DNA Probes

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Chemically Immobilized Single-Stranded Oligonucleotides on Praseodymium Oxide Nanoparticles as an Unlabeled DNA Sensor Probe Using Impedance**

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Nowadays, there is increasing demand for DNA diagnosis in body fluids, food and drink, waste water, etc. A number of accurate DNA diagnostics based upon radiochemical, enzymatic, fluorescent, or electrochemiluminescent methods have therefore been developed. For example, DNA microarrays based on fluorescent methods involving immobilization of biomolecules on solid surfaces form the foundation of high-quality DNA analysis and detection, which has revolutionized the fields of genomics and bioinformatics. Although these techniques are extremely sensitive and quantitative, they require a long analytical time, cumbersome sample preparation, amplification steps, the use of expensive labeled reagents and equipment, and the systems are generally nonportable. On the other hand, the potential advantage of using a simple electrochemical impedance spectroscopy (EIS) method to detect DNA molecules is particularly noted because of its potential to use label-free reagents, the ability to work in aqueous environments that are native to the biomolecules, and they can be integrated with small, fast, and inexpensive microelectronics system. However, as far as we are aware, very limited success has actually been achieved in this area. The key problem that limits further development is the intrinsic poor sensitivity of most tested transducers for biosensing processes despite the fact that various materials including glass, silicon, gold, platinum, and carbon paste electrodes have been explored.[1,2] Furthermore, the problems in the instability of the output signal and poor signal transformation have yet to be solved.[3] A recent extensive screening exercise to search promising transducer materials for various biomolecule attachments has also been initiated.[4]

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In contrast, it has been demonstrated that highly conducting oxides, such as rare-earth oxides, can display excellent sensing properties for the detection of chemical species by simple electrochemical methods.[5] Our interest in this work is to develop rare-earth oxides, especially praseodymium oxide, as a prospective highly conductive sensing material owing to its excellent potential as an alternative material to silicon. Praseodymium oxide has many unique properties, such as a high dielectric constant ($\kappa \approx 26$–30), a large band gap (3.9 eV), high electron affinity (0.962 ± 0.024 eV),[6] and it is also relatively easy to present as a thin film.[7] More importantly, this oxide material intrinsically possesses a relatively high electrical conductivity at low temperatures.[7,8] For example, the electron hopping mechanism between the mixed valence states in the Pr_6O_{11} lattice accounts for its high electrical conductivity.[8] The higher sensitivity/selectivity of the praseodymium oxide compared with tin oxide based sensors for the detection of ethanol vapor has recently been demonstrated.[5] We have also recently noted that physisorption of oligonucleotides onto the surface can induce a shift in the impedance spectrum, which was determined by using two-probe impedance spectroscopy.[9] As a result, an interesting approach would be chemical immobilization in which a single-stranded oligonucleotide is placed on the praseodymium oxide surface as an electrochemical DNA probe. The advantages of such a probe are envisioned to include ease of purification, conservation of materials and reagents, reduction of interference between oligonucleotides, and facilitated sample handling.

DNA-immobilization, surface-modification techniques with silanes are especially regarded as attractive approaches as they are compatible with many of the materials used in the biological context, such as silica gel, glass slides, or silicon wafers.[10] Utilization of a heterobifunctional cross-linking agent is a useful method to stabilize the adsorbed biomaterials. The bifunctional reagent consists of two reactive sites, each with selectivity toward different functional groups (amine or thiol), thus allowing the conjugation of molecules in a defined manner and avoiding notable formation of dimers and polymers.[11]

Thus, we are concerned, herein, with a new attempt at chemical immobilization of oligonucleotides onto the Pr_6O_{11} surface. This is believed to be a first step towards obtaining a reliable, reproducible, and stable DNA functional surface (without being washed out) for DNA detection. Furthermore duplex formation on the surface does not involve label or shuttle reagents. Materials are characterized by FTIR, thermogravimetric analysis (TGA), and AFM. The immobi-

lized oligonucleotide is then evaluated by EIS to probe if there is any change in the electrical properties upon hybridization with complementary base pairs from solution.

