Chapter 15

Diagnostic Applications of Morpholinos and Label-Free Electrochemical Detection of Nucleic Acids

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Abstract

Diagnostic applications of morpholinos take advantage of their unique properties including backbone charge neutrality, a weak impact of ionic strength on their hybridization behavior, and their resistance to enzymatic degradation. This chapter overviews how these properties have advanced transduction and other capabilities useful for the analysis of nucleic acids. In many cases, the benefits stem from electrostatic mechanisms; for example, use of low ionic strengths improves sensitivity of detection while decreasing background signals because only the nucleic acid analyte is charged. While most literature reports focus on in vitro assays in buffer, morpholinos have been also used for biodistribution measurements of species such as fungal rRNA and miRNA. After reviewing the diagnostic applications of morpholinos, the chapter describes preparation of morpholino monolayers on metal supports for electrochemical diagnostics and the procedure for performing label-free detection of DNA from changes in surface capacitance.

Key words Biosensor, Surface, Interface, Microarray, FISH, Fluorescence, Imaging

1 Introduction

Morpholinos bring a number of key advantages to diagnostic applications. Compared to other uncharged DNA analogs, such as peptide nucleic acids (PNA) and methylphosphonates [1], morpholinos offer flexibility with regard to oligomer sequence and length combined with an optimal balance of binding affinity. The binding affinity is well suited to diagnostics using oligomer lengths of around 20 nucleotides. These lengths provide good sequence specificity and affinity to bind analyte nucleic acids (targets) at the fM to nM concentrations of interest without the affinity being so high as to induce strong background signals from shorter and/or mismatched sequences [2]. Like other nucleic acid analogs morpholinos possess excellent resistance to enzymatic degradation.

Diagnostic applications of morpholinos (MOs) were initiated nearly two decades ago. The first example devised a purification strategy in which nucleic acids of interest were hybridized with

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complementary MOs in solution, followed by solid phase capture of the hybrids [3]. This early study already highlighted certain key advantages bestowed by the charge-neutral nature of morpholinos. For example, it was recognized that hybridization between a charge-neutral MO and a nucleic acid has a much reduced dependence on ionic strength, compared to base pairing between two nucleic acids. This insensitivity to salt concentration can be used to devise assays in which low ionic strengths destabilize secondary structure in a nucleic acid analyte and thus improve its availability for detection. This general strategy was exploited in morpholino microarrays [4] as well as nanoparticle-based diagnostics in which thermally induced aggregation of MO-modified gold nanoparticles was used to identify analytes of interest [5, 6]. The nanoparticle aggregation strategy was recently combined with asymmetric PCR to develop a single nucleotide polymorphism assay for warfarin sensitivity [7]. These studies found ionic strengths in the 10–50 mM range as optimal for realizing good diagnostic signals while suppressing analyte secondary structure. The lower limit arises because, in surface hybridization such as encountered in microarrays, overly low ionic strengths decrease signals because of electrostatic penalties to accumulation of charged nucleic acid analyte on the array [4].

Perhaps the broadest impact of morpholinos on diagnostic research has been through advancement of mechanisms of transduction, especially ones based on electrostatic and conductivity principles. Tercero and coworkers demonstrated label-free detection of hybridization between immobilized morpholino probes and solution DNA targets by monitoring changes in interfacial capacitance [8, 9]. The observed capacitive signals were attributed to changes in the near surface ionic strength and dielectric constant that accompanied accumulation of charged DNA strands. The signals were several times higher when uncharged morpholino probes were used instead of DNA probes, illustrating the benefit of restricting electrostatic interactions to just the analyte. Gao et al. took advantage of the accumulation of analyte surface charge in a different way by using it to electrostatically immobilize, once hybridization was complete, cationic polymers [10, 11]. The final extent of hybridization could be detected using amperometric methods in combination with a redox-active, positively charged polymer for the readout step [11]. Alternately, detection could be accomplished through impedance measurements and an insulating polymer as the contrast agent [10]. A somewhat related approach by another group of investigators used Zr^{4+} cations to "label" hybridized morpholino films through association with the bound DNA, followed by complexation of the associated Zr^{4+} with carboxylate rhodamine in a second step [12]. This method provided linear fluorescent readout from 1 pM to 1 nM concentration of analyte. The detection limit was further lowered to 10 fM by switching to hybridization on microspheres instead of a planar support and implementing posthybridization labeling with

