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Colorimetric/fluorescent bacterial sensing by agarose-embedded lipid/polydiacetylene films

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Abstract

Aim: Development of a new chromatic (colorimetric/fluorescence) bacterial sensor, for rapid, sensitive and versatile detection of bacterial proliferation.

Methods and Results: We constructed agarose-embedded chromatic films which produce dramatic colour changes and fluorescence transformations in response to bacterial growth. The sensing constructs comprise glass-supported Langmuir–Schaeffer phospholipid/polydiacetylene films that undergo both blue-red transformations and induction of intense fluorescence following interactions with bacterially secreted amphiphilic compounds that diffuse through the agarose. The agarose matrix coating the sensor film further contains growth nutrients, facilitating signal amplification through promotion of bacterial culture proliferation. The agarose layer also constitutes an effective barrier for reducing background signals not associated with the bacteria. We demonstrate the applications of the new sensor for the detection of Gram-negative and Gram-positive bacteria, and for screening specimens of physiological fluids (blood and urine) and foods (meat) for bacterial contaminations.

Conclusions: The experiments demonstrate that the new agarose-embedded film constructs are capable of bacterial detection through visible colour transitions and fluorescence emission recorded in conventional apparatuses.

Significance and Impact of the Study: This work demonstrated a new simple chromatic platform for bacterial detection, based on the generation of easily recorded colour and fluorescence changes. The new bacterial detection scheme is highly generic and could be employed for varied practical uses, in which, rapid reporting on bacterial presence is required.
understanding of the biochemical and structural properties of the bacterial species sought, limiting applications in case of unknown pathogens or variants (Hobson et al. 1996; Vollenhofer-Schrumpf et al. 2005).

We have recently demonstrated generic bacterial detection approaches based on the signals induced by bacterially secreted molecules and metabolites following their interactions with chromatic vesicles and films containing polydiacetylene (PDA) as a colour and fluorescence reporter (Silbert et al. 2006; Scindia et al. 2007). Cross-linked PDA assemblies exhibit unique chromatic properties, as polymerized PDA appears intense blue owing to the conjugated ene–yne framework (Ringsdorf et al. 1988). Furthermore, PDA aggregates and films have been previously shown to undergo dramatic blue–red changes owing to conformational transitions in the conjugated polymer backbone that are induced by external structural perturbations, such as binding of amphiphilic and membrane-associated hydrophobic molecules (Okada et al. 1998; Kolusheva et al. 2000). The colorimetric transformations of PDA go hand in hand with the induction of fluorescence: no fluorescence is emitted by the initially polymerized blue-phase PDA, while the red-phase PDA strongly fluoresces at 560 and 630 nm (excitation at 485 nm) (Takayoshi et al. 1997).

The bacterial detection technologies developed by us rely upon the amphiphilic properties of membrane-active compounds, which are secreted by bacteria to their environments (Thanassi and Hultgren 2000; Bendtsen et al. 2005). Such bacterially released amphiphilic molecules were shown to bind to lipid/PDA assemblies, consequently inducing chromatic transformations of PDA. The important feature of the polymer in such sensing constructs is its function as a ‘built-in optical/spectroscopic reporter’ undergoing rapid chromatic transformations in response to bacterially secreted molecules. Here we describe a new versatile and simple bacterial sensing scheme based on agarose-embedded lipid/PDA Langmuir–Schaeffer (LS) films. These films undergo colour/fluorescence transformations upon interactions with bacterially secreted compounds which diffuse through the agarose layer.

Materials and methods

Materials and bacterial strains

Dimyristoylphosphatidylcholine (DMPC) and dioleoylphosphatidylcholine (DOPC) were purchased from Sigma. The diacetylenic monomer 10,12-tricosadiynoic acid was purchased from GFS Chemicals (Powell, OH, USA), dissolved in chloroform, and passed through a 0.45-µm nylon filter before use. The bacteria used in the studies were Salmonella enterica serovar Typhimurium (strain ATCC14028), Staphylococcus aureus (strain ATCC6538) and Escherichia coli XL1 (provided by Prof Dudy Bar- Zvi, Ben Gurion University).