It has been shown earlier that an ultrathin layer of praseodymium oxide in the form of Pr_6O_{11} with a high internal surface area can be deposited on a tin-doped indium oxide surface (ITO) by applying a negative sweeping voltage (cathodic electrodeposition) to an aqueous solution containing $Pr(NO_3)_3$ and H_2O_2 by using cyclic voltammetry followed by annealing the film at 500 °C for 1 h. AC impedance measurements revealed that the deposited film (sample A in Scheme 1) shows a much higher electrical conductivity than the pure ITO.[7] In this work, we carried out a close examination of the deposited praseodymium oxide film on ITO before the material was used as the host for the oligonucleotide immobilization. As can be seen from AFM and scanning electron microscopy (SEM) images in Figure 1, this film is assembled with a monolayer coverage of 300–500 nm and with the ellipsoidal praseodymium oxide particles packed closely on the ITO surface (the monolayer packing refers to the oxide particles packed as a 2D film without the particles stacking perpendicular to the film). However, a complete close packing of the regular oxide particles has apparently not yet been achieved.

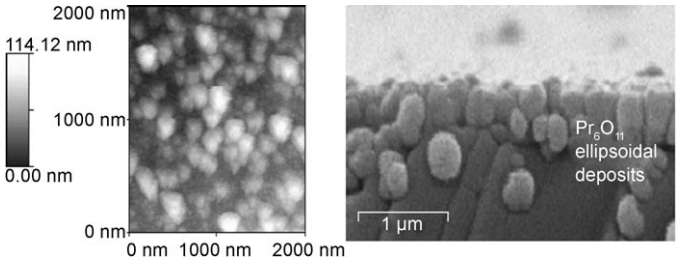
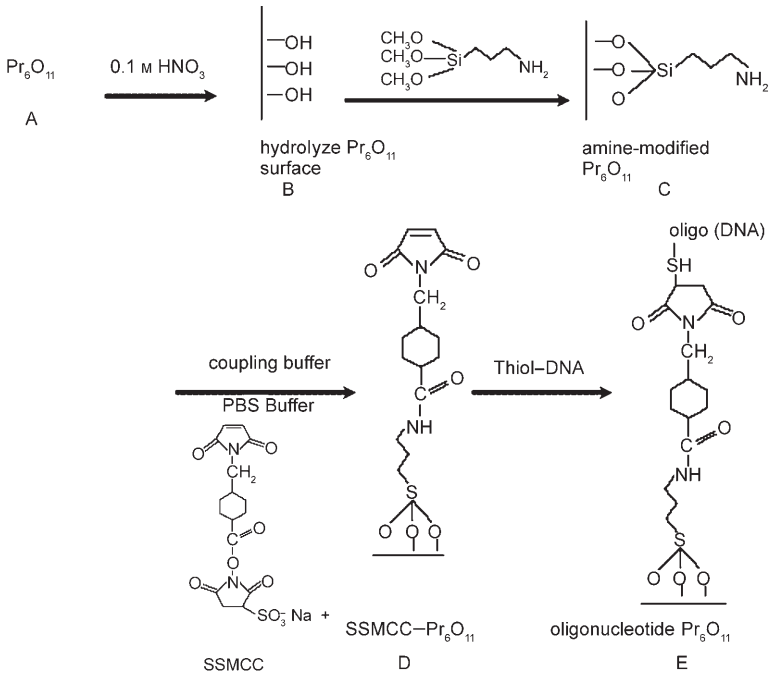


Figure 1. The topographical features of a monolayer Pr_6O_{11} particles deposited on ITO surface (300–500 nm thick). AFM top-view (left) and SEM side view (right) after the electrochemical and heat treatment.