streptavidin/alkaline phosphatase, coupled to a phosphatase-catalyzed reaction cascade for fluorescent detection [13].

Several approaches have relied on unique properties of nanomaterials [14–17]. The narrow pores of porous anodic alumina nanostructures, on the order of a few tens of nm in diameter, were modified with morpholino probes. Because of the narrowness of these pores, subsequent hybridization effectively fills the pores with charged nucleic acids together with mobile countercharge needed to maintain electroneutrality. These changes can be readily measured, especially at lower ionic strengths, to monitor the progress of hybridization. Various approaches have been used, including measurements of the AC impedance across the nanopores [14] and monitoring the through-pore diffusion of charged electroactive species, such as ferricyanide anions, whose partitioning into the pores is affected by the amount of hybridized nucleic acid [15]. Alternately, Gao et al. immobilized MO–DNA heteroduplexes on nanopore walls; if the sample was sufficiently complementary an exchange reaction occurred that transferred the heteroduplex DNA to the solution sample, leading to increase in ferricyanide diffusion [16]. Other nanostructures explored for morpholino diagnostic schemes include nanowires, as reported by Zhang et al., who functionalized silicon nanowires with morpholino probes and detected binding of charged nucleic acids from changes in nanowire conductance [17]. Binding of negatively charged DNA analyte leads to repulsive field effects within the nanowire that lower its conductance. Approximately a 100 fM sensitivity was reported.

The compatibility of morpholinos with hybridization under low ionic strengths enables additional capabilities. For example, Martins et al. exploited low ionic strength buffers to concentrate charged DNA analyte using an electrokinetic ion concentration polarization strategy, reaching 1000-fold increases in analyte concentration within several minutes and accelerating rates of DNA hybridization to immobilized MO probes by over 50-fold [18]. Zhao and coworkers combined morpholino-modified gold nanoparticles with pH-sensitive folding of i-motif DNA to demonstrate plasmonic pH sensors capable of functioning in low ionic strength environments [19].

The synthetic origins of morpholinos endow them with excellent resistance to enzymatic degradation, a consideration that becomes especially relevant when morpholinos are introduced into live cells or tissue matrices. A number of investigators have exploited this advantage for diagnostic purposes. Liong et al. used morpholino linkers to bestow DNase resistance in their approach to magnetic immunolabeling of cells [20]. Cao and coworkers demonstrated that the DNA strand in a MO–DNA duplex is protected against DNase I cleavage in surface hybridization applications [21]. Burki et al. took this notion a step further by developing an assay for morpholino concentrations in tissues and sera based on nuclease digestion of unhybridized DNA

probes immobilized on well plates. In this approach, only MO–DNA hybrids remained undigested and are subsequently detected through an alkaline phosphatase-mediated mechanism that leads to a fluorescent product [22].

Morpholinos have also found significant use in biodistribution measurements. They can be modified with radiolabels and antibodies for PET and SPECT tomography imaging, as demonstrated by Cheng et al. in the context of studying drug distributions in mice [23]. Radiolabeled morpholinos have also been used to detect presence of fungal rRNA as a diagnostic intervention, based on direct hybridization of the morpholino with the rRNA sequences of interest in the lungs of infected mice [24]. Most recently, fluorescein-modified morpholinos were used for in situ hybridization with small RNA in tissue samples to reveal the RNA content of synaptic vesicles, in combination with alkaline phosphatase-based signal amplification [25]. This experimental protocol was previously developed and demonstrated for determining miRNA distributions [26]. The same study demonstrated that morpholino probes are a highly competitive alternative to locked nucleic acid (LNA) probes for in situ hybridization diagnostics. Diagnostic assays have been also developed for the "reverse problem" of quantifying the concentration of morpholino oligomers in tissue and cell samples, using in situ hybridization and fluorescent LNA probes for the morpholino sequences of interest [27].

