DNA Hybridization Detection with 100 zM Sensitivity Using Piezoelectric Plate Sensors with Improved Noise-Reduction Algorithm

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DNA Hybridization Detection with 100 zM Sensitivity Using Piezoelectric Plate Sensors with Improved Noise-Reduction Algorithm†

Ceyhun E. Kirimli,a Wei-Heng Shih,b and Wan Y. Shihc

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We have examined real-time, in situ hybridization detection of target DNA (tDNA) in a buffer solution and in urine using 8 μm-thick lead magnesium niobate-lead titanate (PMNPT) piezoelectric plate sensors (PEPSs) about 1.1-1.2 mm long and 0.45 mm wide with improved 3-mercaptopropyltrimethoxysilane (MPS) insulation and a new multiple-parabola (>50) resonance peak position fitting algorithm. With probe DNA (pDNA) immobilized on the PEPS surface and by monitoring the first width extension mode (WEM) resonance frequency shift we detected tDNA in real time at concentration as low as 1 × 10⁻¹⁹M in urine (100±M) with a signal to noise ratio (SNR) of 13 without DNA isolation and amplification at room temperature in 30 min. The present multiple-parabola fitting algorithm increased the detection SNR by about 10 times from those obtained using the raw data and by about 5 times from those obtained using single parabola fitting. The detection was validated by in situ follow-up detection and subsequent visualization of fluorescent reporter microspheres (FRMs) coated with reporter DNA complementary to the tDNA but different from the probe pDNA.

1 Introduction

Cell-free DNA was first discovered by Mandel and Metais1 in 1948 and became increasingly more important when mutant Ras gene fragments were discovered in the blood of patients.2,3 Since then, circulating DNA in the blood has been studied extensively for its diagnostic and prognostic association with various cancers such as: bladder cancer,4,5 breast cancer,6-9 cervical cancer,10,11 colorectal cancer,12-16 hepatocellular carcinoma,17-20 lung cancer,21-25 lymphoma,26-28 melanoma,29-36 ovarian cancer,37,38 pancreatic cancer,39,40 and prostate cancer41-46. The passage of circulating DNA through the kidney barrier has been overlooked due to the selectivity of the nephron and DNA fragments observed in urine have been mostly thought to have originated from organs and tissues of the urogenital tract. More recently, it has been found that low-molecular weight (LMW) DNA fragments from a distant organ could pass through kidneys47,48. The current standard method for detecting DNA is polymerase chain reaction (PCR). For transrenal DNA detection, PCR has limitations on the amplicon size49 and potential inhibitions by co-isolated factors. Furthermore, the effectiveness of PCR could also be limited by the DNA isolation techniques which are mostly for isolating nuclear DNA from intact cells48, not particularly suitable for isolating short transrenal DNA fragments36,48,50. It would be desirable to have a real-time, label-free method that can detect transrenal DNA fragments in urine that does not depend on the DNA isolation techniques and is not limited by the lengths of the DNA fragments.

Current genetic detection technologies under development rely on fluorescence51-53, quartz crystal microbalance (QCM)54,55, electrochemical56 binding of nano-metal particles57, surface plasmon resonance (SPR)58, silicon-based microcantilever sensor as well as piezoelectric micrcantilever sensor. For DNA detection, nanoparticle amplified QCM exhibited a concentration sensitivity of 1pM59. Nanoparticle enhanced SPR exhibits concentration sensitivity of 10-100 aM60. The electrochemical methods involving nanoparticles and nanotubes also exhibited concentration sensitivity of about 30 fM61. Nanowires62-66 and nanotubes67,68 exhibited concentration sensitivity ranging from 100/fM to 1/M. Microcantilevers coupled with nano-metal particles exhibited 0.01nM concentration sensitivity69. Although methods such as QCM, SPR, silicon-based microcantilever sensor as well as lead zirconate titanate (PZT) piezoelectric microcantilever sensor

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The goal of this study is to investigate how one can use a WEM peak of a PMN-PT PEPS with improved electrical insulation to detect short DNA fragments spiked in urine using a multiple-parabola peak position fitting approach. The hypothesis was that by fitting the WEM peak to more than one parabola with varying number of data points and by averaging the fitted peak positions one would be able to reduce the noise level for more meaningful tracking of the WEM peak frequency shift due to target DNA binding. We will use 1762T/1764A Hepatitis B virus double mutation (HBV-DM) as the model tDNA as it was used in the previous study by

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**Fig. 1** A schematic of (a) the cross section of a piezoelectric plate sensor (PEPS), (b) the first length extension mode (LEM), (c) width extension mode (WEM) vibration of a PEPS where the solid bars illustrated the initial position of the PEPS and the dashdotted shapes illustrate the extended positions, and (d) a top-view optical micrograph.

