenase LinB and the fluorescent pH indicator 5(6)-carboxyfluorescein (*orange asterisk*) conjugated with bovine serum albumin (*BSA*). These recognition elements are cross-linked with glutaraldehyde on the optical fiber (*zigzag line* between protein molecules). *LED* light-emitting diode, *PMT* photomultiplier tube

sensitivity corresponding to the pH of environmental samples, and high fluorescence. Functional groups suitable for immobilization, commercial availability, and a low cost of the indicator are also important factors.

Eight fluorescent pH indicators were tested (Table 1). Most of dyes tested were analogues of fluorescein because they match exactly the excitation and emission wavelengths fixed in the fluorescence photometer. The fluorescence intensity increased with increasing pH for all indicators and the pH dependence had a sigmoidal shape (Fig. S2). The only exception was 5-(4,6-dichloro-*s*-triazin-2-ylamino) fluorescein, which reacted reversely from pH 9.3 to higher pH values. The fluorescent dyes 5(6)-aminofluorescein, 2′,7′-*bis*(2-carboxyethyl)-5(6)-carboxyfluorescein and fluorescein reacted sensitively in the same range from pH 3.0 to 6.6. The indicators CF, 5-(4,6-dichloro-*s*-triazin-2-ylamino) fluorescein, and 6-[fluorescein-5(6)-carboxamido]hexanoic acid are useful for detection of pH changes from an acidic to a slightly alkaline environment (pH ~ 5.0-7.5). Only 8-hydroxypyrene-1,3,6-trisulfonic acid exhibited pH sensitivity from pH 6.1 to 8.5.

The fluorescence intensities of the dyes were recorded under identical experimental conditions, with the same amount of fluorescent pH indicator and the spectrofluorometer setting (Fig. S3). The highest fluorescence intensity was measured for fluorescein, which is often used as a fluorescence standard, with a fluorescence quantum yield of 0.95 [32]. High fluorescence was also detected for CF and 6-[fluorescein-5(6)-carboxamido]hexanoic acid, which makes these indicators potential candidates for construction of the biosensor.

Of the fluorescent pH indicators tested, CF (Fig. 2) was chosen owing to its compatibility with blue-light-emitting diode excitation, appropriate pH sensitivity, and strong fluorescence. This derivative of fluorescein is available as a mixture of 5-carboxy and 6-carboxy isomers [33], which provide the 5-carboxy and 6-carboxy groups for covalent immobilization. In addition, the low price and high water solubility make this dye an attractive indicator for a dehalogenase-based biosensor.

Immobilization of the fluorescent pH indicator

Three methods for immobilization of a pH indicator on the optical fiber are widely used: adsorption, entrapment, and covalent binding. Covalent binding results in sensors which are free of leaching phenomena [34]. Therefore, covalent binding by cross-linking and the combination of cross-linking and entrapment for immobilization of recognition elements on the optical fibers were tested. The dependence of the biosensor response on the pH of solution was investigated (Fig. 3). The biosensor response was significantly dependent on the immobilization method. The

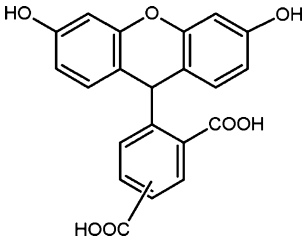

Fig. 2 Chemical structure of the fluorescent pH indicator CF

procedure using a combination of cross-linking and entrapment led to a lower detected signal in comparison with simple cross-linking. Preparation of CLEAs and their entrapment in PVA is not applicable for the biosensor owing to the minimal signal and the low sensitivity to pH changes. Cross-linking of CF–BSA with glutaraldehyde and entrapment in PVA resulted in a higher signal, but the sensitivity to pH changes was still low. Simple cross-linking of CF–BSA provided improved response to pH changes. The potential detected for CF–BSA cross-linked with EDC at lower pH increased with increasing pH, but immobilized conjugates reacted reversely at higher pH. This behavior can pose problems for biosensor applications. The best variant was cross-linking of CF–BSA with glutaraldehyde. Significant potential changes were detected in the pH range from 1.0 to 6.6. This pH sensitivity is larger than that for the indicator in solution (Table 1). The shift is attributed to the influence of the immobilization on the pH response of the indicator. This phenomenon was also observed by others, e.g., for 5(6)-aminofluorescein immobilized by both covalent binding and entrapment [34, 35] or for 8-hydroxypyrene-1,3,6-trisulfonic acid covalently bound [36, 37] and physically entrapped [38]. Cross-linking with glutaraldehyde was used for immobilization of CF–BSA and LinB on the optical fiber in further experiments.

