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Detection of *P. aeruginosa* using nano-structured electrode-separated piezoelectric DNA biosensor

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Abstract

A DNA biosensor for detection of *Pseudomonas aeruginosa* was set up based on the modification of two membranes (nano-TiO_2 and nano-TiO_2–polyethylene glycol hybrid membrane) to the ESPS surface. These two membrane materials were synthesized by sol–gel method. The detection was accomplished by modifying ss-DNA on the sensitive membrane and then hybridizing with their complementary strands from the *P. aeruginosa* in liquid phase. UV spectrum was used to identify the purity and concentration of extracted DNA; IR spectrum and SEM were used to characterize the properties of the membrane. The detection was highly improved by adoption of nanotechnology and hybrid membrane. Less than 3 h was sufficient. The detection linear range was from 10^{-1} to 10^{-3} g l^{-1} and the limit of detection was 10^{-4} g l^{-1}.

Keywords: Nano-TiO_2 membrane; Hybrid membrane; DNA biosensor; ESPS electrode; Hybridization; *P. aeruginosa*

1. Introduction

Pseudomonas aeruginosa had been shown to cause food poisoning or enteric infections to human, especially to babies, sometimes with fatal consequences. *P. aeruginosa* might cause severe and fatal infections to immunodeficient hosts such as trauma and burn victims, patients with long time lung disease and cystic fibrosis [1–4]. This cell consisted of an outer cell wall membrane, an electron transparent space and the cytoplasmic membrane. When cultured in vitro, the biofilm [5] was formed. Drugs effect these bacteria by crossing an ordered and crossing-linked "mat" of polysaccharide chains. And also, *P. aeruginosa* mutated easily, especially in anti-drug mutation. It could also cause anti-engulfing, anti-chemotaxis action. Therefore, great difficulties existed in diagnosis and controlling this disease.

Methods which used to detect *P. aeruginosa* were Gram-stained, incubation and biochemistry reaction identification, blood serum identification, ELISA [6], antigen-antibody reaction method [7] and so on. For Gram-stained method, it could not distinguish from other Gram-negative bacillus, merely given a primary estimation. For incubation and biochemistry reaction method, it made identification by morphology, pigment, particular ginger odor, and oxide enzymatic experiment and cytopigment oxide enzymatic experiment. But it was not suit to those *P. aeruginosa* which gave off non-typical odor. For antigen-antibody reaction method, it was involved in many studies. Generally speaking, experiment was always done on living organisms, so it could cause harm to researchers. For all, they were not only time consuming, but also relatively bad accuracy and low detection limit. Even for the most sensitive quartz crystal microbalance (QCM), the detection limit only reaching to 100,000 cells ml^{-1} [8].

In the 1990s, the expanding availability of nano-particles have attracted wide-spread attention [9]. Nano-particles possess of chemical properties distinguished from either extended bulk solids or single molecules. More noteworthy are the surface charge, high surface area and biocompatibility [10]. Nano-sized membranes have approximately 1 or 2 orders of magnitude more surface area than that found in continuous thin films. It is expected that this large amount of available surface area-to-volume ratio has the potential to provide unusually high sensitivity and fast response time in sensing applications [11].

In this paper, two kinds of nano-structured electrode-separated piezoelectric sensor (ESPS) sensors were set up

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to detect *P. aeruginosa*. Two kinds of sensitive modified membrane: nano-TiO_2 and nano-TiO_2–polyethylene glycol hybrid membrane were synthesized using sol–gel method. Probe ss-DNA was immobilized on the two sensitive membrane. Detection was based on the hybridization reaction between ss-DNA and sample DNA. The detection limit can be down to 10^{-4} g l^{-1}.

2. Experimental

2.1. Materials

A source DNA was extracted from the culture of attenuated *P. aeruginosa* obtained from Institute of Life, Hunan Normal University. Attenuated organisms were incubated into a solid Lowenstein–Jenson medium for 18–24 h at 37 °C, and then picked out for extracting. All reagents and apparatus involved were sterilized at 121 °C for 20 min. All operations were done in super-clean platform. DNA extracted was stored in refrigerator at 4 °C.

