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Polysaccharide Templated Silver Nanowire for Ultrasensitive Electrical Detection of Nucleic Acids

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An ultrasensitive electrical detection method of nucleic acids has been developed on a nanogapped biosensor. In this study, peptide nucleic acid (PNA) probes were immobilized in the gaps of a pair of finger microelectrodes first and were then hybridized with their complementary target DNA. After that, pectin molecules were introduced into the DNA strand via zirconium−phosphate and zirconium−carbonate chemistries and were oxidated by periodate in acetate buffer (pH 3.98). The newly produced aldedyde groups act as a reactant to reduce ammoniacal silver ion to produce silver nanoparticles, which bridged the gap of the interdigitated microelectrode. The conductance of the metallic nanoparticles correlated directly with the amount of the hybridized DNA. A much higher sensitivity was achieved at 3 femtomolar (S/N > 3) under optimal conditions. This biosensor is also applicable to the direct detection of RNA.

The past few years have witnessed an increasing demand for ultrasensitive sensors capable of detecting biomolecules such as DNA and proteins in various areas such as molecular diagnostics, forensic investigations, or environmental monitoring. Traditionally, most detection methods are based on fluorescent probe and expensive optics setups, which are hampered by several drawbacks, such as photobleaching, quenching of molecular fluorophore labels, high cost, complexity, requirement of bulky instruments, and time-consuming operation. The electrochemical detection has attracted particular attention because it provides a simple, inexpensive, accurate, and sensitive platform for the detection of biomolecules. Recently, several new electrochemistry based techniques have been proposed for the detection of DNA and proteins.[1−4] More particularly, metal nanoparticles have been applied to electrical and electrochemical DNA sensors. For example, in 2001, Moller demonstrated a scheme for DNA detection based on conjugated gold nanoparticles, in which silver was used to enhance their conductivity, achieving sensitivities in the pico to femtomolar range.[5−9] Wang et al. reported on an electrochemical assay based on traces made out of quantum dot nanocrystals.[10−12] In 2006, Hansen et al. presented a method to detect DNA at the femtomolar level by using the anodic stripping voltammetry of metal sulfide nanoparticles.[13] This sensor was capable of efficiently detecting down to 0.1 femtomole (33 fM, 3 mL) of the target DNA. However, these methods either suffer from their need of costly equipments, the complexity of preparation of nanoparticles, or the conjugation of DNA with conductive nanoparticles. Some improvements were achieved by introducing an intermediate step to combine the biomolecules to be detected to polymer molecules, which could be used as a template for the synthesis of silver nanoparticles. Advances in this field lie in bringing an aldehyde to the DNA molecules and a site-directed reduction of silver.[14] In this method, aldehyde-derivatization of DNA was obtained by reacting a primary amine group on an adenine base within a DNA molecule with an α, β-unsaturated aldehyde polymer (formed by partially polymerization of glutaraldehyde), leading to DNA derivatization with multiple aldehyde-groups through a stable secondary amine bond. Guanine and cytosine bases also contain primary amine groups that could react in a similar manner. Certain aspects of this method still harbor room for improvement. First, it needs a gold metallization step because the number of aldehyde groups is low with approximately 1 aldehyde group per 30 base pairs. Second, an extra step of polymerization of glutaraldehyde is needed, which makes the analysis procedure more complex.

Those limitations could be overcome by considering pectin molecules. They have a linear backbone composed of units of (1,

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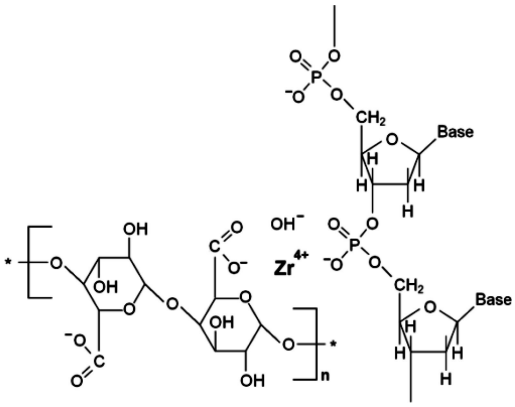

Figure 1. Schematic representation of the attachment of pectin to zirconium-templated DNA.