Having overviewed some of the ways that morpholinos have and continue to contribute to diagnostic applications, the remainder of this chapter will describe a protocol for preparation of morpholino films on electrodes, and of using such films for monitoring binding of nucleic acids through determination of the changes in interfacial capacitance. The protocol foremost derives from studies reported in references [8, 9].

2 Materials

Prepare all solutions using ultrapure water with a resistivity of 18.2 MΩ cm at 25 °C. Use analytical grade reagents and follow all waste disposal regulations when discarding waste materials.

2.1 Components for Oligonucleotide Labeling and Purification

1. HPLC system with a binary mobile phase.
2. Reversed-phase column, e.g., Clarity Oligo-RP column, 100 × 4.6 mm, 3 μm particle size (Phenomenex, Torrance, CA, USA).
3. Vacuum centrifuge, e.g., vacufuge (Eppendorf, Hamburg, Germany).
4. HPLC aqueous mobile phase: triethylamine/hexafluoroisopropanol buffer (TEA-HFIP); 4.5 mM TEA, 100 mM HFIP, pH 8.0.

5. HPLC organic mobile phase: methanol.
6. Morpholino probe solution: 0.25 nmol of morpholino probes (Gene Tools, Philomath, OR, USA) in 1 ml of deionized water. Store at 0 °C until use.
7. Electroactive labels for determining absolute coverage of probes and targets, if desired. These are available commercially and can be amino-reactive such as ferrocene mono-carboxylic acid *N*-hydroxysuccinimide ester (FcCA-NHS) as well as sulfhydryl-reactive such as *N*-(2-ferrocene-ethyl) maleimide (FEM).
8. Disulfide deprotection buffer: 10 mM Tris–HCl, pH 8.0, 10 mM dithiothreitol, 1 mM EDTA. Use solution immediately after preparation.
9. Probe morpholino labeling buffer: 0.5 M sodium carbonate buffer, pH 9.0.
10. DNA target: commercially synthesized and HPLC purified (Eurofins MWG Operon, Louisville, KY, USA).
11. Oligonucleotide purification columns: NAP-10 purification column (GE Healthcare, Chicago, IL, USA) and oligonucleotide purification cartridge (Applied Biosystems, Foster City, CA).
12. Target DNA labeling buffer: 150 mM potassium phosphate buffer, pH 8.0.

2.2 Components for Preparation and Modification of Gold Electrodes

1. Electrochemical etching solution: 0.5 M H_2SO_4.
2. Solution for determination of electrode roughness: 0.1 M NaF.
3. Mechanical polishing solution: 1 μm diamond particle suspension (Bioanalytical Systems, Inc., West Lafayette, IN, USA).
4. Electrode polishing pad (Bioanalytical Systems, Inc., West Lafayette, IN, USA).
5. Passivation solution: 1 mM mercaptopropanol (MCP, 95% purity; Aldrich).

2.3 Materials for Electrochemical Measurement

1. Electrochemical workstation for measuring AC impedance and cyclic voltammetry with a three-electrode cell with ports for an Au working electrode (*see* following **step 3**), a platinum wire counter electrode, and a reference electrode. A typical reference electrode is Ag/AgCl/saturated KCl; 0.197 V vs NHE [normal hydrogen electrode] at 25 °C (*see* **Note 1**). All quoted potentials are expressed relative to this reference.
2. Working electrodes: typically 1.6 mm diameter polycrystalline gold disk electrodes (Bioanalytical Systems, Inc., West Lafayette, IN, USA).