- NAC culture medium: peptone 20 g, K_2HPO_4 0.3 g, $MgSO_4$ 0.3 g, cetrimide 0.2 g, nalidixic acid 15 mg, agar 15 g and distilled water 1000 ml, pH 7.4.
- Saline–EDTA solution (used to dilute bacteria): 0.15 mol l^{-1} NaCl + 0.1 mol l^{-1} EDTA, pH 8.0.
- DNA extraction solution: phenol–chloroform–isoamylol (v/v 25:24:1).
- DNA storing solution: 1 mol l^{-1} NaCl; denaturing solution: 1.5 mol l^{-1} NaCl + 0.5 mol l^{-1} NaOH.
- DNA immobilization solution: 0.05 mol l^{-1} NaCl.
- DNA hybridization solution: 10 mmol l^{-1} Tris–HCl + 0.05 mol l^{-1} NaCl buffer solution, pH 7.9.
- Solution for recovering of membrane: TE buffer solution (0.02 mol l^{-1} Tris–HCl + 1 mmol l^{-1} EDTA, pH 7.4).
- Solution for recovering of electrode: 1 mol l^{-1} HCl.
- The probe and target ss-DNA for detection were all self-extracted.
- *P. aeruginosa* sample was got from sewage treatment plant in hospital.
- All chemical reagents used were analytical pure grade, and double distilled water was used.

2.2. Apparatus

The quartz crystal used in this work was 9 MHz AT cut, silver-coated electrodes (Beijing 707 factory). The measuring system consisted of a transistor–transistor logic (TTL) circuit and a frequency counter, which was controlled by a PC computer (self-made).

Membranes were synthesized by sol–gel method at room temperature, and a magnetic stirring machine was employed during the whole process.

Otherwise, UV spectrum was used to identify the purity and concentration of the extracting DNA. IR spectrum and SEM were used to characterize the synthesizing membrane.

2.3. Procedure

The detection procedure basically consisted of five steps: extraction of whole chromosomal DNA, denaturation, synthesis of sensitive modified membranes, preparation of probe, and detection of *P. aeruginosa* (including real sample detection).

2.3.1. Extraction of whole chromosomal DNA [12]

The bacteria was picked out from solid incubation medium, then diluted with saline–EDTA solution to 1.5 ml, injected into the Eppendorf centrifuge tube, centrifuged for 30 s at 10,000 rpm. Then repeated for three times. Discarded the upper solution, added 200 ul saline–EDTA solution, blended thoroughly to get suspending solution. Added 5 ul 20% SDS, mixed thoroughly, heated 5 min at 60 °C, transparent solution was got. After that, 400 ul of phenol–chloroform–isoamylol (v/v 25:24:1) was added, and span 5 min at 10,000 rpm to get a even solution. Transferred upper solution to a fresh microcentrifuge tube (attention!), added 400 ul icy alcohol (70%) at room temperature, centrifuged 3 min at 10,000 rpm. Discarded the upper solution, added 200 ul 0.25 mol l^{-1} NaCl + 50 mmol l^{-1} Tris buffer solution to completely dissolve DNA. Followed by adding 400 ul icy alcohol to reprecipitate DNA. Centrifuged 3 min at 10,000 rpm. Threw away the upper solution and added 500 ul 70% alcohol. Shaking or reversing centrifuge tube for some times, followed by centrifuging 1 min at 10,000 rpm. Abandoned alcohol, then dried and dissolved in 1 mol l^{-1} NaCl. Stored at 4 °C for following steps.

2.3.2. Denaturation of DNA [13]

Denatured DNA was produced by adding 1.5 mol l^{-1} NaCl and 0.5 mol l^{-1} NaOH solution to DNA solution prepared above. After an hour, heated the solution in a water bath at 100 °C for 4 min. Then rapid cooled in an ice bath to 0–4 °c, stored at that temperature for use.

2.3.3. Synthesis of sensitive modified membrane

2.3.3.1. Synthesis of nano-TiO_2 membrane [14]. Dissolved 3.5 ml $Ti(OBu)_4$ in 0.3 ml acetic acid, then adding 12 ml alcohol, marked solution A. Solution B was composed of 40 ul condensed HCl, 0.5 ml water, 80 ul DMF and 12 ml alcohol. Added solution B to solution A drop by drop, stirring thoroughly for 30 min, an stable and transparent solution was achieved. Placed at room temperature or controlled temperature till the gelatin was formed.

