CHAPTER II

THOERY AND LITERATURE REVIEWS

## 2.1 Deoxyribonucleic acid (DNA)

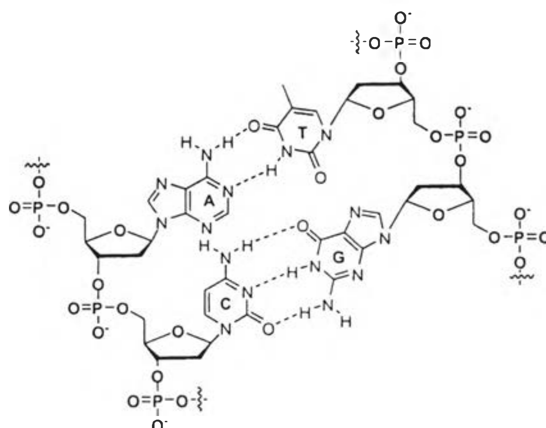


Figure 2.1 Structure of deoxyribonucleic acid (DNA) [44]

Deoxyribonucleic acid or DNA is a biological macromolecule that most living organisms used for storage and transfer of genetic information. It consists of several deoxyribonucleosides linked together between the 5' and 3' positions by a phosphodiester bond, making it a negatively charged molecule. There are four deoxyribonucleosides which are different only at the nucleobase (A, T, C and G). The order of the base ("base sequence") determines the structures and functions of all proteins and other components of the organism that own the DNA. Accordingly, the base sequence is the genetic information itself. DNA normally exists in the form of two "complementary" strands held together to form a duplex by hydrogen bonds. Since 1953, Watson and Crick had proposed that specific hydrogen bonding patterns between adenine and thymine and between guanine and cytosine were responsible for the specific pairing between the bases in the double helical structure [45] Due to the larger number of hydrogen bonds, G-C pairs (3 H-bonds) are generally stronger than A-T pairs (2 H-bonds).

## 2.2 Peptide nucleic acid (PNA)

The main component of nucleotides and DNA is deoxyribose, phosphate and nucleobase. In most analogues of DNA, either the deoxyribose or the phosphate groups are modified. These modifications often results in an increased biological stability at the expense of binding affinity/specificity. Surprisingly, a class of DNA

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analogue, in which the negatively charged deoxyribose phosphate backbone was replaced by an electrostatically neutral peptide-like backbone called peptide nucleic acid or PNA is not only more biologically stable than DNA, but also retains and even shows improvement in binding affinity and specificity to DNA targets [46].

The first PNA was reported by Nielsen and coworkers in 1991 [4]. In this PNA, now known as aegPNA, the deoxyribose phosphate backbone was replaced by 2-aminoethyl glycine (aeg). Owing to the absence of electrostatic repulsion the affinity and specificity interaction between DNA and PNA is higher than between natural DNA. Unlike DNA, the rather flexible structure of aegPNA can bind to DNA in both anti-parallel and parallel binding modes [5]. During the past 20 years, many research groups had attempted to develop new PNA systems. However, only few of these provide significant improvements over the existing aegPNA. In one example, Pokorski and coworkers suggested the development of aegPNA by incorporating one or more *(S,S)-trans*-cyclopentane units into the aegPNA backbone [47]. The new cyclopentane-modified PNA showed significantly increased affinity to complementary DNA as shown by UV-melting analysis. The more *(S,S)-trans*-cyclopentane units incorporated, the more increased melting temperature was observed. In another example, Dragulescu-Andrasi and coworkers attached a small substituent such as methyl group to the $\gamma$-carbon of the original aegPNA backbone in an *S*-configuration. This simple modification resulted in a so-called $\gamma$-PNA with dramatic improvement of the binding affinity and specificity compared to aegPNA [6]. Modification of the $\gamma$-PNA with diethylene glycol further was improved water solubility and reduced non-specific binding [8]. Other modifications of the original aegPNA structures had been extensively reviewed [46, 48].

