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Ultrasensitive electrical detection of nucleic acids by hematin catalysed silver nanoparticle formation in sub-microgapped biosensors

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ABSTRACT

An ultrasensitive electrical detection method of nucleic acids has been demonstrated on sub-microgapped biosensor. In this method, peptide nucleic acid (PNA) probes were firstly immobilized in the gap areas of a pair of interdigited microelectrodes and then were hybridized with their complementary target DNA. After hybridization, hematin molecules were introduced into the DNA strand via zirconium-phosphate and zirconium-carbonate chemistries. The newly attached hematin molecules act as a catalyst to accelerate reducing ammoniacal silver ion to form silver nanoparticles, which span the gap of the interdigitated microelectrode. The conductance of the silver nanoparticles directly correlated with the number of the hybridized DNA molecules. Nearly 1 fM sensitivity was achieved under optimal conditions. This approach is also applicable to the detection of RNA.

1. Introduction

There is a worldwide effort towards the development of bioanalytical devices that can be used for detection, quantification and monitoring of specific chemical species including nucleic acid molecules. For example, it is a critical factor in diagnostics of genetic, bacterial, and viral diseases and environmental monitoring of micro-organisms. The most frequently used methods for determining the presence and concentration of nucleic acids include autoradiography, fluorescence, chemiluminescence or bioluminescence as well as electrochemical and electrical techniques (Odentahl and Gooding, 2007). The search for simple, cost effective and rapid detection methods resulted in techniques such as polymerase chain reaction (PCR) and the fast development in hybridization based DNA chips (Arlinghaus et al., 1997; Christopoulos, 1999).

Typical nucleic acid detection techniques rely on marking of the nucleic acid to be examined. The molecular markers used for this purpose may be fluorescent, luminescent or electrically or magnetically active molecules or particles (quantum dots, magnetic beads). After hybridization with complementary DNA capture molecules in a homogeneous phase or on a microarray, the markers are detected at the bound nucleic acids sites by one of the aforementioned methods. These techniques are highly sensitive and well established but also show many disadvantages, such as photo bleaching of fluorophore labels, quenching, high costs, complicated protocols, the requirement of bulky instruments, and long analytical processing time. For this reason, there is still a need for a simple, reliable and cost-effective detection method for rapid nucleic acid analysis.

An alternative way is to use label-free method for detection. There have been some techniques available for label-free detection, these techniques can be electrochemistry, such as label-free electrochemiluminescence (Wei et al., 2007), label-free impedance detection (Dharuman et al., 2005; Berdat et al., 2008); and optical methods, such as silicon-on-insulator microring resonator method (Vos et al., 2007) and surface plasmon resonance method (Kim et al., 2007a,b); micromechanical technique – dynamic microcantilever sensors (Fang et al., 2005), other technologies – differential transfer function of field-effect transistors (Ingebrandt et al., 2007), nanopore sensor for fast label-free detection (Kim et al., 2007a,b), surface–colloid interaction detection (Sun et al., 2007), and nanogapped sensors.

Several years ago, a paper (Lee et al., 2002) was published concerning the DNA detection by measuring its capacitance changes before and after the DNA hybridization. The gap distance is 50 nm. It is a label-free method, no necessary to use fluorescent label, however, it is very difficult to fabricate a nanogap sensor with the gap distance only 50 nm wide; and meanwhile the signal difference before and after hybridization is not very high. In 2004, there is a report (Moreno-Hagelsieb et al., 2004) to use interdigited electrodes for DNA sensing, its electrode material is Al/Al_2O_3, which replace gold as the electrode material and reduce the cost.

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Its detection limit can reach to 0.2 nM and this method can be compatible with microarray technology. This group in Belgium further developed their method and the detection limit can reach to 50 pM, it also opens the way for correlation studies and noise reduction techniques based on multiple electrical measurements of the same DNA hybridization event with a single sensor (Moreno-Hagelsieb et al., 2007).