(PEMS)\textsuperscript{70,71} were label-free, the sensitivity was still many orders of magnitude away from the attomolar (10\textsuperscript{-18}M) requirement.\textsuperscript{72} Similarly, the 10\textsuperscript{-15}M sensitivity achieved by magnetic beads isolation coupled with electrochemical enhancement was not sufficient.\textsuperscript{73} Nano-scale mechanical imaging by atomic force microscopy (AFM) can differentiate un-hybridized single-stranded DNAs (ssDNAs) from hybridized double-stranded DNAs (dsDNAs) at attomolar sensitivity but it required sophisticated instrument such as AFM.\textsuperscript{74} Although a GaN nanowire extended-gate field-effect-transistor could detect attomolar concentrations of target DNA (tDNA) in situ,\textsuperscript{53} the detection signal (0.2\textsuperscript{°}) at 10\textsuperscript{-18}M was not very different from that (0.3\textsuperscript{°}) at 10\textsuperscript{-6}M making it not suitable for DNA quantification. Streptavidin horseradish peroxidase functionalized carbon nanotubes could detect DNA at 10\textsuperscript{-18}M however it required labeling and was not in situ while label-free carbon nanotube impedance biosensors could only detect DNA at 100 aM, not sufficient for clinical applications.\textsuperscript{76} Electrochemical biosensors have been shown to reach attomolar sensitivity. However, they required electrocatalysis\textsuperscript{77} or magnetic beads amplification\textsuperscript{78} and were thus not label-free or real-time.

Lead magnesium niobate-lead titanate (Pb(Mg\textsubscript{1/3}Nb\textsubscript{2/3})\textsubscript{O\textsubscript{3}}\textsubscript{0.65} – (PbTiO\textsubscript{3})\textsubscript{0.35} (PMN-PT) piezoelectric plate sensor (PEPS) is a new type of piezoelectric sensor consisting of solely a PMN-PT freestanding film 8\textmu m in thickness\textsuperscript{79} thinly coated with a gold electrode on the two major surfaces and encapsulated with a thin 3-mercaptopropyltrimethoxysilane (MPS) electrical insulation layer Figure 1a. Receptor specific to a biomarker is immobilized on the surface of the electrical insulation layer. Binding of the target biomarker to the receptor on the PEPS surface shifts the PEPS length-extension-mode (LEM) (Figure 1b) or width-extension mode (WEM) (Figure 1c) resonance peak frequency, f. Detection of a target protein or target DNA (tDNA) marker is achieved by directly immersing a PEPS in the biological fluid and monitoring the LEM or WEM resonance frequency shift, \Delta f in real time. What is unique about PMN-PT PEPS is that the detection f is a result of binding stress induced polarization switching within the PMN-PT layer\textsuperscript{80}, which was typically three orders of magnitude higher than could be accounted for by the mass change alone.\textsuperscript{81} As a result, PMN-PT PEPS has shown unprecedented concentration sensitivity of 1.6 aM (960 copies/ml) in \textit{in situ} tDNA detection without the need of amplification.\textsuperscript{80} The reason we had different LEM and WEM modes was that we made PEPS longer than it was wide for ease of handling and ease of making. Ideally, one would explore WEM for detection because the higher frequency of WEM could offer better sensitivity. Although, in theory, one could use either the LEM or WEM peak for detection, in past practice, only the LEM peak was usable in liquid. The reason was that the width of the WEM peak was closely related to the transverse electromechanical coupling constant, $\kappa$.\textsuperscript{81} The better the piezoelectric performance of the PMN-PT layer the wider the WEM peak. For a $\kappa$ of about 0.32 the Q value—the ratio of the peak frequency to the width at half the peak height—would be about 10.\textsuperscript{81} Such a wide WEM peak at around 3.5MHz coupled with imperfect electrical insulation and insufficient signal processing made tracking any meaningful peak position shift difficult. Recently, Soylu et al. has found that coating the MPS at pH = 9 and with trace amount of water reduced the conductivity of the insulation layer by three orders of magnitude.\textsuperscript{82} With such improvement, it may be possible to track WEM peak position change with improved peak position fitting algorithm. The advantage of using a WEM peak for detection is that the resonance frequency of a WEM peak is many times higher than that of the LEM peak. As a result, one may be able to further lower the detection concentration limit.

