Label-free detection of bacterial RNA using polydiacetylene-based biochip

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ARTICLE INFO

Article history:
Received 18 November 2011
Received in revised form 27 January 2012
Accepted 30 January 2012
Available online 22 February 2012

Keywords:
Polydiacetylene
Label-free
Immobilization
Multiplex detection
Pathogenic bacteria

ABSTRACT

We developed a simple and effective polydiacetylene-based, label-free multiplex DNA chip for the detection of various pathogenic microorganisms. A novel immobilization method of PDA vesicles on glass slides was exploited using α-cyclodextrin (α-CD). The surface topography of the efficiently immobilized PDA vesicles was confirmed using scanning electron microscopy (SEM) and atomic force microscopy (AFM). Then, oligonucleotides complementary to rRNAs of three pathogenic bacteria were conjugated to the PDA vesicles. Finally, crude lysate of pathogenic bacteria was applied to the PDA biochip. The pathogenic bacteria were specifically detected by DNA–RNA hybridization in an hour. The new PDA sensor was effective in detecting multiple pathogenic bacteria easily and accurately without rigorous purification, amplification, and labeling of their genetic components.

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1. Introduction

Infectious diseases constitute a severe threat to public health. For example, infection by food-borne pathogens causes 6–33 million cases of illness, including about 5000 deaths annually, in the United States alone (Hedberg, 1999; Kodell et al., 2002). For this reason, many researchers have tried to develop methods to detect the pathogenic infections quickly and accurately. Existing methods for multiplex pathogen detection include quantitative polymerase chain reaction (PCR) assay (Welti et al., 2003), immunoassay (Tok et al., 2006) and DNA microarray (Call et al., 2003; Eom et al., 2007; Hwang and Cha, 2008). Especially, the microarray system allows high-throughput and specific identification of pathogenic bacteria (Call, 2005; Szemes et al., 2005). In DNA microarray, genes encoding virulent factors, rDNA, or intergenic regions are usually amplified and fluorescently labeled during PCR, followed by hybridization with oligonucleotide microarrays specific to various pathogens. Up to 39 pathogenic bacteria have been successfully identified by this method (Yoo et al., 2009). Otherwise, 16S rRNAs are used as targets in DNA microarray, wherein amplification by PCR is not required due to the relatively high concentration of rRNA in the cell lysate (Chandler et al., 2003; Wang et al., 2004). However, a common problem of these microarray methods is long hybridization time, about 2–16 h. Therefore, a more rapid and efficient method for labeling and hybridization is needed.

Polydiacetylene (PDA) is considered to be an attractive material for the development of label-free chemosensors based on its unique properties, including a blue-to-red color change and self-fluorescence by external forces (Jelinek and Kolusheva, 2001). PDA vesicles consisting of diacetylene monomers, including 10, 12-pentacosadiynoic acid (PCDA), form stable, liposome-like bilayer structures in aqueous solution. Closely packed and well-ordered diacetylene monomers are polymerized by UV irradiation at 254 nm via 1,4-addition reaction, which changes triple bonds to ene-yne alternated polymer chain, resulting in the formation of the backbone (Charych, 1993; Jelinek and Kolusheva, 2007). The non-fluorescent polymerized PDA is blue in color, but it exhibits red color and fluorescence after being perturbed by external stimuli, such as heat, pH, organic solvents, mechanical stress, and ligand-receptor interactions (Ahn and Kim, 2008; Ahn et al., 2009). In general, there are two types of PDA sensors; liquid-phase sensors and immobilized sensors on solid substrates (Lee et al., 2010). Liquid-phase PDA sensors are very easy to detect, as their color transition is noticeable to the naked eye (Kolusheva et al., 2000; Reichert et al., 1995). However, they cannot easily detect multiplex samples and require a considerable amount of analytes. Therefore, microarray-type immobilized PDA vesicles were developed (Kim et al., 2003, 2005b). Immobilized PDA sensors, also called PDA chips, can be detected based on changes in fluorescence. They provide a few advantages, such as high portability, low sample consumption, and high integration capability for multiplex detection. To make PDA chips, immobilization of PDA vesicles is a crucial step. Immobilization with hydrophobic or charge interactions is plagued by the loss of PDA vesicles during washing steps and decreased efficiency due to insufficient immobilization (Park et al., 2009). For efficient immobilization, PCDA derivatives have been utilized to