2.3.3.2. Synthesis of TiO_2–polyethylene glycol hybrid membrane. The preparation process was similar to

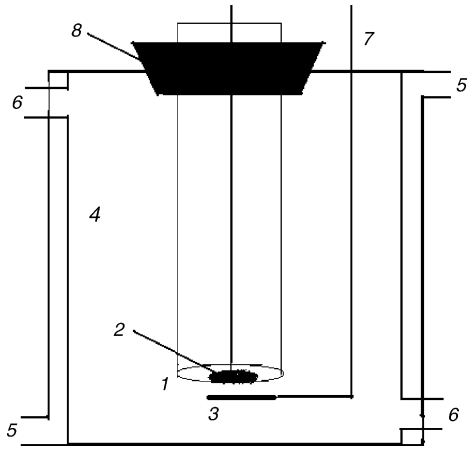

Fig. 1. Configuration of electrode-separated piezoelectric crystal sensor. 1: piezoelectric quartz crystal; 2: Ag electrode of PQC; 3: separated electrode; 4: detection cell; 5: thermostated water jacket; 6: solution drain; 7: leading wire; 8: rubber stopper.

Section 2.3.3.1. At first, dissolved polyethylene glycol into the solution B, then did as stated in Section 2.3.3.1.

2.3.4. Preparation of probe DNA of P. aeruginosa

2.3.4.1. Modification of quartz crystal. One side of the silver electrode was removed by 6 mol l^{-1} HNO_3. Washed with ultrasonic for 1 h, then with organic solvents and double-distilled water, dried for use. Planted it into the self-made cell as shown in Fig. 1. A quartz crystal disc was mounted at one end of the tube. One face of the sensor without silver electrode was exposed to test solution, and the other face of the crystal was in atmosphere. Read the frequency F_0. The solution was then drained off. Dried the cell and electrode. Modified the membrane materials onto crystal by "dip-coating" method, dried for some hours lower than 100 °C. After overnight at room temperature, washed with corresponding solvent and double-distilled water, dried, and read frequency F_1 in 0.05 mol l^{-1} NaCl after 5–20 min. $\Delta F = F_0 - F_1$, ΔF was related to the coated membrane amount.

2.3.4.2. Immobilization of ss-DNA. Different concentration of ss-DNA was directly injected into the cell. After the frequency value did not change with time, drained off the solution. Washed with double-distilled water after standing for 24 h. Now the *P. aeruginosa* DNA sensor was ready for use.

2.3.5. Detection of P. aeruginosa

Added the whole chromosomal DNA target of different concentration (diluting with 10 mM Tris–HCl, 0.05 M NaCl, pH 7.9 buffer solution) into the detection cell, hybridized with well-prepared DNA sensor at 30 °C for 2 h. Read the frequency F_1 every 3 min, respectively. When without target ss-DNA, read F_0, then calculated the frequency shift ΔF ($\Delta F = F_0 - F_1$).

2.3.6. Detection of P. aeruginosa in sewage treatment plant

Preparation of sample [15]: took 300 ml sewage (without sterilizing) from sewage treatment plant in hospital. Added solid $CaCl_2$ to 1 mmol l^{-1}; inoculated five droplets on the NAC culture medium for 18–24 h at 37 °C; followed by DNA extraction and other successive operation. Here, probe DNA was extracted from attenuated *P. aeruginosa* organisms, and target DNA was extracted from sample.

3. Results and discussion

3.1. Identification of purity and concentration of DNA

When set up DNA sensor, high purity extracted DNA was needed. UV spectrum was used to identify the purity and concentration of DNA. According to absorption curve [16], if $A_{260}/A_{280} = 1.8$, pure DNA; if $A_{260}/A_{280} > 1.8$, RNA contamination and $A_{260}/A_{280} < 1.8$, protein in the sample. The maximum absorb wavelength of nucleic acid was 260 nm, the concentration of nucleic acid solution could be found by detecting the absorbance at 260 nm [17], for ds-DNA, $1A_{260} = 50$ ug ml^{-1}; for ss-DNA, $1A_{260} = 33$ ug ml^{-1}. Nucleic acid concentration was calculated by equation C (ug ml^{-1}) = A_{260} × diluting times × 50 (or 33). Fig. 2 was the UV spectrum of 10 times diluted extracted ss-DNA. In the curve, the maximum absorbent wavelength was at 258.5 nm, $A_{258.5} = 1.354$; $A_{260} = 1.349$; $A_{280} = 0.762$. $A_{260}/A_{280} \approx 1.8$ showed be pure DNA and $C = 4.45 \times 10^{-1}$ g l^{-1}.

