Colorimetric Detection and Fingerprinting of Bacteria by Glass-Supported Lipid/Polydiacetylene Films

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Glass-supported films of lipids and polydiacetylene were applied for visual detection and colorimetric fingerprinting of bacteria. The sensor films comprise polydiacetylene domains serving as the chromatic reporter interspersed within lipid monolayers that function as a biomimetic membrane platform. The detection schemes are based on either visible blue—red transitions or fluorescence transformations of polydiacetylene, induced by amphiphilic molecules secreted by proliferating bacteria. An important feature of the new film platform is the feasibility of either naked-eye detection of bacteria or color analysis using conventional scanners. Furthermore, we find that the degrees of bacterially induced color transformations depend both on the bacterial strains examined and the lipid compositions of the films. Accordingly, bacterial fingerprinting can be achieved through pattern recognition obtained by recording the chromatic transformations in an array of lipid/PDA films having different lipid components.

Introduction

The demand for new diagnostic and sensing technologies that can serve as alerts for bacterial contamination has significantly increased in recent years because of incidents of food poisoning, bioterrorism alerts, and anthrax scares. Numerous technologies have been developed.1-5 There are, however, limitations to existing bacterial detection techniques as rapid and generic approaches. Specifically, many methods employed for pathogen sensing provide results after relatively long time spans (several hours to days in the case of culture-based methods).6 Other currently employed technologies often involve complex detection mechanisms that require specialized instrumentation, trained personnel, and the need for active operation (addition of reagents, initiation of chemical reactions, etc.), which overall do not make possible applications in settings other than laboratory environments.5,7 Furthermore, a prerequisite for many detection methods is a detailed understanding of the biochemical and structural properties of the bacterial species sought, limiting applications in the case of unknown pathogens or variants.

Here we describe a bacterial detection platform based on Langmuir—Schaeffer films containing lipids and polydiacetylene (PDA). PDA has attracted significant scientific and technological interest in recent years because of its unique chromatic properties. Specifically, PDA was shown to self-assemble into organized vesicles for bacterial detection.11 Furthermore, we show that film arrays incorporating different biological analytes, essentially making the polymer a “built-in optical reporter” within the mixed lipid/PDA assemblies. The chromatic reactions induced in lipid/PDA films by amphiphilic membrane-active molecules are the primary phenomenon exploited in this work. The detection scheme is based upon bacterially secreted amphiphilic compounds that bind to the lipid/PDA film surface, thereby inducing chromatic transformations in PDA. We demonstrate that the chromatic changes are visible to the naked eye, can be recorded spectroscopically (UV—vis and fluorescence), or can be analyzed by conventional color scanning of the films combined with simple image analysis. Furthermore, we show that film arrays incorporating different

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lipid compositions makes bacterial fingerprinting possible through distinct color patterns corresponding to tested bacteria.

**Experimental Section**

**Materials.** 10,12-Tricosadiynoic acid (TRCDA) was purchased from GFS chemicals (Powell, OH) and was purified by dissolution in chloroform followed by filtration through a 0.45 μm nylon filter. The filtrate was solidified by evaporation of the solvent to obtain the purified form of TRCDA. Lipids 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-dioleoylsn-glycero-3-phospho[Lα(1-glycerol)] (POPG), sphingomyelin, and cholesterol were purchased from Sigma. Chloroform (CHCl₃) was HPLC grade (Frutarom Ltd.).

**Preparation of Lipid/PDA Films.** Film construction was described in detail in previous studies. Briefly, stock solutions (2 mM) of TRCDA and lipids were prepared by dissolving the appropriate amounts of material in CHCl₃. TRCDA/lipid mole ratios in all films were 9:1, except in the TRCDA/sphingomyelin/cholesterol film where the mole ratio was 90:7:3. Lipid/TRCDA mixtures (50 μL) were spread on a water subphase (pH 6.5) within a computerized Langmuir trough (model 622/D1, Nima Technology Ltd, Coventry, UK). Following 20 min of equilibration, the film was compressed at a constant barrier speed of 10 Å/molecule min⁻¹. The equilibration time and compression rate outlined were found to produce optimal films. The compressed films were kept for 5 min at a constant surface pressure of between 16 and 22 mN/m, depending upon film composition. Following equilibration, the films were irradiated with 254 nm light for 10 s (1.8 mW/cm²) and transferred to a glass surface (cleaned thoroughly by placing in ethanol/water) by horizontally placing the slide on the film surface and lifting slowly.

