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Advancement of Nucleic Acid Biosensors Based on Morpholino

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Abstract

Morpholino has drawn considerable attention as a result of its advantageous properties. In the past few decades, morpholino has demonstrated in applications as being the premier knockdown tool in developmental biology because of its cytosolic delivery in the embryos through micro-injection. Morpholino has outstanding affinity for nucleic acids and the destabilizing effect of mismatches in morpholino-containing heterodimers is higher than in a DNA or RNA double strand. Therefore, morpholino-based nucleic acid biosensors have high sensitivity and specificity for nucleic acid detection. In this review, the characteristics of morpholino are briefly introduced, followed by highlights of nucleic acid biosensors based on morpholino, including fabrication, analytical characteristics and biological applications.

Keywords: nucleic acid biosensor; morpholino; hybridization probe; sequence specificity; electrochemistry

1. Introduction

A nucleic acid biosensor is defined as that the probe in which is DNA, RNA or a nucleic acid analogue. A signal is obtained when the probe reacts with the specific targe nucleic acid [1]. The immobilization of the probe on the biosensor is an important step that affects the overall analysis performance. Nucleic acid biosensors are applied in different fields, such as genotyping and gene-expression studies [2], disease diagnosis [3], drug discovery [4], etc. Some of these applications take advantage of the increasing development of nucleic acid analogues, which overcome specific limitations

of natural nucleic acids for biosensing. Specifically, the use of morpholino enables high specificity and sensitivity to be achieved in practice.

Morpholino, an artificial nucleic acid comprised of morpholino rings, non-ionic phosphorodiamidates and nucleic acid bases, was designed by James Summerton in 1985. Morpholino is more cost-effective than most of the other nucleic acid analogs. It is synthesized from the natural rA, rG and rC ribonucleosides and the synthetic rT [5]. The morpholino subunits can be integrated into antisense oligonucleotides via coupling to the nitrogen without the catalysts and oxidation steps, which are required in the production of the great majority of other nucleic acid analogs [6]. Several inter-subunit linkage types of morpholino, including the phosphoryl, carbonyl and sulfonyl linkages [7], have been assessed by Summerton. Although morpholino containing such linkages provides effective binding to target sequences, non-ionic phosphorodiamidate-linked morpholino exhibits more excellent binding to complementary nucleic acids [8,9]. In the sections below, the characteristics of phosphorodiamidate-linked morpholino and the comparison of morpholino and PNA are briefly introduced, followed by highlights of nucleic acid biosensors based on morpholino: including fabrication, analytical characteristics and biological applications.

2. Properties of morpholino

Morpholino has received great attention due to its advantageous properties such as water solubility, resistance to enzymatic degradation, high hybridization specificity and activity in cells [9]. When nucleobases in aqueous solutions are poorly stacked, the oligo displays poor water solubility due to the difficulty of inserting the hydrophobic faces of unstacked bases into the aqueous environment. Morpholino exhibits good base stacking and the hydrophobic faces of stacked bases protect morpholino from the polar solvent, thus showing high solubility in aqueous solutions. Morpholino having the carbamate inter-subunit linkages is much less soluble than a corresponding morpholino containing sulfamide or phosphoroamidate inter-subunit linkages [10]. The solubility difference of morpholino subtypes is possibly due to the restricted rotation of the carbamate inter-subunit linkage which prevents base stacking, whereas the unrestricted rotations of the sulfamide and phosphorodiamidate linkages allow excellent base stacking. Good water solubility of morpholino proves essential for effective access to a target sequence in cells [6].