3.2. Studies on the properties of the membrane

A piezoelectric quartz crystal (PQC) was usually called a quartz crystal microbalance and obeyed the Sauerbrey equation [18] only if the PQC held for a gas-phase and the crystal surface coating was rigid and of negligible thickness compared to that of the crystal itself. Usually, a PQC is configured with electrode on both sides of a thin disc of AT-cut quartz. Mo et al. [19] reported a type of electrode-separated piezoelectric sensor. The electrodes on the surface of the crystal in the ESPS were separated by a liquid layer of a few tenths of a millimeter, and the high-frequency electric field was applied to the quartz disc through the liquid layers. The mass response sensitivity of the ESPS is the same as that of a normal PQC. As coating was on the crystal surface, not on Ag electrode surface, so the most remarkable virtue of ESPS was that Ag corrosion by coating materials can be avoided. In this paper, ESPS sensor was chosen.

IR spectrum of membranes TiO_2 and TiO_2–polyethylene glycol as well as membranes after binding DNA were as shown in Fig. 3A and B correspondingly. In Fig. 3A, curve I was obtained by TiO_2 and curve II was obtained by membrane which binded DNA. 1021.85 cm^{-1} was typical peak for Ti–O–Ti, and 3383.62 cm^{-1} was stretching vibration of O–H. While for curve II, new peaks at 1121.32 cm^{-1} and

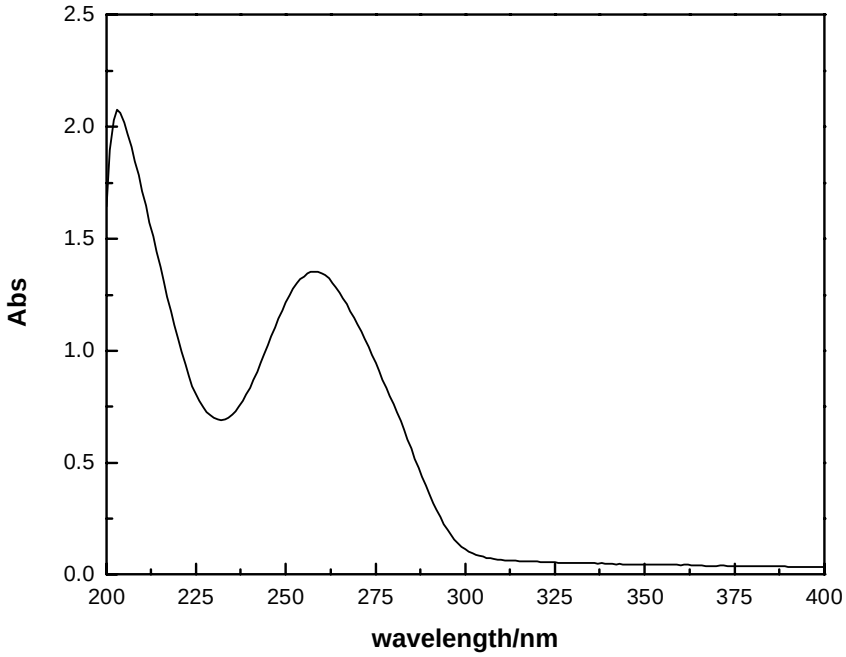

Fig. 2. UV spectrum for extracted DNA.