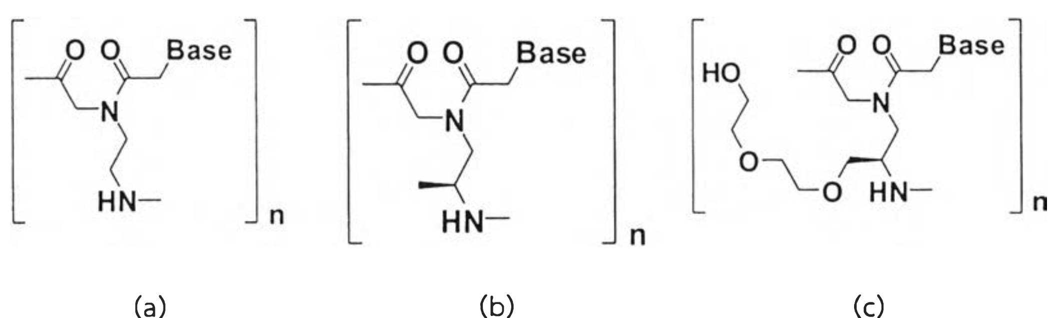


Figure 2.2 Structure of (a) aegPNA, (b) $\gamma$-PNA and (c) diethylene glycol-containing $\gamma$-PNA

Related to the two aforementioned systems, Suparpprom and coworkers introduces a new conformationally-restricted pyrrolidinyl PNA system deriving from

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D-prolyl-*trans*-(1*S*,2*S*)-2-aminocyclopentanecarboxylic acid (*SS*-ACPC) (also known as acpcPNA) [9]. AcpcPNA provided several advantages over aegPNA. These include: 1) pyrimidine-rich acpcPNA can form only duplexes with purine rich DNA 2) acpcPNA bind to DNA and RNA exclusively in antiparallel fashion 3) acpcPNA bind to DNA with higher affinity and specificity than aegPNA 4) the stability of acpcPNA-DNA>acpcPNA-RNA>>acpcPNA-acpcPNA duplexes [10, 49]. From this unique feature, acpcPNA is a promising candidate as a probe for DNA sensing as well as other applications.

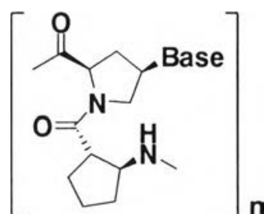


**Figure 2.3 Structure of acpcPNA**

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## 2.3 Electrochemical DNA biosensors employing immobilized DNA or PNA probes

The specific pairing between nucleobases of a detection molecule ("probe") and the target DNA molecule is the basis for the design of many DNA biosensors. In DNA biosensors, the binding event between the "probe" and the target DNA must be translated into a measurable signal by some appropriate signal transduction mechanism.

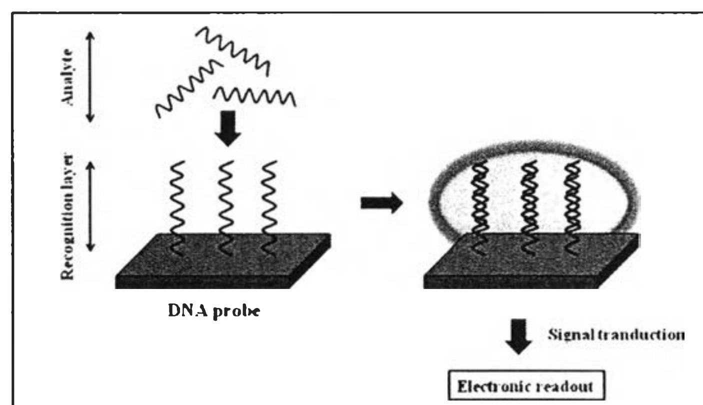


**Figure 2.4 General design of DNA biosensors [50]**

Several signal transduction mechanisms were used in DNA detection. These include optical (fluorescent, surface plasmon resonance and colorimetric), piezoelectric and electrochemical methods. Among these, the electrochemical method shows several unique advantages over other methods due to its rapid

detection, high sensitivity and selectivity, simplicity, portability and inexpensive instrumentation.

