Sol–gel luminescence biosensors: Encapsulation of recombinant E. coli reporters in thick silicate films

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Abstract

A class of sensing elements based on the encapsulation of genetically engineered bioluminescent Escherichia coli (E. coli) reporter strains in sol–gel derived silicates is described. We demonstrate the concept by the immobilization of these bacterial cells in thick silicate films. Heat shock, oxidative stress, fatty acids, peroxides, and genotoxicity reporting bacteria were incorporated in the sol–gel silicates and their luminescence response was compared to that of the non-immobilized culture. All the immobilized bacteria maintained viability and luminescence activity for several months. The bacteria–silicate hybrids can be used either as disposable sensors or in multiple use sensing test-kits, and they can be also integrated in early warning devices operated in continuous flow conditions. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

The use of test-kits and early warning biosensing devices based on recombinant bacteria and other reporting micro-organisms is rapidly increasing [1–3]. Recombinant bacteria-based toxicity tests are suitable for sensing applications that are inadequately addressed by traditional chemical instrumental analysis or by the evolving molecular recognition sensors. In fact, these three fields complement each other. Laboratory instrumental analysis which is often coupled with advanced chromatographic separations allow for the identification of a large number of analytes by a single analytical run. For example, diverse families of organic compounds can be routinely analyzed by gas or liquid chromatographs, and metals are determined by atomic absorption spectroscopy and related techniques. However, most of these instruments require large capital investment and involve fairly large sampling-to-analysis delays. The high selectivity of modern molecular recognition devices, which often rely on enzymatic catalysis or antibody-affinity facilitates field analysis by non-experts since only a mild clean-up treatment is involved. The whole-organism-based ecotoxicity and bio-availability sensors provide a third option since they are usually non-specific and of low cost. Thus, they provide convenient early warning or environmental screening capabilities which would otherwise require either a large number of specific sensors or time consuming laboratory analysis. Indeed, despite the fact that several viable cell reporters for specific elements [3–6], specific organic compounds [7,8] or various endogenous substances [9] were proposed the mainstream of scientific activity in whole-cell biosensing

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is directed toward genetic engineering of bacteria for expression of reporting enzymes in response to physiological stress conditions [3,10]. Researchers are willingly trading off the molecular specificity for early warning of physiological stress such as nutrient deficiency, genotoxicity, membrane cell damage, changes in osmotic constraints, or excitation of the heat shock or the oxidation protective systems.

This very broad and rather simplistic classification of analytical tools provides the incentive for the current research, which involves the immobilization of luminous bacteria in sol–gel silicates. If indeed engineered bacteria are to be used as an early warning on-line devices they should be encapsulated in a supporting matrix that will prevent bacteria wash-out from flow cells and still enable signal transduction and rapid communication with the environment. Ecological field screening by non-expert sample collectors requires minimal wet chemistry and fluid handling, which again points at the practical importance of the encapsulation step.

The mainstream of research in recombinant bacteria sensors is invested in broadening and improving the biological sensing pathways and relatively few research attempts where directed towards physical encapsulation. Notable exceptions include several successful research projects that convincingly demonstrated the ability to encapsulate recombinant bacteria in soft gels such as agarose [11], polyacrylamide or calcium and strontium alginites [12,13]. However, these sensors suffer from the known drawbacks of soft hydrogel supports, which include biodegradation susceptibility, diffusion limitation due to the thick films involved, low physical deformation resistance, and the instability of the alginites in calcium-poor solutions and in the presence of calcium chelates. Encapsulation of cells by a dialysis membrane [14] and a membrane of glycerol–acryl vinyl acetate copolymer latex were also reported [15]. Antibody attachment to glass- and gold-coated glass substrates were also recently reported [16].

In this publication, we describe a luminescence physiological stress sensor based on the encapsulation of viable recombinant E. coli in sol–gel derived silicates. Sol–gel bio-encapsulation matured within a relatively short time from an extreme, and thus, intriguing example of inorganic–organic hybrid formation to a vivid field of technological activity due to the synergism between the favorable physical properties of inorganic matrices and the specificity and versatility of biochemical catalysts and chelating agents [17–19]. Silicate caging is especially attractive for luminescence and optical biosensing due to the rigidity and the transparency of silicates. Several reports have already described successful encapsulation of viable bacteria and yeast in silicates [19–25]. The feasibility of sol–gel encapsulation of luminous bacteria and their ability to report physiological stress during the encapsulation process were recently communicated [26]. Here, we address the preparation and the biosensing application of immobilized luminous bacteria.

