Nanomechanical assay for ultrasensitive and rapid detection of SARS-CoV-2 based on peptide nucleic acid

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ABSTRACT

The massive global spread of the COVID-19 pandemic makes the development of more effective and easily popularized assays critical. Here, we developed an ultrasensitive nanomechanical method based on microcantilever array and peptide nucleic acid (PNA) for the detection of severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2) RNA. The method has an extremely low detection limit of 0.1 fM (10^-15 copies/mL) for N-gene specific sequence (20 bp). Interestingly, it was further found that the detection limit of N gene (pharyngeal swab sample) was even lower, reaching 50 copies/mL. The large size of the N gene dramatically enhances the sensitivity of the nanomechanical sensor by up to three orders of magnitude. The detection limit of this amplification-free assay method is an order of magnitude lower than RT-PCR (500 copies/mL) that requires amplification. The non-specific signal in the assay is eliminated by the in-situ comparison of the array, reducing the false-positive misdiagnosis rate. The method is amplification-free and label-free, allowing for accurate diagnosis within 1 h. The strong specificity and ultra-sensitivity allow single base mutations in viruses to be distinguished even at very low concentrations. Also, the method remains sensitive to fM magnitude lung cancer marker (miRNA-155). Therefore, this ultrasensitive, amplification-free and inexpensive assay is expected to be used for the early diagnosis of COVID-19 patients and to be extended as a broad detection tool.

KEYWORDS

SARS-CoV-2, nucleic acid assay, nanomechanical sensoring, peptide nucleic acid, amplification-free

1 Introduction

After the severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2) was first discovered in December 2019, it demonstrated a more aggressive and infectious nature than the six reported coronaviruses, spreading globally at an alarming rate [1]. The coronavirus disease 2019 (COVID-19) pandemic has now infected about 400 million people and killed 5.76 million, with an average of 500 thousands of new infections per day. Although RT-PCR exhibits good detection limit, its cycle time to generate results is long (hours to days), mainly caused by tedious steps of nucleic acid purification and amplification [7–9]. Most importantly, RT-PCR requires specialized operators and expensive equipment (> $10,000) and can only be operated in laboratories with large stationary equipment and reagents, which greatly limits detecting capacity in resource-poor areas [10,11]. Immunological antibody tests are easy to perform and less device-dependent, but are limited by the fact that the human immune system may take 14–21 days to

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produce the antibody to be detected and may be interfered by cross-reactivity. During this time, most infected patients may already have clinical symptoms, which inadvertently increases the spread of the virus [12]. Obviously, the RNA of SARS-CoV-2 detecting can identify COVID-19 patients earlier and take timely interventions to control the spread of SARS-CoV-2. RT-PCR has a detection limit of only 500 copies/mL even by amplification [7], which would allow early infected individuals (< 500 copies/mL of viral RNA in vivo) to be misdiagnosed as negative. The ideal assay is ultrasensitive and amplification-free, which helps to reduce the false-negative misdiagnosis rate and thus the risk of transmission due to false negatives. At the same time the operation of medical personnel is simplified and the efficiency of the detection is improved. Therefore, ultrasensitive assays without amplification on the basis of ensuring accuracy and economics are in urgent need of development.

Biosensors, with capability of real-time detection, are ideal for clinical diagnosis. Among them, the nanomechanical sensor measures the real-time deflection of the microcantilever caused by the binding of the probe and the analyte. Microcantilevers have gradually developed two modes of operation, electrical readout and optical readout. Electrical microcantilevers that monitor impedance changes are limited by their electrical readout mode, and it is difficult to get a significant increase in sensitivity [13]. And thanks to the amplification of the deflection by the optical lever, the microcantilever based on optical readout demonstrates better detection performance [14–17]. Therefore, microcantilever sensing based on optical readout has been rapidly developed in recent years because of its label-free, sensitive and real-time characteristics, and has been successfully applied in the detection of cancer biomarkers, toxins, cell viability and medicines [18–21].

By differentiated functionalization of microcantilever arrays, multiple experimental and in-situ comparison groups can be performed in a single detection, thus eliminating non-specific signals and obtaining accurate detection signals. These properties of microcantilever sensing make us realize the importance of microcantilever arrays in the development of methods for SARS-CoV-2 detection, so we hope to use it to develop new detection methods that can be easily and widely applied.

Our previous studies have shown that the properties of the probe significantly affect the generation of stress on the surface of the microcantilever, and that the magnitude of the stress is directly related to the sensitivity of the nanomechanical sensor [18, 22]. Therefore, efficient analyte capture is an important prerequisite for ultrasensitive detection, which is mainly determined by the affinity of the probe molecule [23]. Secondly, the activity of the molecular layer (probe density and orientation) formed by the probe molecule on the sensor surface also affects the sensitivity [24]. Ideally, the sensitivity of mechanics-based microcantilever sensors is facilitated when high-affinity probe molecules form a dense and oriented molecular layer on the surface of the microcantilever. As a common nucleic acid hybridization probe in biosensors, single-stranded DNA (ssDNA) can be modified on microcantilevers to capture target nucleic acids, and duplexes repel each other to create stress that bends the microcantilever for detection [25]. However some issues are worth noting, in a liquid environment, ssDNA exhibits an uncontrollable orientation and collapsed conformation on the surface of the microcantilever to maximize its entropy value [26, 27]. Also, the negatively charged sugar-phosphate backbone of ssDNA causes the repulsion between ssDNA [28], which may limit the spacing between ssDNA when it is modified on the gold surface. All these factors induce each ssDNA to occupy a larger spatial area and maintain a larger distance from each other, thus restricting the density of ssDNA modifications on the limited gold surface [15], and affecting the final stress magnitude. Moreover, when hybridizing with a target nucleic acid (negatively charged), the electrostatic repulsion between ssDNA and target nucleic acid affects the ability to bind [29]. In addition, in the actual assay, ssDNA is degraded by nucleases or proteases in the sample, and its activity is also affected by ion concentration, temperature and pH [30]. Clearly, these factors limit the sensitivity and practical application of DNA probe sensors. If a probe molecule has the ability to hybridize complementarily, while being uncharged, possessing higher affinity and stability, then combining it with the sensitive, non-labeling nature of microcantilevers would be expected to greatly hoist the detection capacity for nucleic acid.