**Bacterial Growth.** Bacterial strains *Salmonella typhimurium* (strain C5093) 1a (provided by A. Porgador, Ben Gurion University), *Bacillus cereus*, *E. coli* K-12 strain C600 pMRInv, (provided by E. Gazit, Tel Aviv University), and *E. coli* XL1 (provided by D. Bar-Zvi, Ben Gurion University) were used in this study. The bacteria were grown aerobically at 37 °C on a sterilized solid Luria Bertani (LB) medium composed of 13.5% yeast extract, 27% peptone, 27% NaCl, and 32.5% agar at pH 7.4. The *E. coli* C600 pMRInv strain was grown in LB medium containing 30 μg/mL kanamycin. After overnight growth, a colony from each bacterial strain was taken and added to 0.5 mL of sterilized growth medium. After suspension, two 200 μL aliquots of the sensor films were placed on the glass-supported films for incubation, the films were washed with distilled water, dried gently and emission was recorded at 540 and 640 nm.

**Colorimetric Bacterial Sensing with Lipid/PDA Films.** Solid-supported films comprising phospholipids and PDA have been shown to exhibit unique chromatic properties, undergoing visible blue—red transformations and fluorescence transitions in response to binding of varied biological analytes. Films were prepared by spreading the lipid/diacetylene mixtures at the air/water interface, followed by compression and equilibration, deposition onto glass substrates by the Langmuir—Schaeffer technique, and polymerization (Experimental Section). Figure 1 depicts a representative AFM image of a DMPC/PDA film. Importantly,

![Figure 1. Lipid/PDA film structure.](Image)
at the surface pressures in which the films were transferred onto the glass substrate, both lipids and the diacetylene molecules form distinct domains exhibiting different organizations (Figure 1). Specifically, the lipid molecules form a fluid monolayer, and the diacetylene monomers are compressed into rigid trilayer structures, confirmed by the height profile depicted in AFM analysis, which are dispersed within the phospholipid monolayer. The trilayer organization reduces the relative surface area occupied by the PDA domains (Figure 1).

The domain organization depicted in Figure 1 confers important structural and functional properties to the lipid/PDA as a biosensing platform. Specifically, the creation of separate diacetylene and phospholipid areas rather than a homogeneous mixture of the two components facilitates the polymerization of the diacetylene trilayers, which gives the film its distinctive blue appearance. The rigid PDA moieties further serve as “scaffolding”, imparting high stability to the lipid/PDA film even after exposure to ambient conditions for long periods of time. In addition, the lipid monolayer essentially constitutes a biomimetic membrane platform that is designed to promote the binding of biological analytes to the film surface.

The thrust of the bacterial detection scheme described here is the induction of chromatic transformations in the lipid/PDA films upon film-surface interactions of bacteria and bacterially released amphiphilic substances. Figure 2 depicts scanned images of a glass-supported DOPE/PDA film before (Figure 2A) and after (Figure 2B) the deposition of supernatant solutions extracted at different times after the initiation of bacterial growth (E. coli XL1). Specifically, the scanned image in Figure 2B was recorded following the separation of the pellet (containing the bacterial particles) and supernatant (containing bacterially released substances within the growth media), placing small drops (15 μL) of the supernatant on the film surface, incubating at 33 °C for 30 min, and washing and drying the film surface. The image in Figure 2B clearly shows that blue—red transformations occurred on the film surface. In particular, Figure 2B demonstrates that more pronounced color changes were induced by suspensions that contained higher concentrations of bacteria (i.e., longer times after the beginning of growth). Importantly, similar color transitions were observed after placing on the films the total bacterial suspensions (without separation between bacterial particles and supernatant). However, the use of the bacterial supernatant rather than the total bacterial suspensions gave “cleaner” color transformations, reducing masking by bacterial particles attached to the film surface. Also, an analysis of the resuspended pellets (containing only the bacterial particles) gave rise to less pronounced colorimetric transformations (data not shown).

Overall, the observation that supernatant solutions of the bacterial growth media induced color transformations on the lipid/PDA film (Figure 2) indicates that the color changes are most likely due to film-surface interactions of bacterially secreted membrane-active compounds. This conclusion is supported by a recent analysis of bacterially induced chromatic transformations in lipid/PDA vesicles. Indeed, the release of varied amphiphilic substances by bacteria into their environments is widely encountered in bacterial fauna. Bacterially secreted substances have been identified in processes that have essential functional roles, such as overcoming host-defense mechanisms, allowing colony proliferation, or facilitating bacterial communication.

