Antibody-based immobilization of bioluminescent bacterial sensor cells

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Abstract

Whole-cell luminescent bioreporter sensors based on immobilized recombinant Escherichia coli are described and evaluated. The sensors were prepared by glutaraldehyde-anchoring of nonspecific anti-E. coli antibodies on aminosylilated gold or silica glass surfaces with subsequent attachment of the probe bacteria. We demonstrate the generality of the concept by attachment of several E. coli strains that express luciferase in response to different physiological stress conditions including heat shock, DNA damage (SOS), fatty acid availability, peroxide and oxidative stress. The sensors can be used either as single- or multiple-use disposable sensing elements or for continuous operation. We show compatibility with optical fiber technology. Storage stability of the sensors exceeded 5 months with no measurable deterioration of the signal. Repeatability on exposure in successive days was <15%, as was sensor to sensor reproducibility. Sensitivity and detection limits of the immobilized cells were comparable to that of non-immobilized bacteria. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Genetically modified reporter microorganisms can be used as sensitive and inexpensive analytical tools for laboratory and field toxicity assessment [1,2]. Such organisms can be ‘tailored’ to emit a readily detectable signal in response to pre-determined environmental conditions. This is normally achieved by the selection of a gene induced in response to the desired stress, and the genetic fusion of its promoter to a gene or a group of genes coding for reporter proteins, the activity or presence of which can be easily monitored. In E. coli and other microorganisms, the use of colorimetric, electrochemical, fluorescent or luminescent whole-cell reporting has been successfully demonstrated [1,2]. In this paper, we make use of a set of...
genetically modified E. coli strains that emit a dose-dependent luminescent signal in response to the activation of several global defense circuits [3–5]. The specificity of the different strains was determined by the choice of the gene promoter.

While the high sensitivity and applicability of these and similarly constructed bacterial sensor cells under controlled laboratory conditions has been repeatedly demonstrated, less attention has been devoted to their long-term storage and use in an immobilized form. The successful incorporation of such microorganisms in solid-state matrices is an important step en-route to the conversion of these bioassays into user-friendly sensing devices capable of continuous monitoring or multiple use detection. A few attempts to embed genetically engineered probe bacteria in solid matrices were reported, based on encapsulation in soft gels including agarose [6], acrylamide [7], calcium and strontium alginites [8], and agar [9]. Polyak and coworkers [10] reported that alginate-based matrices suffer from diffusion limitations due to the thick films but did create viable biosensors [11]. Alginites are also unstable in calcium-poor solutions, and deteriorate in the presence of phosphate and other calcium chelators although this has not been shown to be the case in field studies (Polyak and Marks, unpublished results). The low deformation resistance and the biodegradability of most soft gels are additional incentives for a search for an alternative encapsulating procedure. Deposition of a protective dialysis membrane was also reported [12] although this cannot be considered a true encapsulation.

In this study we present a simple and general approach for the immobilization of recombinant E. coli on solid substrates and their use as disposable or multiple use bioluminescent sensors. The procedure is based on the immobilization of the engineered bacteria on antibody modified substrates. The technology for bonding antibodies on different flat or porous substrates is very versatile and well developed and it is often used as a first step in the construction of complex sensing elements. The successful combination of this generic approach with reporting microorganisms paves the way for their incorporation on or in virtually any substrate. So, despite the fact that in this article we address only the immobilization of luminous bacteria on glass and gold substrates, it establishes a way that can be used for the integration of recombinant bacteria in other cell-based-biosensing-architectures as well. In this article, we demonstrate the validity of this generic approach for two types of surfaces: glass and gold-coated glass. We further demonstrate compatibility of the technology with different modes of optical fiber signal transduction.