A relatively fast and real-time analysis can be achieved using electrical and electrochemical biosensors. There are already some reports describing the use of electrical technique detection, such as conductivity measurements (Park et al., 2002) (US patent application 2005/0079533). Generally, most detection methods depend on the binding of an analyte to the capture probe; the binding changes the conductivity or other electrical properties between two electrodes. In addition, field effect transistor (FET) based biosensors, such as ion-sensitive field effect transistors (ISFET) are also used. An example was given by Schöning and Poghossian (2002).

Recently, the potential of metal nanoparticles as an alternative label for ultra-sensitive detection of nucleic acids has been shown (Haick, 2007). As early as in 1999, Velev and Kaler (1999) demonstrated the creation of arrays of biosensors by in site assembly of colloidal particles onto micropatterned electrodes. Their exploratory results showed that a method of interfacing colloidal assemblies with electronic circuits could be used to create functional devices with sensitivities comparable to those of clinical assays. It can be deduced that their method held great promise for creating disposable on-chip arrays of highly sensitive miniature sensors for specific proteins, DNA fragments, or other biomolecules.

In 2001, Möller et al. reported a scheme for an electrical detection of the concentration of bioconjugated colloidal gold particles in solution, based on the use of particles carrying nucleic acid molecules. The particles are immobilized in the gap of microstructured electrodes, followed by a metal enhancement step and electrical measurements. Although, these electrical detection approaches achieved high sensitivity, they are limited by a relatively high background noise due to labelling of biomolecules with metal nanoparticles. Further, a silver enhancement step is required. In 2005 the group presented a revised protocol involving the use of the enzyme horseradish peroxidase (HRP) for catalytic metal deposition from a source of metal ions (Möller et al., 2005).

At present, electrical detection approaches either have low sensitivity or required labelling nucleic acids with gold nanoparticles. Alternative approaches based on the detection of fluorescence resonance energy transfer (Ray et al., 2007) even require the linkage of nucleic acids to both a fluorophore and a gold nanoparticle. Procedures such as gold nanoparticle preparation and functionalisation are tedious and require highly skilled labor; thus, there remains a need for an alternative method for the detection of nucleic acids.

The aim of this work is to develop a method to detect nucleic acid molecules electrically, which avoids these disadvantages just described.

Hematin (hydroxyferriprotoporphyrin) is the stable, oxidized form of the free heme center of the enzyme, HRP. In comparison to HRP, hematin is an inexpensive iron-porphyrin molecule that does not contain any amino acid residues and hence has significantly higher stability in a wider range of pH conditions. Therefore, by introducing a compound hematin to the analytical system to replace HRP, a constant activity can be sustained for silver deposition.

In this paper, a novel, simple, rapid and ultra-sensitive electrical detection method for nucleic acids on sub-microgapped biosensor is developed. It does not require biological ligation, a second probe, amplification or the use of radio-active substances. Also it is ultra-sensitive and non-radioactive. The hematin is bound to target DNA strand using zirconium phosphate chemistry (Buscher et al. (1996) and Mazur et al. (2005).) and the resultant complex catalyses the reduction of a solution containing silver ion to form metallic silver nanoparticles whose presence is detected as a change in the electrical properties of the sub-microgap. Experiments show that specific nucleic acids are detected with high sensitivity and specificity.