A nucleic acid analogue, peptide nucleic acid (PNA), was synthesized by replacing the negatively charged sugar phosphate backbone of DNA with a neutral polyamid backbone. PNA retains the properties of base-specific hybridization and remains effective in identifying complementary targets [31]. Moreover, due to the flexibility of the polyamid backbone and the absence of electrostatic repulsion in hybridization, the binding capacity and efficiency of PNA are significantly improved [29]. In contrast to DNA, PNA exhibits resistance to enzymatic hydrolysis and independence from the environment of the solution in which hybridization is performed (ion concentration, temperature, pH, etc.), which gives PNA the ability to perform stable nucleic acid hybridization under complex conditions [32, 33]. Based on its great potential, PNA has gradually started to replace DNA as a nucleic acid hybridization probe in recent years in localized surface plasmon resonance (LSPR), electrochemical sensors and nanopore sensing [34–36]. The stresses generated by the hybridization of DNA on the microcantilever surface are complex, with stresses toward the gold surface [14], and stresses dorsad toward the gold surface [28]. The neutral backbone allows for significant differences in the physicochemical properties of PNA and DNA, which results in a dramatic change in the environment of the sensor surface when PNA is used as a probe for nanomechanical sensing. This change in environment inevitably affects the generation of stress during hybridization and the sensitivity of the sensor in different ways; however, these issues are unknown. Clearly, addressing these issues promises the development of novel detection technologies for SARS-CoV-2 that meet our expectations.

Here, we developed a one-step method for the detection of SARS-CoV-2 based on optical readout using PNA as a probe. Benefit from the stress enhancement brought by PNA to the microcantilever and differential modification of microcantilevers in an array, the ultrasensitive and accurate detection of SARS-CoV-2 can be accomplished within 1 h by in-situ comparison (Fig. 1). The factors influencing the stresses induced by PNA on the microcantilever surface were investigated. The ability of the method for ultrasensitive quantitative detection of N gene of SARS-CoV-2 was determined, and clinical proof-of-concept was performed with a pseudovirus model. The stress enhancement mechanism brought by PNA to nanomechanical sensors was analyzed, and the effect of the size of the target nucleic acid on sensitivity was explored. The method is a promising option for ultrasensitive, amplification-free, label-free, accurate, and inexpensive screening of virally infected individuals.

2 Results and discussion

2.1 Characterization of self-assembled molecular (SAM) layer of microcantilever surface

The formation of an effective SAM layer on the surface of the microcantilever by the N-gene specific PNA probe is necessary to
achieve SARS-CoV-2 detection. The N-terminal of PNA was designed with a sulfhydryl group (–SH), and the functionalization of microcantilevers was accomplished in one step through gold–sulfur covalent bond. Unfunctionalized microcantilevers and PNA-functionalized microcantilevers were irradiated with X-rays separately to obtain X-ray photoelectron spectroscopy (XPS) spectra to evaluate the functionalization of PNA on the sensor gold surface (Fig. 2(a)). Because the Au atoms on the surface of the unfunctionalized microcantilever were partially oxidized, their binding energies (BEs) increased slightly compared to that with Au(0) (83.9 ± 0.1 eV). And the formation of Au–S bond led to the decrease of BE from 84.09 to 83.36 eV after the PNA completed the immobilization on microcantilevers by the sulfhydryl group (Fig. 2(b)). The unfunctionalized microcantilever without the presence of N atoms showed no absorption peak of N 1s in the XPS spectrum, while after functionalization of the microcantilever with PNA (containing N atoms), a clear absorption peak (400.1 eV) appeared (Fig. 2(c)). Meanwhile, the binding energy of the O 1s for the PNA-functionalized microcantilever underwent a −0.8 eV change (Fig. 2(d)). The BE differences in these atoms are consistent with changes in chemical bonding when PNA is immobilized on the surface of the microcantilever, which tentatively confirms that the SAM layer has been successfully formed on the gold surface.

It has been shown that highly specific detection of SARS-CoV-2 can be ensured and achieved by designing ssDNA probes that are complementarily paired with a partial sequence of the N gene of SARS-CoV-2 (N-gene specific sequence) (Fig. 3(a)) [37]. Therefore, we selected and synthesized a PNA probe with reference to the sequence analysis of ssDNA probes and also synthesized N-gene specific sequence (RNA), and then further verified whether the PNA formed a SAM layer on the gold surface by surface plasmon resonance (SPR). The surface of the chip used for SPR testing was identical to that of the microcantilever, both covered by a nanometer-thick gold film. When PNA was modified on the gold surface, a large amount of PNA was immobilized on the gold surface to form the SAM layer. To further demonstrate that the SAM layer formed by PNA on the
gold surface was active, various concentrations of N-gene specific sequences were added after saturation of the PNA modification. The SAM layer composed of PNA on the gold surface was successfully hybridized in the presence of multiple concentrations of N-gene specific sequences, and analysis of the binding kinetics showed that PNA bound to N-gene specific sequences with a high affinity (KD) of 2.709 nM (Fig. 2(e)). We also designed ssDNA probes of the same sequence and obtained the affinity of ssDNA for N-gene specific sequences to be $2.644 \times 10^{-6}$ nM (Fig. 2(f)). The results of SPR further demonstrate that the gold surface of the microcantilever successfully forms a SAM layer composed of PNA and is molecularly active. Affinity analysis showed that PNA had a prominent strong affinity advantage over DNA in capturing N-gene-specific sequences. Thus, the XPS and SPR data demonstrate that the PNA-functionalized microcantilever is covered by an effective SAM layer, which establishes the basis for achieving SARS-CoV-2 detection.