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doi:10.1016/j.bios.2012.01.043
implement biotin–avidin interactions or covalent bonding between succinimide and amine functional groups (Jung et al., 2006; Lee et al., 2008). However, PCDA derivatives are not widely available and are difficult to synthesize for those who are not familiar with the relevant chemical synthesis principles.

In this article, we first introduce a novel immobilization method of PDA vesicles using α–CD strongly bound with PDA vesicles (Kim et al., 2005a). This new immobilization method no longer requires PCDA derivatives to introduce functional groups onto PDA vesicles. After PDA vesicles were immobilized, label-free detection of nucleic acids by complementary binding was successfully demonstrated. Previously, detection of the hybridization between target and probe DNA using PDA vesicles was reported in a liquid phase PDA sensor, but not an immobilized one (Wang and Ma, 2005; Wang et al., 2006). For multiplex detection of pathogenic bacteria, the complementary DNA probes against specific 16S rRNA of three pathogenic bacteria were conjugated to the PDA vesicles. Then, crude cell lysate of the bacteria without rigorous purification, amplification, and labeling was hybridized to the PDA sensor. After 40 min of hybridization, the pathogenic bacteria were successfully identified by DNA–RNA hybridization.

2. Materials and methods

2.1. Preparation of polydiacetylene vesicles

The PDA vesicles using only PCDA monomers were prepared by following reported method (Kim et al., 2003). SONOPULS ultrasonic homogenizers (HD 2070, Bandelin, Germany) and a 0.8 μm syringe filter were also employed to synthesize PDA vesicles. The filtrated solution was stored at 4 °C over 12 h.

2.2. Immobilization of polydiacetylene vesicles

Succinyl-α-CD was dissolved in 10 mM phosphate-buffered saline (PBS) solution (pH 7.4) to a concentration of 1 mM. Solutions of 50 mM N-ethyl-N’-(dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 430 mM N-hydroxysuccinimide (NHS) in autoclaved DI water were made, respectively. Equal volumes of EDC and NHS solutions were mixed and added to succinyl-α-CD solution at 1:10 volume. After incubation at 4 °C for 2 h, the solution was reacted with amine-coated glass slides (Nuricell, Korea) in a CoverWell perfusion chamber (Sigma–Aldrich, USA) at room temperature for 4 h. After reaction, the substrates were washed three times with autoclaved DI water and then dried with pure N2 gas. The solution of PDA vesicles was spotted onto the substrates with α-CD using Nano-plotter v1.2 (Gesim, Großberkmannsdorf, Germany). The substrates consisted of eight frames, and 64 spots (8 × 8) were arranged for each frame. The PDA vesicles were immobilized for 30 min, and then washed and dried as above. To confirm immobilization of the PDA vesicles, we characterized the surface of the substrates by field emission scanning electron microscopy (FE-SEM; Hitachi S-4700, Japan) and atomic force microscopy (AFM; Nanosurf AG, Switzerland).

2.3. Preparation of polydiacetylene vesicles coupled with capture probes

The DNA probes for multiplex detection were synthesized from Genotech Co. Ltd. (Daejeon, Korea). The sequences of oligonucleotides synthesized as DNA probes were as follows (Hwang and Cha, 2010).

Escherichia coli DNA probe:

5’-GAAGGCACATTTCTCATCTCTGAAAAC-Amino(C7)-3’

Listeria monocytogenes DNA probe:

5’-GCATGCACACCTTTTATCATT-Amino(C7)-3’

Salmonella enteritidis DNA probe:

5’-AGGCACAAATCCATCTGGATT-C-Amino(C7)-3’

These sequences were complementary to strain-specific 16S rRNA. Solutions of 1 μM DNA probes were conjugated with PDA vesicles by adding 1/10 volume of 50 mM EDC, followed by incubation at 4 °C for 4 h. After conjugation, the substrates were washed and dried three times with autoclaved DI water and pure N2 gas. The immobilized PDA vesicles with DNA probes were polymerized by UV light at 254 nm with an intensity of 1.0 mW/cm² for 5 min.