3. DNA target solution: 25 nM complementary DNA target, commercially synthesized and HPLC purified (Eurofins MWG Operon, Louisville, KY, USA) and diluted in deionized water.
4. Hybridization buffer: sodium phosphate buffer, pH 7.0 (*see* **Note 2**).

3 Methods

3.1 Labeling and Purification of Morpholino and Nucleic Acid Oligonucleotides

(*only required if absolute coverages are to be determined*)

1. Label morpholino oligos at the end not used for immobilization with an electroactive label, such as ferrocene. Standard bioconjugate methods are used.

   Example: Combine 150-fold stoichiometric excess of ferrocene mono-carboxylic acid *N*-hydroxysuccinimide ester (FcCA-NHS) with 0.3 mM solution of morpholino probe in 150 mM pH 9.0 sodium carbonate buffer for 1 h at room temperature. Remove unreacted FcCA-NHS using a NAP-10 purification column. Complete purification of recovered morpholino oligos using reverse-phase HPLC with a 20 min linear gradient of 12–100% methanol in triethylamine/hexafluoroisopropanol buffer (4.5 mM TEA, 100 mM HFIP, pH 8.0), followed by 5 min at 100% methanol.
2. Label nucleic acid analyte with an electroactive label. Standard bioconjugate methods apply.

   Example: for amino-modified nucleic acid and amino-reactive electroactive tags, the sample procedure in **step 1** earlier can be followed but using an HPLC gradient for nucleic acids (*see* later). For disulfide-modified nucleic acid and thiol-reactive labels, proceed as follows. Deprotect the disulfide for 2 h in a solution of 10 mM dithiothreitol (DTT), 10 mM Tris–HCl, 1 mM EDTA, pH 8.0. Remove excess DTT on a NAP-10 column. React the recovered solution for 1 h with 50-fold molar excess of a thiol-reactive tag such as *N*-(2-ferrocene-ethyl) maleimide (FEM) in 150 mM potassium phosphate buffer, pH 8.0. Purify the resultant product using a 12–60% gradient of methanol in HFIP-TEA spread over 22 min.
3. Store labeled morpholino and DNA conjugates dry at −20 °C until use.

3.2 Electrode Preparation and Modification

1. Clean a gold disk working electrode by mechanical polishing with 1 μm diamond suspension on an electrode polishing pad; once done rinse well with methanol followed by deionized water. Electrode should have a mirror-like finish. While keeping the electrode covered by a water droplet to prevent contamination from adsorbates in air, transfer it to 0.5 M H_2SO_4 and

perform 60 cycles of cyclic voltammetry electropolishing by cycling from 0.24 to 1.54 V and back to 0.24 V at 0.1 V/s. Electropolishing further cleans the gold through alternate oxidation and reduction cycles. When done, rinse electrode thoroughly with deionized water and transfer, while covered with a water droplet, into 0.1 M NaF for determination of roughness as in **step 2** of Subheading 3.2, or else directly into probe deposition solution as in **step 3** of Subheading 3.2.

2. If desired, determine electrode roughness r and the true area $A = rA_g$ where A_g is the geometric area of the electrode, by measuring the electrode capacitance at −0.8 V in 0.1 M NaF solution. Set up the experiment as in **step 1** of Subheading 3.3. Measure the total electrode capacitance using AC impedance at a bias of −0.8 V, using a superimposed potential modulation of 25 Hz and 5 mV rms amplitude. From the measured in-phase and out-of-phase currents, calculate the electrode capacitance as in Subheading 3.4, making sure to use μF as the units. Divide the electrode capacitance by 22 μF/cm^2 to arrive at the true electrode area A in cm^2. Roughness factor r can be calculated from A and A_g, $r = A/A_g$.
3. After roughness determination, rinse the electrode with deionized water and, without drying, immerse it in 0.25 μM probe deposition solution. A probe solution concentration range of 0.25–0.75 μM is recommended (*see* **Note 3**).
4. After immobilization, rinse electrode surface with deionized water, then passivate the electrode surface with 1 mM mercaptopropanol solution for 150 min (*see* **Note 4**).