2. Materials and methods

2.1. Chemicals and instruments

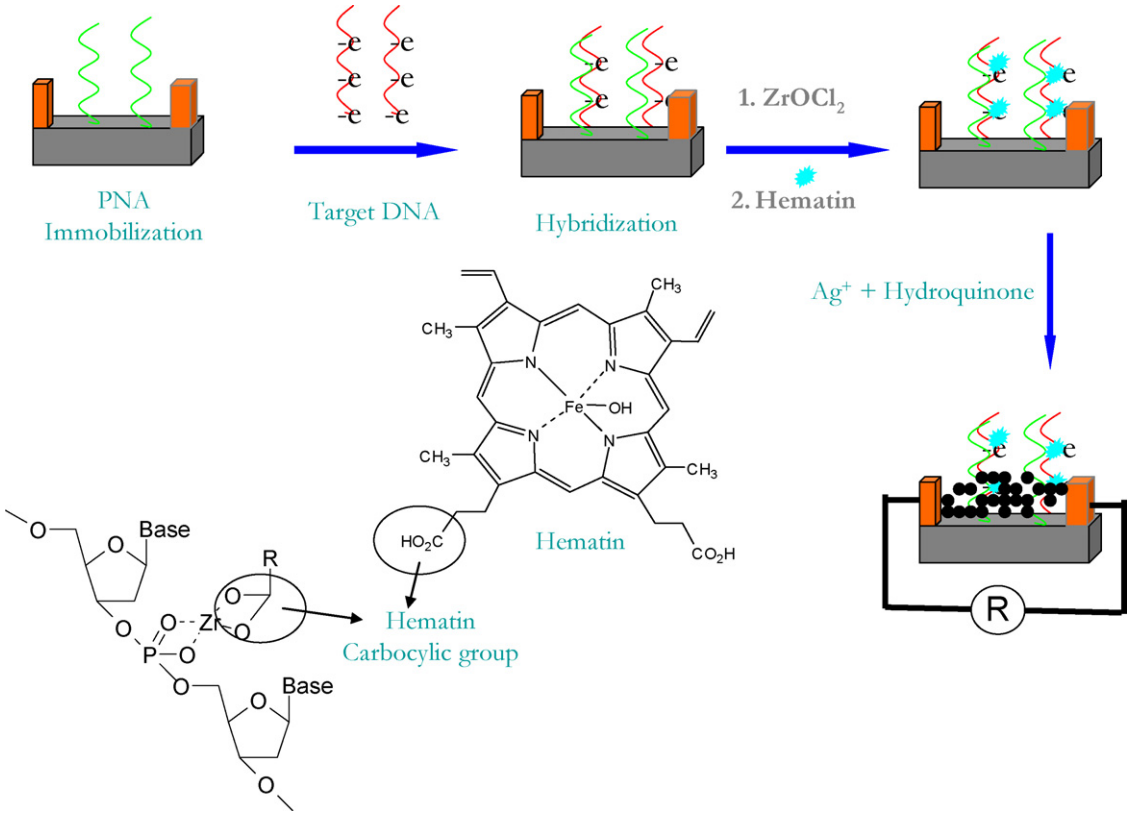
Amino-terminated PNA capture probes with sequence 5′-NH_2-AAC CAT ACA ACC TAC TAC CTC A-3′ were custom-made by Eurogentec (Herstal, Belgium) and used as received. Target complementary oligonucleotide 5′-TGA GGT AGT AGG TTG TAT GGT T-3′, one base mismatch oligonucleotide 5′-TGA GGT AGT AGG TTG TGT GGT T-3′ and control oligonucleotide 5′-GGA AGG GAG TAA AGT TAA TAC CTT TGC TCA TTG ACG-3′ were purchased from 1st Base Pte Ltd. (Singapore). 3-Aminopropyl triethoxysilane (APTES, 99%), hematin, silver acetate and 1,4-phenylenediisothiocyanate (PDITC, 98%), were obtained from Sigma–Aldrich (St. Louis, MO). All other reagents were also obtained from Sigma–Aldrich and used without further purification.