The goal of this study is to investigate how one can use a WEM peak of a PMN-PT PEPS with improved electrical insulation to detect short DNA fragments spiked in urine using a multiple-parabola peak position fitting approach. The hypothesis was that by fitting the WEM peak to more than one parabola with varying number of data points and by averaging the fitted peak positions one would be able to reduce the noise level for more meaningful tracking of the WEM peak frequency shift due to target DNA binding. We will use 1762T/1764A Hepatitis B virus double mutation (HBV-DM) as the model tDNA as it was used in the previous study by
2 Experimental

2.1 PEPS fabrication

Two PEPS (PEPS A and PEPS B) were used in this study. PEPS A was 1.2mm long and 0.45mm wide and PEPS B was 1.1mm long and 0.45mm wide. The geometry of the sensor, about 1mm long and 0.5mm wide, was a compromise between ease of fabrication and sensitivity. Presently, the PEPS were fabricated manually. While making the PEPS smaller can increase the LEM and WEM frequencies and potentially further enhance the detection sensitivity it would be hard to accomplish manually. However, it should be noted that with presently available tools and automation, in the future, it is possible to make smaller PEPS. Briefly, PEPS A and PEPS B were fabricated from PMN-PT freestanding films 8µm thick that was coated with 110nm thick Cr/Au electrodes by thermal evaporation (Thermionics VE 90) and cut into 2.5mm by 0.45mm strips by a wire saw (Princeton Scientific Precision, Princeton, NJ). Gold wires 10µm in diameter were glued to the top and bottom electrodes of each strip using conductive glue (XCE3104XL, Emerson and Cuming Company, Billerica, MA). The rear end of the strip was fixed on a glass substrate by a nonconductive glue (Loctite 1C Hysol Epoxy Adhesive) to form the PEPS geometry. It was then poled at 15KV/cm at 90°C for 60 min in an incubator (Digital Control Steel Door Incubator 10 – 180E, Quincy Lab). The dielectric constant of the PEPS was measured using an electrical impedance analyzer (Agilent 4294A) to be about 1800 with a loss factor of 2.5% – 3.7% at 1kHz.

2.2 Electrical Insulation

A PEPS was electrically insulated to stabilize the resonance peaks for in-liquid detection by a new 3-mercaptopropyltrimethoxysilane (MPS) (Sigma-Aldrich Co. LLC.) solution coating scheme involving improved MPS cross-linking at pH=9.0 and with water. First, the PEPS was cleaned in a Piranha solution (two parts of 98% sulfuric acid (Fisher) with one part of 30% hydrogen peroxide (Fisher)) for 1 min, followed by rinsing in water and ethanol. Before coating the PEPS with MPS at pH=9.0, we dipped the PEPS in 50ml of a 0.01-mM MPS solution in ethanol (Fisher) with 0.5% of de-ionized (DI) water for 30 min to promote hydrolysis followed by rinsing with water and ethanol. It was then subject to 5 12-hr of MPS coating in 50ml of a 0.1% MPS solution with 0.5% of DI water in ethanol at pH = 9.0 (adjusted by adding KOH (Fisher)). For each 12-hr of MPS coating, the PEPS was always rinsed with water and ethanol first before being immersed in a fresh 0.1% MPS solution at pH = 9.0 with 0.5% water. At the end of the 5th round of MPS coating, the PEPS was rinsed with DI water and ethanol before further coating with receptors for detection. After
insulation, the resonance spectra of the PEPS were measured using a portable AIM 4170C impedance analyzer (Array Solutions).

2.3 Resonance peak frequency determination
The phase-angle-versus-frequency resonance spectra of PEPS A and PEPS B in air (black) and in phosphate buffer saline (PBS) solution (red) are shown in Figure 2a and b. Note that PEPS A had $a - k_{31} = 0.32$ and PEPS B had $a - k_{31} = 0.33$ and the WEM peak of PEPS A and PEPS B were both at around 3.5 MHz with a Q of around 10, reminiscent of the high $-k_{31}$ of both PEPSs. Also note that the baselines of the in-air and in-PBS spectra of the PEPSs were less than 1° – 2° apart, indicative of the effectiveness of the new insulation scheme.