Determination of equilibration and response time

To monitor the biosensor response, 1,2-dibromoethane was selected as a model compound. This chemical was used as a leaded-gasoline additive and soil fumigant. Currently, it is still a widely used solvent, chemical intermediate, and gauge fluid. 1,2-Dibromoethane belongs to hazardous contaminants in groundwater and soil [39] owing to its toxicity [40] and persistence in nature [41]. Haloalkane dehalogenase LinB is able to catalyze the conversion of 1,2-dibromoethane to a bromide ion, an alcohol, and a proton, the last being responsible for the biosensor signal change.

Signal stability was tested before monitoring the biosensor response to 1,2-dibromoethane. An optical fiber with immobilized LinB and CF–BSA was immersed in 1 mM HEPES buffer (pH 8.2). The biosensor signal was recorded for 60 min to determine the equilibration time (Fig. 4). The

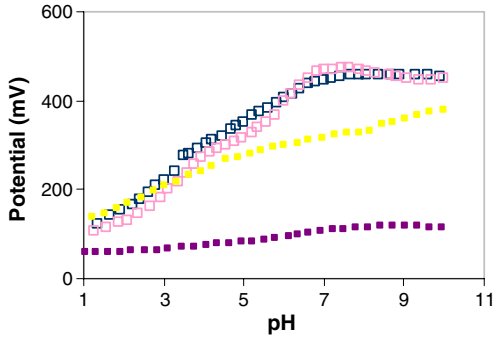

Fig. 3 Response of CF–BSA to pH changes. CF–BSA was immobilized on the tip of the optical fiber by cross-linking with glutaraldehyde (*blue squares*), cross-linking with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (*pink squares*), cross-linking with glutaraldehyde and entrapment in poly(vinyl alcohol) (PVA) (*yellow squares*), and preparation of cross-linked enzyme aggregates and entrapment in PVA (*purple squares*)

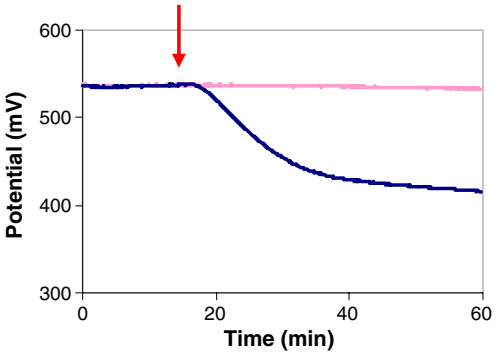

Fig. 4 Time course of the biosensor response in 1 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, pH 8.2: determination of signal stability under constant conditions (*pink line*); determination of response time after addition of 1,2-dibromoethane (*blue line*) (5 mM; indicated by the *arrow*)

measured potential was stable and the signal noise was ±5 mV. The biosensor response to the halogenated substrate was recorded after an equilibration time of 15 min (Fig. 4). Addition of 1,2-dibromoethane to a final concentration of 5 mM led to a significant decrease in fluorescence intensity of CF-BSA as a result of the dehalogenase reaction lowering the local pH in immobilized layer. A 2-min delay was observed after analyte addition before the biosensor response. This could be attributed to the starting pH of the reaction buffer (pH 8.2), which is higher than the pH range of the immobilized indicator. When the local pH reached the active range of CF, the response of the biosensor is significantly improved. The response to the addition of 1,2-dibromoethane was monitored for 45 min; most of the signal amplitude was detected within the first 20 min of the reaction. An optical fiber with BSA cross-linked with conjugates was tested in the same manner and was used as a negative control. No detectable signal was observed after addition of 1,2-dibromoethane (Fig. S4).

The biosensor response to decreasing concentrations of 1,2-dibromoethane was investigated (Fig. 5). The signal amplitude decreased with decreasing substrate concentration. The response time was significantly shorter at higher analyte concentration.

