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Detecting and correlating bacterial populations to visual color change of polydiacetylene-coated filters

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ABSTRACT

Membrane filters were coated with 10,12-pentacosadiynoic acid (PCDA) then polymerized on the filter for rapid bacterial detection and quantification. The polymerized PCDA (pPDCA)-coated filter changed color in response to *Salmonella* Typhimurium and *Escherichia coli* but not to *Listeria innocua*. The time required for color change of pPCDA-coated filters was determined by a visual panel. A simple linear regression model was generated to fit the observed data and was validated with goodness of fit analysis and residual analysis. The pPCDA-filter method estimated *Salmonella* Typhimurium populations of 8 to 3 log CFU ml^{-1} within 1.5–7.5 h, respectively.

1. Introduction

Conventional bacterial detection methods rely on culturing bacteria in media to enumerate viable bacterial cells [1]. This method is sensitive and reliable but requires dilutions for sampling and incubation to permit cell colony growth. The reported incubation time for most bacteria is 18–24 h but may take up to 72 h [2]. Rapid bacterial detection methods generally can be classified into immunological-based, biosensor-based, and nucleic acid sequence-based methods. A well-known immunological-based method is enzyme-linked immunosorbent assay (ELISA) [3,4]. Biosensor-based methods include optical [5], electrochemical [6], mass-based [7], and biochemical biosensors [8]. These methods often require physicochemical instruments such as infrared and fluorescence spectroscopy, flow cytometry, or chromatography [9]. Examples of nucleic acid sequence-based methods include various types of polymerase chain reaction (PCR) [10] and DNA microarrays [11]. Among all of the rapid detection methods, PCR is the most widely applied method for detection of bacteria. This rapid detection method is sensitive and less time consuming than conventional culture dependent method but is limited to determining presence and not enumeration. PCR requires a pure sample, specific primers, substantial work to enumerate, access to specific equipment, and expertise in molecular biology. There is no single method without limitations that fits all applications. In this study, the proposed method combines conventional culturing and biosensor methodology for rapid unaided visual detection of bacteria by using polydiacetylene as a color indicator.

Polydiacetylenes are highly conjugated polymers which consist of diacetylene-containing monomers possessing the unique chromatic property of changing color from blue to pink in response to triggering stimuli. Color change of the polydiacetylene from blue to pink arises from conformational changes in the polydiacetylene backbone. Because of this chromatic property, polydiacetylene have been widely explored as chemosensors [12] and biosensors [13]. Published reports of polydiacetylene-based biosensors are mostly applied in liquid phase [14]. There have been some reports of solid phase [15–17] and novel semi-solid phase [18,19] polydiacetylene-based biosensors with the solid phase biosensors mainly focusing on surface immobilization of polydiacetylene liposomes on substrates such as glass [17] and paper [13,15] using chemical reactions [17,20]. The current study reports a solid phase polydiacetylene sensor created by physically coating pPCDA directly on a filter surface. The objective of this study was to develop a method using a pPCDA coated filter for visual bacterial detection and quantification.

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Table 1
Bacteria population on pPCDA-coated filter and time needed for color change.

Bacteria type	Bacteria concentration (Cells ml^{-1})	Bacteria population on filter (Cells)	Bacteria density (Cells mm^{-2})	Log bacteria density log (Cells mm^{-2})	Time for color change (h)
Salmonella Typhimurium	10^8	$10^8 * 1 = 10^8$	$10^8/86.49 = 1.156*10^6$	6.06	1.5
	10^7	$10^7 * 1 = 10^7$	$10^7/86.49 = 1.156*10^5$	5.06	2.5
	10^6	$10^6 * 1 = 10^6$	$10^6/86.49 = 1.156*10^4$	4.06	4–4.5
	10^5	$10^5 * 1 = 10^5$	$10^5/86.49 = 1156$	3.06	5.5
	10^4	$10^4 * 5 = 5*10^4$	$5*10^4/86.49 = 578.1$	2.76	5.5
	10^4	$10^4 * 1 = 10^4$	$10^4/86.49 = 115.6$	2.06	6–6.5
	10^3	$10^3 * 5 = 5*10^3$	$5*10^3/86.49 = 57.81$	1.76	6.5–7
	10^3	$10^3 * 1 = 10^3$	$10^3/86.49 = 11.56$	1.06	7.5
	10^2	$10^2 * 5 = 5*10^2$	$5*10^2/86.49 = 5.781$	0.76	7.5–8.5
	PBS	0	0	NA	NA
Escherichia coli	10^8	$10^8 * 1 = 10^8$	$10^8/86.49 = 1.156*10^6$	6.06	2.5–3
	10^7	$10^7 * 1 = 10^7$	$10^7/86.49 = 1.156*10^5$	5.06	3.5–4.5
	10^6	$10^6 * 1 = 10^6$	$10^6/86.49 = 1.156*10^4$	4.06	6–7
	10^5	$10^5 * 5 = 5*10^5$	$5*10^5/86.49 = 5780$	3.76	6.5–7
	10^5	$10^5 * 1 = 10^5$	$10^5/86.49 = 1156$	3.06	8.5
	PBS	0	0	NA	NA