Film preparation

Phospholipid/diacetylene films were constructed through the LS technique. The lipids and diacetylene solutions were each prepared at a concentration of 2 mmol l⁻¹ by dissolving in chloroform, and subsequently mixed in a 1:9 mole ratio (lipid:diacetylene). The mixed monolayers were formed on an aqueous subphase through drop-wise addition of the lipid mixture and compression in the Langmuir trough. Surface compression was ceased following the occurrence of the monolayer–trilayer transition of the diacetylene (Volinsky et al. 2002), and the mixed lipid/diacetylene films were then transferred onto the glass substrate. Polymerization of LS films was carried out by irradiating the glass-supported films for 2 min at 254 nm in an ultraviolet cross-linker (UV-8000; Strattegene, CA, USA). The liquefied agarose matrix containing Luria–Bertani (LB) growth medium was kept at approximately 30°C, poured over the polymerized films, and subsequently solidified. Preparation of agarose films with different thicknesses (1 and 5 mm) was carried out by specially constructed aluminium template, in which the agar was solidified prior to transfer into the multiwell plate.

Physiological fluids and food specimens

Blood and urine specimens were provided by the microbiology laboratory of the Soroka Medical Center, Beer Sheva. In the meat analysis experiments, a 20-g sample of bacterially contaminated meat was added to 180 ml of sterilized potassium phosphate buffer (pH 7.2) 1:10 diluted and homogenized. This parent solution was further diluted (1:100 and 1:1000) prior to testing. Two hundred ul of each meat dilution was poured into a well containing the chromatic films prior to addition of the liquefied LB agarose and solidification.

Multiwell fluorescence spectroscopy

Agarose/phospholipid/PDA assemblies were constructed in cells of multiwell (six- and 12-well) plates. Fluorescence experiments were carried out in the desired temperatures on a fluorescence plate reader (Fluoroscan Ascent, Thermo, Finland). All measurements employed 485-nm excitation and 555-nm emission using LP filters with normal slits (3 mm). Acquisition of data was automatically performed every 30 min. All experiments were carried out at least three times to establish statistical significance.
Raman spectroscopy

The Raman spectroscopy measurements were conducted on a Jobin-Yvon LabRam HR 800 micro-Raman system, equipped with a liquid-N₂-cooled detector. A He–Ne laser was used for excitation (633 nm). The excitation line had its own interference filter (for filtering out the plasma emission) and a suitable Raman notch filter (for laser light rejection). The laser power on the sample was reduced by neutral density (ND) filters to 1–100 μW to prevent photo-degradation of the samples.

Monolayer adsorption

Adsorption experiments were carried out at 25°C using a Nima-611 Teflon trough. Solutions of chloroform-dissolved DMPC were spread at the air/water interface of the dipping well (total volume of 50 cm³) and allowed to equilibrate for 20 min, reaching the desired initial surface pressure (πi). Gram-negative and Gram-positive bacteria were grown overnight and centrifuged to separate the supernatant from the bacterial pellet. Aliquots (100 μl) of the supernatant solutions were gently injected close to the magnetic stirrer, approximately 2 cm beneath the lipid monolayer. Adsorption curves were recorded in several initial surface pressures of the lipid monolayer. The final surface pressures were recorded 1 h after injection of the supernatant into the water subphase (allowing equilibration). The error in the measurements was 0.5 mN m⁻¹.

Results

General properties

A schematic description of the sensing scheme and representative images are shown in Fig. 1. The glass-supported LS films appear blue to the naked eye (Fig. 1a), and the agarose-embedded sensor films (Fig. 1b) was produced by the solidification of the KB agarose at room temperature on the polymerized film (see Materials and methods). Growth of bacteria (E. coli XL1) on the agarose surface resulted in visible blue–red transformations of the lipid/PDA film (Fig. 1c).