2. Experimental

2.1. Preparation of the recombinant E. coli

The five E. coli strains used in this study are listed in Table 1. Each contained a multi-copy plasmid in which a different gene promoter was

<table>
<thead>
<tr>
<th>Strain designation</th>
<th>E. coli host strain</th>
<th>Promoter</th>
<th>Stress sensitivity</th>
<th>Inducer used in this study</th>
<th>Concentration (M)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TV1061</td>
<td>RFM443</td>
<td>grpE</td>
<td>Heat shock (general stress)</td>
<td>Ethanol</td>
<td>0.43&lt;sup&gt;a&lt;/sup&gt;</td>
<td>[3]</td>
</tr>
<tr>
<td>DPD2794</td>
<td>RFM443</td>
<td>recA</td>
<td>SOS (genotoxicity)</td>
<td>NA&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.9 × 10&lt;sup&gt;-6&lt;/sup&gt;</td>
<td>[4]</td>
</tr>
<tr>
<td>DPD2511</td>
<td>RFM443</td>
<td>katG</td>
<td>Oxidative (peroxides)</td>
<td>H&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>2.9 × 10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>[13]</td>
</tr>
<tr>
<td>DPD2544</td>
<td>W3110</td>
<td>fabA</td>
<td>Fatty acid availability (general stress)</td>
<td>Phenol</td>
<td>5.2 × 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>[14]</td>
</tr>
<tr>
<td>DPD2515</td>
<td>W3110</td>
<td>micF</td>
<td>Oxidative (superoxide)</td>
<td>MV&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.9 × 10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>[15]</td>
</tr>
</tbody>
</table>

<sup>a</sup> 2% (v/v).  
<sup>b</sup> Nalidixic acid.  
<sup>c</sup> Methyl viologen
fused to the *Vibrio fischeri luxCDABE* genes. The use of the 5 *lux* genes under the same promoter alleviates the need for externally supplying a long-chain aldehyde, the substrate for bacterial luciferase. Construction of the different plasmids has been described in detail in the past in the respective references in Table 1.

2.2. Preparation of bacterial cultures

Prior to immobilization, the bacterial strains were grown overnight with shaking at 37 °C in Luria–Bertani (LB) broth (NaCl 5 g l⁻¹, Yeast extract 5 g l⁻¹, Tryptone 10 g l⁻¹) [16] containing 100 µg ml⁻¹ ampicillin to ensure plasmid maintenance. The stationary culture was diluted to ca. 10⁷ cells per ml with fresh LB medium and then regrown under the same conditions. Growth was monitored using a Klett-Summerson colorimeter (Monostat Corporation) and the cells were harvested at early exponential growth phase (20 Klett units, corresponding to about 10⁸ cells per ml as previously described [3,5,13].

2.3. Luminescence determination

The effects of different inducers on non-immobilized bacteria were measured by mixing early-exponential growth cultures aliquots (50 µl) with equal amounts of LB broth containing the appropriate inducer concentration. The mixture was placed into duplicate wells of a 96-wells opaque white microtiter plate (Costar Europe, Bdhoevedrop, The Netherlands). The microtiter plates were incubated in a temperature controlled (26 °C) shaker, and the luminescence was periodically measured by a microtiter plate luminometer (model Victor³, EG&G Wallac 1420, Turku, Finland). LB broth and bacterial samples without the inducer served as controls.

The luminescence response of the immobilized bacteria was measured by a similar procedure, with the solid substrate (glass or gold), along with its attached bacteria placed into a 100 µl LB containing the appropriate inducer concentration in duplicate microtiter plate wells. In all cases, the signal is reported in the instrument’s arbitrary Relative Luminescence Units (RLU).

2.4. Optical fiber measurements

Multimode optical fibers, PUV 400 BM (CermaOptec, GmbH, Bonn), were used in these experiments. They present a pure core diameter of 400 µm, with a refractive index of 1.4571 (at 633 nm) and a cladding diameter of 440 µm, with a refractive index 1.4011 (at 633 nm). Their black nylon jackets were stripped away from 1-cm long optical fiber tips, which were then used for immobilization of the bioluminescent cells. The optical fiber was placed in a 0.5 ml conical tube (Jonplast, Italy) within the test sample solution or in a 1-cm in diameter opaque flow cell (an opaque 0.5 cm plastic T junction). The optical fiber was fixed at the epicenter of the light proof tube via a 100 µl pipette tip to avoid ambient light interference. The far end of the fiber was held by a fiber holder (FPH-DJ, Newport) and placed into an adjustable fiber mount (77837, Oriel). The photon counting system was comprised of a light-tight box and a Hamamatsu HC135-01 photomultiplier tube sensor module, as was described elsewhere [10,11]. The response is reported in the instrument’s relative response units (photon counts per second).