2. Material and methods

2.1. Materials

Mixed Whatman™ cellulose ester membrane filters (GE Healthcare Life Science, Buckinghamshire, UK) (white with grid) with pore size of 0.45 μm and a diameter of 47 mm were coated with 10,12-pentacosadiynoic acid (PCDA) (GFS Chemicals, Columbus, OH). Isopropyl alcohol (BDH, Radnor, PA) was used to dissolve PCDA and coat the filters. *Salmonella* Typhimurium 14028, *Escherichia coli* HB101, *Listeria innocua* (ATCC 33090) of various concentrations were used in this experiment. Tryptic soy agar (TSA) without dextrose was purchased from Becton Dickinson (Sparks, MD). Scotch® magic tape was used to create a square pattern on membrane filters. Ultrascan Pro spectrophotometer (Hunter Associates Laboratory Inc., Reston, VA) and a chromameter CR-400 (Konica Minolta Sensing Americas Inc., Ramsey, NJ) was used to characterize pPCDA-coated filters.

2.2. Coating PCDA on filters

PCDA was dissolved in isopropyl alcohol at a concentration of 3 mg ml^{-1}, by stirring for 1 h followed by filtering through a 0.45 μm filter to eliminate the small amount of pPCDA that is a minor impurity in the stock monomer. Removal of pPCDA would slightly reduce the concentration to less than 3 mg ml^{-1}. Thus, subsequent dilutions from this filtered solution will be slightly lower in PCDA concentration. The solution was stored at 4°*C* until use and was further diluted with isopropyl alcohol before coating on filters. A pPCDA-coated filter was created using a rubber paint-roller as previously reported to cover the whole filter and UV irradiated for 1min [21]. A blue color square area of a pPCDA-coated filter was also formed using the following procedure: A 9.3 mm by 9.3 mm square area was created using tape and this area of the filter was coated by placing 15 μl of a PCDA solution on the filter in the square followed by air-drying for 2 min then exposed to UV at 254 nm for 1 min to polymerize PCDA. To characterize and standardize the pPCDA-coated filters, filters were scanned (area view 0.190 in) using a HunterLab UltraScan® Pro dual-beam spectrophotometer.

2.3. Comparison of whole filter coating to square area coating

The whole filter and square area pPCDA-coating approaches were compared as to the time needed to respond to bacterial growth with a visual change in color. *S.* Typhimurium, approximately 10^8 cells ml^{-1} in phosphate buffered saline (PBS), was filtered through both the whole filter or the square area of pPCDA-coated filters and incubated on TSA (without dextrose) at 37°*C* for 1.5 h. The purpose of using a smaller square area rather than the whole filter was to increase the concentration of bacterial cells interacting with pPCDA with the goal of shortening the time for visual color change.

2.4. Optimizing the coating concentration

PCDA at concentrations of 0.5, 1, 2, and 3 mg ml^{-1} were coated on a 9.3 mm by 9.3 mm square area of the filters. The density of coated PCDA on the square area was estimated by multiplying the concentration of each PCDA coating solution by the volume of solution placed on the filter, then dividing this product by the surface area. A chromameter was used to characterize various concentrations of pPCDA-coated filters with CIE L*a*b* values. A *S.* Typhimurium culture of approximately 10^8 cells ml^{-1} in PBS was filtered through pPCDA-coated filters and incubated on TSA (without dextrose) at 37°*C* for 1.5 h. Color change of pPCDA coated filters at 1.5 h was recorded and evaluated to optimize the coating PCDA concentration on filters for bacteria detection.