4)-linked α-D-galacturonic acid and its methyl ester. The galacturonic acid units may be in the salt form, galacturonate, making pectin an anionic polymer. The carboxyl groups of galacturonic acid units in pectin can bind to DNA via zirconium ions by zirconium−phosphate−carboxylate (ZPC) chemistries.[15,16] The association of pectin to DNA can be illustrated in the following mode (as shown in Figure 1).

By utilization of the Tollen's reduction of the silver ion, silver can be deposited on the pectin backbone through a chemical reduction with lower background noise. The aldehyde groups on pectin are produced by periodate oxidation of vicinal diols in pectin,[17] an established technique for aldehyde production on polysaccharide.

MATERIALS AND METHODS

Chemicals and Instruments. Amino-terminated PNA capture probe with sequence 5′-H_2N-ATGGTGGGCATGGGTCAGA-3′ was custom-made by Eurogentec (Herstal, Belgium). Target complementary oligonucleotide 3′-TACCACCCGTACCCAGTCTTCCTAAGGATA CACCCGCTGC TCCGGGTCTC GTTCTCTCCG TAGGAGTGGG ACTTCATGGG GTAGCTCGTG CCGTAGCAGT-5′ (Proligos, Sigma-Aldrich), one base mismatch oligonucleotide 3′-TACCA CCGGT ACCCA GTCTT CCTAAGGATA CACCCGCTGC TCCGGGTCTC GTTCTCTCCG TAGGAGTGGG ACTTCATGGG GTAGCTCGTG CCGTAGCAGT-5′ (Proligos, Sigma-Aldrich), 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%), aniline (99.5%), 1,4-phenylenediisothiocyanate (PDITC, 98%), potassium pectate (mean molecular weight 95 500), and silver nitrate as well as all other reagents were obtained from Sigma-Aldrich (St. Louis, MO) and used without further purification.

Fabrication of Nanogapped Biochip. Nanogapped chips in this experiment were fabricated with the lift-off method. There are 100 interdigited electrodes on each chip. Each electrode has 170 pairs of fingers, which are 500 nm wide and 195 μm long. The gap distance between each pair of the interdigited electrodes is 500 nm; these electrodes consist of a 10 nm thick titanium capped with a 15 nm thick gold. They were fabricated as a 10 × 10 array on a silicon chip with 500 nm silicon oxide by standard microfabrication procedures. Surface modification of the chips was performed according to the method described elsewhere[18] with minor modifications. Briefly, the chips were first thoroughly cleaned with chloroform and acetone to remove any possible organic contaminants, dried with nitrogen, treated in oxygen plasma for 5 min, and washed in isopropanol and absolute ethanol. Next, the dried clean chips were incubated for 4 h in a solution of 1% APTES and 0.5% H_2O (v/v) in absolute ethanol for silanization. They were washed then with absolute ethanol and allowed to dry under mild nitrogen flow before aging at 110 °C for 10 min prior to the PDITC activation. The bifunctional coupling agent PDITC was employed to activate the chip surface via its surface amino groups. The chips were immersed in a solution containing dimethylformamide (18 mL), pyridine (2 mL), and 40 mg of PDITC for 2 h, washed with dimethylformamide and dichloromethane, and subsequently dried under mild nitrogen flow. The commercial product of PNA capture probes were dissolved in 0.1% trifluoroacetic acid solution according to the product instructions and diluted to 10 μM with a 0.50 mM sodium carbonate buffer (pH 9). An amount of 100 μL of the PNA capture probe solution was spotted onto the chip surface, and the reaction was allowed to last for 3 h in a humid chamber at 37 °C. The unreacted PNA capture probes were removed by a thorough wash with water and methanol separately. Lastly, the chips were treated with dimethylformamide solution containing ethanolamine and diisopropylethylamine to passivate the chip surface.

Hybridization and Electrical Detection. Hybridization was performed in TE buffer (10 mM Tris-HCl, 1.0 mM EDTA, and 0.1 M NaCl) at room temperature for 60 min. The DNA strand was activated via zirconium−phosphate/carboxyl chemistry. Pectin attached to the DNA strand by doing so, and the chips were then incubated for 2 h in the dark at 4 °C in a freshly prepared solution of 25 mM of $NaIO_4$ in 0.2 M sodium acetate buffer (pH 3.98). In the ensuing step, the newly formed aldehyde groups reduced ammoniacal silver nitrate ($Ag(NH_3)_2NO_3$) solution (pH 9.3) for 60 min to silver in the dark at room temperature. A continuous silver nanowire formed and the electrodes were bridged by the silver nanowire, dramatically reducing their electrical resistance. This is illustrated in Scheme 1.