Though resistance to enzymatic degradation can be improved by introducing special groups or nuclease-resistant inter-subunit linkages to the terminal of DNA, a backbone which is resistant to a broad range of enzymes in cells is preferred [11,12]. Moreover, there is no need for concern that the degradation product might be toxic or result in mutation of cellular genetic materials. Morpholino is completely resistant to many enzymes and other degradation factors in the physiological environment [13]. In order to increase the capability of morpholino to invade target DNA, thymine is used instead of uracil in the synthesis of morpholino. Morpholino is also stable in strong bases, but is cleaved by strong acids such as trifluoroacetic acid. Morpholino can get entry into cellular compartments where the target sequence is synthesized and processed for biological activity [14]. In addition, it has been reported that the phosphorothioate DNA (S-DNA) stimulates tumor necrosis factor-α (TNF-α) production, while morpholino exhibits specific inhibition of TNF-α production [15,16].

Minimum inactivating length (MIL) was defined as the shortest length of oligo of a given structural type which achieves substantial inhibition at concentrations typically achieved in cells. The measured MIL value for a given structural type varies somewhat as a function of sequence, G+C content and concentration of the tested oligo [17]. The oligo should have a MIL sufficiently long enough to avoid inactivating non-target sequences. In addition, the length of the oligo must be sufficiently larger than its MIL. In order to achieve high specificity in a human, a 25-mer oligo with MIL value of about 14 or greater is required for this scenario. Thus morpholino, which has a MIL of about 15, exhibits excellent sequence specificity.

In solution, morpholino binding affinity for complementary genetic sequences is relatively independent of counterion concentration, indicating that the interaction of counterion with the unhybridized strand is comparable to that with the corresponding morpholino-DNA duplex [18]. On a solid surface, the intrinsic reactivity of hybridization between immobilized morpholino and complementary target DNA from solution depends both on ionic strength and probe coverage, with the fastest kinetics obtained at high ionic strength and low probe coverage. The influence of probe coverage is due to the reduced association between morpholino probes at low coverages. As hybridization proceeds, distinct kinetic stages appear and the hybridization rate exhibits dependency on the hybridization extend not captured by classical Langmuir kinetics. It is consistent with an interfacial organization in which the unhybridized morpholino localize near the solid support, while the well-soluble Morpholino-DNA hybrids segregate to the solution phase [19]. The main differences between surface hybridization of Morpholino-DNA and DNA-DNA on solid phase are consistent with the molecular organization influenced by probe solubility. Hybridization on solid phase is more complicated than in solution due to interactions of solid surface and probes [18].

3. Morpholino and PNA comparison

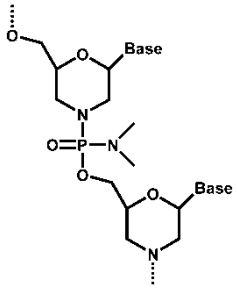
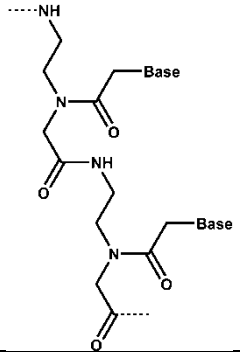
Peptide Nucleic Acid (PNA), another advanced artificial nucleic acid shares a number of key properties with morpholino, such as non-ionic backbones whose structures differ radically from that of nucleic acid, resistance to enzymatic degradation, insensitivity to ionic strength of the medium, high affinity for complementary DNA sequence and low toxicity in vivo [20,21]. In spite of these similarities, morpholino and PNA also exhibit significant differences which result in different advantages in particular applications (Table 1). PNA subunits are synthesized from the standard purine (A, G) and pyrimidine (C, T) nucleobases. Morpholino subunits are synthesized from the natural rA, rG and rC ribonucleosides. The synthetic rT is used in the synthesis of a morpholino subunit instead of the natural rU due to the positive impacts T bases have on hybridization affinity of the resulting oligos. The PNA backbone is stable in strong bases and strong acids. The morpholino backbone is stable in strong bases, but is cleaved by strong acids, such as trifluoroacetic acid. PNA has significantly greater backbone flexibility whereas morpholino has a more rigid backbone. Morpholino shows high solubility in aqueous solutions, generally 5-30 mM for 25 mer, depending on sequence, while PNA is typically hundreds-fold less soluble. PNA has higher affinity for DNA than morpholino has, though both morpholino and PNA form duplexes with DNA which are more stable than corresponding DNA-DNA duplexes [22]. The high-MIL morpholino (MIL~15) exhibits substantially better specificity over a larger concentration range than the low-MIL PNA (MIL~10). Morpholino achieves high specificity over a concentration range two orders of magnitude more than DNA [23]. As a consequence of these different properties, it appears that PNA excels in high-affinity applications such as hybridization with short sequences or discrimination of single base differences, as in single nucleotide polymorphisms (SNPs). Conversely, morpholino synthesis is flexible in sequence length or composition and thus it is able to use longer morpholino as a capture probe for hybridization with longer target DNA while PNA can not. In addition, morpholino is better suited for applications which require high water solubility and exquisite discrimination, like in vivo applications with developing embryo and other complex systems [22].