2.2. Fabrication of sub-microgapped biochip

Sub-microgapped chips used in this experiment were fabricated by a lift-off method. A single chip contained 10×10 interdigited microelectrodes each formed of 170 pairs of 195 μm long and 500 nm wide fingers; the gap distance between each pair of finger-like electrodes was 500 nm. These electrodes consisted of a 15 nm thick layer of gold on top of a 10 nm thick layer of titanium. They were fabricated on silicon wafers with 500 nm thick silicon dioxide by standard microfabrication procedures. The chip surface was treated according to a method described elsewhere (Liu and Bazan, 2005) with a few modifications. Briefly, the chips were thoroughly cleaned with chloroform and acetone to remove any possible organic contaminants, then were dried with nitrogen and exposed to 5 min oxygen plasma, before being washed in isopropanol and absolute ethanol and dried. Next, the clean chips were incubated for 4 h in an absolute ethanol solution of 1% APTES and 0.5% H_2O (v/v) for silanization, then they were washed with absolute ethanol and allowed to dry under mild nitrogen flow before aging at 110 °C for 10 min prior to the PDITC activation. PDITC, a bifunctional coupling agent, was exploited for chip activation via its surface amino groups. 40 mg of PDITC was added to a mixed solution of pyridine (2 mL) and dimethylformamide (18 mL) and the chips were immersed in this solution for 2 h, followed by washing with dimethylformamide and dichloromethane, then drying under mild nitrogen flow. The commercial PNA capture probes were dissolved in 0.1% trifluoroacetic acid solution according to product instructions and diluted to 10 μM in 0.50 mM sodium carbonate buffer (pH 9). A 100 μL PNA capture probe solution was spotted onto a chip surface, and allowed to stand for 4 h in a humid chamber at 37 °C. Unreacted PNA capture probes were removed in the next step by a thorough wash with water and methanol. Lastly, the chips were treated with a dimethylformamide solution of ethanolamine and diisopropylethylamine to passivate their surface.

2.3. Hybridization and electrical detection

Hybridization was carried out in a 10 mM Tris–HCl, 1.0 mM EDTA, and 0.1 M NaCl (TE) buffer for 60 min at room temperature. After that, the hybridized DNA strand was activated via zirconium-phosphate chemistry. By doing so, hematin molecules



Scheme 1. Illustrative diagram of measure mechanism.

were attached to the DNA strand, to act as catalysts in the ensuing step of silver ion reduction producing silver nanoparticles. A freshly prepared solution of mixed silver acetate and hydroquinone in the ratio of 2:1 (both dissolved in sodium nitrate buffer, pH 3.62) was added to the chip surface and incubated for 5 min (balancing the time and measuring efficiency—result not shown), to produce a continuous silver nanowire, which would electrically short the electrodes. The illustrative diagram for the measurement mechanism is shown as Scheme 1.

Conductance measurements were performed under ambient conditions by using Alessi REL-6100 probe station (Cascade Microtech.) equipped with an Advantest R8340A ultrahigh resistance meter (Advantest Corp., Tokyo, Japan). Data were reported as an average of the responses from 20 measurements.

3. Results and discussiones

3.1. Choice of silver ion concentration

Of several factors which affect the conductance between the pair electrodes in a sub-microgapped sensor, one of the important factors is the silver concentration. Therefore, we investigated the effect of silver concentration used in the silver reduction step by hematin. In this experiment, hematin concentration and DNA concentration as well as the reaction time are fixed at 250 μM in DMF, 10^{-12} M in TE buffer and 5 mins respectively; the only change is the concentration of silver acetate. From Fig. 1 it can be seen that at low silver ion concentrations, 12 mM and below, conductances of both control and sample groups were small, showing little difference. Between silver ion 13 mM and 14 mM the conductance of the sample groups was much greater than the control groups. While above 14 mM, the conductance of control groups increased leading to an increase in background noise and a decrease in sensitivity. Based on these results, it was concluded that 14 mM of silver acetate would be the most appropriate silver concentration and it was adopted for the following experiment.

3.2. Choice of hematin concentration

We also investigated the effect of hematin on the conductance results of the sub-microgapped sensor. In this experiment, 14 mM silver acetate solution and 10^{-12} M DNA sample were used and the reaction time is 5 min. It can be seen from Fig. 2 that the conductance reached a plateau above the hematin concentration of 300 μM, indicating that the response of DNA to hematin saturated at this concentration which was therefore used for all the following experiments.

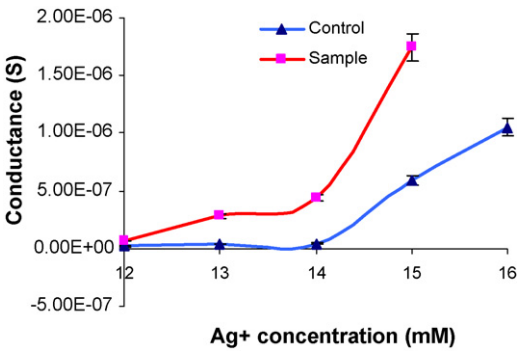


Fig. 1. Effect of silver ion concentration on conductance. Experimental condition: the hematin concentration 250 μM in DMF and DNA concentration 10^{-12} M, reaction time 5 min.