Conductance measurements were performed under ambient conditions on an 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.

RESULTS AND DISCUSSION

Several factors involved in the system may affect the conductance of the pair of electrodes in the nanogapped biosensor. Therefore the sensitivity of the whole system can only be evaluated after essential independent studies of each of these parameters to obtain optimized conditions for its operation.

Choice of Silver Ion Concentration. The concentration of ammoniacal silver ions (or diaminesilver (I) complex cations) is an important factor in the silver reduction step and directly determines the number of nanoparticles formed at the end of the silver amplification. We investigated its effect on the conductance

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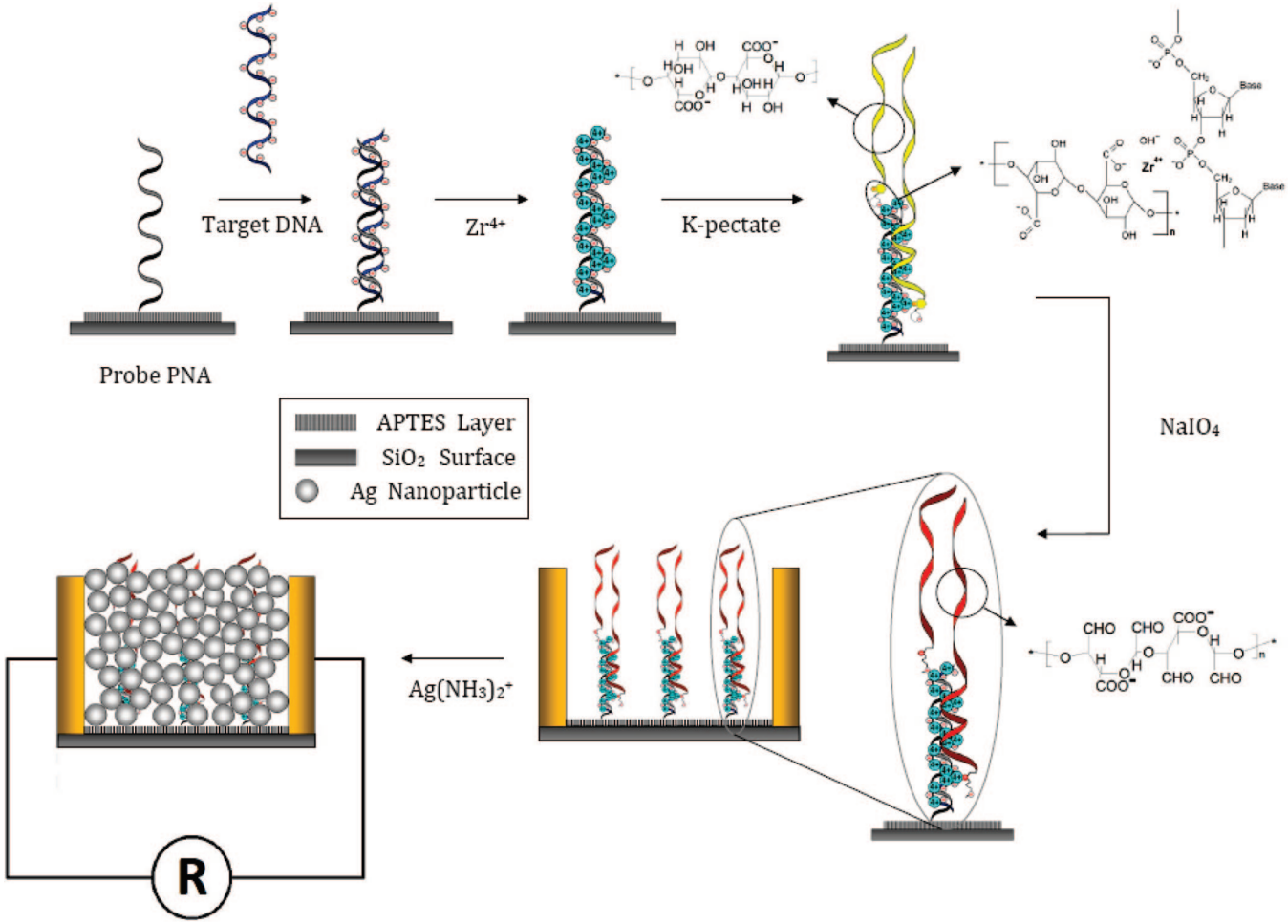
Scheme 1. Schematic Representation of the Pectin Templated Silver Nanowire Formation