Scheme 1 summarizes the four key steps and the intermediate product materials for the chemical attachment of thiol-modified oligonucleotides on a praseodymium oxide surface (see the Supporting Information). It was interesting to find that the AFM image of the Pr_6O_{11} film after amine attachment clearly revealed a dramatic change in its surface topography (sample C in Scheme 1). Figure 2a and b show that a much smoother surface was observed after amine attachment. This was previously noted in samples after silanization[12] and is attributed to the presence of organic groups filling the void space within and between particles after silanization. As a result, the smooth surface on the amine-modified Pr_6O_{11} film formed an ideal platform for the imaging of subsequent anchored oligonucleotides. Interestingly, detailed topographical examination (Figure 2b) of sample E from Scheme 1 clearly revealed regular, long, hairlike striped patterns on the sample surface. The measured dimension was 10 Å thick, which was the thickness previously assigned to the cross-section of the oligonucleotides.[13,14] The observed length was about 250 Å, which may be related to the length of the oligonucleotide lying flat on the surface. However, this should be cautiously considered as there is still a possibility of a surface artifact being introduced from the scanning-probe-microscopy probe, despite the reproducible observation from different scanning directions.



Scheme 1. Representation of a thiol-modified oligonucleotide (oligo-1) attached to an amine-modified praseodymium oxide surface. PBS = phosphate-buffered saline solution. SSMCC = sulfo-succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carbonate.

As seen in Figure 3 (and also in Table S1 in the Supporting Information), a large and distinctive absorption peak owing to the presence of OH groups at 3490–3600 cm^{-1} in the hydrolyzed praseodymium oxide sample (sample B in Scheme 1) is clearly evident as compared with the untreated oxide. This may be due to the physical adsorption of water molecules (OH

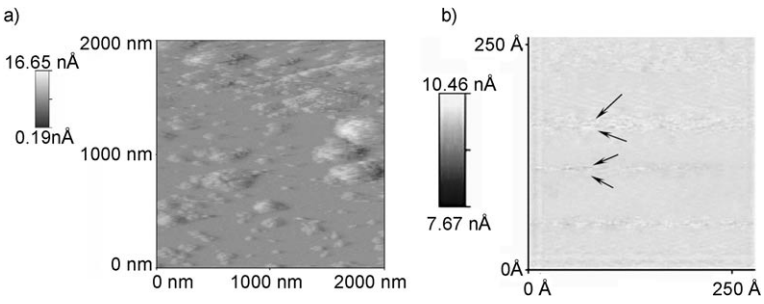


Figure 2. a) General AFM image showing the topographical feature of the Pr_6O_{11} nanoparticles treated with APTMS and b) detailed AFM image showing the topographical feature of the oligonucleotides immobilized on Pr_6O_{11}.

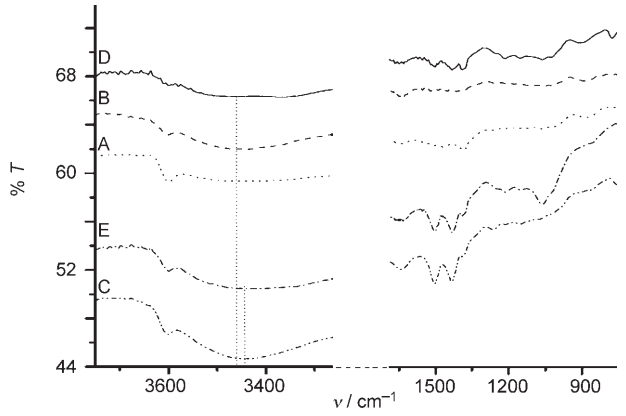


Figure 3. FTIR spectroscopy of different modification steps (sample A: Pr_6O_{11}; sample B: Pr_6O_{11}–OH; sample C: Pr_6O_{11}–NH_2; sample D: Pr_6O_{11}–SSMCC; sample E: Pr_6O_{11}–oglionucleotides) for attachment of DNA molecules to the praseodymium oxide surface. *T* = transmittance. The dotted lines indicate the red shift of the peak.