For detection, phase angle-versus-frequency resonance spectra of a PEPS were measured continuously using the AIM 4170C electrical impedance analyzer controlled by a laptop with a custom program written in MatLab. After each resonance-spectrum scan, the MatLab program recorded, analyzed the obtained spectrum, and determined the peak frequency as described below. A resonance peak frequency shift, $\Delta f$, versus time plot on the computer screen was refreshed after each resonance spectrum scan in real time. The program also used the obtained peak frequency shift to adjust the start and stop frequencies for the next resonance-spectrum scan such that the next expected resonance frequency was roughly at the center of frequency window. To determine the peak frequency, the raw resonance spectrum (black full squares in Figure 3) was first smoothed by weighted-linear-least-square regression as illustrated by the red curve in Figure 3. The smoothed curve was then fitted to multiple (about 50) parabolas each with a different frequency range centered at the apparent peak frequency of the smoothed curve. As an example, one of the fitted parabolas is shown as the blue curve in Figure 3 with its peak position denoted by the blue triangle. Each parabola generated a fitted peak frequency. The final fitted peak frequency was the average of all the fitted parabola peak frequencies with outliers excluded.

2.4 Target DNA, probe DNA, and reporter DNAs
The tDNA used was the same 200-nucleotide (nt) long single-stranded DNA (Integrated DNA Technologies) used in the previous study containing the nucleotide sequence of the Hepatitis B virus genome (GeneBank Accession #X04615) centered around the 1762T/1764A double mutations. Part of the sequence of the tDNA that contained the double mutations is shown in Table I where the two mutation sites were underlined. The probe DNA (pDNA) was a 16-nt long synthetic single-stranded DNA (Sigma) complementary to the 16-nt sequence of the tDNA centered on the double mutation sites as shown in Table 1. The pDNA had a biotin with a 12-polyethylene glycol (PEG) spacer at the 5’ end. The melting temperature of the pDNA to the tDNA was 47°C as estimated under the experimental conditions and listed in Table1. To immobilize the biotin-activated pDNA on the PEPS surface, the MPS-coated PEPS was first immersed in 200 µl of 5 mg/ml of maleimide activated biotin (Maleimide-PEG11-Biotin) (Pierce) in PBS for 30 minutes. The maleimide reacted with the thiol group on the MPS surface to immobilize the biotin on the PEPS surface. It was then followed by immersing the PEPS in 200 µl of 1 µM of streptavidin in PBS to bind streptavidin to the biotin on the PEPS surface. Afterwards, the PEPS was immersed in 200 µl of a 10 µM solution

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**Table 1** The sequences of the tDNA, pDNA, upstream urDNA and downstream drDNA and the melting temperature, $T_m$, of the tDNA with pDNA, that of the tDNA with urDNA, and that of the the tDNA with drDNA

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<td>5’…GGTTAAATGATCTTTGT…3’</td>
<td>-</td>
</tr>
<tr>
<td>pDNA</td>
<td>Biotin-5’-ACAAAGAT</td>
<td>47</td>
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<tr>
<td></td>
<td>CATTAACC-3’</td>
<td></td>
</tr>
<tr>
<td>Upstream rDNA</td>
<td>Amine-5’-ACAGACCAATTATG</td>
<td>76.3</td>
</tr>
<tr>
<td>(urDNA)</td>
<td>CCTACAGCCTCCTTAG-3’</td>
<td></td>
</tr>
<tr>
<td>Downstream rDNA</td>
<td>Amine-5’-AATCTCCTCCCCCAA</td>
<td>77.4</td>
</tr>
<tr>
<td>(drDNA)</td>
<td>CTCCCTCCAGTCTTT-3’</td>
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<sup>a</sup>Mutation sites are indicated by underlined bases.
stream rDNA was amine-activated with a 7-PEG spacer at the 3' end. The sequence of the upstream rDNA and that of the downstream rDNA are also shown in Table 1. Figure 4(a) is a schematic that illustrates the relationships between the tDNA, the pDNA, the urDNA, and the drDNA. In real DNA fragments, the mutation sites may be located anywhere in the fragments and in some cases the mutated sites may be too close to the edge for strong enough rDNA binding. Under such conditions, rDNAs in the opposite stream would permit the binding of the rDNA to captured tDNA on the sensor surface.