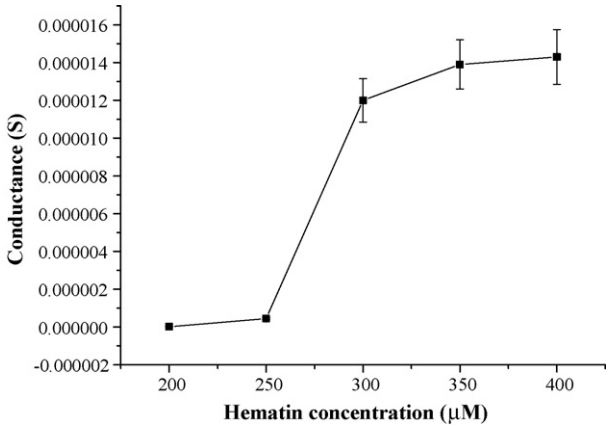


Fig. 2. Effect of hematin concentration on conductance. Experimental condition: silver ion concentration 14 mM and DNA 10^{-12} M, reaction time 5 min.

3.3. Evidences of sub-microgap bridging with silver nanoparticle

In Fig. 3, a scanning electron microscope (SEM) image shows that a silver nanowire formed after hematin and silver acetate treatments bridging across the sub-microgapped biosensor hybridized with complementary DNA. By contrast, in the control sample no such nanowire was observed. These results proved that hematin indeed act as a catalyst catalysing the formation of silver nanoparticles, which is similar to the work of Möller et al. (2005) by using of the HRP for catalytic metal deposition from a source of metal ions in on an interdigited microelectrode sensor.

3.4. Calibration curve

The conductance of a pair of sub-microgap electrodes on a chip primarily depends on the number of silver nanoparticles formed along gap. This number is in turn dependant on the amount of hematin bound to the target DNA strands in the gaps. Provided the saturation concentration of hematin is used, this is directly proportional to the amount of target DNA molecules hybridized.

The hybridization of PNA and target DNA is in the ratio of 1:1, thus the amount of the PNA capture probes immobilized in the gaps and the hybridization efficiency will ultimately determine the number silver nanoparticles formed in the gaps of the biosensor. If the number of the target DNA molecules hybridized in the sub-microgaps is directly related to the conductance, then a simple linear relationship between the conductance and the amount of target DNA (or concentration) is to be expected.

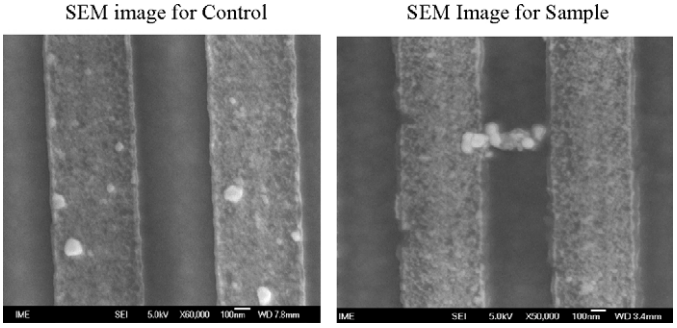


Fig. 3. SEM images of control and sample. Experimental condition: silver ion concentration 14 mM, hematin concentration 300 μM in DMF, DNA concentration 10^{-12} M, reaction time 5 min.