2.2 Detection capability assessment

The advantage of the array sensing is its in-situ comparison capability, which can be fully revealed by manipulating the microcantilever array on capillary differential modification devices (step C in Fig. 1 and Fig. S1 in the Electronic Supplementary Material (ESM)). We functionalized microcantilevers 1, 3, 5 and 7 of an array with PNA (experiment microcantilevers) and microcantilevers 2, 4, 6 and 8 as non-functionalized controls (reference microcantilevers) (Fig. 3(b)). The array was subsequently blocked with 6-mercapto-1-hexanol (MCH) and then mounted in the reaction chamber. After the array reached a balanced baseline in saline-sodium citrate (SSC) buffer with a stable flow rate, the samples containing N-gene specific sequence (RNA) were injected into the reaction chamber (step D in Fig. 1). Eight semiconductor lasers sequentially emit a stable beam focused on the tip of each microcantilever in an array, while a position sensitive detector (PSD) is responsible for monitoring the deflection of each microcantilever in real time through measuring the movement of reflected light (step E in Fig. 1). Subsequent experiments were all performed as described above. We first injected a higher concentration of N-gene specific sequence solution (1 nM) to explore the feasibility of proposed method. Experiment microcantilevers underwent rapid deflection after injection of the solution, with deflections as high as about 155 nm (Figs. 3(b) and 3(c)). But the reference microcantilevers showed only about 25 nm at 60 min, which was mainly a non-specific signal caused by adsorption, external environmental interference, etc. Both the experimental and reference microcantilevers in an array exhibited good agreement, which shows that the proposed method has good intra-batch reproducibility. The significant difference between the deflection of experiment microcantilevers and reference microcantilevers indicates that deflections of experiment microcantilevers are caused by the binding of the PNA to the N-gene specific sequence. By subtracting the average deflection of reference microcantilevers from the average deflection of experiment microcantilevers, the interference of non-specific signals can be eliminated, and an
accurate differential deflection, namely specific signals caused by the binding of PNA and N-gene specific sequences can be obtained (Fig. 3(c)). The microcantilevers showed such a large (~128 nm) differential deflection in the presence of a 1 nM N-gene specific sequence, which demonstrates the feasibility of the proposed method for SARS-CoV-2 detection and the potential of the proposed method for ultrasensitive detection. The subsequent experimental results are all shown with differential deflections.

### 2.3 Optimization of probe

In the new micro-nano environment consisted of PNA and microcantilever, the immobilization of PNA is an important step in the construction of detection methods. The surface density of the probe plays a crucial role in the fabrication of efficient biosensors and closely affects the strength of the detection signal [24, 33]. And the modification concentration and immobilization time of the probes significantly affect the probe density on the sensor surface [24]. In terms of mechanical sensors, the probe density and status inevitably affect the stress generation. Then exploring the effect of the modification of the probe PNA on stress generation is critical to achieve ultrasensitive detection. So we expected to optimize the probe density and status on the microcantilever surface by evaluating the signal due to different concentrations and immobilization time of PNA. The PNA concentrations used for functionalization of different microcantilever arrays were 0.1, 1, 5 and 10 μM, respectively, for the same concentration of N-gene specific sequence, the differential deflection increased with increasing concentration at PNA concentrations less than 5 μM, while at PNA concentrations of 10 μM, the differential deflection showed a certain decrease (Fig. 3(d)). With a PNA concentration of 1 μM as a differentiation point, the PNA forms two different molecular orientations on the gold surface, flat-bottom arrangement and upright conformation [38]. So when the microcantilever was modified with 0.1 and 1 μM, the PNA was mainly arranged in a flat bottom arrangement and could not form a high-density SAM layer. And the 5 μM PNA exhibited an upright ordered structure on the surface of the microcantilever, forming a dense and saturated SAM layer. However, at higher PNA concentrations (~10 μM), the high packing density of PNA blocked the adsorption sites, limiting the binding to the complementary chains and thus affecting the activity of the SAM layer [38]. Therefore, the 5 μM PNA forms the most active SAM layer on the surface of the microcantilever, which is most conducive to the capture of target molecules, resulting in a larger stress. Obviously, the arrays functionalized by 5 μM PNA exhibited the greatest differential deflection, and we chose a modification concentration of 5 μM to continue the study afterwards. Differences in differential deflection were evaluated after different microcantilever arrays were immersed in 5 μM PNA solution for 1, 4, 8 and 12 h, respectively. It is clear that the maximum differential deflection was observed at 4 h of PNA immobilization, after which there was a decrease in differential deflection with increasing immobilization time (Fig. 3(e)). PNA gradually covered the gold surface as the modification time increased, and a more suitable coverage area was generally formed at around 210 min [38]. After 1 h of PNA immobilization, only part of the surface of the microcantilever was covered by PNA, and the limited coverage area limited the capture of target molecules. And at 4 h of PNA immobilization, the surface of the microcantilever was basically covered completely, and the ideal coverage area was favorable for the capture of target molecules. When the PNA immobilization time continued to increase, individual PNAS may crowd together to form PNA ribbons, and this structure was not conducive to hybridization. As time increased, there were fewer individual PNAS on the surface of the microcantilever, more PNA ribbons were formed, and the capture of target molecules became more difficult. Therefore, when the PNA fixation time was 4 h, the injection of target molecules caused the most prominent differential deflection of the microcantilever array. Overall, the microcantilever functionalized with 10 μM PNA for 4 h formed a dense and ordered layer of highly reactive molecules, which may facilitate the generation of large stresses on the surface of the microcantilever.