4. Applications for a nucleic acid biosensor

Morpholino is applied primarily for antisense applications in complex systems, such as extracorporal treatment of cells from thalassemic patients and correcting splicing errors of pre-mRNAs in cultured cells. The widest use of morpholino is in developmental biology because of its antisense application [24,25]. A particular attraction in developmental biology is using morpholino to selectively target zygotic RNA

without inhibition of material RNA coded by the same gene. Morpholino has great applications as the premier knockdown reagent in developmental biology because of its cytosolic delivery in embryos [26-28]. In recent years, morpholino is getting into utilization in nucleic acid biosensors. The unique characteristics of morpholino make it an attractive capture probe for development of nucleic acid biosensors. The applications of morpholino in nucleic acid biosensors are mainly based on electrochemical, fluorescent, microelectronic and microarray methods, especially electrochemical methods. These methods have excellent specificity and sensitivity for nucleic acid due to the prominent properties of morpholino.

Table 1. Comparison of the properties of morpholino and PNA

Morpholino	PNA
	
Non-ionic backbones; Resistance to enzymatic degradation; Insensitivity to the ionic strength; High affinity for complementary DNA sequence; Low toxicity in vivo.	
1. Synthesized from the natural rA, rG, rC ribonucleosides and synthetic rT;	1. Synthesized from the standard purine (A, G) and pyrimidine (C, T) nucleobases;
2. Stable to strong bases, cleaved by strong acids;	2. Stable to strong bases and strong acids;
3. Rigid backbone;	3. Flexible backbone;
4. High solubility;	4. Low solubility;
5. High affinity for DNA;	5. Higher affinity for DNA than morpholino;
6. MIL of about 15;	6. MIL of about 10;
7. High flexibility of synthesis in sequence length;	7. Low flexibility of synthesis in sequence length;
8. Better sequence specificity than PNA.	8. Good sequence specificity.

4.1 Electrochemical

Electrochemical biosensors have many advantages over other biosensors, as they are simple, rapid, sensitive, inexpensive and easy to miniaturize for application. Consequently, there has been great interest in developing electrochemical biosensors based on morpholino for different applications [29]. Tercero's group has reported the formation of morpholino monolayers on gold electrodes and the surface hybridization between morpholino and DNA. Morpholino monolayers on gold supports was prepared with a method similar to what is employed for DNA monolayers, in which thiolate bonds served to anchor one terminal of morpholino to the support, and the rest of this surface was passivated against nonspecific absorption with a short alkanethiol. Hybridization of uncharged morpholino with charged target DNA changed the ionic strength and dielectric characteristics of the surface. Negative charge was increased on the probe layer upon