2.5. Applying pPCDA-coated filter for bacteria quantification

Overnight cultures of *S.* Typhimurium and *E. coli* were centrifuged and washed twice with PBS and diluted to various concentrations in PBS, specifically 10^8, 10^7, 10^6, 10^5, and 10^4 cells ml^{-1}. Samples of 1 ml or 5 ml (Table 1) were filtered through 1 mg ml^{-1} pPCDA-coated square area filters drop by drop (to ensure that only the square area with pPCDA was covered) using vacuum filtration system (Fig. 3 b) and incubated at 37°*C* on TSA (without dextrose). The 1 ml samples were used to create the 10^8, 10^7, 10^6, 10^5, and 10^4 cell populations; and the 5 ml samples were used to create the $5*10^5$, $5*10^4$, $5*10^3$, $5*10^2$ cell populations. PBS without bacteria was also filtered through 1 mg ml^{-1} pPCDA-coated square patterned filters as a control group. Photos were taken every half hour with Nikon D3400 digital camera (1/40,000, F 5.6, ISO-A 18000) on black background to record color of pPCDA-coated filters.

2.6. Visual sensory panel and statistical analysis

A threshold visual panel of 20 participants was randomly selected to determine the time needed for color change of pPCDA-coated filters. Photos of square sections of pPCDA-coated filters at different incubation times were evaluated by the sensory panel for identification of the presence of pink color. An example of visual sensory survey can be found in supplementary material. Statistical analysis was performed with JMP pro 14. A linear regression model was generated to fit data with ANOVA, using R^2, residual standard error, lack of fit test and residual plot to validate the fitted model. The time for color change was also correlated to the log cell density on the filter using the proc corr command

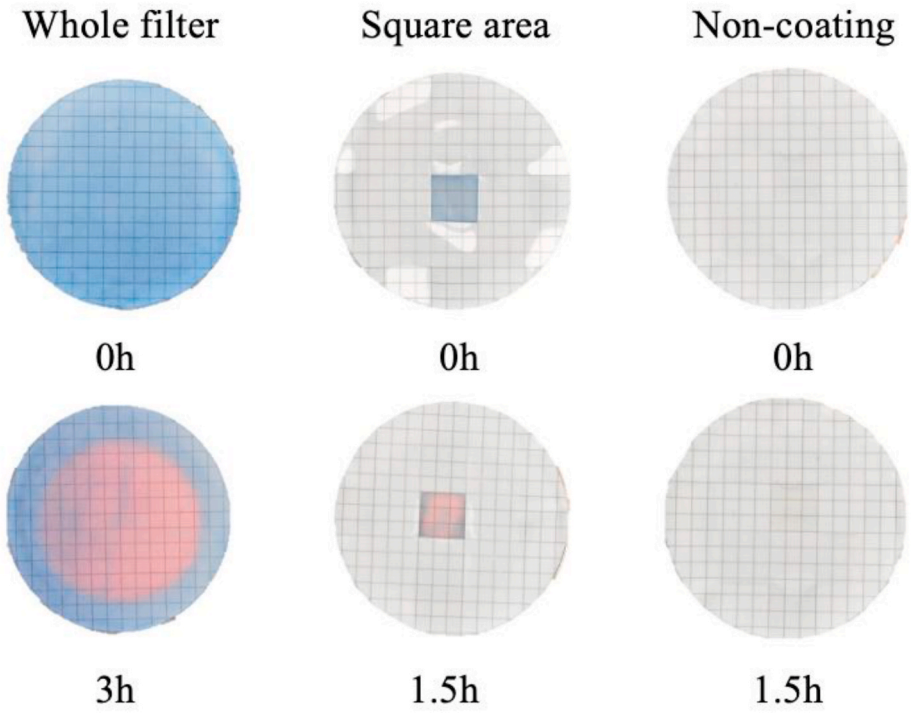


Fig. 1. Time needed for color change of pPCDA-coated filters (square area coated filters and whole filter coated filters) and non-coated filters to *Salmonella* Typhimurium. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

generating Pearson's Correlation Coefficients.