Stress effects have a significant impact on nanomechanical sensors. Previous studies have shown that stress loss occurs when the forces generated in the binding region of the probe and target molecule are transferred to the surface of the microcantilever. And the increase in probe length leads to greater stress loss, which reduces the sensitivity of the microcantilever sensor [22]. So, exploring the relationship between the chain length of PNA and stress transfer can help design high-performance nanomechanical sensors. To avoid the effect of long chain PNA stability, PNAs are commonly designed with 10–30 bases. We respectively designed PNA with chain lengths of 10, 20 and 30 bp (all complementary to the N-gene specific sequence) and respectively investigated the differential deflection of the microcantilever in the presence of various concentrations of N-gene specific sequence (1, 50, 100 and 1,000 pM) for three chain lengths of PNA to optimize the chain length of PNA (Fig. S2(b) in the ESM). The microcantilever functionalized with 20 bp PNA showed the most prominent differential deflection in multiple sets of concentrations, while the differential deflection of the microcantilever respectively functionalized with 30 and 10 bp PNA decreased sequentially (Fig. S2(a) in the ESM). Although the binding region of the 10 bp PNA to the N-gene specific sequence was the closest to the gold surface and theoretically has minimal loss when transferring stress, the 10 bp PNA probe can only complete complementary pairing with a portion (10 bp) of the N-gene specific sequence (20 bp) (Fig. S2(b) in the ESM). Incomplete complementary pairing made the formation of duplexes even more difficult, and thus may result in fewer duplexes on the microcantilever surface, reducing less repulsive forces. So even though the loss of stress transfer is minimal, it can only cause a small deflection of the microcantilever. The 30 bp PNA probe was longer, which may potentially cause the binding site to be blocked, thus affecting the formation of the duplexes. More importantly, its bonding region was the farthest from the microcantilever surface, which led to a greater loss of stress transfer and thus attenuated the stress variation at the microcantilever surface (Fig. S2(b) in the ESM). In contrast, the 20 bp PNA completed the complete complementary pairing and the distance from the binding region to the microcantilever surface was moderate. These made the stresses generated on the microcantilever surface the most desirable (Fig. S2(b) in the ESM). Obviously, 20 bp should be the optimal chain length of the PNA probe for N gene detection. These results suggest that the chain length of PNA probes is not the longer the better, but has the most efficient stress effect within a reasonable range. This has important value for nucleic acid hybridization based on stress principles.

### 2.4 Real-time nanomechanical detection of N-gene specific sequence

We now explored the sensitivity of the proposed method in the presence of N-gene specific sequence (RNA) and further confirmed the feasibility of the method. The functionalized microcantilevers all deflected after the injection of different concentrations of samples (Fig. 4(a)). The higher the concentration of the sample, the more rapid the response of the microcantilever and the greater the differential deflection that occurred. Only after 50 min of sample injection, the
microcantilever deflections caused by different concentrations of samples were already easily distinguishable. These demonstrate the ability of our method to rapidly quantify N-gene specific sequences. More importantly, the differential deflection of the microcantilevers was as high as 73 nm in the presence of 50 pM of N-gene specific sequence, while the differential deflection of the microcantilevers was still 9 nm even when the concentration of N-gene specific sequence was as low as 0.1 fM (Fig. 4(a)). The appreciable signal at ultra-low concentrations indicates that our method can achieve ultrasensitive detection of N-gene specific sequences. The differential deflection of the microcantilever in the control experiment (Ref), done entirely in the SSC-only solution, approached a horizontal line, which further suggests that the differential deflection of the microcantilevers represents a specific binding signal for PNA and N-gene specific sequence. After fitting analysis, we found a good linear relationship between the differential deflection of the microcantilevers and the N-gene specific sequence at concentrations ranging from 0.1 to 1,000 fM (Fig. S3(a) in the ESM). Also, to visually assess the advantages of our method in terms of detection capability, we explored the sensitivity of the ssDNA functionalized microcantilever in the presence of N-gene specific sequences. As expected, the sample injection did induce the specific deflection of the functionalized microcantilevers (Fig. 4(b)). However, when the concentration of N gene-specific sequences was 10 nM, the microcantilevers also showed only a differential deflection of 67 nm. At N-gene specific sequence concentrations diluted to 0.1 nM, the differential deflections of the microcantilevers were not observed, indicating that the detection limit of DNA-functionalized microcantilever is greater than 0.1 nM. It is obvious that there is a significant gap between DNA-based microcantilevers and PNA-based microcantilevers in terms of detection limits (Fig. 4(c)). All the above-mentioned results fully demonstrate that the proposed method can quantitatively detect N-gene specific sequences with excellent sensitivity and accuracy within 1 h.

2.5 Detection of SARS-CoV-2 in pharyngeal swabs and serum samples

Our method showed excellent sensitivity and accuracy in detecting N-gene specific sequences. To further explore the potential in clinical diagnosis, we respectively mixed the N-gene of SARS-CoV-2 with human throat swab samples and serum to simulate clinical samples after patient collection by adopting the proof-of-concept method that has been widely used [7, 39, 40]. The First Affiliated Hospital of Anhui Medical University provided us with pharyngeal swab samples (16 groups) and serum samples (16 groups) from volunteers who were all identified as negative by RT-PCR by the Laboratory Department (Fig. S4 in the ESM). Each negative sample was tested individually with the proposed method and none of them caused differential deflection of the microcantilever (Figs. 5(a)–5(c)). The mean of the differential deflections of the 16 pharyngeal swab negative samples and the mean of the differential deflections of the 16 serum negative samples are shown and labeled as negative samples in Figs. 5(a) and 5(b), respectively. Then the RNA of the pseudovirus (containing the intact N gene) was mixed with the negative samples and configured to the appropriate concentration as clinical samples. After injection of pharyngeal swab clinical samples, the microcantilever showed a rapid deflection and was able to clearly differentiate sample concentrations at 50 min after sample injection. A concentration of 800 copies/mL of pharyngeal swab sample caused a differential deflection of up to 116 nm of the microcantilever and a differential deflection of 17 nm in the microcantilever even when the concentration of pharyngeal swab sample was diluted to 50 copies/mL (Fig. 5(a)). The serum clinical sample concentrations were still rapidly differentiated within 50 min after injection. The differential deflections of microcantilevers caused by concentrations of 100, 400 and 800 copies/mL serum samples were 28, 92 and 118 nm (Fig. 5(b)), respectively, which were almost the same as the differential deflections of 27, 94 and 120 nm caused by the same concentration of pharyngeal swab samples. Considering that the complex composition of serum causes a significant increase in assay noise, the proposed method has a reasonable detection limit of 100 copies/mL for serum clinical samples. To ensure reproducibility of the method, we performed four tests on each positive sample (five positive pharyngeal swab samples and four positive serum samples). The statistics of 32 times negative sample tests and 36 times positive sample tests visually showed that the positive samples of the same concentration showed good inter-batch reproducibility between the positive samples, and there was a clear difference between the