hybridization, which was compensated by variation in local concentration of small ions, namely by accumulation of cations or expulsion of anions. The adjustment mechanism that dominates is tunable through the applied surface potential. For example, at negative biases, when anions concentration on the surface is small, cations accumulation is dominant. At positive potentials, anions expulsion is the primary response mechanism. These responses of hybridization can be monitored by surface differential capacitance C_d, where C_d was increased or decreased depending on the relative populations of cations and anions [30]. According to these hybridization phenomena, morpholino surface hybridization was used for label-free DNA analysis. Based on a 10:1 signal-to-noise ratio, C_d measurements demonstrated quantification limits down to 3×10^{10} cm^{-2} DNA. This performance is comparable with other label-free methods, such as quartz crystal microbalance and surface plasmon resonance techniques [31,32]. In the case of capacitive transduction, sensitivity can be improved at lower ionic strengths, under a condition which is more dilute than the 0.2 M PBS used for the process. This method can be promisingly adapted to microelectronic biochip platforms to develop a fully integrated chip for electrochemical DNA assays. Hereafter, this group also reported that C_d measurements produce a strong hybridization response under conditions of PBS concentration from 0.001 to 0.5 M and probe coverage from 1×10^{12} to 7×10^{12} cm^{-2}. The detection sensitivity, shown as the capacitive variation per hybridization event, increased about 5-fold as buffer concentration decreased from 0.5 to 0.01 M. Further increase was obvious in 0.001 M but could not be exactly quantified as a result of the fact the hybridized analyte coverage was too low to independently confirm. At high buffer concentration, the capacitive response to hybridization is dominated by thinning or, depending on probe coverage, morpholino breakup as a collapsed probe becomes converted to solvated duplexes. At low buffer concentration, the capacitive response can be explained as being dominated by ionic effects originating from accumulation of target charge [33]. Low ionic strengths, except for amplified diagnostic sensitivity, are expected to mitigate biasing influence on an assay by destabilizing the secondary structure of nucleic acid analytes. The capability to amplify electrostatic detection through measurement under low ionic strengths may be applicable for other uncharged nucleic acid analogues, such as PNA. Low ionic strengths have been exhibited to attractively improve detection effect with some of these approaches [34].

In addition, electrochemical DNA biosensors based on a porous anodic alumina (PAA) membrane modified with morpholino have been developed. The surface charge effect in controlling ionic conductance through a nanoporous alumina membrane modified with neutral silanes and morpholino has been investigated for label-free DNA detection. PAA membrane was modified with 3-aminopropyltrimethoxysilane (APTES) or 2-(carbomethoxy)ethyltrichlorosilane (ETS). A gold film electrode was deposited on the opposite side of the membrane at a 15° angle to the membrane. The surface of the membrane was activated by glutaraldehyde. Then the membrane was assembled into a homemade cell, where the following modifications were performed: the cell held the membrane between two PMMA pieces with matching holes for solution flow and 5'-aminated morpholino was immobilized on the formyl groups activated membrane, which was already assembled into the cell. The corresponding electrode on the opposite side of the membrane was connected to a potentiostat for impedance measurements. Finally, a strong surface charge effect on ionic conductance variation upon morpholino-DNA hybridization was yielded, exceeding an order of magnitude. This surface charge effect can be applied in fabrication of inexpensive electrical DNA sensors [35]. Another method that has been proposed for DNA detection is by measuring the morpholino-DNA hybridization hindered diffusion flux of $Fe(CN)_6^{3-}$ through nanochannels of a PAA membrane. Similarly, the PAA was also modified with APTES followed by deposition of a gold film electrode on the opposite side. The gold film-coated membrane was held between two

poly(ethyleneterephthalate) (PET) sheets. A copper wire was attached in electrical contact with the membrane by silver(Ag) conductive epoxy. The surface of the membrane was further activated by glutaraldehyde. Then, the homemade cell held the membrane between two poly(dimethylsiloxane) (PDMS) films. The gold(Au) film coated side of the membrane was connected with the cell. The Au film was set as the working electrode, a platinum wire as the counter electrode and the Ag/AgCl as the reference electrode in the cell formed a three-electrode system for electrochemical detection. After the membrane was placed inside the cell, amino-modified morpholino reacted with the formyl groups. As soon as $Fe(CN)_6^{3-}$ was added into the cell, a significant current increase was observed as a result of $Fe(CN)_6^{3-}$ flowing through the channel to the gold film electrode surface (Fig. 1). Hybridization of the morpholino with target DNA formed a negatively charged morpholino-DNA complex, hindering $Fe(CN)_6^{3-}$ from diffusing through the nanochannels. The hindered diffusion process was dominated by the electrostatic effect between morpholino and DNA. The concentration of target DNA can be recognized from the signal change upon hybridization and the detection limit was 0.1 nM [36].