2.4. Hybridization with synthetic RNAs

To examine the changes in fluorescence of PDA vesicles by specific DNA–RNA hybridization, two different target RNAs were synthesized from Bioneer Co. (Dea-jeon, Korea).

Matched target RNA:

5’-GUUUCAGAGAUGAUGUGCCUC-3’

Negative control RNA:

5’-GAAAGUCUCUCUCUACACCGGAAG-3’

Matched target RNA was complementary in sequence to E. coli DNA probe while negative control RNA had the same sequence as the E. coli DNA probe, but with thymine changed to uracil. The matched target RNAs at various concentrations (0, 100 pM, 1 nM, 10 nM, 100 nM, 1 μM) and 1 μM negative control RNA in MOPS buffer containing 0.02% of ProtectRNA™ RNase inhibitor (Sigma–Aldrich) were hybridized with PDA chips at 40 °C for 40 min.

2.5. Preparation of bacterial lysates

Three pathogenic bacteria, E. coli (American Type Culture Collection (ATCC 25922)), L. monocytogenes (ATCC 15313), and S. enteritidis (Institute for Fermentation (IFO 3313)), were cultivated for multiplex detection. L. monocytogenes were cultured in BHI medium at 37 °C, and E. coli and S. enteritidis were cultured in LB medium at 37 °C until the optical density (OD) at 600 nm reached 0.6–0.8. The OD values were determined by a UV-Vis spectrophotometer (UV-1800, Shimadzu, Japan). The pellets of cells were harvested by centrifugation for 5 min at 13,000 rpm, followed by washing three times with autoclaved DI water. Cell pellets were resuspended in 10 μl lysis buffer (1 M of NaOH, 0.1% of Triton X-100, 2 mM of ECTA in 20 mM Tris–HCl (pH 8.0), and 1 mg/ml of lysozyme for gram-negative bacteria or 20 mg/ml of lysozyme for gram-positive bacteria). Vortexing of the samples was performed for 2 min, followed by incubation for 5 min at room temperature. After adding 190 μl diethanol pyrocarbonate (DEPC)-treated water, 10 μl HCl solution (1 M), and 20 μl RNA fragmentation buffer (100 mM of ZnCl2 in 100 mM Tris–HCl (pH 7.5)), the bacterial lysates were incubated at 70 °C for 5 min. RNA fragmentation step was terminated by adding 20 μl EGTA (0.5 M, pH 8) and incubating at room temperature for 5 min. Then, 250 μl cell lysates was mixed with 100 μl MOPS buffer containing 0.02% of ProtectRNA™ RNase inhibitor and then immediately hybridized with the PDA chips in a chamber at 40 °C for 40 min. All experimental apparatus were used after being treated with RNase remover (TaKaRa, Japan), and all buffers were...
2.6. Scanning of fluorescence intensity and analysis

After hybridization, PDA chip for multiplex detection was washed with DEPC-treated DI water and then dried with pure N₂ gas. The fluorescence image of the chip was scanned by ArrayWoRx Biochip Reader (Applied Precision, USA) with Cy3 filter (excitation at 540 nm and emission at 595 nm). Imagene software (v.6.1 BioDiscovery, USA) was employed to analyze the changes in fluorescence intensities.

3. Results and discussion

Fig. 1 is a schematic representation of the PDA biochip capable of label-free and multiplex detection of pathogenic bacteria. First, succinyl-α-CD activated with EDC/NHS was covalently attached to the amine-coated substrate. Then, PDA vesicles were spotted and immobilized by coupling terminal carboxylic acid of PDA vesicles and α-CD on the glass substrates. Sequence-specific DNA probes were then conjugated on the surface of the PDA vesicles. Subsequently, PDA vesicles were polymerized by UV light at 254 nm. The samples of crude cell lysates containing target 16S rRNAs were placed on the PDA chip. Finally, the fluorescent signals of the PDA vesicles were monitored at 595 nm to detect the hybridization of DNA probes and target RNAs.