Figure 4 Nanomechanical signal of microcantilevers for SARS-CoV-2-specific sequence detection. (a) Real-time differential deflection of PNA-functionalized microcantilever induced by the presence of N-gene specific sequences. (b) Real-time differential deflection of ssDNA-functionalized microcantilever induced by the presence of N-gene specific sequences. (c) Differential deflections of PNA-functionalized and ssDNA-functionalized microcantilevers as a function of N-gene-specific sequence concentration, respectively. In all figures the average of replicates (n = 4) is shown.
amplification-free, label-free and reagent-free, and the process very complicated. In contrast, our method is 

required for the assay are also greatly increased, making they require amplification and labeling, and the materials and 

still not as sensitive as ours (Table S2 in the ESM) [42]. However, signal amplification and thus increase sensitivity, most of them are 

transmission from missed detections. Currently, the reported methods are based on ssDNA to detect the N gene of SARS-CoV-2 [11, 37, 41], and although they use other materials to achieve 

accuracy. Clinically, samples with a cycle count greater than 35 and no amplification curve are judged as negative, while samples with a cycle count less than or equal to 35 and a significant amplification curve are judged as positive. RT-PCR successfully 

patients, and confirms the universality of the method for nucleic acid detection. Among the published detection methods with high sensitivity for miRNA-155, atomic layer deposition (ALD)-based electrochemical methods reach a detection limit of 1 fM [44]. They prepared ultra-thin molybdenum disulfide (MoS2) based on ALD to support more gold nanoparticles, thus enhancing conductivity and improving sensitivity. However, the preparation of MoS2 films was carried out at 450 °C and 5.3 hPa pressure, which is very demanding for the operating environment and complex. More importantly, our approach is more sensitive (0.1 fM). In another work, a DNA three-dimensional (3D) probe reached the same detection limit of 0.1 fM on the electrochemical

Figure 5  Performance evaluation of the method. Real-time differential deflection of PNA-functionalyzed microcantilevers in the presence of different concentrations of N gene of SARS-CoV-2 in pharyngeal swab samples (a) and serum samples (b). The negative sample curves presented in (a) and (b) are the average of 16 negative pharyngeal swab samples and 16 negative serum samples, respectively. (c) Statistical analysis showed that the reproducibility of the method was satisfactory, not only for significant differentiation between infected and healthy individuals, but also for rapid quantification. (d) Nanomechanical assay for ultrasensitive and rapid detection of clinical marker of lung cancer (miRNA-155). (e) Cross-reactivity of microcantilever functionalized with specific PNA of N gene of SARS-CoV-2 in the presence of N gene of MERS-CoV.

positive samples of different concentrations (Fig. 5(c)). The significant difference analysis revealed that the difference between the test results of the method for positive samples and those for negative samples was highly significant, and the method was able to steady distinguish healthy individuals from patients (Fig. 5(c)). These indicate that our method is satisfactorily reproducible for detecting the N gene of SARS-CoV-2 in a complex clinical diagnostic setting and is very sensitive and capable of rapid quantification. The differential deflections of microcantilevers still show a good linear relationship with the clinical sample concentration ($R^2 = 0.9897$) (Fig S3(b) in the ESM). To further validate the reliability of our method, we detected the same batch of pharyngeal swab clinical samples with RT-PCR, and three replicate wells were set up for each group of samples to ensure accuracy. Clinically, samples with a cycle count greater than 35 and no amplification curve are judged as negative, while samples with a cycle count less than or equal to 35 and a significant amplification curve are judged as positive. RT-PCR successfully 

concentration (miRNA), a class of single-stranded RNA molecules encoded by endogenous genes, has been verified to be an early clinical marker for cancer. Considering the high incidence of lung cancer, we studied the ability of the method to detect low concentrations of the lung cancer marker—miRNA-155. As shown in Fig. 5(d), after functionalization of the microcantilever with PNA designed for miRNA-155, the injection of miRNA-155 rapidly stimulated the deflection of the microcantilever. At 60 min, the differential deflection of the microcantilever induced by different concentrations of miRNA-155 could be clearly distinguished. With the increase of miRNA-155 concentration, the differential deflection of the microcantilever increased successively, and there was a good linear relationship between the differential deflection of the microcantilever ($D$) and miRNA-155 concentration ($C_{miRNA-155}$). The regression equation was expressed as $D = 9.6 \log_{10} C_{miRNA-155} + 17.5$, and the correlation coefficient ($R$) was 0.9997 (Fig. S3(c) in the ESM). The above results demonstrate that our method can accomplish ultrasensitive and simple detection of miRNA-155, which helps to achieve early diagnosis of lung cancer patients, and confirms the universality of the method for nucleic acid detection. Among the published detection methods with high sensitivity for miRNA-155, atomic layer deposition (ALD)-based electrochemical methods reach a detection limit of 1 fM [44]. They prepared ultra-thin molybdenum disulfide (MoS2) based on ALD to support more gold nanoparticles, thus enhancing conductivity and improving sensitivity. However, the preparation of MoS2 films was carried out at 450 °C and 5.3 hPa pressure, which is very demanding for the operating environment and complex. More importantly, our approach is more sensitive (0.1 fM). In another work, a DNA three-dimensional (3D) probe reached the same detection limit of 0.1 fM on the electrochemical

2.6 The universality of the method

We also focused on the universality of the proposed method in nucleic acid detection. Early diagnosis of cancer is clinically important and helps to improve patient survival [43]. MicroRNA (miRNA), a class of single-stranded RNA molecules encoded by endogenous genes, has been verified to be an early clinical marker for cancer. Considering the high incidence of lung cancer, we studied the ability of the method to detect low concentrations of the lung cancer marker—miRNA-155. As shown in Fig. 5(d), after functionalization of the microcantilever with PNA designed for miRNA-155, the injection of miRNA-155 rapidly stimulated the deflection of the microcantilever. At 60 min, the differential deflection of the microcantilever induced by different concentrations of miRNA-155 could be clearly distinguished. With the increase of miRNA-155 concentration, the differential deflection of the microcantilever increased successively, and there was a good linear relationship between the differential deflection of the microcantilever ($D$) and miRNA-155 concentration ($C_{miRNA-155}$). The regression equation was expressed as $D = 9.6 \log_{10} C_{miRNA-155} + 17.5$, and the correlation coefficient ($R$) was 0.9997 (Fig. S3(c) in the ESM). The above results demonstrate that our method can accomplish ultrasensitive and simple detection of miRNA-155, which helps to achieve early diagnosis of lung cancer patients, and confirms the universality of the method for nucleic acid detection. Among the published detection methods with high sensitivity for miRNA-155, atomic layer deposition (ALD)-based electrochemical methods reach a detection limit of 1 fM [44]. They prepared ultra-thin molybdenum disulfide (MoS2) based on ALD to support more gold nanoparticles, thus enhancing conductivity and improving sensitivity. However, the preparation of MoS2 films was carried out at 450 °C and 5.3 hPa pressure, which is very demanding for the operating environment and complex. More importantly, our approach is more sensitive (0.1 fM). In another work, a DNA three-dimensional (3D) probe reached the same detection limit of 0.1 fM on the electrochemical
sensor as our method [26]. But DNA 3D probes require multiple ssDNAs to be individually oriented to achieve a settled structure, which is expensive to produce. Additional reagents (Helper1, Helper2) and markers (beacons ferrocene, methylene blue) are also required for the assay. In our method, PNA spontaneously arranges vertically under suitable conditions, and the whole process is label-free and reagent-free, which makes the assay simpler and inexpensive. It is clear that our method is expected to gain popularity in the early diagnosis of cancer.