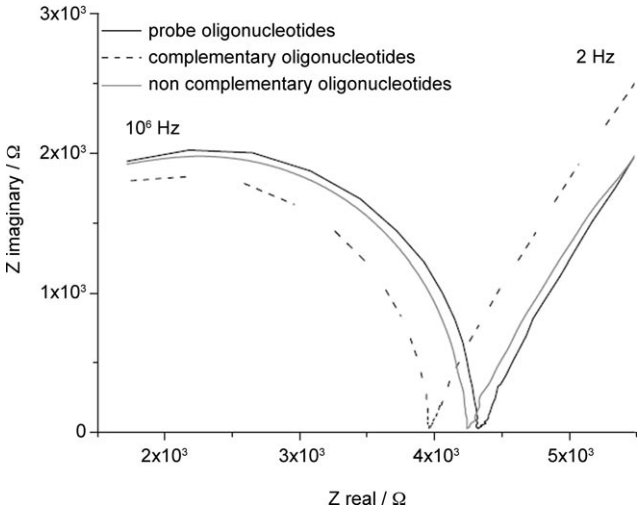


Figure 4. A typical left shift in the impedance spectra after addition of complementary oligonucleotide (oligo-2; 250 μL) to the probe oligonucleotide immobilized on Pr_6O_{11}/ITO (the AC impedance measurement was obtained by using a sweeping frequency from 10^6 Hz to 2 Hz with an applied voltage of 25 mV on a working electrode with the oligonucleotide immobilized on Pr_6O_{11}/ITO and a 1.5-cm^2 rectangular platinum disc as the counter electrode. See the Supporting Information for a detailed setup).

stretching at 3486 cm^{-1}) merged with the characteristic surface hydroxy-group stretching (at 3599 cm^{-1}). After the silanization treatment of the hydrolyzed praseodymium oxide with 3-aminopropyltrimethoxysilane (APTMS), a slightly red shift in the absorption peak to 3400–3550 cm^{-1} was observed (see Figure 3 and the samples C and D in Scheme 1 for the relevant stages), which is attributed to the presence of surface amine or amide groups (amine symmetric stretching at 3434 cm^{-1}, amide asymmetric stretching at 3464 cm^{-1}, see Table S1 in the Supporting Information). In addition, the distinctive peaks of -CH_2- bending at 1428 cm^{-1} as well as Si–CH_2 bending at 1499 cm^{-1} and C–N stretching at 1374 cm^{-1} were also present in the spectra. Consequently, the attachment of the linker molecule, SSMCC, showed the presence of absorption bands, including C=O stretching at 1628 cm^{-1}, -CH_2 at 1400–1500 cm^{-1}, and C–N stretching at 1374 cm^{-1} (sample D in Scheme 1). Equally, the red shift in the absorption peak to 3400–3550 cm^{-1} is owing to amide interaction at 3464 cm^{-1}, C=O stretching at 1632 cm^{-1}, C–N stretching at approximately 1400 cm^{-1}, N–H bending of thymine at 1202 cm^{-1}, and P–O stretching of phosphate in the range of 1000–1200 cm^{-1} (owing to the presence of the phosphate backbone on the single-stranded oligonucleotide) were clearly observed on the Pr_6O_{11} surface modified with oligonucleotides (sample E in Scheme 1). Finally, the successful implementation of each modification step was concluded. (Refer to Table S1 in the Supporting Information, which summarizes the FTIR peak assignments[15–17] and the thermogravimetric analysis methods that were employed to characterize the organic groups on the Pr_6O_{11} particles from their oxidative decompositions in air.)