2. Experimental

The bacterial strains and the inducers used in this study are listed in Table 1. Each contained a multi-copy plasmid in which a different gene promotor was fused to the Vibrio fischeri luxCDABE genes. Prior to the encapsulation, the bacterial strains were grown overnight in LB broth [27] mixed with 100 μg/ml ampicillin to ensure plasmid maintenance. The culture was continuously shaken at 37 °C. The culture was diluted with fresh LB medium and then re-grown under the same conditions to a cell density of about 10^8 cells/ml. 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 10^8 cells/ml, as previously described) [28,29].

For the measurement of the effect of the different inducers on the luminescence response of suspended bacteria, early exponential growth cultures were exposed to the inducer and then a sample from the culture was mixed with an equal amount of LB medium. 100 μl of the mixture were placed in an opaque white microtiter plate (Costar Europe, Bdhoevedrop, The Netherlands). The microtiter plates were incubated in a temperature-controlled (26 °C) incubator and the luminescence was periodically measured by a microtiter plate luminometer (Victor2, EG&G Wallac 1420, Turku, Finland). Luminescence values are presented in the instrument’s arbitrary relative luminescence units (RLUs). Neat LB and 1:1 mixtures of the culture and the bacteria without the inducer served as controls. Luminescence responses of the encapsulated bacteria
Table 1
Bioluminescent bacterial strains and the inducers used in the present study

<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 RPM443 grpE</td>
<td>Heat shock (general stress)</td>
<td>Ethanol</td>
<td>0.43(^a)</td>
<td>[28]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DPD2794 RPM443 recA</td>
<td>SOS</td>
<td>NA(^b)</td>
<td>5.9 \times 10^{-6}</td>
<td>[30]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DPD2511 RPM443 katG</td>
<td>H₂O₂</td>
<td>Phenol</td>
<td>5.2 \times 10^{-6}</td>
<td>[31]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DPD2544 W3110 fabA</td>
<td>Fatty acid availability (general stress)</td>
<td>Phenol</td>
<td>5.2 \times 10^{-6}</td>
<td>[31]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DPD2515 W3110 micF</td>
<td>Oxidative (superoxide)</td>
<td>MV(^c)</td>
<td>3.9 \times 10^{-3}</td>
<td>[32]</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) 2 vol.%.  
\(^b\) Nalidixic acid.  
\(^c\) Methyl viologen.

were measured by a similar procedure, with the encapsulated bacteria placed in 100 μl solution of LB and inducer in duplicate microtiter wells. The luminescence spectra were obtained by using a Luminescence Spectrometer (model LS 50 B of Perkin Elmer, UK).

Glass slides (1 mm thickness) were purchased from Paul Marienfeld GmbH & Co. KG Laboratory Glassware Company, Germany and cut to 4 mm \times 4 mm pieces. The glass slides were cleaned successively by a 1:1:1 acetone:ethanol:chloroform mixture, soap solution, piranha solution (H₂SO₄:H₂O₂ 4:1) solution, distilled water and 1 M NaOH. Then, the clean glass pieces were dried for an hour in a 80 °C oven. The sol–gel mixtures were prepared by mixing 4 ml of tetramethylorthosilicate (TMOS, Aldrich) with 2 ml of distilled water and 0.5 ml of 0.1 M HCl. The mixture was sonicated for 10 min to ensure uniformity and left to age at 4 °C for a day.

An amount of 3 ml of the E. coli suspension (20 Klett units, 10⁷ cells/ml) in LB was thoroughly mixed with 0.5 ml of the sol–gel solution, and a known amount (unless otherwise stated we used 10 μl which contained approximately 10⁶ cells) of the mixture (E. coli suspension with sol–gel precursors at pH 7) was then coated on the glass plate and dried for 5 min under ambient conditions. The E. coli-sol–gel films were washed with phosphate buffer and then by LB medium, both at pH 7, and kept in the microtiter plate in phosphate buffer.