Determination of storage conditions

The biosensors prepared by immobilization of LinB and CF–BSA on the optical fibers were stored under various conditions. The effect of storage on the biosensor response was investigated (Fig. 6). Storage of the biosensor for 24 h in air at 4 °C led to an unstable signal. The biosensor exhibited no response to the addition of a substrate (Fig. 6a). When the biosensor was stored for 24 h in air at 4 °C and then for 24 h in 50 mM phosphate buffer (pH 7.5) at 4 °C, a steady signal was detected during equilibration. Addition of substrate led to a gradual signal decrease. A reaction time of 45 min was not long enough to obtain a stable signal. The difference between the initial and the final potential was 69±1 mV from triplicate measurements (Fig. 6b). Storage of the biosensor for 24 h in 50 mM phosphate buffer (pH 7.5) at 4 °C led to a steady signal during equilibration. Addition of a substrate led to a significant signal decrease (130±14 mV). Minimal potential changes were detected after 45 min of enzymatic reaction (Fig. 6c). These conditions were therefore selected for storage of the prepared biosensor tips.

Determination of detection limit

The biosensor response to 1,2-dibromoethane and 3-chloro-2-(chloromethyl)-1-propene in the concentration ranges 0-1.2 mM and 0-0.8 mM, respectively, was tested (Fig. 7). The response of the biosensor was calculated as the difference between the initial and the final signal after

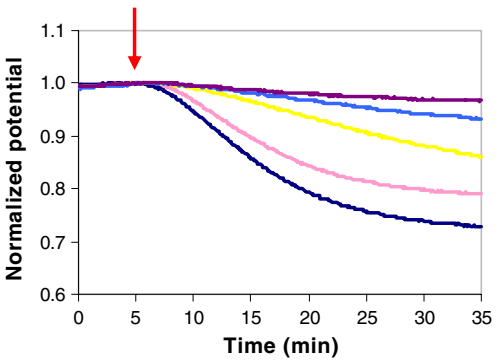

Fig. 5 Response of the biosensor in 1 mM HEPES buffer, pH 8.2, to decreasing final concentrations of 1,2-dibromoethane (addition indicated by the *arrow*): 5.0 mM (*dark-blue line*); 1.0 mM (*pink line*); 0.5 mM (*yellow line*); 0.3 mM (*light-blue line*); 0.1 mM (*purple line*)

30 min of incubation. The response of the biosensor increased linearly with increasing analyte concentration for both 1,2-dibromoethane (R^2=0.9702) and 3-chloro-2-(chloromethyl)-1-propene (R^2=0.9997). Detection limits of 0.133 and 0.014 mM were determined for 1,2-dibromoethane and 3-chloro-2-(chloromethyl)-1-propene, respectively. The difference of an order of magnitude in the detection limits corresponds to the catalytic property of haloalkane dehalogenase LinB, in particular with different Michaelis constants for 1,2-dibromoethane (1.90 mM) and 3-chloro-2-(chloromethyl)-1-propene (0.08 mM).

Different amounts of immobilization mixture (0.5, 1.0, 2.0, 3.0, and 5.0 μl) applied on the fiber tip were tested to improve the sensitivity of the biosensor. Higher enzyme loading was expected to increase the biosensor response to 1,2-dibromoethane, but the amplitude of the biosensor response was similar (approximately 70 mV) for all variants tested except for 0.5 μl of immobilization mixture (approximately 100 mV). The data indicate that the detected signal is generated from the thin layer, which is in close contact with the optical fiber. The reduction of the thickness of the biosensitive layer can improve mass transport of analyte and thus increase the magnitude and the rate of the biosensor response.

An optical biosensor based on haloalkane dehalogenase as a biorecognition element is specific to halogenated aliphatic hydrocarbons. Its selectivity therefore corresponds to the compounds within this class of substrates, allowing their quantitative detection. The response of the biosensor exposed to a mixture of 1,2-dibromoethane and 3-chloro-2-(chloromethyl)-1-propene spiked at 0.5 mM for each compound was 93 mV, whereas for individual compounds spiked at 0.5 mM the response was 29 and 39 mV, respectively, after 30 min of incubation.