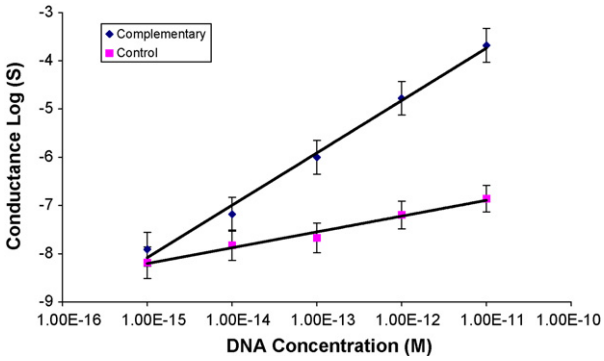


Fig. 4. Calibration curve for complementary DNA and control sample. Experimental condition: silver ion concentration 14 mM, hematin concentration 300 μM in DMF, reaction time 5 min.

Based on previous experimental results, the optimal conditions for analysis were determined to be: silver acetate 14 mM, hematin concentration 300 μM, reaction time 5 min.

Under such conditions, sub-microgapped biosensors were treated with DNA samples, their concentration ranging from 10^{-11} M to 10^{-15} M with 20 measurements at each concentration. The obtained calibration curve of the average of the conductance versus the DNA concentration is shown in Fig. 4. As expected there is a linear relationship between DNA concentration and conductance with a regression coefficient of 0.993, which shows that a good linear relationship between DNA concentration and conductance exists, proves that the number of the target DNA molecules hybridized in the sub-microgaps is directly related to the conductance.

The detection limit is near 1 fM, 2 orders of magnitude better than the one achieved using silver-enhanced gold nanoparticle labelling methods for detection of nucleic acids (Park et al., 2002).

3.5. Detection specificity—single base mismatch

To test the capability of this new method for discriminating against single base mismatch (SBM), we applied two different samples to the same PNA-immobilized biosensor, a complementary sequence and a single base mismatch sequence, both at a concentration of 1×10^{-12} M. Optimised conditions were used (silver acetate = 14 mM, hematin concentration = 300 μM, reaction time = 5 min). As shown in Fig. 5, the conductance for

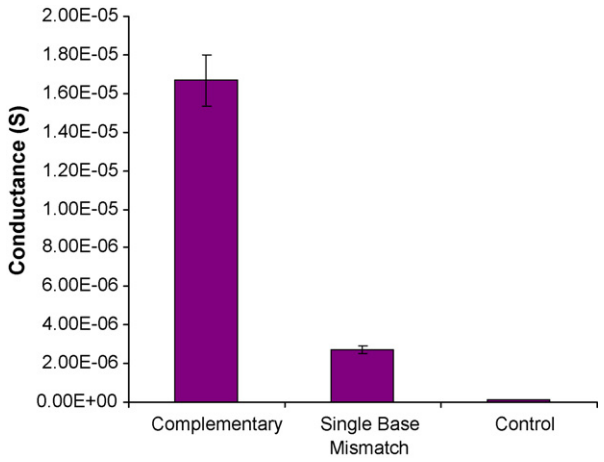


Fig. 5. Comparison of complementary DNA and one base mismatch sample. Experimental condition: silver ion concentration 14 mM, hematin concentration 300 μM in DMF, reaction time 5 min, sample concentration 10^{-12} M.

complementary sequence and a single base mismatch sequence are 1.65×10^{-5} S and 2×10^{-6} S, respectively. Thus the selectivity factor for detection of the SBM sequence on this biosensor is 8.25, higher than that of the conventional molecular beacon method (4:1) (Rosi and Mirkin, 2005), which allows it to discriminate against the fully matched complementary sequence and single base mismatch sequence. The higher specificity of this biosensor enables it to be used in many different fields—for instance, diagnostics of genetic, bacterial, and viral diseases, as well as environmental micro-organisms monitoring.

4. Conclusions

Ultrasensitive detection of DNA on a sub-microgapped biosensor has been demonstrated in this paper. For the first time, hematin was used to catalyse the silver nanoparticle formation in a sub-microgapped sensor for DNA detection, which has greatly reduced the background noise and simplified the detection procedure. It can be expected that a narrower gap width will provide the potential for higher sensitivity. Meanwhile, since the metallic silver formed in the sub-microgapped sensor is stable, it can be exposed to the outside environment for longer time, which would be an advantage for developing multiplexed detection of different DNA samples.

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