3. Results and discussion

Five E. coli strains where selected for this study as depicted in Table 1. All the strains harbored a fusion of different stress promoter genes to the Vibrio fischeri luxCDABE genes. Thus, all five strains express luciferase and emit luminescence in the presence of pre-determined groups of chemicals: (a) strain TV1061 [28], harboring a fusion of the heat-shock gene grpE to the Vibrio fischeri luxCDABE genes was shown to be a good general toxicity sensor; (b) strain DPD2794 [30], harboring the promoter of the recA gene is a genotoxicity reporter; (c) strain DPD2544 containing the fabA promoter is induced by fatty acids and was also shown to be a general toxicity indicator [31]; (d) strain DPD2515 containing the micF gene promoter is induced by superoxide-driven oxidative stress [32]; and (e) strain 2511 containing the katG gene is induced by peroxides [33].

The model inducing chemicals used in this study were ethanol, nalidixic acid, phenol, methyl viologen (MV) and hydrogen peroxide for strains TV1061, DPD2794, DPD2544, DPD2515, and DPD2511, respectively. In all cases, we obtained similar luminescence spectra with a peak at 483 nm for the suspended and encapsulated bacteria (Fig. 1). We attribute the red shifting of the radiation of the free culture to fluorescence, though we did not make specific efforts to determine the fluorescent species. We do not know what is the reason for the noisier spectra that are obtained in our tests for the free cells compared to the encapsulated ones.

3.1. Dynamic response

The time course of luminescence of the five immobilized (solid lines) and non-immobilized (dotted lines) E. coli strains exposed to the different inducers (Table 1) are presented in Fig. 2A E. The light response is presented in RLUs, as read by the
The luminescence increase during the 4.5 h experiment was not caused by bacterial growth in the silicate matrix or in the solution. This conclusion is based on the following observations: (1) the magnitude of the luminescence response is much higher compared to the control experiment (lower curves in Fig. 2A–E) though the same nutrients were supplied to the control bacteria; (2) thorough rinsing of the cell–silicate hybrid decreased the luminescence by less than 5%, suggesting that the cells were indeed solidly embed- ded in the sol–gel matrix; (3) there was no residual luminescence in the medium after removal of the E. coli encapsulated glass plates from the microtiter wells, indicating that luminescence was generated by the caged bacteria and not by suspended cells.

3.2. Dose–response curves

The concentration–response dependence of the genotoxicity (DPD2794) and heat shock (TV1061) reporters are depicted in Fig. 3A–C. The figure shows the response to nalidixic acid and ethanol after a 108 min exposure. Similar trends were obtained for other induction periods as well. In all cases, the “calibration”
Fig. 2. Time course of the evolution of luminescence of different *E. coli* reporters (as of Table 1) after addition of the respective inducers. Luminescence response in blank LB medium is also shown (lowest curves in all figures). (A) TV1061–silicate sensors exposed to ethanol (0.43 M). (B) DPD2796–silicate sensors exposed to nalidixic acid (6 μM). (C) DPD2544–silicate sensors exposed to phenol (0.5 mM). (D) DPD2515–silicate sensors exposed to methyl viologen (3.9 mM). (E) DPD2511–silicate sensors exposed to hydrogen peroxide (3.9 mM). The response of the suspended cells to the same condition is represented by the dotted lines. Each test was conducted with 10^6 cells. The response intensity of the hybrid sensors (dashed lines) was multiplied by two in order to apply the same scale for the suspended and glass sensors.
curves for the suspended (dotted line) and the encapsulated *E. coli* cells were similar. All five strains exhibited bell shape luminescence–concentration dependencies. The induced luminescence reached a maximum and then decreased, probably due to inactivation of the *E. coli* cells by the high concentration of the stress inducers. In some cases, we could observe a logarithmic dynamic range such as that depicted in Fig. 2C for ethanol induction of strain TV1061. Note, that in this case, the specific luminescence responses of the suspended and encapsulated cells were very similar and can be obtained by dividing the y-axis by 10^6, because an equal number of cells was used for the suspended and immobilized cells tests.