Bacterial detection using the new sensor constructs can be achieved both via observation of the colour transition of the film (Fig. 1) and also by monitoring the fluorescence emission. Figure 2 depicts the application of agarose-embedded chromatic films for bacterial detection using fluorescence scanning in a conventional multiwell plate (ELISA) reader. The bacteria tested in Fig. 2 (E. coli XL1) were streaked onto the agarose surface (through deposition of 1 μl aliquots) from a bacterial solution containing 10⁷ cells per ml (determined from the UV-vis absorption at 600 nm). The topographic maps in Fig. 2 show the fluorescence intensities recorded at specific times within the well area (excitation 485 nm, emission 555 nm) during incubation at 35°C. The initial film fluorescence was minimal (background signal, Fig. 2a) as blue PDA is nonfluorescent (Takayoshi et al. 1997). However, approximately 9 h after streaking, an area of increased fluorescence clearly appeared in the film (Fig. 2b), becoming prevalent in the entire film area after 24 h (Fig. 2c). Importantly, the induced fluorescence in the film was detected earlier than the appearance of visible bacterial colonies on the agarose surface, emphasizing the potential utilization of the film/agarose sensor for early bacterial detection.

Figure 3 presents an experiment designed to confirm that the chromatic transformations in the agarose-embedded sensor films were indeed induced by bacterially secreted substances diffusing through the agarose matrix and interacting with the film surface. Specifically, we grew E. coli XL1 up to the stationary phase and separated the supernatant from the bacterial pellet. Aliquots (400 μl) from the supernatant solution were then placed on agarose surfaces deposited upon DOPC/PDA films (Fig. 3). Importantly, we placed the supernatant suspensions in two types of wells: one in which the chromatic film was covered with a thick agarose layer [5 mm, Fig. 3(ii)], and...
a film construct in which the agarose coating was much thinner [1 mm, Fig. 3(iii)].

The marked colour transformations (Fig. 3a), and time-dependent fluorescence data (Fig. 3b) provide compelling evidence that the colour/fluorescence transformations in the films were induced by bacterially secreted substances diffusing through the agarose layer. Importantly, no bacterial colonies appeared in the cells examined in Fig. 3, confirming that the supernatant suspensions were indeed the factors causing the chromatic transitions. Furthermore, the difference in colours and kinetic profiles between the thick-agarose and thin-agarose assays point to the role of molecular diffusion in the induction of the chromatic transformations. In particular, the thicker agarose matrix is more likely to retain a greater percentage of the diffusing molecules, resulting in the lesser blue–red transformation as indeed recorded in Fig. 3a. Moreover, the passage of the secreted compounds present in the supernatant is expected to be faster when the agarose layer was thin, giving rise to the earlier fluorescence induction observed in Fig. 3b.

Chromatic transitions induced by Gram-negative and Gram-positive bacteria

Figures 4 and 5 present comparisons of the responses of the new bacterial sensor to Gram-negative and Gram-positive bacteria, respectively. Figure 4 depicts an experiment in which different bacterial concentrations were placed on the films prior to addition and solidification of the agarose within the multiwell plate (the ‘pour-plate’ method (Michael et al. 2003), and the fluorescence emission was then monitored during incubation at 30°C. The experiments depicted in Fig. 4 were repeated at least three times, reproducing the data presented.

Specifically, we compared in Fig. 4 the fluorescence induced by Gram-negative bacteria (S. Typhimurium, Fig. 4a) and Gram-positive bacteria (S. aureus, Fig. 4b). Essentially, each curve in Fig. 4 corresponds to the average fluorescence intensity recorded from a particular cell
in which the indicated initial bacterial concentrations were added to the well.

Several important observations are apparent in Fig. 4. First, the increase in fluorescence emission was not gradual, but rather abrupt jumps in the fluorescence curves were observed. Moreover, the fluorescence ‘steps’ were correlated to the bacterial concentrations examined. For example, when a suspension of $10^{7}$ S. Typhimurium cells per ml was placed in the well, the increase in the fluorescence occurred after approximately 7 h, while adding a solution of $10^{5}$ S. typhimurium cells per ml gave rise to fluorescence induction only after 12 h (Fig. 4a). The relationship between the chromatic transitions and initial bacterial concentrations was apparent for both bacterial strains, although significant differences between the two bacterial species can be clearly discerned (Fig. 4). Specifically, the fluorescence transformations induced by S. Typhimurium (Fig. 4a) occurred considerably earlier than the corresponding transitions induced by the same concentrations of S. aureus (Fig. 4b).