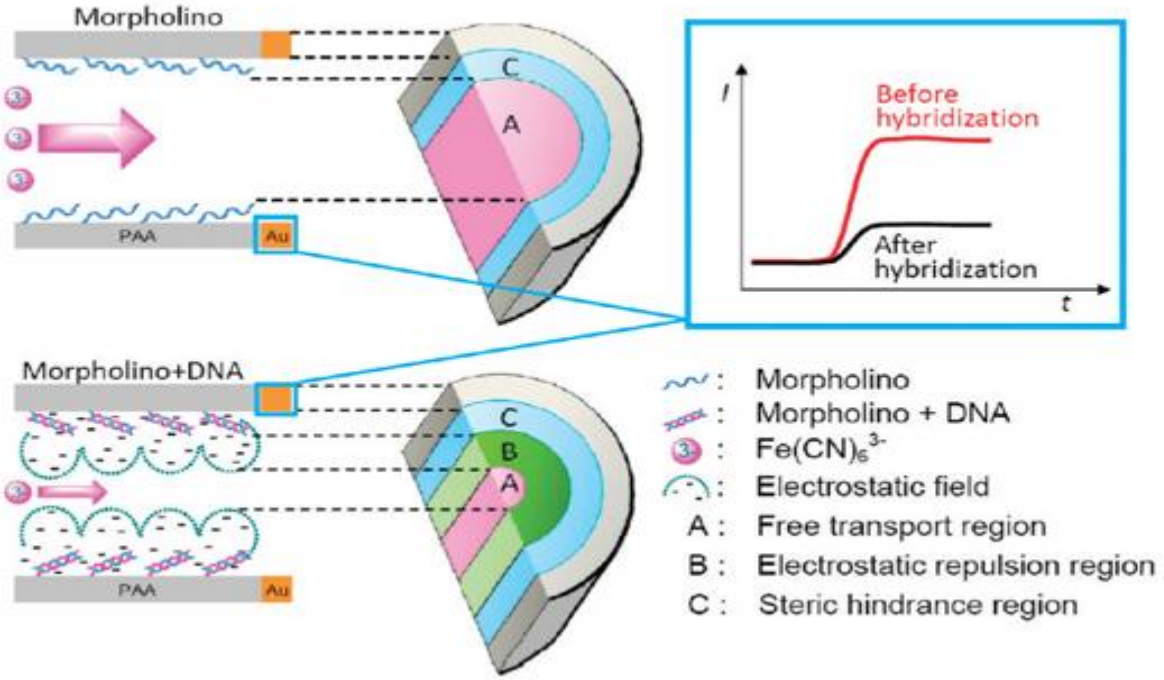


Figure 1. Schematic illustration of the working principle for DNA analysis based on the electrochemical detector.Reprinted with permission from ACS Nano.