end point using a pectin concentration of 12 mg/mL (near saturation), a DNA concentration of 10^{-12} M, and a reaction time of 60 min.

Results are shown in Figure 2. The conductance started to increase dramatically for ammoniacal silver ion concentrations above 10.0 mM and reached a plateau around 13.5 mM. This is in agreement with previously reported results by Dagan-Moscovich et al., wherein the silver nitrate concentration used to deposit silver on the surface of enzyme molecules was 10 mM.[19]

Repeated experiments between 13.0 and 15.0 mM enabled one to narrow down on the turning point which was finally obtained at 13.25 mM. The ammoniacal silver ion concentration for the subsequent studies was fixed at 13.25 mM.

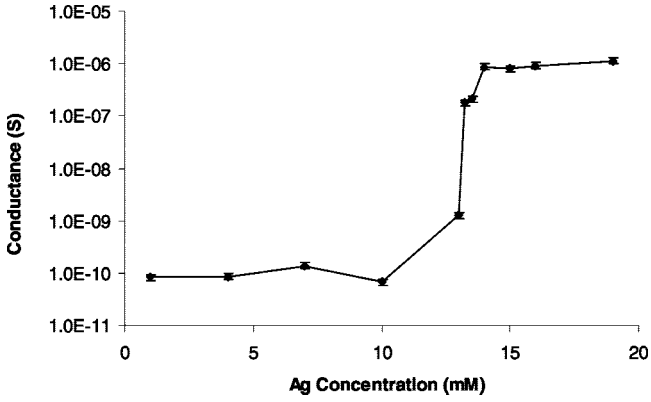


Figure 2. Effect of silver ion concentration on conductance Experiment conditions: pectin 12 mg/mL, DNA concentration 10^{-12} M, and reaction time 60 min.

Silver Ions Reduction Reaction Time. The formation and growth of silver nanoparticles in the chip surface was caused by the silver ion reduction by aldehyde groups derived from pectin combined with the target DNA strand and then enlarged by Ostwald ripening, a phenomenon first described by Wilhelm Ostwald in 1896.[20] The definition of Ostwald ripening in the O.B. 86, IUPAC Compendium of Chemical Terminology (second edition, 1997) is "The growth of larger crystals from those of smaller size which have a higher solubility than the larger ones."

In Ostwald ripening, larger particles consume smaller ones which are within their vicinities in order for them to grow even larger.[21] This is a spontaneous occurrence because larger particles are thermodynamically more stable than smaller ones due to their higher volume to surface area ratio. Since physical systems always attempt to lower their overall energy level, atoms or molecules on the surfaces of smaller particles will diffuse to join the surface of larger ones.[21,22] As a result, smaller particles continue to shrink, while larger particles continue to grow.

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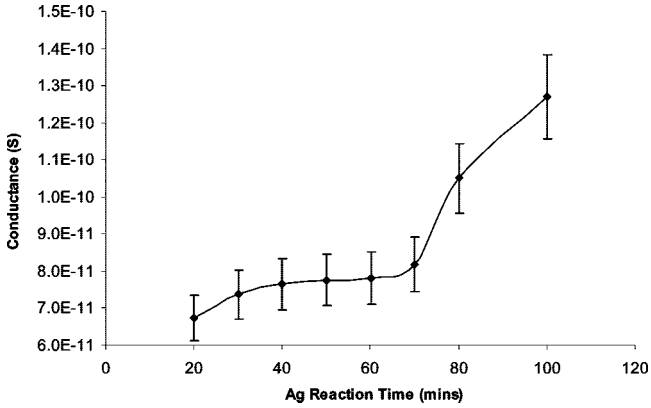

Figure 3. Effect of silver reduction time on conductance. Experiment conditions: pectin 12 mg/mL, DNA concentration 10^{-12} M, and silver ion concentration 10 mM.