For this reason, we included both upstream and downstream rDNA in the study even though in the present synthetic tDNA the mutation sites were centrally located. The melting temperature for binding the upstream rDNA (urDNA) to the tDNA was 76.3°C and that of the downstream rDNA (drDNA) to the tDNA was 77.4°C, which are also listed in Table 1. Both drDNA and urDNA are immobilized on 6 μm size fluorescent report microspheres (FRM) as described previously80,89 for in situ validation as well as for visualization of the detection80. In Figure 4(b), we plot the Δf/Δt versus time of PEPS A during the various steps of pDNA immobilization followed by tDNA detection and the subsequent FRM detection. Steps of immobilization are illustrated in inset in Figure 4(b).

2.5 Urine Sample Preparation

Urine samples were collected in a 50 ml centrifuge in a first morning sample collection fashion after emptying the bladder the previous evening. Samples are kept in 4°C refrigerator for detection. Blocking of non-specific binding is accomplished by dissolving 3% BSA in urine equilibrated to room temperature.

2.6 Flow setup

All the tDNA detections were carried out in a flow. A schematic of the flow system consisting of a polycarbonate detection chamber 18.5 mm long 3.5 mm wide and 5.5 mm deep (volume = 356 μl), three reservoirs, and a peristaltic pump (Cole-Parmer 77120 – 62) interconnected with 0.8 mm wide tubing is shown in Figure 5a. The PEPS was vertically placed in the center of the flow chamber with its major faces parallel to the flow as illustrated by the schematic shown in Figure 5b. In each detection event, only one reservoir was connected to the detection chamber. The total volume of the liquid was 50 ml including the liquid in the reservoir, the detection chamber and the connecting tubing. In what follows, all detections were carried out with a flow rate of 1 ml/min corresponding to an average flow velocity of 1.4 mm/s at the PEPS surface. Furthermore, in this setup, the detection could transition from one detection experiment involving the sample in one reservoir to another detection experiment involving the sample of
3 Results

The theoretical first WEM and the first LEM resonance peak frequencies could be calculated using $f = c/2w$ and $f = c/4L$, respectively where $c = \sqrt{Y_11/\rho}$ was the sound velocity in the piezoelectric layer with $Y_{11} = 81$ GPa and $\rho = 7800$ kg/m$^3$ being the Young's modulus in the length and width directions and $\rho$ the density of the piezoelectric layer, respectively and $w$ and $L$ the width and the length of the PEPS, respectively. The theoretical first LEM and WEM peak frequencies were estimated to be 623 kHz and 3.44 MHz, respectively for PEPS A and 718 kHz and 3.42 MHz, respectively for PEPS B as indicated by the vertical dashed lines in Figures 2(a) and 2(b).

tDNA detection was carried out using PEPS A in PBS with tDNA spiked at various concentrations to compare the signal-to-noise ratio (SNR) of the detection resonance frequency shift obtained by fitting the resonance peak frequency using the present multiple-parabola algorithm to those for the same detection but obtained using the raw data or using a single-parabola algorithm. The tDNA detection was immediately followed by FRM detection at a concentration of $10^5$ FRMs/ml as described previously. The tDNA detection $\Delta f/f$ versus time of PEPS A and the corresponding $-\Delta f/f$ at $t = 30$ min versus tDNA concentration are included in the supplemental materials. In the present signal-to-noise (SNR) analysis, the signal was the average tDNA detection $\Delta f/f$ between $t = 25 - 30$ min and the noise was the standard deviation of $-\Delta f/f$ of the blank sample (i.e., without tDNA). The resultant SNR versus tDNA concentration is shown in Figure 6. Note all data points in Figure 6 were the average of three independent runs for each concentration. As can be seen, the SNR obtained by the present multiple-parabola fitting (full squares) were larger than 10 down to the tDNA concentration of $10^{-19}M$. By convention, the lowest acceptable SNR is 3. The fact that the present detection with the multiple-parabola fitting algorithm exhibited an SNR of 10 at $10^{-19}M$, indicating the high sensitivity of the PEPS detection. For comparison, we
also plot the SNR obtained with the raw data (full triangles)
and by single-parabola fitting (full circles). As can be seen,
the present multiple-parabola fitting algorithm improved the
SNR by about ten times from those obtained by the raw data
and by about 5 times from those obtained by single-parabola
fitting. Note the drop in SNR obtained from the raw data at
10⁻¹⁷ M was not meaningful, as the SNR at concentrations be-
low 10⁻¹⁷ M were already below 3 - an indication that SNR
values at below 10⁻¹⁷ M were not reliable. The positive tDNA
detection with an SNR of 10 even at a tDNA concentration as
low as 60 copies/ml was validated by the FRM detection im-
mediately following the tDNA detection as described in the
supplemental materials.