Morpholino is completely resistant to many enzymes in the physiological environment. The hybridized double strand of morpholino-DNA exhibits higher stability than DNA-DNA, both on electrode surfaces and in solution due to the lack of electrostatic repulsion [19]. Cao et al. have studied the anti-enzymolysis ability of surface attached DNA, morpholino and DNA-DNA/morpholino-DNA duplexes using cyclic voltammetry. DNase I, an endonuclease which can nonspecifically cleave single-strand or double-strand DNA, was used as the model enzyme [37]. Ferrocene was covalently attached at the terminal of morpholino as an electroactive reporter. Results obviously show that the presence of morpholino in the morpholino-DNA double strands significantly improves the anti-enzymolysis ability of DNA. The protection effect is attributed to the steric hindrance of morpholino toward the enzymolysis reaction,

leading to considerably decreased enzymolysis speed even though DNase I can still attach on the morpholino-DNA double strand [38]. The effect of morpholino on the resistance of DNA to enzymatic degradation on a solid surface provides useful information on the fabrication and application of DNA biosensors and molecular electronic devices.

Gao et al. have applied an electrochemical biosensor, in which morpholino functions as the capture probe and a cationic redox polymer as the signal generator, for direct DNA detection. It is based on the modification of the morpholino and formation of a DNA/cationic redox polymer bilayer structure on an indium-tin oxide (ITO) electrode for amperometric detection of target DNA. After hybridizing the target DNA to the morpholino probe, the cationic redox polymer was immobilized on the ITO electrode through electrostatic interaction with the DNA. The deposited cationic redox polymer showed excellent electrocatalytic activity for the oxidation of ascorbic acid (AA), allowing for voltammetric and amperometric detection of DNA. A linear relationship between current and concentration up to 500 pM and detection limit of 1.0 pM were obtained in amperometry. The high sensitivity and significantly improved signal-to-noise ratio were attributed to the efficient catalytic oxidation of AA at 0.15 V as well as the high electron-exchange rate of $Os(dmbpy)_2$ (where, dmbpy= 4,4′-dimethyl-2,2′-bipyridine) redox moieties. The association of the electrostatic formation of DNA/cationic redox polymer bilayer with electrocatalysis provided a highly sensitive label-free method for DNA quantification and mismatch discrimination [39]. Gao et al., have also reported on an ultrasensitive label-free microRNA (miRNA) biosensor through electrochemical impedance spectroscopic detection, based on electrodeposition of an insulating polymer film (Fig. 2). Non-ionic morpholino used as the capture probe was modified on an ITO glass. The neutral surface was converted to anionic form upon hybridization of morpholino to miRNA. In the presence of peroxide, the horseradish peroxidase catalyzed the polymerization of 3,3'-dimethoxybenzidine and the poly(3,3'-dimethoxybenzidine) (PDB) was electrodeposited onto the ITO glass. The sensitivity was significantly enhanced due to the PDB electrodeposition process. This allows the electrochemical detection of miRNA to be performed in the concentration range from 5.0 fM to 2.0 pM with a detection limit of 2.0 fM [40].

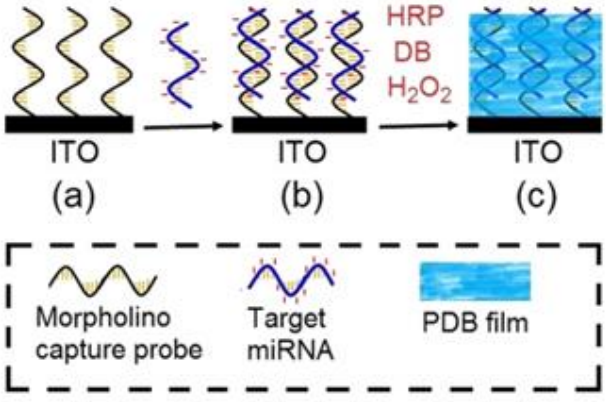


Figure 2. Schematic illustration of the principle for miRNA analysis based on morpholino-modified ITO glass. Reprinted with permission from Analytical Chmistry.