Eventually, the size of the nanoparticles created can be controlled by the duration of this process.[23] Hence the duration of silver deposition needs to be optimized in order to obtain the formation of nanoparticles large enough to be in contact with one another and form a physically stable nanobridge. This works hand in hand with an optimal silver concentration which determines the amount of reducible silver[24] initially deposited on the pectate chain. While a high silver concentration may provide a great number of silver nuclei, their growth into physically stable entities depends essentially on the time given to them to expand. If too small, the bridge could easily collapse under hydrodynamic forces. In the opposite, if the silver reaction is allowed to last very long while the concentration is kept low, large silver entities will be sporadically dispersed. A large distance between neighboring entities may not be favorable to the formation of a bridge. Hence, an optimal amount of silver is needed to produce sufficient starting points for particles to grow and ample time is required for the particles to grow large enough to contact the microelectrodes.

The initial time taken for silver reaction is 20 min. Series of 3−5 sets of varying times with 20 min intervals were performed repeatedly. The values were collated, and the plot obtained is shown in Figure 3. It can be observed that the conductance increased gradually from 20 to 60 min and dramatically increased thenceforth. The first part of the trend may be due to surface adsorption kinetics while the dramatic increase may be due to the continuous growth of silver particles according to Ostwald ripening kinetics. In fact, there may also be an overlapped crossover of trend occurring between 40 and 70 min. As the aim of this experiment is to develop a sensor with a fast response rate, it was important to choose a short silver reaction time. Hence 60 min, which represents the saddle point in the plot and a compromise between fast response and sensitivity, was chosen to be the silver reaction time.

Sodium Periodate Concentration and Incubation Temperature. Assuming an excess in ammoniacal silver, the amount of silver reduced then depends on the number of free aldehyde groups, which in turn depends on the number of diols oxidized. Therefore it is essential that, in the presence of abundant diols, excess sodium periodate is used to ensure that all of the diols across the system are oxidized. To confirm that 25 mM of sodium periodate was sufficient to achieve the complete oxidation of pectate, its concentration was doubled from 25 mM to 50 mM. No significant changes were observed (results not shown). Moreover, the resistance change when the chip was immersed in sodium periodate at 4 °C from 20G to 4.3G was slightly higher than from 20G to 4.6G at room temperature.

Sensitivity of DNA Detection Using an Optimized Procedure. When using pectin-templated silver nanoparticles as the signal amplification method for the electrical detection of DNA, the conductance of a pair of nanogapped electrodes mainly depends on the amount of the silver nanoparticle formed along the target DNA strands hybridized with the probes in the gaps. The conductance reflects the quantity of nanoparticles, which is linked to that of pectin, which in turn depends on the amount of DNA molecules.

One molecule of PNA can hybridize to one molecule of DNA only, and the number of pectin molecules attached is directly linked to the number of DNA molecules. Thus the amount of the PNA capture probes immobilized in the nanogaps and the hybridization efficiency will ultimately determine the number of silver nanoparticles formed in the biosensor. We can deduce that the target DNA molecules hybridized in the nanogaps are directly related to the conductance; therefore, a simple relationship between the conductance and target DNA number (or concentration) would be a logical expectation.

On the basis of the results presented previously, the optimal conditions were set as an ammoniacal silver nitrate concentration of 13.25 mM, a saturated pectin concentration in water, and a reaction time of 60 min. Under such conditions, the biosensor was treated with a DNA concentration ranging from 10^{-11} to 10^{-15} M and 20 measurements were carried out for each concentration to obtain the average of the conductance for plotting a calibration curve. It shows a linear relationship between DNA concentration and conductance with a satisfactory regression coefficient of 0.99 (Figure 4).

From Figure 4, it can be observed that cDNA can be detected within a dynamic range of 1.0 fM to 10.0 pM (4 orders of magnitude) with the regression coefficient R as 0.99. The plot also shows that the curves of a single-base mismatch and the control are very close, in fact almost overlapping at low DNA concentrations. Although the system may not distinguish between a complementary and a single-base mismatched target effectively at 10^{-15}M, it is remarkably effective in doing so at slightly higher concentrations implying an increasing specificity.