Bacteriocins, for example, are cytolytic peptides that induce membrane leakage and are released by wide variety of bacterial species. Secretion of pore-forming exotoxins is also abundant, and endotoxins such as lipopolysaccharides, often released by gram-negative bacteria, strongly interact with membrane components of host cells. Importantly, previous studies have shown that lipid/PDA assemblies are highly sensitive to varied peptides and toxins and undergo chromatic transformations following the binding of such molecules. Specifically, the interactions of the secreted compounds with the lipid/PDA surface occur either through electrostatic attraction to the polymer surface and/or binding to and insertion of hydrophobic peptides and proteins into the biomimetic lipid layers within the films. Overall, these interactions give rise to the structural and (chromatic) transformations in polydiacetylene.

The bacterially induced changes in the lipid/PDA films are also manifested spectroscopically (Figure 3). Figure 3A features UV—vis spectra of DOPE/PDA films placed in contact with supernatant solutions extracted at different times after the initiation of bacterial growth (S. typhimurium). Figure 3A demonstrates that the spectral component at around 650 nm progressively decreases while the signal at approximately 500 nm increases as the bacteria proliferate in the growth media. This spectral change corresponds to the blue—red transformation of the film.

The blue—red changes in the lipid/PDA films following interactions with the bacterial supernatant solutions go hand in hand with the induction of fluorescence emission (Figure 3B). The spectra depicted in Figure 3B demonstrate that significantly higher fluorescence emission signals are recorded after supernatants extracted from solutions containing greater bacterial concentrations were placed on the film. The enhanced fluorescence emission is ascribed to the transition of the nonfluorescence blue PDA phase to the fluorescent red PDA, induced by bacterially secreted compounds present in the supernatant solutions. Fluorescence microscopy can also be applied to the detection of bacterially induced chromatic changes. The microscopy images

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in Figure 3C highlight the contrast between the bright (fluorescent) film surface onto which bacterial suspensions were added and the darker, nonfluorescent background. The fluorescence microscopy images that depict very small areas within the bacterially covered film surface demonstrate qualitatively that there is a clear difference between the control surface and the surface after the deposition of bacterial solutions. The use of fluorescence microscopy could facilitate the examination of a large number of samples at minute volumes.

The bacterially induced chromatic transformations occur within minutes after the deposition of bacterial supernatants on the film. Figure 4 depicts the kinetic profile of the fluorescence transformation induced in a DOPE/PDA film by a supernatant extracted from a suspension containing $10^8$ S. typhimurium bacterial particles. The curve in Figure 4 indicates that a significant fluorescence signal appears almost instantly after the addition of the bacterial solution, reaching a plateau after approximately 30 min. The evolution of the chromatic transformation is most likely determined by the diffusion and binding of the bacterially released substances on the film surface.

**Colorimetric Bacterial Fingerprinting.** The bacterially induced color transitions can be recorded and analyzed by conventional color scanning and digital image analysis (Figure 5). Recent studies have demonstrated that digital colorimetric analysis (DCA) of the blue–red transformations on lipid/PDA films is a powerful and simple method for the evaluation of color changes occurring on such films. DCA measures the abundance and average intensity of transformed red pixels within a defined film surface area (generally the surface covered by the sample.
tested) and thus provides a quantitative value for the colorimetric transformation occurring on the film. The DCA analysis is applied using a conventional desktop scanner and readily available image analysis software (Experimental Section).

Figure 5A depicts red chromaticity shift (RCS) curves calculated after placing supernatant aliquots of several bacterial strains, extracted at different times after the initiation of bacterial growths, on a sphingomyelin/cholesterol/PDA film (mole ratio 7:3:90). RCS values are essentially a measure of the blue—red transformations occurring on the film surface, with higher values corresponding to a more pronounced red appearance of the film (greater blue—red transformation) and low values reflecting a less pronounced color transition (Experimental Section). Thus, each data point in Figure 5A mirrors the extent of the blue—red transformation induced by a specific bacterial sample on the film. As expected, bacterial proliferation in the growth media gave rise to more pronounced blue—red transitions in later stages of the growth; this result was apparent for all bacterial species examined. However, Figure 5A also demonstrates a divergence among the RCS curves, reflecting a variation in the extent of blue—red transformations induced by each bacterium. For example, *E. coli* XL1 suspensions gave rise to faster and consistently more pronounced blue—red transformations (squares in Figure 5A) compared to *Bacillus cereus* (circles in Figure 5A).