4.2 Other biosensors

Additional nucleic acid biosensing methods based on morpholino have also been developed in the past few years, including fluorescent, microelectronic and microarray techniques. Nucleic acid biosensors based on fluorescence have received great attention due to their high sensitivity, low background noise and uncomplicated instrumentation [41]. We have developed an efficient fluorescent method for the sequence-specific detection of DNA, based on a morpholino-functionalized silicon chip platform (Fig. 3). Morpholino was first immobilized to the surface of the silicon chip by using 3-Aminopropyltriethoxysilane (APTES) as the silane coupling agent and 1, 4-Phenylenediisothiocyanate (PDITC) as the cross linker, then hybridized with DNA in the ensuing step. The fluorescence label was introduced by strongly binding rhodamine B, which contains a terminal carboxylic group, with DNA by means of a phosphate-zirconium-carboxylate coordination reaction. Under the optimal

conditions, a morpholino-functionalized silicon chip presented a great linear relationship between the fluorescence intensity and logarithm of target DNA concentrations in the range from 1.0 pM to 1.0 nM with a detection limit of 4.5 pM. Furthermore, fully complementary DNA versus single-base mismatched, three-base mismatched and non-complementary DNA could be effectively identified under these conditions. The chip rendered satisfactory analytical performance for detection of DNA in serum samples, thus exhibiting practical significance [42].

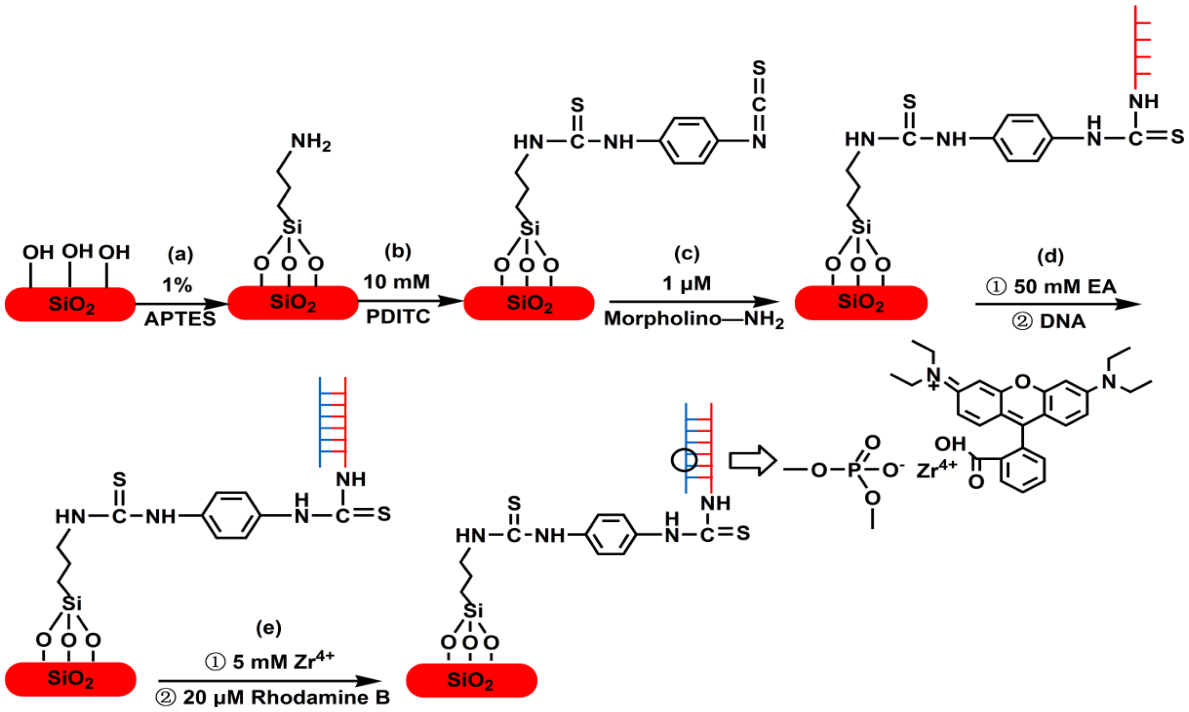


Figure 3. Schematic diagram of the fluorescent DNA detection with morpholino-functionalized silicon chip. (a)The silicon chip was coated with APTES. (b)PDITC reacted with the amino group. (c)The morpholino probe was linked to the isothiocyanate group. (d) After blocking unreacted isothiocyanate group with EA, DNA hybridized with the morpholino probe. (e)After binding Zr^{4+} to the phosphate on DNA backbone, rhodamine B bound strongly to the active zirconium phosphate.