3. Results and discussion

3.1. Comparison of whole filter coating to square area coating

The size of the small square area coated on pPCDA-coated filters was A_s= 3.1 mm * 3.1 mm * 9 = 86.49 mm^2 while the filtration area of the whole filter was A_w = 3.1 mm * 3.1 mm * 100 = 961 mm^2. Thus, the small square area on the pPCDA-coated filters occupied 9% of filtration area of whole filter. As stated earlier, the rationale for using a small area for coating was to increase the number of bacteria in proximity to the pPCDA molecules to shorten the time required for a visual color change. This was accomplished since the bacterial cell density on square area of the filter was D_s ~10^8/86.49 = 1.156203*10^6 cells mm^{-2} while the bacterial cell density on whole pPCDA-coated filter was D_w = 10^8/961 = 1.04058 *10^5 cells mm^{-2}. D_s/D_w ~11.11 and therefore, the smaller square was approximately a little over 11 times more concentrated with bacterial cells than the whole filter. As expected, the time for color change of the smaller square area of pPCDA-coated filters was shorter than the pPCDA-coated whole filter, specifically 1.5 h for smaller square area and 3 h for pPCDA-coated whole filter (Fig. 1). Moreover, PCDA on the small square area can be estimated to be 15 μl * 1 mg ml^{-1} = 15 μg while quantification of the pPCDA-coated whole filter is difficult because of the different coating methods used. Estimating the amount of PCDA is helpful for consistency of pPCDA-coated filter and reproducibility of results.

Non-coated filters did not change color (Fig. 1) while the color changed for the pPCDA-coated filter from blue to pink after incubation at 37°*C* occurred after 1.5 h.

3.2. Optimizing the coating concentration

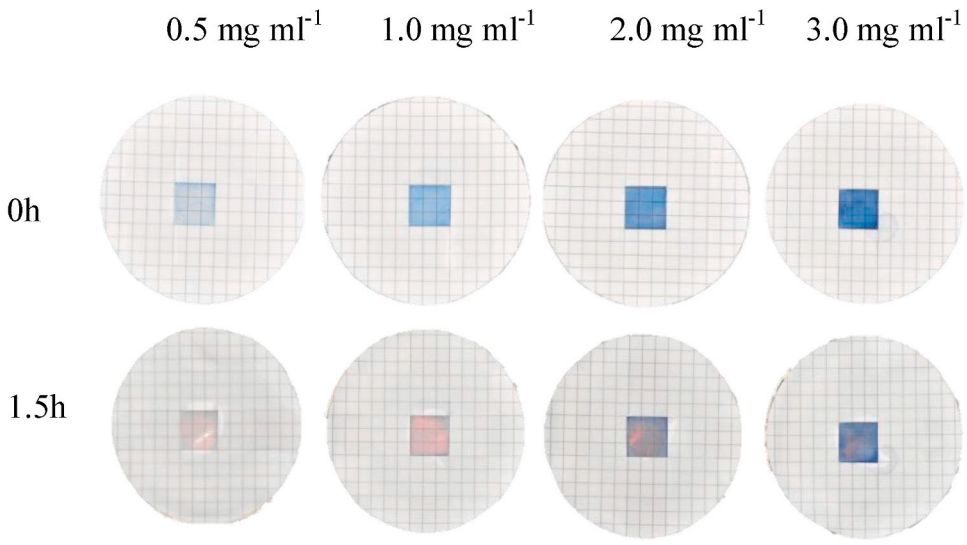
Different concentrations of PCDA on filters from 0.5 to 3.0 mg ml^{-1} were tested resulting in different intensities of blue color after UV polymerization (Fig. 2). Estimation of PCDA density on the square sections of the filters is shown in Fig. 2. Each concentration had some blue color ranging from very light for the 0.5 mg ml^{-1} PCDA to very dark for the 3 mg ml^{-1} concentration (Fig. 2 at 0 h). After bacteria were incubated on the pPCDA-coated filters for 1.5 h, all filters showed color change to some degree. However, the pPCDA concentration of 1 mg ml^{-1} showed a more intense color change from blue to pink compared to the other concentrations and therefore was selected for further testing (Fig. 2). Furthermore, reflectance to transmission spectrum of 1 mg ml^{-1} pPCDA-coated filter was measured using HunterLab UltraScan® Pro dual-beam spectrophotometer and showed in Fig. 3.