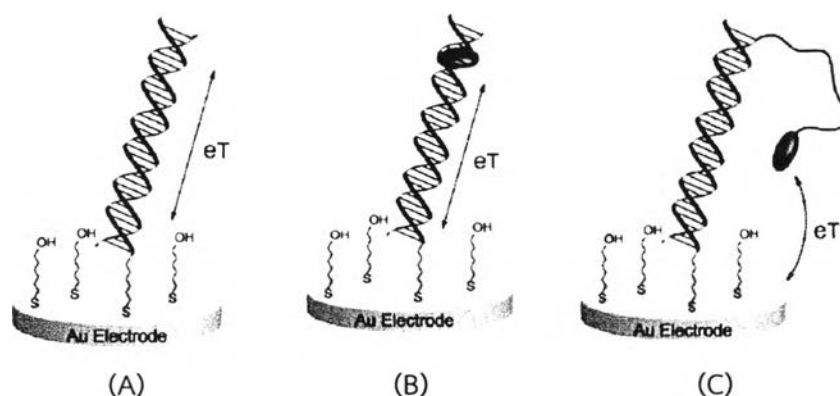


**Figure 2.5 Principles of electrochemical DNA detection based on immobilized probes (A) label free (B) redox indicator (C) redox-labeled probe [33]**

To allow electrochemical detection of DNA, either the probe, the DNA target or their hybrids should be electrochemically active or should be able to transform into an electrochemically active species. In the simplest scenario, the electrochemical signals can derive from electron transfer process of one or more nucleobases that can be oxidized or reduced (**Figure 2.6(A)**). Guanine had been shown to be the most readily oxidized base and the guanine oxidation signal can be used for indicator-free DNA sensing [23, 51-53]. The obvious advantage of this approach is that it requires no other labels. However, a guanine-free probe is often required [23]. Normally, the limit of quantification of this approach is in the micromolar range, but could be inproved to nanomolar for graphene modified electrodes [53].

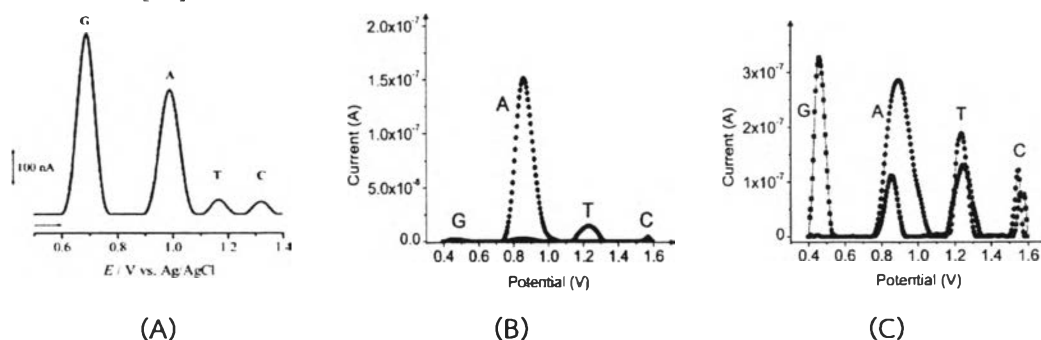


**Figure 2.6 (A) DPV of nucleobase A, T, C and G [52] (B) DPV of nucleobase A, T, C and G before and after MB intercalation in the case of (B) complementary and (C) single base mismatch DNA [53]**

In a more frequently employed approach, a redox-active indicator is added after the hybridization between the probe and the target (**Figure 2.5(B)**). The indicator is often an intercalator, groove binder or metal complex that carries one or more positive charges that can bind specifically to the probe-target duplexes. Frequently used redox intercalator such as $Ir(bpy)(phen)(phi)^{3+}$, daunomycin (DM) and methylene blue (MB) and groove binder such as $Ru(NH_3)_5Cl^{2+}$ and $Fe(CN)_6^{4-}$. Kelley and coworkers had shown that the redox intercalators can more effectively discriminate single base mismatched DNA than groove binders. The charge from the redox-active intercalator was transduced through the DNA base stack [28]. Wong and Gooding employed anthraquinone 2,6-disulfonic acid (AQDS) as an intercalative indicator to detect target DNA. The negative charges on AQDS repel the negatively charged on dsDNA and 6-mercapto-1-hexanol modified on electrode so intercalation predominantly occurred only at the end of strand [29]. The same group also compared the performances of redox cationic and anionic intercalator species in long range electron transfer. Cationic intercalators including methylene blue and $Rh(phi)_2(dmp)^{3+}$ can intercalate quickly but non-specifically to dsDNA. On the other hand, anionic intercalators gave slower binding kinetics but more specific [30].