2.7 Specificity for SARS-CoV-2

To further determine the specificity of the method, we explored the differential deflection of the microcantilever (functionalization with specific PNA of the N gene of SARS-CoV-2) in the presence of the N gene for Middle East respiratory syndrome coronavirus (MERS-CoV) (a similar genomic structure to SARS-CoV-2). Remarkable and different differential deflections were observed in the microcantilever in the presence of different concentrations of the N gene of SARS-CoV-2 (1,600, 800 and 400 copies/mL, Fig. 5(a)), while the differential deflection of the microcantilever caused by different concentrations of the N gene of MERS-CoV were insignificant and not outstanding different (Fig. 5(e)). This demonstrates that the method accomplishes screening of SARS-CoV-2 without cross-reactivity, which lays the foundation for the feasibility of complex sample detection.

2.8 Mutation identification of SARS-CoV-2

SARS-CoV-2 mutates at a high frequency to mediate its ability to infect and spread. Effective identification of SARS-CoV-2 mutations facilitates early deployment of prevention and control measures against the mutant strains [45]. Since its discovery, the D614G mutant strain (accompanied by a multisite C-U mutation) has become more prevalent with the spread of the pandemic, appearing in more than 74% of all published sequences [46]. Considering the high prevalence of the D614G mutant strain, we investigated the ability of the proposed method to identify SARS-CoV-2 mutations based on the mutational characteristics of D614G. First, we tested N-gene specific sequences and N-gene specific sequences where single-base mutations (C-U mutation) had occurred. In the presence of a standard sequence of 1,000 pM, the microcantilever showed a differential deflection of 128 nm, whereas in the presence of a mutant sequence of 1,000 pM, the microcantilever showed a significant reduction in differential deflection of only 76 nm (Fig. 6(a)). Even at lower concentrations (50 pM and 10 fM), there were significant differences in the differential deflections caused by standard and mutant sequences, respectively (Figs. 6(b) and 6(c)). The microcantilevers still showed different differential deflections for standard and mutant sequences of 10 fM, which again illustrates the ultra-sensitivity of the method. To further investigate the ability of the method to identify SARS-CoV-2 mutations, we designed several mutation types of N gene to be tested separately. All assays were performed in pharyngeal swab samples. I: N gene with N-gene specific sequence fully mutated (N-gene specific sequences were all mutated and no mutations occurred in other regions). N gene with N-gene specific sequences fully mutated at concentrations of 800, 400, 200 and 100 copies/mL all caused no differential deflection of microcantilever (Fig. 6(d)). II: N gene with scrambled sequence (no mutations occurred in N-gene specific sequence and mutations occurred in other regions). The injection of the N gene with scrambled sequence at concentrations of 800, 400, 200 and 100 copies/mL quickly caused differential deflection of the microcantilever and reached 117, 96, 52 and 26 nm after 50 min, respectively (Figs. 6(d) and 6(f)). It can be noted that the differential deflections of the microcantilever caused by the same concentration of the N gene with scrambled sequences and the N gene are almost identical (Fig. 6(f)). III: N gene with D614G mutant type (single base mutations occurred in N-gene specific sequence and no mutations occurred in other regions). After injection of N gene with D614G mutant type at concentrations of 800, 400, 200 and 100 copies/mL, differential deflections also occurred for microcantilevers (67, 50, 25 and 13 nm), but the deflection values were reduced compared to the N gene without the mutation (Fig. 6(f)). The assay results of the N gene with N-gene specific sequence fully mutated and N gene with scrambled sequence (Figs. 6(d) and 6(f)) fully showed that PNA captured the N gene of SARS-CoV-2 with high specificity through complementary pairing with N-gene specific sequences.

Figure 6 Identification of SARS-CoV-2 mutations. (a)–(c) A single base mutation in a N-gene specific sequence was used as an example to measure the ability to recognize viral mutations at different concentrations. (d) Real-time differential deflection of PNA-functionalized microcantilevers in the presence of different concentrations of N gene with N-gene specific sequence fully mutated and N gene with scrambled sequence in pharyngeal swab samples, respectively. (e) Detection signals of pharyngeal swab samples containing N gene with D614G mutant type. (f) Histogram of assay results for N-gene, N gene with N-gene specific sequence fully mutated, N gene with scrambled sequence and N gene with D614G mutant type. In all figures the average of replicates (n = 4) is shown.
differential deflections of the microcantilevers both showed a certain degree of attenuation in the presence of the mutant sequence (Figs. 6(a)–6(c)) and N gene with D614G mutant type (Fig. 6(e)), respectively, which reflects the good recognition of SARS-CoV-2 mutation, and also demonstrates again the excellent specificity of PNA for the N gene of SARS-CoV-2. The presence of mismatches reduces the melting temperature ($T_m$) of PNA/RNA duplexes considerably compared with fully complementary PNA/RNA duplexes, affecting the stability of the duplexes [31]. On microcantilevers, the duplex formed by PNA and mutant sequences may reduce. Then the stress on the surface of the microcantilever reduced accordingly, which makes the deflection of the microcantilever at the same concentration of unmutated sequences and mutant sequences appear different.