3.3. Applying pPCDA-coated filter for bacteria detection

Based on results of different pPCDA concentrations tested, 1 mg ml^{-1} pPCDA coated filter to quantify various concentrations of *S.* Typhimurium, *E. coli*, and *L. innocua* was investigated in this study. The TSA medium provides nutrients for many types of bacteria to grow but in the absence of dextrose supplement, bacteria that can utilize amino acid will decarboxylase amino acid to produce amines that in turn increase the pH of the surrounding environment. This pH increase will change the conformation of pPCDA resulting in a color change of pPCDA-coated filters from blue to pink [21]. For bacteria that cannot decarboxylate amino acids, growth will occur with no color change. As expected, *S.* Typhimurium and *E. coli* changed the color of pPCDA-coated filters from blue to pink while *L. innocua* did not produce a color change in pPCDA-coated filters despite visible colonies of *L. innocua* (Fig. 4). This observation revealed that pPCDA-coated filters can detect bacteria that decarboxylate amino acids sufficiently to increase the surrounding environmental pH.

3.4. Visual panel and data analysis

A visual panel of 20 participants evaluated the time needed to detect a color change of pPCDA-coated filters in response to various concentrations of bacteria. The minimum level of agreement to determine the time for color change was defined as ≥ 90% of the participants



PCDA amount (μg)	7.5	15	30	45
PCDA density (ug mm^{-2})	0.087	0.173	0.347	0.520
L*	81.88	72.91	62.54	56.84
a*	2.08	3.50	5.59	7.15
b*	-11.52	-19.21	-21.65	-22.57

Fig. 2. Color of various concentration pPCDA-coated filters for *Salmonella* Typhimurium detection at 0 h and 1.5 h of incubation at 37 °C. L*, a* and b* values are for 0 h samples. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

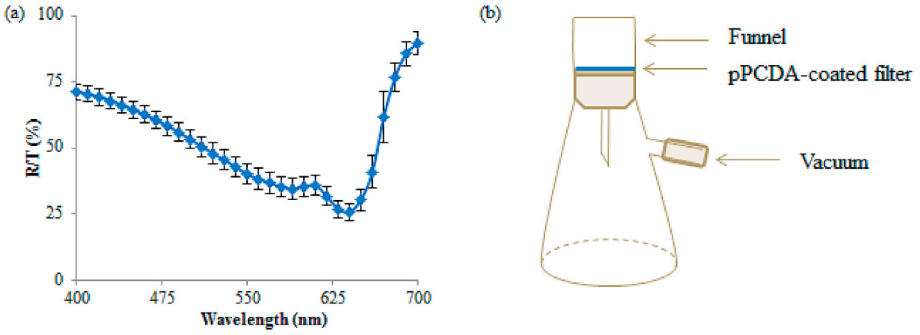


Fig. 3. a. Reflectance to transmittance spectrum of 1 mg ml^{-1} square patterned pPCDA-coated filter. b. Scheme of vacuum filtration system.

identifying the same incubation time for demonstrating sufficient color change. Color change of pPCDA-coated filter for the 10^8 cells ml^{-1} *S.* Typhimurium was clearly observed within 1.5 h which demonstrated a quick detection for high populations of *S.* Typhimurium. Color change of relatively low concentrations of bacteria such as $5*10^2$ cells ml^{-1} was observed after 8.5 h and prior to the appearance of colonies which implied that this PCDA-coated filter gave a fast response to low concentrations of *S.* Typhimurium (Table 1). For *E. coli*, color change of pPCDA-coated filter to 10^8 cells ml^{-1} bacteria was observed clearly within 2.5–3 h and 8.5 h for 10^5 cells ml^{-1} which indicated that pPCDA-coated filters fit for rapid detection of high concentration *E. coli* (Table 1). Control group of PBS was shown at the right corner of Fig. 5, which remained blue indicating no false positives from other factors that may cause color change of pPCDA-coated filters.

To determine the relationship between the time for color change with bacterial population a simple linear regression line with 95% confident interval was generated to fit data model using JMP Pro14 (Fig. 5). For both bacteria, this relationship, the ANOVA test had a p-value << 0.01 indicating the time for pPCDA-coated filter color changed was significantly related to log cell density.