The first use of PNA as a probe for electrochemical DNA detection was reported by Wang and coworkers. $Co(phen)_3^{3+}$ was employed as the redox indicator. This complex was known to associate with the DNA double helix through intercalation and groove binding. The PNA probe showed better performance than DNA probe in terms of both sensitivity and specificity [27]. Hashimoto and Ishimori also used PNA to detect mismatch mutation of cancer gene by using Hoechst 33258 as a redox indicator. The system can readily discriminate between complementary, single base mismatch and non-complementary DNA [54]. In 2006, Kerman et al. used $[Co(NH_3)_6]^{3+}$ to study the interaction between DNA and immobilized PNA. DsDNA shows the highest signal due to electrostatic interaction while dsPNA gives very low signal [31]. Ahour and coworkers used immobilized PNA probe in combination with methylene blue as the redox indicator to detect universal region of all HCV genotypes [55]. Hejazi and coworkers also used methylene blue as indicator in order to discriminate single base mutation in dsDNA without requiring the denaturation step by triplex formation with PNA probes [56]. However, the reproducibility and effectiveness of this method requires loosely packed DNA films to allow effective diffusion of the intercalators into the duplex monolayer. Limit of quantification is generally lower than labeled-free method (0.1-1 μM) [29, 30].

In yet another approach, the probe is labeled with a redox-active reporter prior to the immobilization (**Figure 2.5(C)**). The hybridization causes a change in the signal, which can be increased (signal on) or decreased (signal off), depending on the design of the probe. The intensity of the signal is controlled by the distance between the redox-active reporter and the surface of electrode as well as the arrangement of the reporter (stacked or not stacked within the duplex) [33]. Aoki and Tao employed immobilized ferrocene-labeled PNA to detect DNA target. The ferrocene signal was decreased when the PNA-DNA duplex was formed due to the increased rigidity which inhibited the motion of the ferrocene label [57]. Kumamoto and coworkers used anthraquinone-labeled DNA for DNA detection with single mismatch specificity. When the position of the single base mismatch position is between the anthraquinone label and the electrode, the charge transfer rate is much lower than the complementary DNA or single mismatched DNA with the position of mismatched base beyond the antharquinone label [17]. Husken and coworkers synthesized various ferrocene-labeled PNA that showed different redox potentials depending on linker between them. These labeled PNA are potentially useful as probes for multiplex electrochemical DNA sensing [58]. Paul and coworkers reported the effect of distance to the charge transfer rate of ferrocene-labeled PNA probe immobilized onto gold electrodes [59]. Kang and coworkers compared the properties of different redox-active tags (ferrocene and methylene blue) on immobilized DNA probes. The signals were suppressed upon hybridization with the correct DNA target for both tags, but the methylene blue tag was far more stable for repeated use than ferrocene (**Figures 2.7(A)** and **(B)**) because of the sensitivity of the oxidized form of ferrocene to nucleophilic attack [60]. Farjami and coworkers designed a methylene blue-labeled hairpin probe that can switch the signal change from on-off to off-on modes depending on the length of the probe [36]. Chalatein and coworkers designed quadruply-ferrocene-modified hairpin DNA probe in order to improve the sensitivity. In this system, a limit of detection of 3.5 pM was obtained [61]. Limit of detection of this probe immobilized method is 0.25-2 μM for bare electrode and 0.02-0.1 μM for positively charge modified electrode [21] and can decreased to 0.04-1 nM for amplified technique [61].

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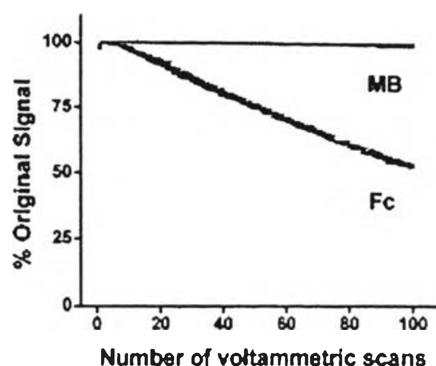


Figure 2.7 Performance of ferrocene and methylene blue labeled probe immobilization after various scans [60]

### 2.4 Immobilization-free electrochemical DNA biosensors

One issue of the use of immobilized probe is the requirement for the time consuming, and often wasteful, process of probe immobilization. Attempts were there for made to avoid this immobilization step. Kobayashi and coworkers observed that the electrochemical signal of the intercalator Hoechest 33258 decreased dramatically in the presence of dsDNA and could be used for immobilization-free DNA detection [62]. Fang and coworkers employed a similar approach to monitor and quantify PCR in real time in a micro-fluidic device by using methylene blue as an intercalator [63].