The sensitivity of the biosensor can also be improved by lowering the Michaelis constant or by increasing the rate constant of the enzyme employed by protein engineering. The selectivity can be modified by restricting or broadening its substrate range. The use of engineered enzymes in the biosensors has already been described in several studies and was recently reviewed [42]. The reported biosensing

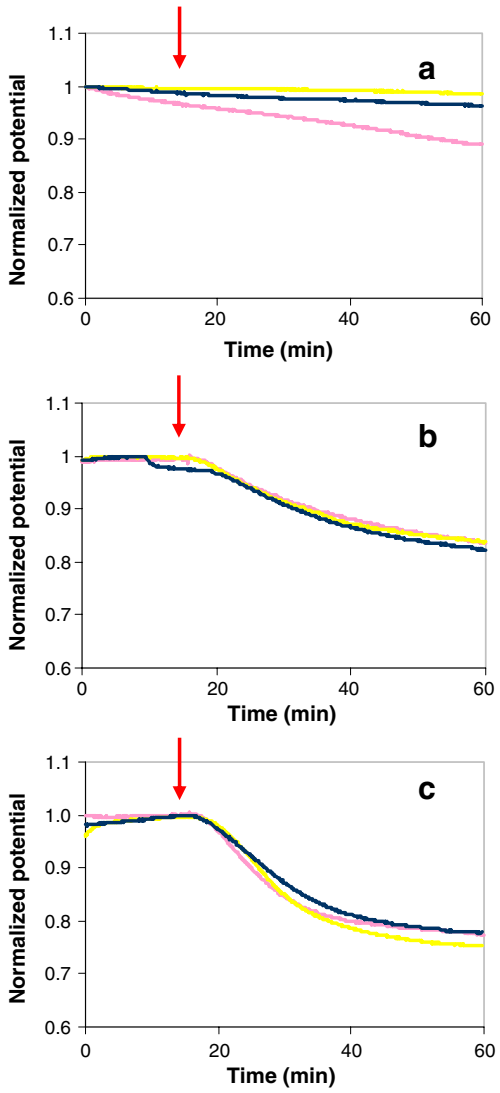

Fig. 6 Response of the biosensor after storage for 24 h in air at 4 °C (**a**), for 24 h in air at 4 °C and then for 24 h in 50 mM phosphate buffer (pH 7.5) at 4 °C (**b**), and for 24 h in 50 mM phosphate buffer (pH 7.5) at 4 °C (**c**). The *arrows* indicate addition of 1,2-dibromoethane to a final concentration 5 mM

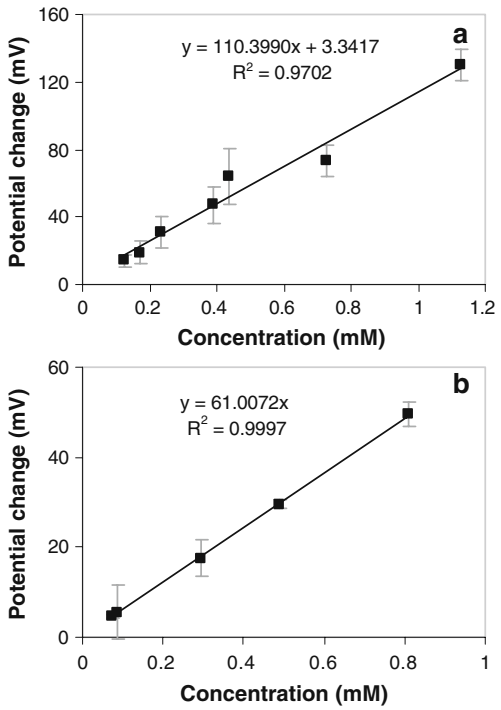

Fig. 7 Calibration plot for the biosensor with immobilized LinB and CF–BSA for 1,2-dibromoethane (**a**) and 3-chloro-2-(chloromethyl)-1-propene (**b**). The standard errors were calculated from three to five independent measurements

devices exhibited increased sensitivity. Furthermore, the concept utilizing genetic modification enables selection or design of enzyme variants that are specific for the detection of individual compounds.

Conclusions

We reported the construction of a novel fiber-optic biosensor for the detection of halogenated compounds. The biosensor is based on purified haloalkane dehalogenase LinB and the fluorescent pH indicator CF immobilized on the optical fiber with a poly(methyl methacrylate) core. The measuring method consisted of storage of the biosensor for 24 h in 50 mM phosphate buffer (pH 7.5) at 4 °C, its equilibration for 15 min, and recording the signal after the addition of a halogenated substrate. The biosensor was validated with 1,2-dibromoethane and 3-chloro-2-(chloromethyl)-1-propene; the response was linear in the concentration ranges 0-1.2 mM and 0-0.8 mM, with detection limits of 0.133 and 0.014 mM, respectively. The kinetic characteristics of the biorecognition element were identified as the main limitation for the sensitivity of the biosensor. Improvement of these kinetic characteristics of the haloalkane dehalogenase using protein engineering is currently ongoing in our laboratory.

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