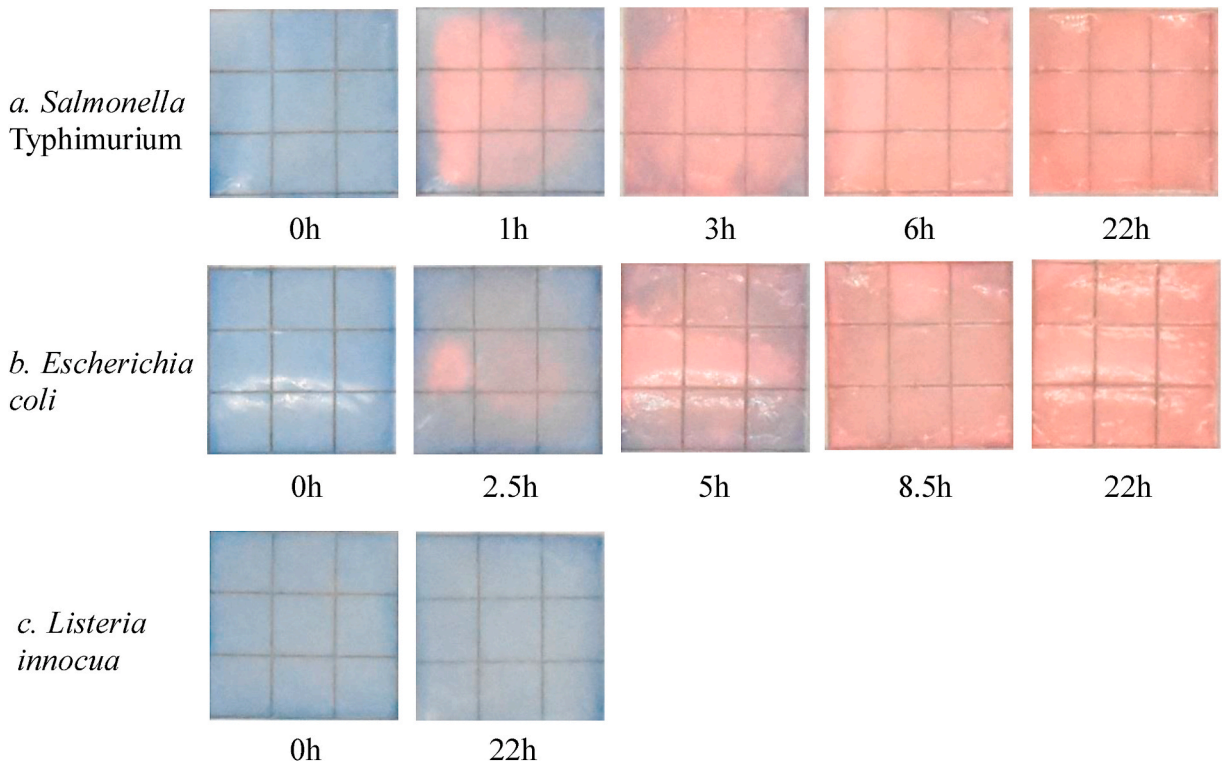


Fig. 4. pPCDA-coated filter gradually changes color from blue to pink in response to incubation with 10^8 cells ml^{-1} a. *Salmonella* Typhimurium and b. 10^8 cells ml^{-1} *Escherichia coli.* c. pPCDA-coated filter did not change color in response to incubation with 10^8 cells ml^{-1} *Listeria innocua.* (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