2.9 Mechanism of surface stress enhanced by PNA

The excellent hybridization capability of PNA was demonstrated on the SPR, fluorescence resonance energy transfer (FRET) sensor and graphene field effect transistor (G-FET) biosensor and brought 3-fold, 10-fold and 3 orders of magnitude improvement in sensitivity for the SPR, FRET and G-FET sensors, respectively (compared to DNA-based SPR, FRET and G-FET sensors, respectively) [47–49]. Clearly, the performance improvements that PNA brings to multiple sensors are different, and this is inextricably linked to the nature of the sensor itself and the fit of the PNA to the sensor. The sensitivity of mechanics-based nanomechanical sensors is directly determined by the magnitude of the stress change on the microcantilever surface. The factors that interfere with surface stress can be divided into (i) electrostatic repulsion force between charged molecules, and (ii) the repulsive hydration forces due to the perturbation of the hydrogen network of water by charged molecules [50–52]. The PNA improves the sensitivity of our nanomechanical sensor by nearly six orders of magnitude compared to DNA-based nanomechanical sensor. Then, PNA distinctly brings a great gain to the stress on the surface of the microcantilever compared to the DNA probe. So it is important to analyze the mechanism of stress enhancement to design high-performance nanomechanical sensors. First, in our above probe optimization (Figs. 3(d) and 3(e)), when the microcantilever was functionalized with a probe concentration of 5 μM and a modification time of 4 h, PNA formed a dense and ordered layer of highly active molecules on the surface of the microcantilever in a nearly upright and oriented conformation ([1] and [2] in Fig. 7(c)). However, the DNA formed a sparse and disordered layer of low-activity molecules in a collapsed and disoriented conformation ([1] and [2] in Fig. 7(d)). Additionally, there was a clear difference in the SPR response when PNA and DNA were immobilized on the gold surface of the SPR chip, respectively (Figs. 7(a) and 7(b)). At 20 min, there was about 2 orders of magnitude difference between the two. The significant gap directly indicates that PNA forms molecular layers at a denser density than DNA on the gold surface, and the high density of PNA molecular layers provides the basis for sensitive nanomechanical sensors.

Secondly, the unique physicochemical properties of PNA (uncharged) allow it to have no electrostatic repulsion and lower spatial resistance in nucleic acid hybridization [29]. Furthermore, the more rigid PNA was in a favorable conformation, allowing its bases to bind better during hybridization ([3] in Fig. 7(c) and [3] in Fig. 7(d)) [53]. These made the PNA have a stronger affinity, as confirmed by the results of our SPR experiments (Figs. 2(e) and 2(f)). The high density and strong affinity of PNA enhanced the capture efficiency of target nucleic acids, and more PNAs eventually formed duplexes with the target nucleic acids ([4] in Fig. 7(c)). Although when the number of RNA is high, PNA forms more duplexes on the surface of the microcantilever, effectively reducing the distance between the duplexes. The electrostatic force and the hydration force can act synergistically to change the stress on the surface of the microcantilever. However, it should still be taken into account that the spacing between PNA/RNA duplexes can still be large when the amount of RNA is extremely low (e.g., 100

![Figure 7](https://www.theNanoResearch.com) Schematic diagram of the mechanism of nanomechanical signal enhancement brought by PNA. (a) and (b) Amount of PNA and DNA immobilized on the gold surface of the chip. (c) Mechanism of stress generation in PNA functionalized microcantilever. Thanks to the formation of more PNA/RNA duplexes and the tight arrangement between the duplexes, a significant repulsive force is generated. (d) Mechanism of stress generation in DNA functionalized microcantilever. Limited by the formation of fewer DNA/RNA duplexes and the sparse arrangement of duplexes, only weak repulsive forces are generated.
copies/mL). In this case, the electrostatic force is negligible and the stress change in the microcantilever is controlled by the hydration force [54]. Therefore, the mechanism of stress enhancement brought by PNA on the surface of microcantilevers should be explored categorically depending on the concentration of the sample to be measured. In high concentration samples, relatively more PNA/RNA duplexes were formed with a relatively small spacing (d) between each other, so they were sufficient to generate electrostatic forces. Simultaneously, a relatively large number of PNA/RNA duplexes caused a relatively large perturbation of the hydrogen network of water and generated a large hydration force. The electrostatic force and the hydration force together produced a strong repulsive force (F) on the surface of the microcantilever ([5] in Fig. 7(c)). Conversely, low density and low affinity DNA did not guarantee efficient capture of the target nucleic acid ([4] in Fig. 7(d)), which resulted in relatively large spacing between DNA/RNA duplexes even in highly concentrated samples. At this point, although sufficient to generate electrostatic force, but the generated electrostatic force was small. The small amount of DNA/RNA duplexes also caused only a small perturbation of the hydrogen network of water, generating only a small hydration force. Smaller electrostatic force and smaller hydration force together only produced a weaker repulsive force (F) on the surface of the microcantilever ([5] in Fig. 7(d)). In low concentration samples, PNA, even with its efficient capture performance, was limited by the small number of target nucleic acids and still only a small number of PNA/RNA duplexes can be formed. The large spacing between PNA/RNA duplexes made electrostatic force almost impossible to generate. Only a small hydration forces generated by a small amount of PNA/RNA duplexes acted on the surface of the microcantilever, so the deflection of the microcantilever was small. At this point, it was difficult for DNA to capture RNA to form the DNA/RNA duplex, generating tiny hydration force and electrostatic force. Therefore, tiny stress changes on the surface of the microcantilever are not sufficient to deflect the microcantilever. More importantly, the increased sensitivity that PNA brings to other sensors is due to the strong affinity of PNA allowing more binding to the target nucleic acid (n) to occur, thus directly enhancing the detection signal. When PNA was used as a nanomechanical sensor probe, the number of captured RNA (n) was also increased. However, what makes PNA unique for stress-based nanomechanical sensors is that the efficient trapping capability of PNA allows for a significant increase in the resulting repulsive force (F). When above two factors are combined, considerable stress is generated (n*F). These repulsive forces gave a gain to the stress-based sensor, causing a significant deflection of the microcantilever ([6] in Fig. 7(c)). In contrast, DNA captured less RNA and generated less repulsive force ([6] in Fig. 7(d)). These factors together limited the variation of stresses on the surface of the microcantilever and affected the deflection of the microcantilever.