Importantly, the concentrations of the different bacterial species examined were similar throughout the experimental time frame (Supporting Information). Accordingly, the divergence of the RCS curves in Figure 5A is intimately related to the underlying mechanism of colorimetric changes (i.e., the interaction of bacterially secreted substances with the lipid/PDA film surface). Indeed, because each bacterial strain exhibits a distinct profile of metabolic pathways and secreted substances, it is expected that different interactions and consequent colorimetric transitions would be induced by each bacterium.

The divergence of the RCS curves in Figure 5A is the key to the colorimetric bacterial fingerprinting method. Specifically, the colorimetric reactions of the tested bacterial strains can be

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**Figure 5.** Color response induced by several bacterial strains on different films. (A) Color transition (RCS) curves of different bacteria placed on a sphingomyelin/cholesterol/PDA film (7:3:90 mole ratio). ▼ LB medium; ● *B. cereus*; ● *E. coli* C600 pMRInv; ■ *E. coli* XL1; and ▲ *S. typhimurium* 1a. (B) RCS values calculated for aliquots extracted 7 h after the start of growth (bacterial concentration of $1 \times 10^9$/mL, see Supporting Information), placed on films with different lipid compositions. (i) *E. coli* XL1, (ii) *B. cereus*, (iii) *S. typhimurium*, and (iv) *E. coli* C600 pMRInv.
recorded on films having different lipid compositions. Figure 5B, for example, depicts the RCS values calculated for the four bacterial strains deposited on films having different lipid compositions. The diagram in Figure 5B features the RCS data calculated 7 h after the initiation of growth, a time point in which the bacteria are in the stationary growth modes (concentration of $1 \times 10^9$ particles/mL). Importantly, the choice of this time point reflects the experimentally significant differences in the RCS values induced by the bacterial species examined (Figure 5A). Figure 5B confirms that the extent of blue-red changes induced on the films varied between both of the bacterial strains and among the lipid compositions examined. For example, E. coli XL1 induced approximately 70% RCS (reflecting the pronounced blue-red transformation of the film surface) when placed on a DOPE/PDA film but only 30% RCS on a DMPC/PDA film. S. typhimurium, however, gave rise to 70% RCS in the DOPE/PDA film and 40% RCS following deposition on the DMPC/PDA film.

The variations in color changes among both bacterial strains and film compositions were anticipated. Specifically, because the color transformations correspond to interactions of the secreted molecules with the lipid/PDA film surface, the distinct pool of molecules released by each bacterial species to its surroundings should interact in a rather distinctive way with films having different lipid head groups, sizes, structures, surface charge, organization of the lipid molecules, and so forth, overall leading to disparities in the colorimetric response depending upon film composition.

The bar diagram in Figure 5B can be also represented as a means of pattern recognition or bacterial fingerprinting utilizing an array of different lipid/PDA films (Figure 6). Figure 6 presents, in a color-code display, the cumulative RCS values recorded for each bacterial species deposited on the films 7 h after the initiation of growth. Figure 6 shows that distinct colorimetric fingerprints are obtained for the four bacterial species examined. The fingerprints are dependent upon the bacterial concentration selected; when the color induced on the films by other bacterial concentrations is tested, the bacterial fingerprints might not be unique. However, the significant result depicted in Figure 6 is that at a certain time point in the bacterial growth curves (identified here as 7 h after the initiation of growth) the supernatant solutions will provide unique patterns, or fingerprints, for the bacterial species examined. It is plausible that a selection of an optimal array of different lipid compositions would produce individual colorimetric signatures for many bacterial strains.

Conclusions

Colorimetric technology exhibits important strengths as a bacterial-sensing and discrimination platform. An important feature of the system is that bacterial detection is not achieved through the use of specific recognition elements but rather is achieved through the affinity of bacteria and bacterially secreted compounds for phospholipid bilayers. In particular, the presence of phospholipids is critical for achieving bacterial discrimination; experiments investigating the response of pure PDA films to bacterial secretions from different strains yielded no experimentally significant differences among the different strains.

The sensing platform also exhibits practical benefits. Film preparation is straightforward and inexpensive, and the films can be stored for long periods of time before use. The new film assay facilitates bacterial detection at a threshold concentration of around $10^6$ particles/mL, a concentration that is reached after short time spans under regular bacterial growth conditions. The colorimetric transformations occur over a wide bacterial concentration range and can be detected by the naked eye or through conventional desktop scanners. Analysis can be carried out by employing either supernatant solutions or the total nonseparated bacterial suspensions. Utilization of the colorimetric bacterial film sensor in high-throughput screening and parallel analysis of metabolic disruption should be feasible.

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Supporting Information Available: Bacterial growth curves. This information is available free of charge via the Internet at [http://pubs.acs.org](http://pubs.acs.org).

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