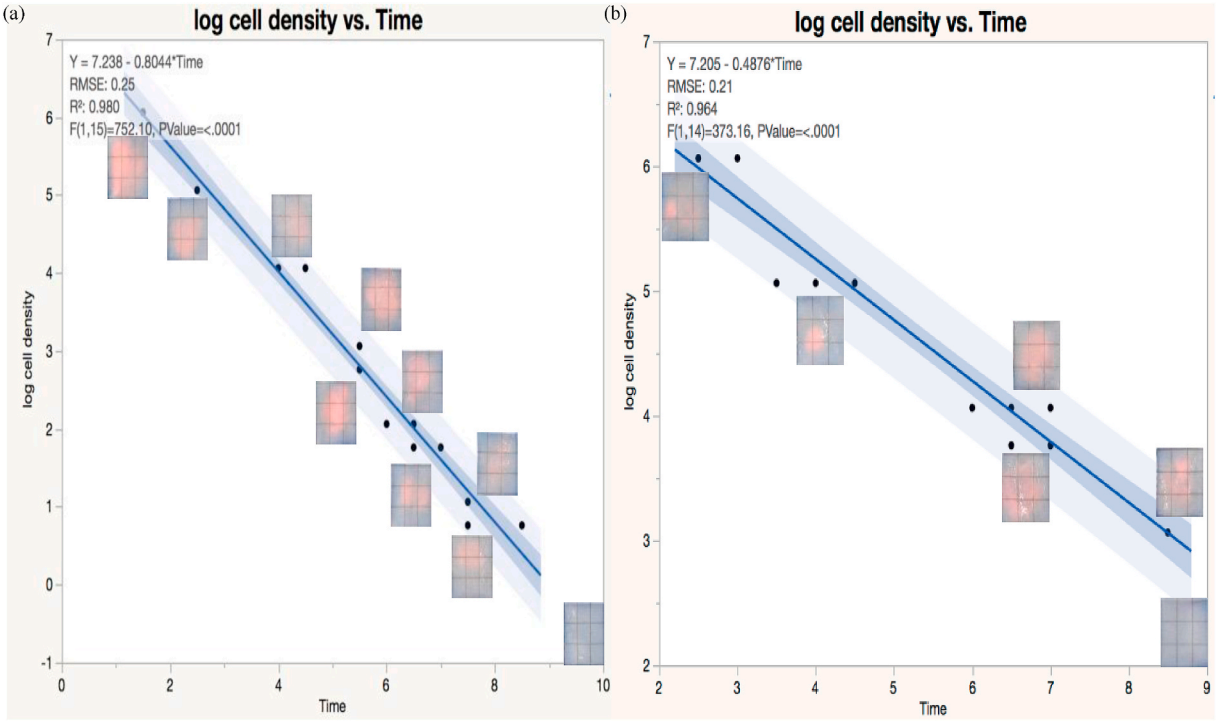


Fig. 5. Time needed for color change of pPCDA-coated filters and simple linear regression model for a. *Salmonella* Typhimurium (n = 2) b. *Escherichia coli* (n = 4). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.5. Validation of statistical model

The correlation between log cell density and time for color change was highly significant ($P < 0.0001$). Furthermore, goodness of fit was indicated by a coefficient of determination (R^2) ≥ 0.98 (*S.* Typhimurium) and 0.96 (*E. coli*), along with similar adjusted R^2 and a small mean