The data presented in Figs 3–4 point to the direct relationship between the chromatic transformations of the films and bacterial secretions. To corroborate this relationship and to further probe the difference between the chromatic transformations induced by Gram-negative and Gram-positive bacteria, we carried out an isothermal adsorption experiment on a phospholipid monolayer at the air/water interface (Brockman 1999; Maget-Dana 1999) (Fig. 5). In this type of experiment, aliquots of the phospholipid solution are deposited on the water subphase in a Langmuir trough to create monolayers at different initial pressures. The examined molecule (or molecular mixture) is then injected into the aqueous subphase, at different initial surface pressures of the monolayer, and the change in surface pressure is monitored. An increase in the recorded surface pressure indicates penetration (or adsorption) of the injected molecule (or mixture) into the monolayer (Brockman 1999; Maget-Dana 1999).

Figure 5 depicts the results of an isothermal adsorption experiment in which supernatant suspensions extracted from overnight growth suspensions of Gram-negative (E. coli XL1) or Gram-positive bacteria (S. aureus), respectively, were injected into the water subphase below a phospholipid (DMPC) monolayer at different initial pressures. The experimental data in Fig. 5 demonstrate that the supernatant solutions of both bacterial species gave rise to an increase in surface pressure, indicating adsorption into the lipid monolayer of molecular species in the supernatants. Indeed, the linear relationship between the increase in surface pressure ($\Delta \pi$) and the initial pressure of the DMPC monolayer prior to injection ($\pi_0$) (Fig. 5) is a hallmark of monolayer adsorption by molecules dissolved at the water subphase (Bhakoo et al. 1982).

An important result apparent in Fig. 5 is the significantly greater increase in surface pressure induced by the supernatant collected from the E. coli bacterial growth (diamonds, Fig. 5) compared with the supernatant extracted from the S. aureus growth solution (circles, Fig. 5). This result indicates that E. coli release more active and/or greater quantity of amphiphilic molecules than S. aureus, resulting in a higher increase in surface pressure, apparent in Fig. 5.

**Raman spectroscopy**

To further probe the relationship between the structural/chromatic transformations of PDA and the film interactions of the amphiphilic compounds secreted by the bacteria, we carried out Raman spectroscopy experiments to characterize the agarose-embedded lipid/PDA films (Fig. 6). Figure 6a and b depict the spectral changes in the Raman spectra of a glass-supported DOPC/PDA film before (Fig. 6a) and after (Fig. 6b) undergoing a complete blue–red transformation induced through immersion in ethanol. Figure 6b displays the pronounced shifts of the Raman peaks at 2100 nm (the acetylene stretching) and 1490 nm (the carbon–carbon double bond stretching) following the blue–red transformation of PDA (Baughman et al. 1974).

Figure 6c depicts the Raman spectrum of a microscopic area within an agarose-embedded DOPC/PDA film following a bacterially induced blue–red transformation (the agarose layer was removed and the film was washed with...
distilled water prior to the Raman measurement). Clearly, the Raman spectrum in Fig. 6c features partial structural transformations of the PDA framework. Specifically, peaks corresponding to the red-PDA phase appeared at around 2100 and 1490 nm; however, the signals corresponding to the polymerized blue PDA were apparent as well in Fig. 6c. The incomplete structural/chromatic transitions of the PDA network can be probably explained by the fact that the blue PDA domains in the lipid/PDA LS films exist in trilayer structures (Volinsky et al. 2002). Accordingly, binding of the bacterially secreted molecules to the film surface would mostly affect the exposed upper PDA layers rather than the entire polymer structure, giving rise to the partial structural transformations observed in Fig. 6c.