2924.97 cm^{-1} appeared. They were for P–O–C and N–H. 1022.43 cm^{-1} was still for Ti–O–Ti. In Fig. 3B, curve I was obtained by TiO_2–polyethylene glycol and curve II obtained by this membrane binding with DNA. 1109.01 cm^{-1} was absorbent vibration of Ti–O–C, and 3383.91 cm^{-1} was expand and contract vibration of O–H for polymer alcohol. Other peaks for $–CH_2–$ and $–CH_3$ also appeared. In curve II, another two new peaks at 1103.22 cm^{-1} and 2928.26 cm^{-1} also appeared. They were for P–O–C and N–H. These results showed that: (1) two different kinds of membrane were got (I line in curve A and B, respectively); (2) DNA bind with two membranes, and belonged to chemical absorbent. Because a new linkage of P–O–C was formed.

Before and after binding DNA, the vibration peaks were slightly moved to lower wave number because of the introduction of DNA. Lowered down the density of electron clouds of the polymer molecular chain and force constant between atoms declined.

For nano-TiO_2 membrane when it was dipped into the detection cell, water reacted with it and a lot of O–H was formed on its surface [20]. Phosphoric acid group at 5′ end of the ss-DNA reacted with O–H and formed phospholipid linkage. The ss-DNA covalently and tightly bound to the surface of electrode. For hybrid membrane, binding mechanism was the same as nano-TiO_2 membrane. The difference was that O–H group was highly increased by introduction of polyethylene glycol and binding sites with ss-DNA was also increased.

SEM was also used to see the image of the membrane. It was shown in Fig. 4. Fig. 4a was for nano-TiO_2, while Fig. 4b was nano-TiO_2–polyethylene glycol. In Fig. 4a, particles were impacted tightly, single particle was seldom seen. In Fig. 4b, the white global particles were TiO_2 encapsulated by polyethylene glycol. Obviously, the sizes of particles were largely increased. Consequently, introduction of polyethylene glycol into TiO_2, the inorganic phase was encapsulated by organic phase, and bigger ostensible size of particles was

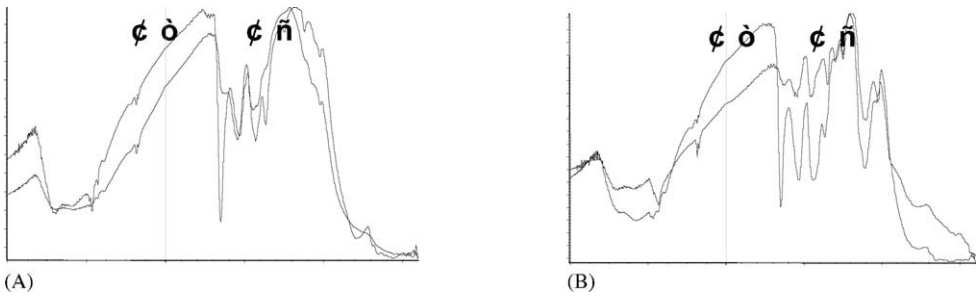

Fig. 3. Corresponding IR spectrum for nano-sized TiO_2 (A) and nano-TiO_2–polyethylene glycol (B) membrane and their reaction product with DNA. In A, I line for nano-sized TiO_2, II line for its reaction product; in B, I line for nano-TiO_2–polyethylene glycol, II line for its reaction product.

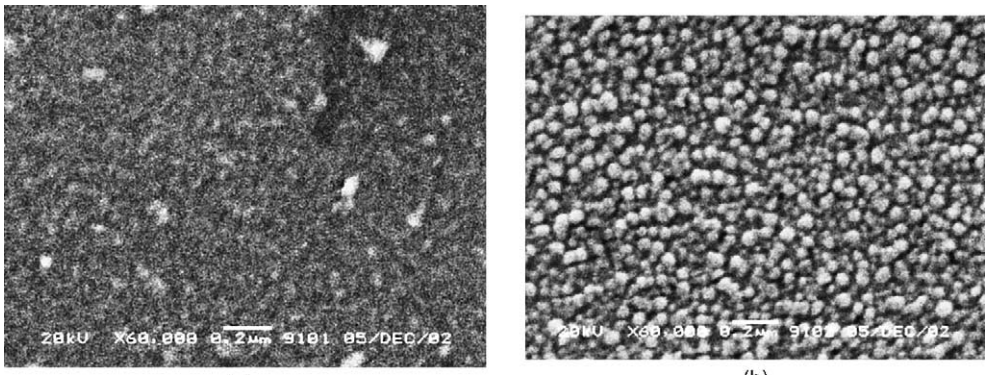

Fig. 4. SEM photograph for nano-TiO_2 (a) and nano-TiO_2–glythelene glycol hybrid membrane (b).

formed. The introduction of TiO_2 particles changed the original network space structure of organic polymer.