3.1. SEM and AFM analysis of immobilized PDA vesicles

Conventional immobilization of PDA vesicles using EDC/NHS results in the formation of unstable multilayers composed of covalent and non-covalent interactions (Park et al., 2009). The formation of an unstable multilayer makes it more difficult to conjugate probes onto PDA vesicles. Further, it is difficult to reduce the loss of PDA vesicles during washing. Therefore, we developed a new method for the immobilization of PDA vesicles using α-CD, a strong conjugator with terminal group of PDA vesicles. The performance of the new immobilization method was compared with that of the conventional covalent immobilization method by observing surface topography using SEM and AFM. Fig. 2a and c are the SEM and AFM images, respectively, of PDA vesicles immobilized by the conventional method, whereas Fig. 2b and d are those of the new method using α-CD. All images were taken after washing three times with DI water. From the SEM and AFM analysis, we demonstrated that PDA vesicles were much more stably immobilized by the new method than the conventional method. In another SEM image with 10,000 × magnification, the diameters of PDA vesicles were about 100–200 nm (data not shown). This was in accordance with the height of immobilized PDA vesicles as measured by three-dimensional AFM (Fig. 2d), suggesting that the majority of the immobilized PDA vesicles formed a monolayer on the solid substrates. In the SEM image in Fig. 2b, several PDA vesicles were clustered and immobilized on the substrate. Moreover, we could reduce the immobilization time to within 30 min since α-CD was promptly and strongly coupled with the terminal carboxyl groups of the PDA vesicles (Kim et al., 2005a). From these analyses, we confirmed that the new immobilization method using α-CD was highly effective to fabricate a PDA sensor chip.

3.2. PDA-based biochip with new immobilization method

The feasibility of detecting specific targets without loss of the unique characteristics of PDA vesicles was investigated by reacting with three different types of cyclodextrins, α, β, and γ-CDs. First, the immobilized PDA vesicles were polymerized by UV light at 254 nm for 5 min. Then, the three cyclodextrins were dissolved in DI water to a concentration of 10 mM (pH 7.0), followed by incubation with PDA chip. The first row of Fig. 3a and b shows the fluorescent images and their average intensities, respectively, after incubation with the three different types of cyclodextrins for 20 min. As expected, only α-CD was bound to the PDA vesicles and elevated their fluorescence levels. The second row of Fig. 3a and c show the fluorescent images and intensities, respectively, of the same PDA chips after heating at 110°C for 5 min to confirm the presence of PDA vesicles. Based on these results, we proved that the PDA chip immobilized by the new method did not lose its property as a label-free sensor.
Fig. 2. SEM images (a and b) and three-dimensional AFM images (c and d) of immobilized PDA vesicles on solid substrates. (a and c) The immobilized PDA vesicles with EDC/NHS method. (b and d) The immobilized PDA vesicles with succinyl-α-cyclodextrins.

Fig. 3. (a) Fluorescent images of the immobilized PDA vesicles after incubation with three different cyclodextrins. (b) Comparison of fluorescence intensities of the images after 20 min incubation at room temperature. (c) Fluorescence intensities after heating the PDA chips at 110°C for 5 min.

Fig. 4. Fluorescence intensities of the immobilized PDA vesicles after incubation with different concentrations (0 pM, 100 pM, 1 nM, 10 nM, 100 nM, 1 μM) of target or control RNAs at 40°C for 40 min. Control represents the experiment with no DNA probe conjugation on PDA chip.