3.3. Luminescence dependence on the sol–gel preparation protocol

The effect of the sol–gel preparation protocol on the viability of the encapsulated cells was investigated using the heat shock reporter, TV1061 as a model strain. As an indication of cell viability in the encapsulated state, we used luminescence after exposure to LB medium containing 2% ethanol for a specified amount of time. The induced luminescence was therefore a combined measure for the viability and the specific luminescence intensity of the cells. Fig. 4 demonstrates the dependence of the luminescence of TV1061–silicate matrix on the initial pH of the LB medium used to produce the hybrid plates. We prefer to specify the pH of the LB medium rather than the pH of the organic mixture because the latter is not...
well defined. Fig. 4 shows that optimal preparation conditions were around pH 7.5 with residual viability throughout the pH range 5–9, in accordance with the behavior of native E. coli. E. coli is known to regulate its internal pH at 7.5 for ambient pH 5–9 [34]. We used pH 7 for the preparation of different TV1061–silicate hybrids with varying water to silicon ratio, \( r \) (\( r \) represents the molar H\(_2\)O:Si ratio in the sol–gel precursors, including the H\(_2\)O that was added with the LB before film casting). Except for the \( r \) value, the preparation conditions were identical to previous protocols and each plate contained the same number of bacterial cells (10\(^6\) cells per plate). We used 60 min induction by 2% ethanol in order to obtain a significant response that allowed quantitation of the viable immobilized bacteria. Fig. 5 shows that there is a low threshold value around, \( r = 5.1 \), below which the encapsulation procedure leads to complete inactivation. For very high \( r \) values, the drying period (5 min under ambient conditions) was probably insufficient, leading to silicate films wash-out during the rinsing stage. This is the reason why the luminescence curve of Fig. 5 was terminated at \( r = 22 \); beyond this point, the films lost mechanical stability. We believe that partial destabilization of the film occurs also at lower \( r \)-values (\( r > 12 \)), which explains the slight luminescence decrease for \( r > 12 \).

The preparation conditions were repeated for different film thickness. This test was carried out by deposition of different volumes of the sol–gel precursors containing the same concentration of cells (10\(^8\) cells/ml) on glass slides. The glasses were then partially dried and aged according to the conventional preparation procedure. After aging in phosphate buffer (pH 7), the coated glass slides were exposed to 2% ethanol in LB and the luminescence was recorded as a function of time. Fig. 6 depicts two typical luminescence–thickness plots taken after 33 and 57 min ethanol induction. The linear dependencies support our conclusion that the active bacteria are embedded in the bulk of the silicate and are not confined to glass support–sol–gel film interface or to the outer film surface. The exposed surface of the films was identical for all the films, and thus, interface confined immobilization should have resulted in thickness independent response. The linear response shows that the bacteria where incorporated throughout the film thickness.

The luminescence response curves of Fig. 6 intercept the x-axis at approximately 0.1 mm. Thinner films, as well as dip-coating procedures did not provide any residual luminescence. These results support our hypothesis that partial drying of an almost constant-0.1 mm thick-film takes place during the 5 min drying period. Thus, the cells in the uppermost layer are inactivated by the physical constraint imposed by the drying process and film shrinkage.
Further support for this conclusion is presented in Fig. 7 which shows the effect of drying time on the viability of the cells in the silicate matrix as a function of time. Again, ethanol induction was taken as a measure for viability. Short drying periods (<3 min) resulted in mechanically deformed or washed out films during the subsequent rinsing stage. Longer drying periods (>8 min) showed a gradual decrease of the induced luminescence, indicating lower residual cell viability in the dried films. Five minutes drying time was therefore selected for further studies.

3.4. Metrological characteristics

One of the perceived advantages of the silicate system is the ability to deposit thin films which do not impose substantial mass transport barrier. In order to determine whether the silicate film is rate limiting and delays the luminescence response, we have traced the time course of luminescence evolution of silicate films as depicted in Fig. 8. The inset in Fig. 8 shows the normalized curves, corresponding to the six different thicknesses used in this study. The identical normalized time-dependent response shows that the responses of all six sensors are kinetically controlled. Significant diffusion barriers should have induced a delayed response, which should have been positively dependent on film thickness. In this case, the (bio)kinetic controlled response is caused by the very long response time of enzyme expression by the bacteria rather than by a truly fast diffusion in absolute values.

Several metrological characteristics of the heat shock based toxicity sensors (silicate-TV1061 hybrids) were investigated. The in-batch reproducibility of the sensors reflects the reproducibility of the preparation protocols. The relative standard deviation of the luminescence response of five toxicity sensors that were prepared under identical conditions (1 mm film thickness, pH 7, 5 min drying time, and induction by 2% ethanol in LB for 60 min) was less than 15% (not shown). Fig. 9 depicts the multiple use of the same sensing elements in consecutive works. The shelf life and reproducibility of all five recombinant E. coli strains were studied. Each test began with rinsing of the sensing element, introducing it into LB solution containing the respective inducers (see Table 1 for the type and concentration of the different inducers). The time course of the luminescence signal was recorded for 4h. At the end of the cycle, the sensors were rinsed with fresh LB and stored in
Fig. 8. Time course of the luminescence after induction of TV1061 by 2% ethanol as a function of film thickness. Preparation conditions are identical to that of Fig. 6. The inset shows the normalized response where each curve is normalized by the RLU at $t = 80$ min.