Luo and coworkers used ferrocene-labeled PNA probe for immobilization-free electrochemical DNA detection based on electrostatic interactions [21]. The modes for signal change (on-off or off-on) can be controlled by the charge on the electrode surface. The electrostatic repulsion between the negatively charged electrode and the negatively charged PNA-DNA duplex resulted in an on-off detection mode. On the other hand, if the surface of electrode is modified to become positively charged, the PNA-DNA duplex is attracted to the electrode and the detection mode was switched into off-on mode (**Figures 2.8(A)** and **(B)**). Subsequently, the same group applied this method for real time electrochemical monitoring of DNA-PNA dissociation by melting curve analysis [22].

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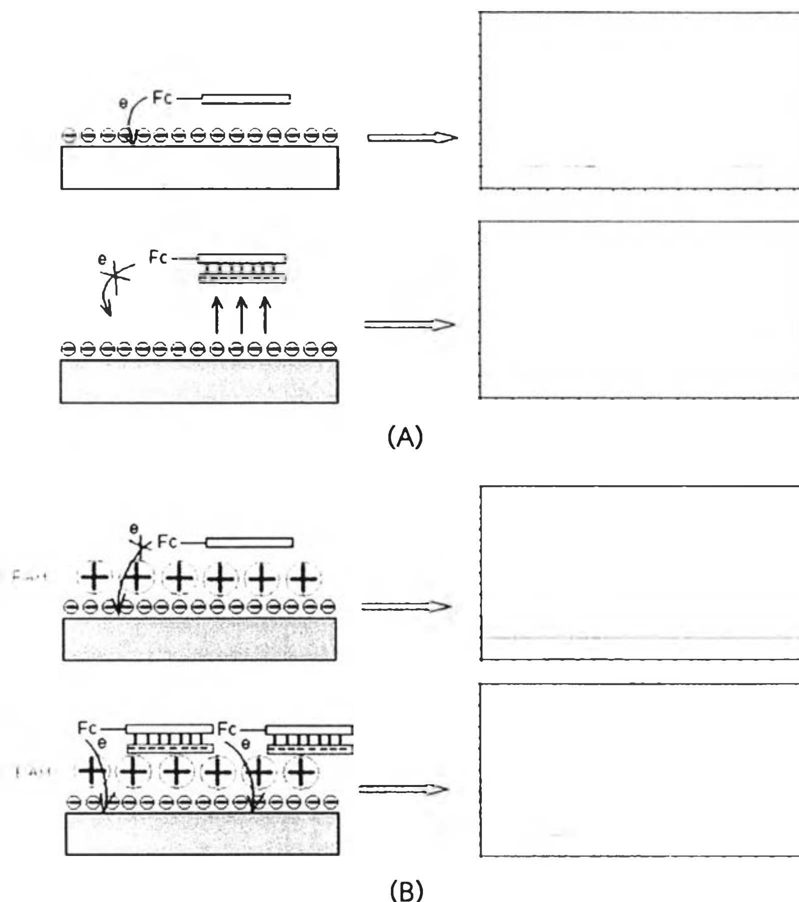


**Figure 2.8 DNA detection by using immobilization free method based on (A) negatively charged and (B) positively charged modified electrode [21]**

Fang and coworkers studied on host-guest recognition technique for immobilization-free electrochemical DNA detection. α-Cyclodextrin was used as a host, which was fabricated on the surface of the electrode. A hairpin DNA that was labeled at one end with a dabcyl group (guest) and gold nanoparticle was employed as a probe. In the closed state, no interaction between the probe and the α-cyclodextrin was observed. In the presence of DNA target, the exposed dabcyl group of the opened probe formed an inclusion complex with the α-cyclodextrin, resulting in a signal change [35]. Hsing and coworkers employed ferrocene-labeled PNA probes in combination with an isothermal circular strand-displacement DNA polymerization reaction for highly sensitive electrochemical DNA sensing by target recycling (**Figure 2.9(A)**) [20]. The same group also employed exonuclease III-assisted target recycling for sensitive DNA detections in signal-on modes (**Figure 2.9(B)**) [64]. Similar principle had been applied to linear probe (**Figure 2.9(C)**) [65].