Practical applications

The new chromatic bacterial detection assay can be readily employed for varied diagnostic and microbiological applications. Figure 7 depicts experiments designed to demonstrate the potential use of the sensor system for the detection of bacterial presence in physiological fluid specimens, such as blood and urine (Fig. 7a), or in foods (Fig. 7b). Specifically, Fig. 7a presents scanned images of DMPC/PDA films embedded in agarose, onto which we streaked 1 μl blood and urine specimens provided by the microbiology laboratory of the Soroka Medical Center, Beer Sheva. The fluid samples were tested in a ‘double blind’ experiment, both in the medical centre using conventional culture methods, and by the new chromatic sensor. Specifically, one of the blood samples was extracted from a healthy individual (no bacterial presence), while the other one was diagnosed to be bacterially infected (Klebsiella pneumoniae isolated, >10 000 CFU as determined by conventional culture methods at the Microbiology Laboratory, Soroka Medical Center).

Figure 7a clearly demonstrates that red areas appeared within a few hours on the embedded film only in the well in which the bacterially contaminated blood was streaked [Fig. 7a(ii)], while the sterile blood specimen did not induce any colour change [Fig. 7a(iii)]. Dramatic blue–red transitions were also observed in the scanned well in which bacterially contaminated urine specimen was placed [Fig. 7a(iv)] (E. coli isolated, >10 000 CFU as determined by culture methods at the Soroka Medical Center).

Figure 7b depicts the application of the agarose/film sensor for monitoring bacterial presence in a meat speci-
men. The experiments were conducted in the conventional microbiological food-testing method (pour plate) in which the meat, ground and homogenized in phosphate buffer, was mixed within the liquefied agarose and subsequently deposited and solidified on the DOPC/PDA film. The curves shown in Fig. 7b correspond to the fluorescence induced in the agarose-embedded films by different dilutions of the buffer-homogenized meat specimen. Similar to the experiments summarized in Fig. 2, the time-dependent curves in Fig. 7b exhibit the abrupt jumps in fluorescence emission ascribed to the proliferation of bacterial cells, initially present in the contaminated meat sample. Indeed, the progressive increase in fluorescence response times were clearly related to the dilution of the meat specimens. An independent microbiological evaluation carried out using conventional plating methods (done at the Institute of Food Microbiology, Ltd.) confirmed the presence of *E. coli* bacteria in the meat sample tested.

The experiments depicted in Fig. 7 emphasize two important aspects of the new bacterial sensor system. First, the blue–red transformations occurred several hours before the appearance of visible bacterial colonies on the agar surface. This result underlies the potential practical application of the film/agarose detection system for health-oriented microbiological screening and food monitoring. Second, the chromatic data in Fig. 7 emphasize the filtering capabilities of the agarose layer, e.g. placement of blood specimens directly on top of a lipid/PDA film would result in an instantaneous chromatic transformation induced by the ions, proteins and other amphiphilic substances in the blood. The agarose layer however constitutes an effective barrier to these substances and provides an intrinsic amplification vehicle for the bacterially secreted molecules.

**Discussion**

This work presents a new bacterial sensing scheme based on agarose-embedded glass-supported lipid/PDA films. The experimental data demonstrate that bacterially secreted amphiphilic molecules diffuse through the porous agarose scaffolding, subsequently inducing colour and fluorescence transformations upon interactions with the lipid/PDA film surface. In the context of biosensor applications, the lipid/PDA film essentially constitutes the reporter element within the sensing assembly, undergoing blue–red transitions and emitting fluorescence signals in response to the lipid-active substances released by bacteria to their outer environment.

The agarose-embedded lipid/PDA sensor exploits the chromatic transformation induced in lipid/PDA assemblies towards membrane-interacting biological molecules secreted by bacteria to their environment (Okada *et al.* 1996; Kolusheva *et al.* 2000). Varied membrane-active bacterially secreted molecules have been identified, and some of those are in fact universal bacterial markers and well-known virulence factors. Among such systems are the cell-wall lipopolysaccharides (LPS), short bacterially secreted amphiphilic peptides, such as haemolysins (and Ostolaza 1998), α-toxin released by *S. aureus* that causes membrane damage (Tomita *et al.* 1992), and pore-forming toxins produced by numerous bacteria (Geny and Popoff 2006). Such compounds have been shown before to induce the chromatic transformations in the lipid/PDA assays (Kolusheva *et al.* 2000).