### 2.10 Effect of the size of the target nucleic acid on sensitivity

The proposed method has a detection limit of 0.1 fM (10^9 copies/mL after unit conversion) for N-gene specific sequences (20 bp) and an even lower limit of 10^8 copies/mL for the N-gene (1,260 bp). Apparently, there is a 3 orders of magnitude difference between the detection limits of the nanomechanical sensor for N-gene specific sequences and N gene, according to which we speculate that the size of the target nucleic acid could potentially affect the sensitivity of the nanomechanical sensor. This is an interesting scientific question worth exploring, so we designed 240 bp sequence of N-gene (sequence length between N- gene and N-gene specific sequence, but containing N-gene specific sequences) to be detected by nanomechanical sensors and RT-PCR, respectively. All tests were performed in pharyngeal swab samples. First we injected 240 bp sequences of N-gene at a concentration of 800 copies/mL, and the PNA-functionalized microcantilever showed no differential deflection. When the concentration was increased to 1,000 copies/mL, the microcantilever reached a differential deflection of about 10 nm after 50 min of sample addition. As the concentration of the injected samples increased, the differential deflection of the microcantilever also increased sequentially (Fig. 8(b)). Thus, the detection limit of the nanomechanical sensor for the 240 bp sequence of N-gene is about 10^9 copies/mL, which is between the detection limit for the N-gene specific sequence (10^8 copies/mL) (Fig. 4(a)) and the detection limit for the N gene (50 copies/mL) (Fig. 5(a)). Although the twisting and bending of RNA in space as the size of RNA increases has the potential to obscure regions of binding, the assay results show that the size of the RNA clearly positively correlated with the sensitivity of the nanomechanical sensor (Fig. 8(c)). However, the total amount of RNA that can be captured in a low concentration sample solution was inherently small, which likely made the distance between the formed PNA/RNA duplexes relatively large (Fig. 8(a)). We assumed that PNA/RNA duplexes were uniformly distributed on the surface of the microcantilever and estimated the spacing (d) between PNA/RNA duplexes at several very low concentrations (50, 100 and 200 copies/mL) of our tests. In extremely low concentration samples, the lengths of the target nucleic acid molecules of the three different sizes were all smaller than the spacing (d) between PNA/RNA duplexes (Table S3 in the ESM). Therefore, the electrostatic force between PNA/RNA duplexes is relatively weak or even negligible at this time. The negatively charged PNA/RNA duplexes affects the stability of the hydrogen network of water and thus the resulting hydration force becomes the dominant factor in the change of stress on the surface of the microcantilever. The larger the size of the RNA, the greater the amount of negative charge the PNA/RNA duplexes carry. Then the perturbation of the hydrogen network of water by the PNA/RNA duplexes becomes stronger and a greater hydration force follows. Ultimately, the larger size of the RNA generates greater repulsive forces, resulting in greater deflection of the microcantilever, which further improves sensitivity (Fig. 8(a)). This also fits in with our proposed mechanism of PNA enhanced surface stress (Figs. 7(c) and 7(d)). Different concentrations of N gene and 240 bp sequence of N gene were tested simultaneously using RT-PCR and three replicate wells were set up for each concentration of samples (Fig S6 in the ESM). The cycle threshold (CT) values of the 240 bp sequence of N gene at concentrations of 32,000, 16,000, 8,000 and 4,000 copies/mL were 14.89, 18.21, 20.99 and 22.83, respectively, while the CT values for the N gene with concentrations of 32,000, 16,000, 8,000 and 4,000 copies/mL were 14.75, 17.51, 20.18 and 22.77, respectively. It is clear that the CT values measured for the two pharyngeal swab samples are almost identical when the concentrations are the same. This suggests that the sensitivity of RT-PCR is based on the quantitative relationship of the target nucleic acid, independent of the size of the target nucleic acid. However, the sensitivity of nanomechanical sensors working on the stress principle is closely related to the size of the target nucleic acid. This unique characteristic of nanomechanical sensors combined with the unique advantages of PNA as a nanomechanical sensor probe is what makes PNA bring more significant performance improvements to nanomechanical sensors than SPR, FRET and G-FET sensors [47–49].

We would like to emphasize here that both PNA and microcantilever are indispensable for the huge increase in...
sensitivity. The gains brought by the properties of PNA (spatial orientation, polyamide backbone) to nucleic acid detection are fully amplified when combined with a microcantilever that works on mechanical principles, ultimately creating a large repulsive force on the surface of the microcantilever. The changes brought about by the PNA in the hybridization so cleverly meet the factors required to increase the surface stress variation of the microcantilever, which brings about the effect that cannot be reflected by a simple superposition. Ultimately the PNA brings a much better increase in sensitivity to microcantilever than it does to the other sensors [47–49]. Thus, the new environment created by the PNA and the microcantilever is unique, and replacing either of them would obstruct a huge increase in detection sensitivity.

3 Conclusions

In conclusion, we developed a PNA-based nanomechanical method to achieve the ultrasensitive and amplification-free detection of SARS-CoV-2 using an optical readout mode. The high affinity and oriented upright conformation of PNA not only enhances the ability to capture RNA, but what makes PNA unique for nanomechanical sensor is that more PNA/RNA double-stranded leads to a stress variation on the surface of the microcantilever considerably increased. The combination of these factors greatly improves the sensitivity of microcantilever sensor. Also, thanks to the differential modification of the microcantilever array, the method is label-free, fast and accurate. The detection limit for the N-gene of SARS-CoV-2 is as low as 50 copies/mL, which is lower than the detection limit of RT-PCR requiring amplification. This phenomenon is unique to our stress-based nanomechanical sensor, which effectively improves the detection performance for the N gene of SARS-CoV-2. In general, our technology is expected to be rapidly promoted without geographical restrictions in the current situation where the virus is rampant, and used for the early detection of SARS-CoV-2. Moreover, there is the ability to be used more extensively for multiple types of nucleic acid detection.

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