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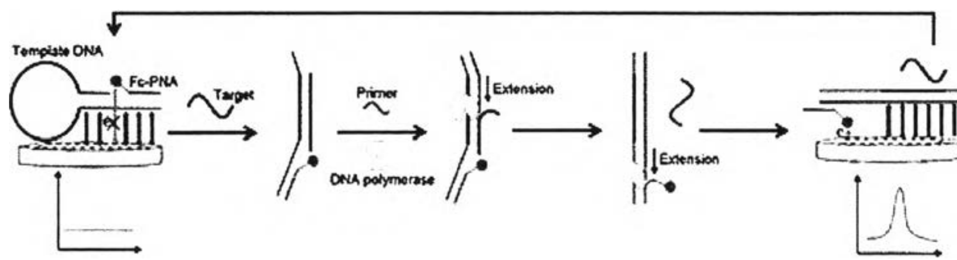
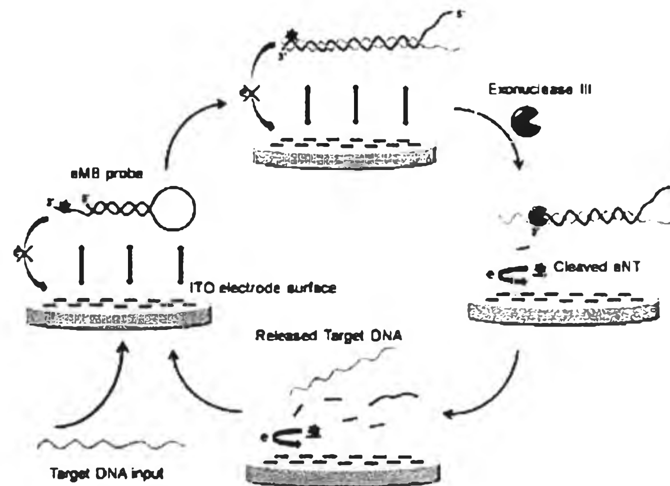



(A)

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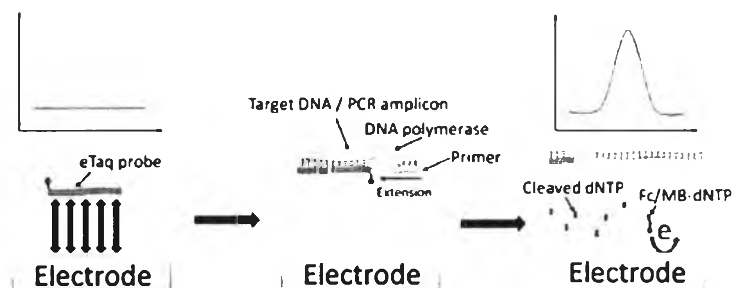


(C)

Figure 2.9 Application used in immobilization-free electrochemical DNA sensing method employing (A) labeled PNA probe and isothermal circular strand-displacement DNA polymerization [20] (B) labeled DNA beacon and exonuclease II-assisted target recycling [64] (C) same as (B) but with linear labeled DNA probe [65]

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### 2.5 Applications of acpcPNA as a probe for electrochemical DNA biosensors

The high DNA binding affinity and specificity of acpcPNA make it a promising candidate as a probe DNA biosensors. Thavarunkul and coworkers used immobilized acpcPNA on gold electrodes for highly specific and sensitive electrochemical detection of DNA by capacitive technique with a detection limit in picomolar range [66, 67]. Jampasa and coworkers recently employed immobilized anthraquinone-labeled acpcPNA on screen print carbon paste electrode (SPCE) for detection of HPV type 16 virus [26]. The signal was completely suppressed in the presence of complementary DNA target corresponding to HPV type 16 virus. However, the preparation of the electrode was time-consuming and required a large quantity of labeled PNA. Furthermore, the detection was in an on-off mode and required rather high concentration of DNA (in micro to nanomolar range).

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