A clear correlation was apparent between the chromatic transformations of the film/agarose construct and the number of bacteria placed on the agarose surface. Specifically, the rapid fluorescence increases recorded in Figs 3 and 4 were different for each initial bacterial concentration streaked on the plates. These ‘steps’ coincided with the appearance of the visible blue–red transitions in the films, and are ascribed to the exponential growth of bacterial populations in colonies, surpassing concentration thresholds of membrane-binding substances that give rise to the chromatic transformations (Silbert *et al.* 2006). This interpretation is supported by the different time periods in which the ‘steps’ in the fluorescence curves occurred – which essentially depend upon the initial concentrations of the bacteria incorporated in the agarose.

Several control experiments confirmed the proposed relationship between the chromatic phenomena and film interactions of bacterially released molecules diffusing through the agarose layer. Specifically, monolayer-adsorption analysis (Fig. 5) and Raman spectroscopic measurements (Fig. 6) yielded evidence that amphiphilic molecules produced by growing bacteria indeed associate to a significant extent with lipid moieties at the air/water interface, and that this binding is most likely the primary factor affecting the structural transformations of the PDA framework.

The monolayer adsorption experiment (Fig. 5) pointed to a significantly more pronounced membrane activity of molecules secreted by *E. coli* vs *S. aureus*. This phenomenon might point to a general difference between Gram-negative (*E. coli*) and Gram-positive (*S. aureus*) bacteria, and is consistent with the kinetics of chromatic transformations induced by the two bacterial families shown in Fig. 4.

Utilization of either visual colour changes observed by the naked eye or fluorescence emission that can be recorded by conventional multiplate readers as viable detection methods is an important advantage of the new bacterial sensing system. While the pronounced colour changes should facilitate bacterial detection by nonexperts
in diverse settings, the fluorescence properties of the chromatic matrix could additionally open the way for employing the platform for high throughput screening applications requiring high sensitivity, large sample quantities or automation.

The intrinsic sensitivity of the fluorescence phenomena, in particular, confers to the new assay enhanced sensitivity. The utilization of fluorescence emission of PDA translates into shorter detection times compared with conventional microbiology methods (i.e. based upon colony identification). Depending upon the type of bacteria analysed and initial number of bacterial cells streaked upon the agarose surface, the new assay could report upon bacterial presence between 3 and 20 h earlier than colony-based screening.

The agarose-film bacterial sensor exhibits other practical advantages. The agarose-film composites are stable for long time periods and can be stored at room temperature. In contrast to a previously reported agar-embedded lipid/PDA vesicle bacterial sensors (Silbert et al. 2006), the agarose-film assemblies reported here can be operated at 37°C without undergoing temperature-induced chromatic transformations. This feature facilitates faster bacterial proliferation and higher sensitivity, and would make possible operation of the new assay in conventional microbiology protocols. The agarose layer, in particular, has a dual role. Specifically, the LB agarose facilitates bacterial proliferation, constituting an inherent enhancement mechanism of the bacterially induced signals. Additionally, the agarose layer essentially serves as an effective ‘molecular barrier’, minimizing the interactions of external molecules with the embedded film, thus reducing background signals unrelated to bacterial growth.

An important feature of the new assay, facilitating its broad potential application as a generic bacterial sensor, is that, prior knowledge of the biochemistry and metabolic profiles of the bacteria tested is actually not necessary for its utilization. The agarose-embedded chromatic film sensor is not capable, at this stage, to differentiate among bacteria. However, the generality of the detection concept and non-specificity of the chromatic platform can, in fact, be an advantage in applications in which reporting on the presence of any type of bacteria is required, e.g. in monitoring sterile environments, evaluation of food freshness, screening of bacterial resistance to existing and new antibiotic compounds, and others.

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