The stability of the membrane would affect the lifetime of the sensor. Some experiments were run to check the membrane stability. Socked the electrode in acidic, neutral and alkaline solutions for 15 min, 1, 3, 6, 24 h, 2 days, 1 week, and some weeks. The results were shown in Table 1. It can be seen that the two kinds of membrane were very stable in neutral and alkaline solutions. But, in acidic solution, they were not stable. After 2–3 h, some parts of the membrane would be peeled off. If together with a heating, the erosion velocity was speed up. Just this, we chose 1 mol l^{-1} HCl to recover the crystal surface.

3.3. Detection of *P. aeruginosa*

The nano-constructed *P. aeruginosa* DNA sensor was used to detect the *P. aeruginosa* DNA concentration based on the Sauerbrey equation. Immobilized fixed amount of ss-DNA on the membrane, then hybridized with complementary ss-DNA in liquid phase. Fig. 5 was the real-time frequency-response curves, at different target DNA concentrations (1: 0.445 g l^{-1}; 2: 0.0089 g l^{-1}; 3: 0.00445 g l^{-1}; 4: 0.00223 g l^{-1}; 5: 0.00148 g l^{-1}; 6: 0 g l^{-1}). Fig. 5A was DNA binding on hybrid membrane (frequency shift was 750 Hz), Fig. 5B for DNA binding on nano-TiO_2 membrane (frequency shift was 4000 Hz). From the picture we can see that the hybridization time was merely 1 h or so. Obviously, the sensitivity for hybrid membrane was much higher. Although probe DNA binding on nano-TiO_2 was more five times than on hybrid membrane, the maximum hybridization amount was approximately the same.

Table 1
Results obtained by examining the stability of membrane in acidic, neutral and alkaline solution

Time	Solution		
	Acidic (Hz)	Neutral (Hz)	Alkaline (Hz)
15 min	82	2	0
1 h	346	17	6
3 h	750	30	12
6 h	4000	47	23
24 h	–	67	31
2 days	–	83	42
1 week	–	98	49
4 weeks	–	103	53

The relationship between the mass change on the crystal surface (Δm) and the response of frequency shift (ΔF) can be explained with Sauerbrey equation [21]:

$$\Delta F = -\frac{F_0^2}{A} \times (\rho_q \times \mu_q)^{-1/2} \times \Delta m$$

where ΔF is the frequency change of the coated crystal, F_0 the resonant frequency of the crystal, A the electrode surface area of the crystal that is coated with material, Δm the mass change due to the deposited coating, ρ_q the density of quartz crystal and μ_q is the shear modulus of quartz crystal. "−" indicates that the addition of mass to the surface of the piezoelectric sensor results in a lower oscillating frequency. Among them, F_0, A, ρ_q, μ_q were constant. So, there is a linear relationship between ΔF and Δm. When DNA sensor hybridized with complementary DNA in solution, the mass of hybridizing on the sensor surface was dominantly dependent on the concentration of DNA in solution. Detect ΔF of different concentration and shown in Fig. 6.

The linearity equation is $\Delta F = 8.1972 \times 10^4 C_{DNA} + 18.4879$ (A), $R = 0.9687$; and $\Delta F = 9.5165 \times 10^3 C_{DNA} + 30.7298$ (B), $R = 0.9238$, respectively. The detection limit is 10^{-4} g l^{-1} with a working range of 10^{-1} to 10^{-3} g l^{-1}. If the concentration went on rising, the linear relationship between ΔF and C_{DNA} would disappear because of its hybridizing saturation for DNA sensor.