The sensors showed good reproducibility and very good storage stability even after multiple use. The relative standard deviation of the signal after 240 min exposure was less than 20% for all sensors. Even after 3 months, the sensors still maintained the same sensitivity (depicted in the last column of Fig. 9), reflecting the excellent shelf life of the cell based sensors.

3.5. Sensor characteristics

It was demonstrated that many environmental toxic compounds induce heat shock gene expression and thus, *E. coli* bacteria engineered with a fusion of heat shock gene promotor can serve as a general early warning sensor [28,29]. Strain TV1061 indeed maintains this ability in its encapsulated state. Fig. 10 demonstrates that 0.1 mM of different pesticides (DDT, aldicarb and malathion) induce the heat shock protection mechanism. In all cases, more than 1 h was needed in order to obtain a reliable response. The response time was similar to that of suspended bacteria. Freeze-drying resulted in disintegration of the films and formation of silicate powder. Moreover, even the powder material was inactive or at least lost its luminescence capability. We believe that the inactivation step occurs during or after the drying step. In contrast, the TV1061–silicate film regained full viability and luminescence power after immersion in liquid nitrogen and subsequent exposure to 2% ethanol–LB (Fig. 11). Freeze-drying of the nitrogen-cooled bacteria led to loss of activity. In this respect, the silicate sensor is still inferior compared to the native bacteria that maintained the luminescence capacity after several freeze-drying and wetting cycles.

An important advantage of the solid silicate-*E. coli* sensing elements compared to suspended bacterial cultures is their ability to operate under continuous flow conditions which makes them potential candi-
Fig. 9. Multiple use reproducibility and shelf life stability studies of different recombinant E. coli reporter sensors. The E. coli strains used for each test are depicted at the upper right-hand corner of each frame and the inducer’s type and concentration are depicted in Table 1. The sensors were prepared under the preparation conditions of Fig. 2. The weekly tests began with rinsing of the sensing element with LB followed by immersion of the sensing elements into LB solution containing the inducer (as of Table 1) and recording the signal for 4 h at 25°C. At the end of the cycle, the sensors were rinsed with fresh LB and stored in the same medium at 4°C. 10^6 cells were used for each test.
Fig. 10. Toxicity test of TV1061–silicate sensors exposed to 0.1 mM of (A) DDT, (B) aldicarb and (C) malathion. The lower lines correspond to TV1061 sensor in blank LB solution.

Fig. 11. Luminescence time course of silicate–TV1061 sensor before (A) and after (B) freezing by immersion of the sensor in liquid nitrogen. The response was measured in 2% ethanol-LB medium at 25°C. 10^6 cells were used for each test.

dates for on-line monitoring schemes, and pave the way for their integration in early warning systems. Fig. 12 demonstrates the ability to monitor toxicity (heat shock stress) under continuous flow conditions. The response of a TV1061–silicate sensor to two step changes of 2% alcohol feeding is indicated by the upward arrows in the figure. The downward arrows indicate a switch to alcohol-free LB feed. The figure demonstrates that the sensor can report physiological heat shock evolution continuously. Note, however, that the sensitivity of the sensor after the second slug of the inducer was substantially higher. We attribute the increased response to the physiological accommodation of the bacteria to the inducer. Some of
the RNA messengers and the enzymes that participate in the luminescence were retained in the cells even after hours of exposure to inducer-free environment.

4. Conclusions

This study demonstrates a new hybrid sensor based on a combination of engineered luminous bacteria and sol–gel matrices. The sensors integrate the improved physical and optical properties of the sol–gel silicates with the special capabilities of engineered bacteria. Unlike most conventional sensors the whole-cell-based sensors report true bio-availability of the targeted inducers rather than the overall concentration of specific chemicals in the environment. This property constitutes a significant advantage for general, early warning detection systems and a drawback for specific regulation-oriented sensing devices. We showed that the encapsulated bacteria maintain the favorable biological properties of the free culture and their metrological characteristics including repeatability, shelf life stability, sensitivity, and broad range response to a wide class of toxic compounds. Additionally, the silicate encapsulated bacteria can be used under continuous operation or in a multiple use sensing elements.

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