Microelectronic biosensors have excellent sensitivity due to their small dimension and large surface-to-volume ratio. Zhang et al. have employed morpholino-functionalized silicon nanowires as platforms for sequence-specific detection of DNA (Fig. 4). Amino-modified morpholino was attached to the silicon surface through APTES and glutaraldehyde. Real-time detection of the morpholino-functionalized SiNW biosensor showed a reduction in a time-dependent conductance upon addition of complementary and mutant DNA. The resultant morpholino-functionalized surface exhibited high recognition specificity for DNA at a concentration down to 100 fM. Fully complementary versus mismatched DNA could be identified through this biosensor. Furthermore, the yielded morpholino silicon surface is stable towards the denaturation and re-hybridization circle [43].

Microarrays, namely biochips, are analytical devices with thousands of probes immobilized on a solid substrate. The probes are arranged in miniaturized two-dimensional arrays of dots. The samples to be analyzed are hybridized to these probes and the interactions between the probes and target are detected by a high-resolution scanner. Microarrays provide possibility of rapid, sensitive and high-throughput analysis in genomics and proteomics [1]. Morpholino

microarrays were fabricated by Qiao et al. through contact printing of 5'-aminated probes onto the aldehyde slides. Besides a covalent attachment, morpholino was found to be effectively immobilized by physical adsorption. Post-printing wash with surfactant solutions, such as nonionic surfactant Tween 20, could be used to remove physically adsorbed morpholino. The surfactants suppressed morpholino-DNA hybridization, which was attributed to the complexation between morpholino and the surfactants. Morpholino-DNA hybridization was dependent on ionic strength and the hybridization reached the maximum at intermediate ionic strengths. At lower ionic strengths, the decline in hybridization was attributed to the electrostatic obstruction accumulated from target DNA, and that was due to the stabilization of the target secondary structure in solution at higher ionic strengths [44].

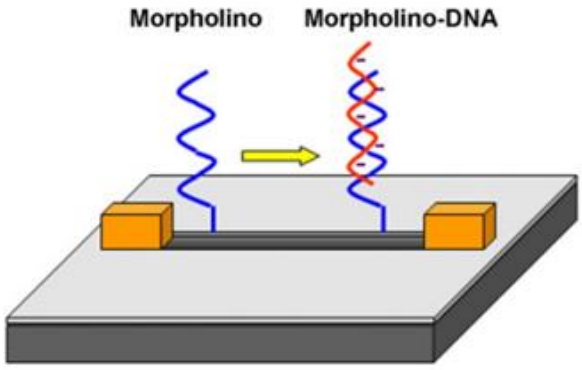


Figure 4. Schematic illustration of the SiNW sensing device. Reprinted with permission from Biosensors and Bioelectronics.

5. Conclusions

In this review, the recent development of nucleic acid biosensors based on morpholino has been discussed. Morpholino has received great attention due to its advantageous properties including water solubility, nucleases and proteases resistance, high hybridization specificity and activity in cells. Morpholino has outstanding affinity for nucleic acids and the destabilizing effect of mismatches in morpholino-containing hererodimers is higher than in DNA or RNA double strands. Morpholino-based nucleic acid biosensors have high sensitivity and specificity for nucleic acid detection. These would provide an increasing number of analytical tools with applications in SNPs and disease diagnosis. Nowadays, manufacturing of integrated and automated devices, reduction of cost and improvement of sensitivity will be significant issues for using morpholino in biomedical applications. Undoubtedly, new advances will emerge rapidly in the next few years that will benefit numerous areas.

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