3.3 Electrochemical Measurements

1. Set up the electrochemical workstation with a three-electrode cell filled with the hybridization solution and connect the Au working electrode, a platinum wire counter-electrode, and an Ag/AgCl/saturated KC1 (or other suitable) reference electrode.
2. To determine capacitance C_d, make an AC impedance measurement. Typical procedure: Set the DC bias to −0.1 V, at which sensitivity is optimal. On top of this bias, apply a sinusoidal potential modulation of frequency f (*see* **Note 5**) and 5 mV rms amplitude. From the measured in-phase and out-of-phase currents, calculate C_d as in Subheading 3.4. Changes in C_d as a function of time are used to monitor the progress of hybridization. To more accurately estimate C_d, correct for baseline drift by extrapolating drift from points measured prior to addition of target. Alternately, add 1 mM mercaptopropanol to the hybridization solution to decrease baseline drift by better maintaining electrode passivation, or use a second working electrode with a nonhybridizing probe sequence to serve as baseline reference.

3. To determine absolute probe and target coverages, make a cyclic voltammetry measurement over a potential range that spans the electroactive region of the tags used. Typical procedure for ferrocene labels: scan the potential at 20 V/s from 0 to 0.6 V and back to 0 V. The high scanning speed decreases likelihood of ferrocene degradation. Calculate probe and/or target coverage from the measured current vs potential voltammogram as in Subheading 3.5.

3.4 Derivation of Differential Capacitance (C_d) from AC Impedance Data

1. Calculate the out-of-phase impedance $Z'' = -V_{AC}I_O/(I_I^2 + I_O^2)$, where V_{AC} is amplitude of the imposed sinusoidal potential modulation and I_O and I_I are the measured amplitudes of the out-of-phase and in-phase components of the current, respectively.
2. Calculate the differential capacitance from $Cd = 1/(2\pi f|Z''|)$, where f is the frequency of the imposed potential modulation and the vertical bars "| |" signify absolute value. The capacitance can be expressed per area by dividing C_d by the total area A (**step 2** of Subheading 3.2).

3.5 Derivation of Target and Probe Coverages from Cyclic Voltammetry Data

1. Integrate areas of the peaks in the CV voltammogram that correspond to the probe and/or the target tag. The area is given by the total current minus baseline current. The baseline current under the peak can often be well estimated by a sum of a linear and a stretched exponential function.
2. Calculate the corresponding probe or target coverage Γ from the area P of the integrated peak using $\Gamma = P/(eA\nu)$ where $e = 1.602 \times 10^{-19}$ C is the elementary charge, A is the measured electrode area (**step 2** of Subheading 3.2), and ν is the scan rate used for measurement. Confirm dimensional consistency by checking all units carefully.

4 Notes

1. Reference electrodes can be bought commercially or fabricated by enclosing a 5–6 cm piece of 0.5 mm diameter silver wire coated with AgCl within a glass sleeve with a porous glass junction and filled with a saturated KCl solution.
2. A recommended concentration range for the pH 7.0 sodium phosphate hybridization buffer is 0.001–0.5 M (molarity of phosphate).
3. The probe coverage that results from the described protocol is typically between 2×10^{12} and 5×10^{12} probes/cm^2.

4. Mercaptopropanol passivation improves reproducibility of baselines in electrochemical experiments and suppresses non-specific adsorption of nucleic acid analyte.
5. The AC frequency should correspond to a phase angle of about 45° for the chosen cell geometry and hybridization buffer. Caution should be exercised to avoid any secondary capacitive charging that may arise at high frequencies.

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