2.5. Preparation of anti-*E. coli* modified glass slides

Glass plates (GP) (1 mm in thickness) were purchased from Paul Marienfeld GmbH & Co. KG Laboratory Glassware Company (Germany). The plates were cut into squares (4 × 4 mm) which were cleaned successively by ethanol, soap solution, piranha solution (H₂SO₄:H₂O₂ 4:1), and finally with NaOH (1 M). The clean plates were dried at 80 °C and then aminosylilated by immersion for 3 h in N-[3-(trimethoxysilyl)propyl]ethylenediamine (EDAS) at 80 °C. The amine-coated samples were rinsed with distilled water and then immersed in 2% (v/v) glutaraldehyde for 30 min. Excess glutaraldehyde was rinsed off and the glass plates were immersed for 30 min in an aqueous antibody solution containing 13 g protein l⁻¹ (AB, Rabbit Anti-*E. coli*, DAKO, Denmark). The plates were rinsed again in distilled water and immersed in the *E. coli* suspension (ca. 2 × 10⁸ cells per ml) for 30 min. The
coated glass plates were washed with LB medium (pH 7), and kept in microtiter plates (Dynatech, Chantilly, VA, USA) in the same solution. Attachment of the bacteria to the optical fibers was carried out in an identical manner. The bacteria were applied only to the exposed upper 10 mm of the fiber.

2.6. Preparation of anti-\textit{E. coli} modified gold-coated glass slides

Gold-coated glass slides (1 mm in thickness) (home made by electrodeposited gold on ITO slides) were cut in squares (4 × 4 mm) and dipped into 0.1 M 2-aminoethanethiol hydrochloride for 3 h. They were then rinsed with distilled water and immersed in 2% glutaraldehyde for 30 min. Excess glutaraldehyde was rinsed off and the gold coated glass slides were immersed in the aqueous antibody solution (13 g protein l$^{-1}$) for 30 min. The plates were rinsed again in distilled water and immersed in the \textit{E. coli} suspension (ca. 2 × 10$^8$ cells per ml) for 30 min. The coated glass slides were washed with LB medium (pH 7), and were kept in microtiter plates (Dynatech, Chantilly, VA, USA) in the same solution.

2.7. Chemicals

Glutaraldehyde, 2-Aminoethanethiol hydrochloride, N-[3-(trimethoxysilyl)propyl]-ethylenediamine, nalidixic acid and phenol were purchased from Aldrich. ACS grade hydrogen peroxide (Merck), ethanol (J.T. Baker), malathion (American Cyanide Company), aldicarb (Union Carbide) and DDT (Union Carbide) were used.

3. Results

Microscopic observation of the immobilized \textit{E. coli} cells attached to antibody modified glass and gold-coated slides revealed that the average surface density of the bacteria was ca. 10$^5$ cells per cm$^2$ corresponding to ca. 2% surface coverage. In a series of attempts to increase the density of the attached bacteria, we have changed the duration of the silanization procedure, increased the bacte-

Fig. 1. Luminescence of TV1061 (\textit{E. coli}) glass sensors that were prepared by immersion of the antibody-modified glass in an \textit{E. coli} culture (10$^8$ cells per ml) for the specified time. The sensors were stored overnight in LB medium and then exposed to a 2% alcohol solution in LB medium for 1 h before the signal was measured.

Fig. 1 depicts an example of such a study. We immersed the antibody coated glass slides in the bacterial culture (10$^8$ cells per ml) for the specified amount of time, then exposed the slide to a solution of 2% alcohol in LB medium for 30 min, and finally recorded the induced luminescence. Fig. 1 shows that ca. 10 min immersion time of the antibody-coated glass in the bacterial suspension was sufficient to attain maximal response. The maximum that appears in Fig. 1 for a 25 min deposition probably reflects data scatter rather than true optimal operating conditions. Therefore, we used 30 min antibody binding duration in all subsequent studies. The rather low surface coverage may be attributed to incomplete coverage of the solid surface by the antibodies or to the nonspecificity of the anti-\textit{E. coli} polyclonal immunoglobulins used in this study, which allows for competition of cell lysis products for antibody binding sites.