Theoretically, ss-DNA in solution complementary to DNA sensor could be detected. But in practice, non-complementary strand response also might be detected in owing to non-specific binding. But according to dynamics, the latter was far behind the former, that is, using DNA sensor to detect complementary and non-complementary ss-DNA, the response signal was

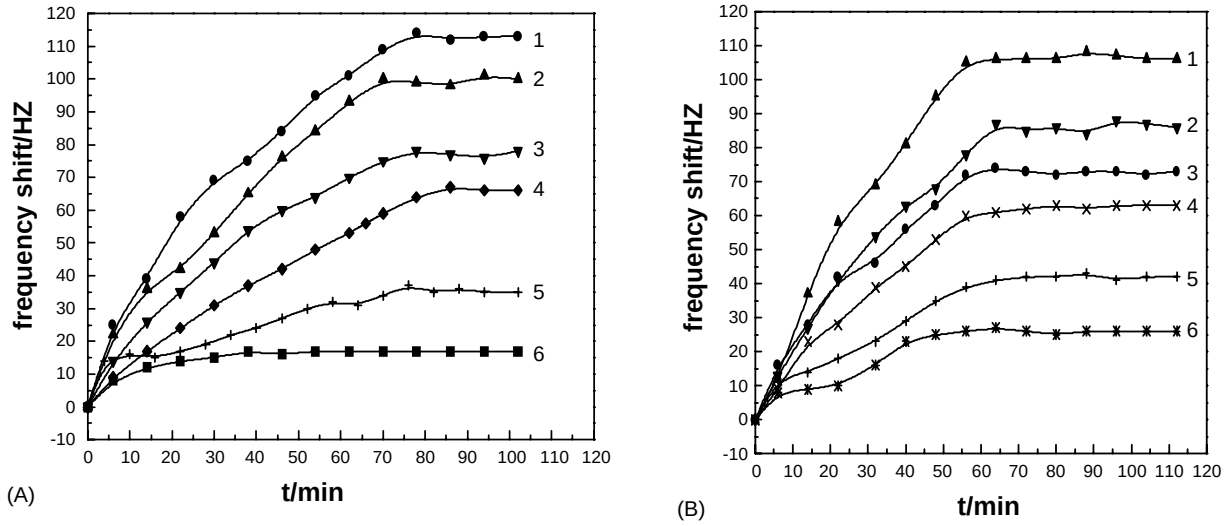

Fig. 5. Real-time frequency-response curves, at different target ss-DNA concentrations (1: 0.445 g l^{-1}; 2: 0.0089 g l^{-1}; 3: 0.00445 g l^{-1}; 4: 0.00223 g l^{-1}; 5: 0.00148 g l^{-1}; 6: 0 g l^{-1}), for the fixed amount probe ss-DNA (for A: 750 Hz, for B: 4000 Hz) immobilized on the different sensor surface (A for hybrid membrane, while B for nano-TiO_2 membrane).

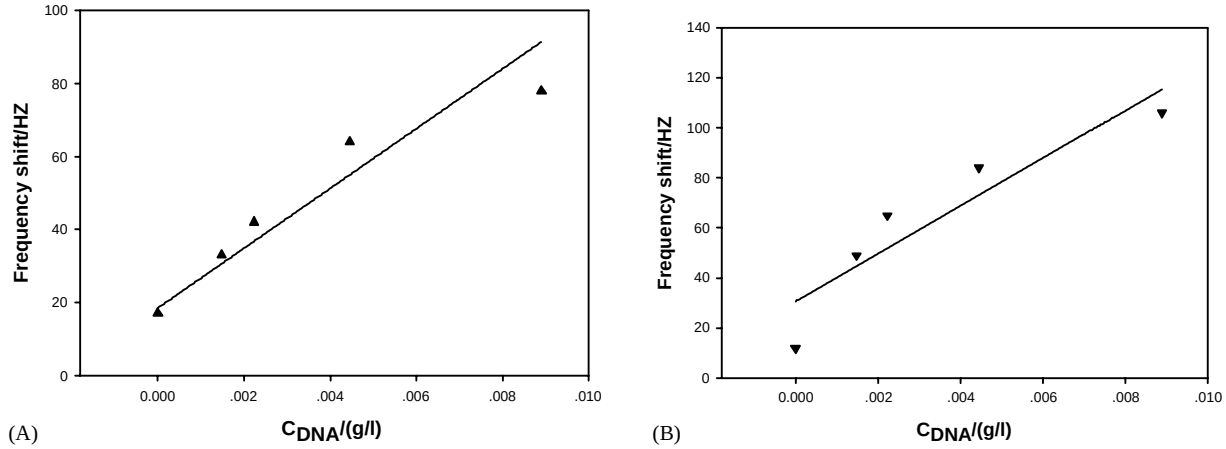

Fig. 6. Typical concentration–frequency shift curve for target ss-DNA hybridization with probe ss-DNA in liquid phase. A indicates with the nano-TiO_2 membrane modified sensor (frequency shift was 4000 Hz); B indicates with the nano-TiO_2–polyethylene glycol hybrid membrane modified sensor (frequency shift was 750 Hz).