Figure 4 shows the complex impedance spectra of the final probe oligonucleotide, oligo-1, chemically immobilized on Pr_6O_{11} and the biointeraction with complementary base pairs (oligo-2). It is interesting to note the distinctive shift of the semicircle to the left (decrease in impedance values at all frequencies) upon addition of the complementary oligonucleotide (oligo-2) from solution. An equivalent effect was not clearly observed with the noncomplementary base pairs (oligo-3). It is known that the overall charge density on the DNA duplex is significantly lower than the single-stranded entity as the charge is effectively neutralized by the counterions located between the duplex strands.[18,19] As a result, the observation of impedance attenuation can be attributed to the charge effect of the surface hybridization.[20,21] Alternatively, this could be assigned to the formation of double-helix DNA structures on the praseodymium oxide surface that have intrinsically lower impedance than the corresponding single-stranded oligonucleotide, which facilitates a better electron transfer through π stacking and leads to longer range charge transport. Experimental evidence presented by Barton and co-workers clearly suggested that electrons can be preferentially transported intrastrand through the DNA π stacks with a lower impedance than the interstrand pathway.[22] Consequently, the formation of double-stranded nucleic acids from surface-attached oligo-1 with oligo-2 in solution on the highly conducting oxide surface clearly reduces the overall impedance of the oxide.

A simple equivalent circuit model (see Figure 5) has been built to represent the electrical interfaces between the oligonucleotide-modified Pr_6O_{11} with ITO electrode. As seen in Table 1, there have been significant changes in the capacitor and resistance values. In particular, the C2 value accounted for the largest change (>12% change, see Figure 6) when the probe oligonucleotide on praseodymium oxide was immersed into the solution with its complementary oligomers (oligo-2). As can also be seen from Figure 4, the effect on impedance after the addition of the noncomplementary oligo-3 is not as large, and only a minor degree of fluctuation in the impedance signal was detected. As noted previously, alteration in the dielectric properties of the

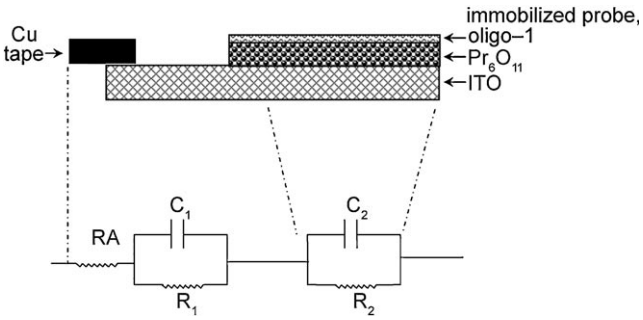


Figure 5. The envisaged electrical interfaces between ITO and the oligonucleotides immobilized on Pr_6O_{11} (represented by R2/C2) with a corresponding equivalent circuit model (R1/C1 could be linked to the counter electrode) with respect to the Pt counter electrode.

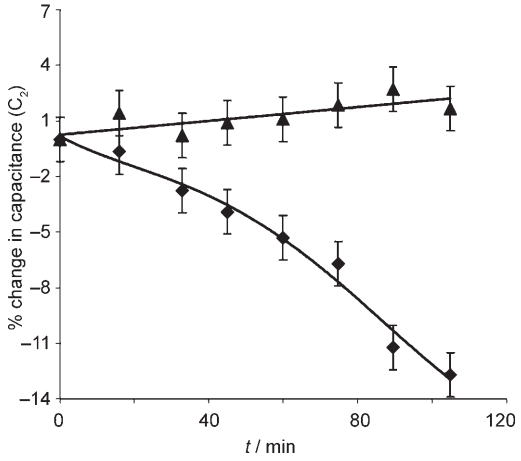


Figure 6. Comparison of the percentage changes in capacitance (C_2) between equivalent circuits of the oligo-1 probe (Figure 5) with either complementary oligo-2 (◆) or noncomplementary oligo-3 (▲). The *x* axis represents the time after the addition of 250 μL of oligonucleotide in minutes.

biolayer can be detected by a major change in capacitance of the underlying semiconducting layer.[9] This clearly confirms that the electrical properties of the chemically immobilized probe oligo-1 can be greatly altered through the hybridization process with its complementary counterparts, indicating the ultrasensitivity of this modified oxide film towards detection of the biomolecule.