3.1. The general immobilization concept

Fig. 2A–E compare the evolution of the bioluminescence signal of the immobilized recombinant bacterial strains in Table 1 in response to their
model inducers. In all cases but one, no signal was obtained in the absence of inducer (always the lowest line in the respective figures). The one exception was strain DPD2511 (Fig. 2E), reacting to the presence of hydrogen peroxide. This phenomenon was already described, and was shown to be caused by H$_2$O$_2$ formation in non-inoculated LB medium [13].

Fig. 2A depicts the stimulation of luminescence in strain TV1061, which reflects the activation of the bacterial heat shock system. It has previously been shown [3,5] that this global circuit is induced in response to a very broad variety of environmental insults and, therefore, induction of luminescence in this strain is an excellent indication of general toxicity. The inducer used in this case and during the rest of this study, ethanol, is routinely employed as a general heat shock activator. Fig. 2A shows the response of the two solid sensors (TV1061 deposited on glass (i) and gold substrates (ii)). The dynamic response of the two was very similar and both were also similar to the photoresponse of suspended (non-immobilized) bacteria curve (iii) in Fig. 2A. Fig. 2B–E similarly depict the response of the other *E. coli* sensor strains to their respective inducers using the glass-immobilized bacteria only. The responses of immobilized DPD2794 (a genotoxicity sensor) to nalidixic acid, DPD2515 (a sensor of superoxide radicals) to methyl viologen, DPD2544 (an additional sensor of general toxic stress) to phenol and DPD2511 (a sensor of peroxide stress) to hydrogen peroxide are all in line for those reported for the non-immobilized cultures [4,13–15]. Sensitivity and detection limits of the immobilized cells were comparable to that of non-immobilized bacteria.

**Fig. 2.** Time course of the evolution of luminescence of different *E. coli* reporters (as of Table 1) in response to the addition the respective inducers. Luminescence response in blank LB medium is also shown (lowest curves in all figures). (A) (i) Glass-based, (ii) Gold-based TV1061 sensors and (iii) TV1061 suspension exposed to 0.434 M ethanol (right axis). (B) Glass-based DPD2794 sensors exposed to nalidixic acid (6 μM). (C) Glass-based DPD2515 sensors exposed to methyl viologen (3.9 mM). (D) Glass-based DPD2544 sensors exposed to methyl viologen (3.9 mM). (E) Glass-based DPD2511 sensors exposed to hydrogen peroxide (2.9 mM).
3.2. Pesticide assays

It was demonstrated that heat shock gene expression [17] is induced by numerous water contaminants and thus *E. coli* harboring a fusion of a heat shock promoter to the *lux* genes (e.g. of *Vibrio fischeri*) can serve as a general early warning ecotoxicity sensor. In an attempt to examine the applicability of strain TV1061 in its immobilized form, we have monitored its response to several pesticides. Three such examples are presented in Fig. 3, following exposure to aldicarb, malathion, and DDT (0.1 mM each). In all cases, the increase in luminescence was apparent shortly following the addition of the pesticide, and reliable signals could be obtained in 1 h. A similar response was obtained for non-immobilized bacteria (not shown). The sensitivity and detection limits of the immobilized cells were comparable to that of non-immobilized bacteria.

3.3. Concentration-dependence of the luminescent response

Fig. 4 depicts the dose-dependency of the luminescent response of immobilized TV1061 cells (several identical sensors) to ethanol. Regardless of the sampling time, maximal signal was obtained in the presence of 2% (0.43 M) of this inducer. Higher concentrations imposed a toxic effect on cell viability and on the bioluminescence reaction, causing a decrease in the overall signal. Similar response was observed for the suspended bacteria as well.