3.3. Detection of nucleic acid hybridization with PDA chip

Next, we demonstrated the feasibility of PDA chip for detecting DNA–RNA hybridization. In Fig. 4, a control experiment was conducted in the absence of DNA probe on PDA vesicles in order to investigate the effect of the RNA target solution on fluorescence change. All other experiments were performed after conjugating *E. coli* probe DNA on PDA vesicles as previously described. After polymerization by UV light, various concentrations of the matched target RNA were hybridized. Finally, 1 μM of mismatched control RNA was also examined as a negative control. Fig. 4 shows successful detection of DNA–RNA hybridization and a positive correlation of the target concentrations with fluorescence intensities. The optimal hybridization time and temperature that can minimize RNA degradation and prevent the formation of secondary rRNA structures was determined to be 40 min and 40°C, respectively, after repeated experiments. The limit of detection (LOD) of RNA was estimated to be about 100 pM.
trigger fluorescence changes of PDA vesicles (see Supplementary Fig. S1). Indeed, none of the components in the lysis buffer stimulated PDA vesicles, but a pH higher than 9 did. However, the lysis buffer neutralized by adding HCl in DEPC water did not stimulate color or fluorescence changes. Further, 0.5 M ethyleneglycoltetraacetic acid (EGTA) instead of ethylenediaminetetraacetic acid (EDTA) was used to terminate the reaction of RNA fragmentation since EGTA exerts a much weaker influence on PDA vesicles according to our previous study (Park et al., 2011). As a result, cell lysis and hybridization solution was successfully optimized to not stimulate PDA vesicles.

Fig. 5a represents a chip design for multiplex detection. Immobilized PDA vesicles in each frame were conjugated with DNA probes against different target bacteria as follows: frame 1, 2 (the absence of the DNA probes), frame 3, 8 (DNA probe for E. coli), frame 4, 6 (DNA probe for L. monocytogenes), and frame 5, 7 (DNA probe for S. enteritidis).

Next, the target RNA in crude cell lysate from each strain was prepared as previously described in materials and methods. Frames 1, 3, 4 were incubated with crude cell lysate of L. monocytogenes at 40 °C for 40 min. Similarly, those of S. enteritidis and E. coli were reacted with frames 2, 5, 6 and frames 7, 8, respectively. No fluorescence was observed in frames 1, 2 treated with L. monocytogenes and S. enteritidis due to the absence of any DNA probes. In the first row of Fig. 5b, increased fluorescence intensities were clearly observed in frames 4, 5, 8, when the targets were matched with the probes. Although slight non-specific signals were detected in frames 3, 6, 7, the levels of fluorescence were much lower than the matched frames. The fluorescence intensities of each frame in the first image of Fig. 5b were calculated and presented in Fig. 5c. A similar result was obtained when cell lysates were hybridized in different frames (see Supplementary Fig. S2). We also performed the same experiments with RNA from 10^1–10^6 CFU/ml bacterial samples and the detection limit of this method was determined as 10^2–10^3 CFU/ml (see Supplementary Fig. S3). By using PDA vesicles, we successfully eliminated the labeling step in the DNA microarray procedure and decreased the hybridization time to 40 min.

4. Conclusion

We developed a PDA-based biochip for the detection of pathogenic bacteria. A new immobilization method without modification of the terminal group of PCDA was successfully developed with α-CD. This new immobilization method overcame the limitation of previous immobilization methods, such as loss of PDA vesicles during washing step. After conjugated with DNA, the PDA chip was successfully employed as a DNA chip by hybridization with complementary RNA. Finally, pathogenic strain-specific DNA probes were conjugated with the PDA vesicles, and the bacterial total RNAs in crude cell lysates were hybridized without purification or amplification. Label-free detection of the bacteria was successfully demonstrated using PDA biochip by direct hybridization between nucleic acids having complementary sequences. A simple and reliable detection method of pathogens was successfully developed with this PDA biochip.

Acknowledgments

This work was supported by the Seoul R&BD Program (10920) and by a National Research Foundation of Korea (NRF) grant funded by MEST (Nos. K20903001812–11E0100–017H00, 20110016488, 2011K000955) and MKE (10033477-2011-13) of the Korea government. We would like to thank Prof. Hyung Joon Cha and Dr. Byeong-Hee Hwang at POSTECH, Korea, for helps in the experimental protocols.
Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2012.01.043.

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Nucleic Acids Res. 33, e70.