In conclusion, we have shown that chemical immobilization of oligonucleotides on a highly conducting praseodymium oxide electrode was successfully achieved. Our results indicate that each surface-modification step during immobilization of thiol-modified oligonucleotide on the praseodymium oxide can be carefully monitored by FTIR, TGA, and AFM. As a result, a prominent shift in the impedance value was observed for the first time by electrochemical AC impedance measurements owing to the biorecognition process of the chemically immobilized probe nucleotide on praseodymium oxide with complementary oligonucleotides in solution without the use of label reagent. No equivalent effect on impedance was obtained upon the addition of the slightly mismatched based pairs in oligo-3. Through simulation with an equivalent electrical circuit model, it was shown that alternation in the capacitance value of the probe oligonucleotide upon addition of the complementary oligonucleotide is the main reason accounting for the major change in the impedance value.

Experimental Section

Praseodymium oxide (Pr_6O_{11}) material was first deposited on ITO electrodes by applying a negative sweeping voltage (cathodic electrodeposition) to the aqueous solution containing $Pr(NO_3)_3$ and H_2O_2 by using cyclic voltammetry followed by annealing the film at 500 °C for 1 h.[7] Detailed microscopic characterization of the praseodymium oxide on ITO surface was conducted before the oligonucleotide was chemically immobilized. SEM was conducted by using a Cambridge Stereoscan 360. AFM was carried out by using an Explorer AFM in noncontact mode. Scheme 1 summarizes the four key steps and their product samples (A–E) for the chemical attachment of thiol-modified oligonucleotide on praseodymium oxide surface.

Synthetic oligonucleotide primer and the probe oligonucleotide, oligo-1 (AAC-GAT-CGA-GCT-GCA-A), was chemically immobilized onto a praseodymium oxide nanoparticle on an ITO surface. To achieve the required biointerface, four key surface-functionalization steps including 1) hydrolysis of praseodymium oxide to increase free surface hydroxy groups, 2) conversion to surface amine functional groups, 3) attachment with a heterobifunctional cross-linker, SSMCC, and 4) immobilization of thiol-terminated oligo-1 were performed. AC impedance measurements of surface-immobilized oligo-1 before and after the contact of a complementary oligonucleotide, oligo-2 (TTG-CTA-GCT-CGA-CGT-T), or noncomplementary oligonucleotide, oligo-3 (CGT-ACC-AAG-ATG-AAC-G), were performed by using an impedance analyzer (Solartron SI 1260/gain-phase analyzer). The impedance measurement was taken by sweeping the frequency between 10^6 to 0.5 Hz at a voltage of 25 mV and a constant temperature of 43 °C and by employing the two-probe system in which the working electrode for this study was the oligonucleotide-immobilized praseodymium oxide/ITO, and a square disk of 1.5 cm^2 Pt acted as the counter electrode. Details of the experimental procedure for surface derivations, detection, and equipment are found in the Supporting Information.

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Table 1: Simulated resistance (RA, R1,and R2) and capacitance values (C1 and C2) based on the equivalent circuit model in Figure 5.[a]

Sequential treatments	RA (4.08×10^3 Ω)	R_1 (1.51×10^3 Ω)	C_1 (2.10×10^{-5} F)	R_2 (11.99×10^5 Ω)	C_2 (4.76×10^{-5} F)
oligo-2	−0.46%	+2.44%	−6.21%	+41.45%	**−12.7%**
oligo-3[b]	−2.48%	−2.64%	−5.91%	+40.89%	**+0.95%**

[a] The change in these values (in percent) after treatment of the thiol oligonucleotide (oligo-1) immobilized on Pr_6O_{11} with oligo-2 or oligo-3 is also given. [b] The change in resistance and capacitor values are referenced to the chemically immobilized, oligo-1 sample. The significant changes in capacitance are shown in bold.

Keywords: DNA structures · electrochemistry · label-free reagents · praseodymium oxide · surface chemistry

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