Pectin Size Impaction on Detection. The pectin used in our experiment is potassium pectate (BioChemika 51 186, mean MW = 95 500) with only 0.5% esterification; this enables potassium pectate to supply enough carboxyl groups for conjugation with the DNA strand. The bigger is the pectin size, the more vicinal diols in pectin, and then more aldehyde groups will be produced after periodate oxidation; therefore, bigger pectin facilitates the DNA detection with higher sensitivity.

SNP Detection Specificity. To evaluate the capability of the proposed method in SNP discrimination (single nucleotide polymorphism), cDNA, 3′-TACCACCCGT ACCCAGTCTT CCTAAG-

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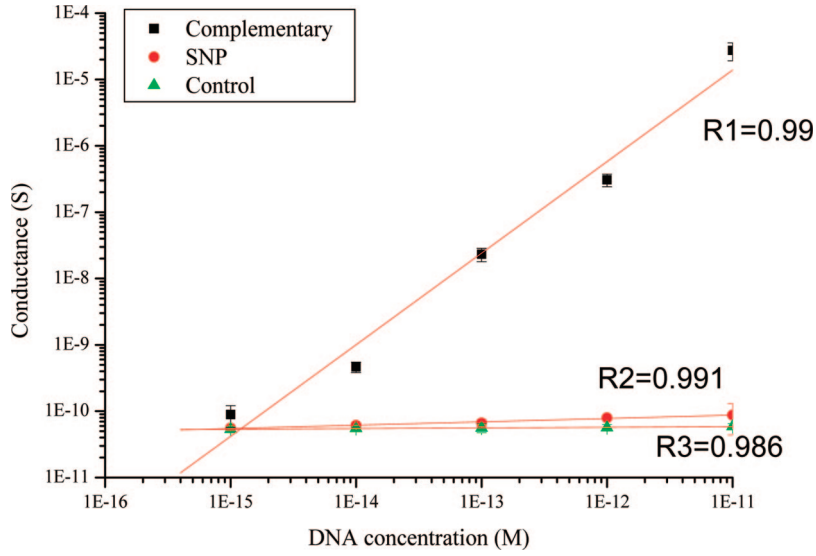

Figure 4. Calibration curve of cDNA and SNP. Experiment conditions: pectin 12 mg/mL, silver ion concentration 13.25 mM, and reaction time 60 min.

GATA CACCCGCTGC TCCGGGTCTC GTTCTCTCCG TAG-GAGTGGG ACTTCATGGG GTAGCTCGTG CCGTAGCAGT and SNP mismatch DNA, 3′-TACCA CCGGT ACCCA GTCTT CCTAAG-GATA CACCCGCTGC TCCGGGTCTC GTTCTCTCCG TAG-GAGTGGG ACTTCATGGG GTAGCTCGTG CCGTAGCAGT, were tested at 10 and 1.0 fM on the biosensor arrays, respectively. As shown in Figure 4, the increases in conductance for the SNP sequence, at 10 and 1.0 fM, were 1.8% and 7.9% of that of cDNA at the same concentration, respectively. It demonstrates that the detection of the SNP mutations is possible using the biosensor array with a SNP selectivity factor of 50:1, which is much higher than that of the conventional molecular beacon method (4:1),[25] the gold nanoparticle replaced molecular quencher molecular beacon method (25:1),[25] and the other previously reported method;[26] readily allowing the discrimination between the perfectly matched and mismatched DNA. The high specificity of the biosensor array suggests that each quantified result represents a specific quantity of a single DNA member, offering unique advantages over optical arrays and much broader applications than for DNA detection.

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CONCLUSIONS

Ultrasensitive detection of DNA with a nanogapped biosensor, enhanced by the pectin templated formation of silver nanoparticle was demonstrated in this paper. For the first time, pectin was directly utilized as a template for the silver nanoparticle formation in a nanogap sensor for DNA detection, which greatly reduces the background noise and simplifies the detection procedure. Principally, it is postulated that if the target DNA strand was longer, an even lower detection limit could be expected because longer DNA will attach more pectin molecules, leading to a bigger template with much more aldehyde groups. Surely this will raise the reaction efficiency for silver ion reduction. As this is an electronic-chip based detection, multiplexed detection capabilities can be built using standard microfabrication techniques, which is our current focus.

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