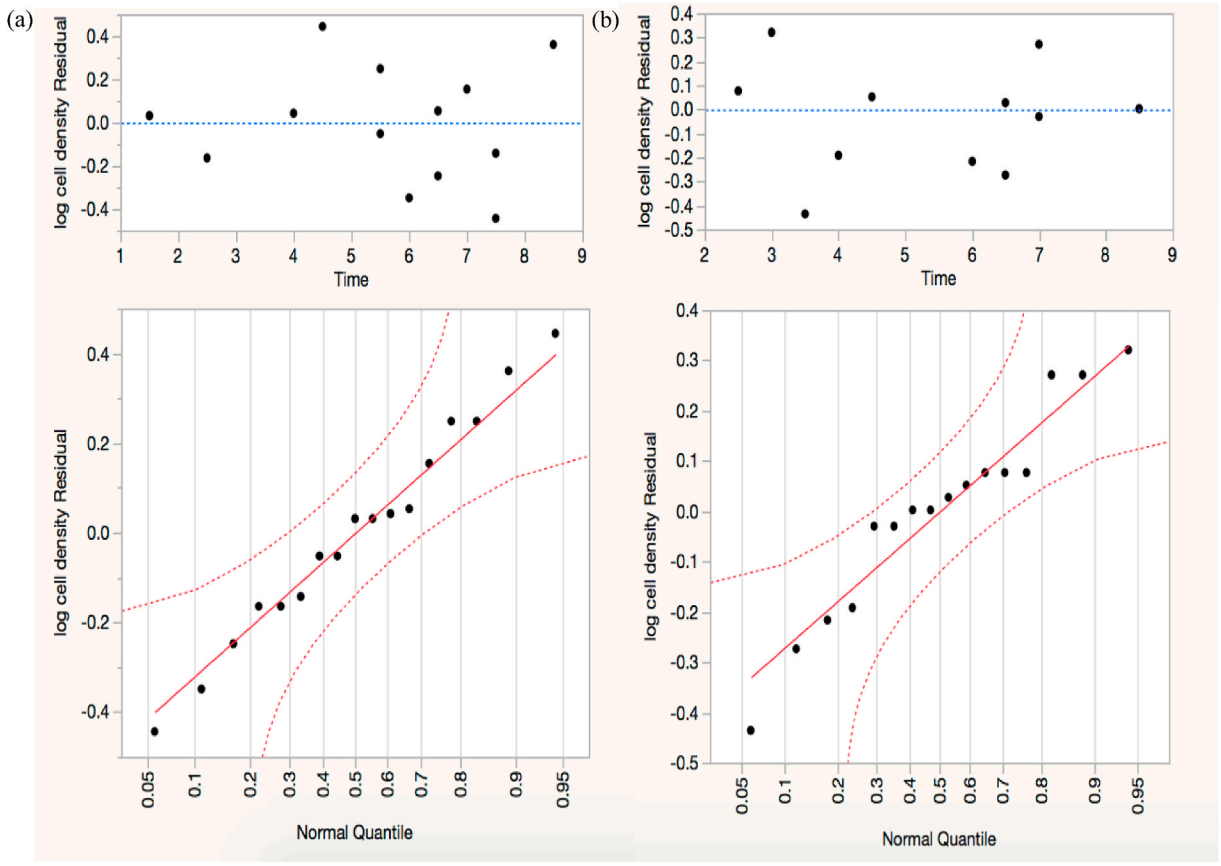


Fig. 6. Residual plot of simple linear regression model for a. *Salmonella* Typhimurium b. *Escherichia coli*.

residual standard error (RMSE) as shown in Fig. 5. Residual diagnostics were first analyzed by residual plot as shown in Fig. 6. Residual plot appears to be random and generally fit a normal distribution. Quantitative analysis of residuals was assessed using lack of fit test which revealed that both models have no lack of fit issue. These statistical tests validated the fitted simple linear regression model and revealed that this statistical model can be applied for estimation of bacteria concentration based on time observed for color change of pPCDA-coated filters.

4. Conclusion

A modified multi-layer pPCDA-coating method was proposed in this study to shorten the time for detection and improve consistency of pPCDA-coated filters. The pPCDA-coated filters changed color non-reversibly in response to *S.* Typhimurium and *E. coli* but exhibited no color change with *L. innocua*. A simple linear regression model was generated to estimate bacterial population based on time for color change of pPCDA-coated filters. Applying pPCDA-coated filter for bacterial enumeration has the following advantages: (1) less time for detection since color change is obvious and faster than the appearance of colonies; (2) direct sampling with no need to make sample dilutions since estimation of bacteria concentration was determined by time needed for color change rather than countable colonies; (3) low-cost due to visual detection since color change is obvious and relies on phase change of pPCDA rather than more expensive instruments; (4) minimized training is required with no specialized background in molecular biology. Limitations include a poor selectivity since it is based on monitoring microbial metabolism and fitted linear regression model can only be applied for prediction of well-defined samples. However, with the ability to modify PCDA molecules with specific moieties, this method may be applied to quickly detect and enumerate bacteria and inspire other pPCDA-based biosensor design in the future.

CRediT authorship contribution statement

Yueyuan Zhang: Investigation, Methodology, Validation, Visualization, Writing - original draft, Writing - review & editing. **Paul L. Dawson:** Investigation, Methodology, Validation, Writing - original draft, Writing - review & editing, Project administration, Resources, Supervision. **Timothy W. Hanks:** Methodology, Validation, Writing - original draft, Writing - review & editing. **Julie K. Northcutt:** Methodology, Validation, Writing - original draft, Writing - review & editing. **Tzuen-Rong Tzeng:** Methodology, Writing - review & editing. **William T. Pennington:** Methodology, Validation, Writing - original draft, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.

org/10.1016/j.talanta.2020.121482.

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