In the following, we applied the multiple-parabola fitting al-
gorithm in the detection of DNA hybridization in urine using
PEPS B. To determine the appropriate amount of BSA needed
to block the PEPS surface from nonspecific binding, PEPS
was first treated with a BSA solution of concentration ranging
0 – 3% followed by inserting the PEPS in flowing urine at a
1 ml/min flow rate for 30 min followed by flowing a phos-
phate buffer saline (PBS) solution at a 6 ml/min flow rate for
30 min. The resultant Δf/f versus time in urine and the sub-
sequent PBS washing with various amounts of BSA blocking
is shown in Figure 7. As can be seen, without BSA blocking,
Δf/f decreased in urine and was subsequently recovered af-
ter PBS washing, indicating that the Δf/f in urine was due
to nonspecific binding by urine which could be washed off
by flowing PBS. The nonspecific binding decreased with an
increasing concentration of BSA blocking and with 3% BSA
blocking it appeared that nonspecific binding by the urine no
longer occurred, i.e, there was no resonance frequency down
shifting in urine and no resonance frequency up shifting in
PBS. In what follows, all detection will be carried out with

Fig. 7 Relative frequency shift, Δf/f, of PEPS B in urine at 1
ml/min for 30 min followed with PBS washing at a flow rate of 6
ml/min after the PEPS was initially blocked with 0, 1, 2 and 3
percent BSA.

Fig. 8 (a) Relative resonance frequency shift, Δf/f versus time of
tDNA detection at various concentrations in urine and (b) –Δf/f at
t = 30min (tDNA hybridization) and at t = 60min (tDNA
hybridization plus FRM detection) versus tDNA concentration. (c)
SNR versus concentration of tDNA graph plotted using data in part
(a)
3% BSA blocking.

Fig. 9 Fluorescent micrographs of the PEPS surface after FRM detection followed by (a) 0 M, (b) 5 × 10^{-20} M, (c) 1 × 10^{-19} M, (d) 1 × 10^{-18} M, (e) 1 × 10^{-17} M, and (f) 1 × 10^{-15} M of tDNA detection. The width of PEPS B as denoted by the parallel dashed lines was 450 μm. Clearly the number of FRMs captured on the PEPS surface increased with an increasing tDNA concentration in a dose responsive fashion validating the tDNA detection at a concentration as low as 5 × 10^{-20} M. (g) Average number of FRMs captured on the PEPS surface versus tDNA concentration in urine.

Discussion

That a 60 copies/ml analytical sensitivity was achieved by both PEPS A in PBS and PEPS B in urine may be attributed to the similar −k_{31} between PEPS A (0.32) and PEPS B (0.33). The sensitivity of a PEPS was related to its −k_{31}: The higher the −k_{31} the more sensitive the PEPS. With results from both PEPS B and PEPS A it suffices to say that with improved MPS insulation and using the present multiple-parabola fitting algorithm a PEPS with −k_{31} ≥ 0.32 exhibit an analytical sensitivity of better than 60 copies/ml, which was ≥ 16 times lower than that of the previous same tDNA detection in PBS using a PEPS of a similar −k_{31} but with only single-parabola fitting.
5 Conclusions
We have examined real-time, in situ hybridization detection of tDNA in a buffer solution and in urine using 8 μm-thick PMN-PT PEPSs about 1.1 – 1.2 mm long and 0.45 mm wide with improved MPS insulation and a new multiple-parabola (> 50) resonance peak position fitting algorithm. With pDNA immobilized on the PEPS surface and by monitoring the first width extension mode (WEM) resonance frequency shift we detected tDNA in real time at concentration as low as 1 × 10^{-19} M in urine (100 zM) with an SNR of > 10 without DNA isolation and amplification at room temperature in 30 min. Note there was no incubation time. 30 min was between the time when the sample was loaded and the time when we stopped the monitoring. The present multiple-parabola fitting algorithm increased the detection SNR by about 10 times from those obtained using single parabola fitting. The detection was validated by in situ follow-up detection and subsequent visualization of FRMs coated with reporter DNA complementary to the tDNA but different from the pDNA.

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