3.4. Reproducibility, repeatability and stability

Several metrological characteristics of the heat shock based toxicity sensors were investigated. Fig. 5 demonstrates the time course of 4 different glass-based sensors, prepared independently under similar conditions and exposed to 2% alcohol. The four sensors exhibited similar behavior, demonstrating good in-batch reproducibility. Fig. 6 depicts typical multiple use of the same sensing elements in consecutive days (Fig. 6A) and weeks (Fig. 6B). The sensors were stored in LB medium at 4 °C between tests. Each test began with rinsing of the sensing element with LB and introducing it into 2% alcohol in the same medium. The time course of the luminescence signal was recorded daily or weekly. At the end of the cycle, the sensors were rinsed with fresh LB and stored in the same medium at 4 °C. The sensors showed good repeatability even after multiple use (Fig. 6B).
placed the optical fiber in close proximity to a 4 × 4 mm *E. coli* TV1061 glass- or gold-bound sensor and used the optical fiber as a wave-guide. In another approach, we immobilized the bacteria onto the optical fiber tip according to the procedure delineated in the experimental section. In the latter configuration, the optical fiber was used both as a sensing element and as a signal transducer.

As a preliminary step towards the application of optical fibers as wave-guides, we have attempted to optimize the position of the fiber tip in respect to the glass elements harboring the cells. Fig. 7 delineates the sensor response as a function of the distance between the flat end of the optical fiber and glass- and gold-based TV1061 sensors. In both cases, actual contact with the bacterial plain was sub-optimal. Elsewhere, the signal decreased very gradually with the vertical (upward) distance, at approximately 1% mm⁻¹. Only when the fiber reached the water surface and a meniscus was formed between the fiber and the liquid (at ca. 15 mm) the signal dropped rapidly and reached close to zero RLU when the fiber was pulled away from the solution. The gold-based sensor showed a slightly higher response compared with the glass-based one, which can be attributed to increased collection efficiency due to light reflection from the mirror like gold-coated surface. Otherwise, the signal–distance curve was very similar for the gold and glass based sensors. The signal was practically independent of the horizontal location of the optical fiber as long as it was located above the surface of the sensor (not shown). In subsequent studies we located the optical fiber at a distance of 2 mm from the center of the sensing element.

One of the most important advantages of solid sensing elements compared with suspended bacterial cultures is their compatibility with continuous monitoring schemes. Fig. 8A and B demonstrate the ability to monitor toxicity (heat shock) stress under continuous flow conditions. Fig. 8A demonstrates the response of a coated optical fiber that was exposed to 2% alcohol feed at the times indicated by the upward arrows in the figure. The downward arrows indicate a switch to alcohol-free feed. The figure demonstrates that the
Fig. 6. Multiple use and stability studies of Glass-based TV1061 sensors exposed to 2% ethanol for the specified duration and then stored in pure LB medium at 4°C. (A) Luminescence time course after exposure of the sensors to alcohol at consecutive days. (B) Luminescence time course after exposure of the sensors to alcohol at consecutive weeks. The last frame corresponds to 20th week. (C) Stability curve denoting the luminescence after weekly consecutive exposure to 2% alcohol solution for 6 h (last point denotes 20 weeks old sensor).

Sensor can sense heat shock evolution (Fig. 8A) continuously. Fig. 8B demonstrates a continuous response of a wave-guide that was placed 2 mm from a 4 × 4 mm glass-based-TV1061 sensor in 2% alcohol feed. The figure demonstrates that the sensor can sense heat shock evolution (Fig. 8B) continuously. The response of the cell modified optical fiber sensor was very similar to the response of the combined glass sensors and optical wave-guide. Fig. 8 demonstrates the technical ability to perform continuous monitoring in the two modes of operation. The difference between the responses of the two sensors (Fig. 8A and B) to the second slug of 2% alcohol feed may stem
from different histories of the biological elements; it clearly illuminates the need for further research on the standardization of the proposed technique.

4. Conclusions

The study described in this communication outlines a possible configuration for the incorporation of genetically engineered microorganisms into a functional biosensor. It is demonstrated that antibody-attached \textit{E. coli} can serve for the monitoring of toxicity, as well as other stress conditions, either as a disposable sensing element, as a multiple use sensing element, or in a continuous monitoring mode of operation. The assays are very simple, the results are rapidly available, and the sensitivities are at least in the same order of magnitude as those of suspended bacteria. However, more research should be devoted to the standardization of this sensing technique; one of the principal thrusts should be towards the minimization of signal dependence on sensor history.

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