stronger than the latter (keeping detecting situations constant). In order to prove it, a control assay was performed. Detect calf thymus ss-DNA (0.2 g l^{-1}) in solution with *P. aeruginosa* DNA sensor. The frequency shift was only 12 Hz, so it can be neglected comparing to total response signal.

3.4. Comparison between nano-studied and non-nano-studied sensor

For sensor, it was expected to have high selectivity and sensitivity, quickly response time and long lifetime. In order to reach these, researchers utilized new technologies that offered improved sensitivity for detection with high specificity at the molecular level (10^{-18} molar or better, a factor of 10^6 over currently available techniques). Among them, nano-sized particles with high chemical reactivity, big surface area and high surface free energy attracted more attention [22]. Common TiO_2 thin film was got by hydrolysis with heating, then coated and detected. Results showed as follows (see Table 2).

Table 2
Comparison between different kinds of films in binding amount and sensitivity

Film type	Binding amount (Hz)	Sensitivity (ug ml^{-1})
TiO_2 thin film	15 ± 5	1
Nano-TiO_2 thin film	80 ± 10	0.35
Nano-TiO_2 thin hybrid film	120 ± 5	0.1

Table 3
Reusability of membrane by washing with TE buffer solution (0.02 mol l^{-1} Tris–HCl + 1 mmol l^{-1} EDTA, pH 7.4)

Washing times	Frequency for nano-TiO_2 membrane (Hz)	Frequency for nano-TiO_2 hybrid membrane (Hz)
0	9021797	9020683
1	9021816	9020682
2	9021832	9020667
3	9021789	9020677
4	9021826	9020717
5	9021807	9020693
6	9021783	9020686
7	9021811	9020656

0: the frequency value after modification; 1–7: the frequency value after recovery.

From the table, we obviously see that applying nano-sized thin film, the binding amount increased 3–5 times and sensitivity improved about three times. While applying nano-sized hybrid thin film, the binding amount increased 5–7 times and sensitivity improved 10 times. Therefore, we could say that using the functional, intelligent and smart materials to make micro-sensors is one of the focus research trends, and these improvements will permit chemical sensors and biosensors to become more competitive.

3.5. The recovery of the crystal

When constructing a piezoelectric biosensor, we must take the reusability into consideration to judge the sensor was better or not. Two choices were chosen to reuse the sensor: either washing the binding DNA or getting the membrane away. TE buffer solution (0.02 mol l^{-1} Tris–HCl + 1 mmol l^{-1} EDTA, pH 7.4) was used to remove the binding probe and target DNA, the good results were shown in Table 3. And 1.0 mol l^{-1} HCl was chosen to get away the membrane after the whole experiment was completed.

3.6. Detection of P. aeruginosa in sewage

As stated in Section 3.6, the amount of DNA of *P. aeruginosa* was determined. Two above sensitive membrane were employed. Probe ss-DNA immobilized was extracted from attenuated organisms, while target ss-DNA was extracted from *P. aeruginosa* in sewage. When there was response in liquid hybridization, we could conclude that there was *P. aeruginosa* in the sewage. And according to the frequency-response, the concentration of the *P. aeruginosa* could be rough estimated.

4. Conclusions

A nano-structured ESPS DNA biosensor was developed for detection of *P. aeruginosa*. Nonspecific results were deleted by using a control group. The sensor was stable and reusable. Using nanotechnology and hybrid membrane, the response sensitivity was highly improved. And this sensor had high specificity, the detection time was greatly shortened and good results were obtained. It can be concluded that this method might have further potential for application in the study of other environmental toxicants and bacteria.

Acknowledgements

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