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Direct and Rapid Detection of RNAs on a Novel RNA Microchip

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Direct, rapid, and accurate detection of pathogen RNAs is essential for controlling the spread of infectious diseases, such as H1N1 influenza or other infectious agents that can cause epidemics.^[1-3] Nucleic acid-based detection systems, such as real-time PCR and DNA microchips (or microarrays), offer high detection specificity and sensitivity and are able to detect single-nucleotide differences in pathogens, genes, and diseases.^[4-14] Though these technologies are powerful, RNAs from viable pathogens are not directly detected, and must be indirectly analyzed by cDNA amplification and detection. It is also difficult to use these technologies to detect the partially degraded pathogenic RNAs that can provide information on pathogen viability. Since it is difficult to amplify and label RNAs, it is extremely challenging to directly detect RNAs, let alone fragments. In addition, a few of the current methods for RNA direct detection, such as the Northern blot, RNase protection assay, microRNA profiling, and direct RNA sequencing,^[15] are labor intensive, costly or instrument-intensive. They are not well suited to rapid and high-throughput RNA detection, especially not for point-of-care diagnosis and other applications.

The direct and rapid detection of RNA remains an unsolved challenge. To face this problem, our research group has developed a methodology for accurate RNA detection by taking advantage of nuclease and polymerase activities. Herein we report for the first time the proof of principle of a novel RNA microchip strategy for the direct and rapid detection of RNAs (Scheme 1). Our simple strategy is fast (ca. 20 min detection time) and of high specificity (single-nucleotide discrimination) and high sensitivity (the low-femtomole level). Furthermore, we have successfully demonstrated the selective detection of multipathogen RNAs in a synthetic mixture and an individual mRNA in total RNA from a biological sample.

Our direct strategy for RNA labeling and detection takes advantage of the DNA lagging synthesis in gene replication,^[18] whereby a DNA polymerase naturally extends an RNA primer with dNTPs on a DNA template. In the same way, we use DNA polymerase to incorporate labeled dNTPs directly into a target RNA on a DNA template immobilized on a

microchip (Scheme 1). Prior to the polymerase extension, the RNA is cleaved by RNase H, which serves as a guided “RNA-endonuclease” on the DNA template. RNase H exposes an internal sequence of the target RNA (e.g., by removing the poly(A) tail of mRNA) for the specific detection and extension with the hapten-labeled dNTPs (such as biotin-dNTPs) by DNA polymerase;^[19] this was demonstrated in a well-plate format. Through the specific interaction with a binder-enzyme conjugate (e.g., streptavidin–horse radish peroxidase), the hapten labels were converted to enzymatic labels that catalyzed chemiluminescent reactions and amplified signals for detection by a CCD camera with high sensitivity.^[20]

Furthermore, to simplify the cleavage and extension procedures, we designed and easily synthesized chimeric probes (by solid-phase synthesis) [5'-DNA-(2'-Me-RNA)-DNA-3', or 5'-DNA-(2'-Me-RNA)-3'] to consolidate these three steps: the target RNA hybridization, RNase H digestion, and DNA polymerase extension (Scheme 1 and Figure 1). The 5'-DNA sequence serves as both the DNA guide for RNase H digestion and the DNA template for DNA polymerase extension. The 3'-DNA sequence serves as another DNA guide for RNase H digestion to cleave the 5'-region of target RNAs. Because of the double digestion, only a very short (even less than 20 nt) piece of RNA is required for the actual labeling and detection; this allows detection of partially degraded RNAs and identification of pathogen viability. Since RNase H does not digest RNA/RNA duplexes,^[21] the 2'-Me-RNA portion in the chimeric probe prevents the excessive digestion of the target RNA, and so the target is retained on the probe for the DNA polymerization. The 2'-methylation of the probe RNA portion also stabilizes the probe RNA sequence against RNases. In addition, the chimeric probes are modified with 3'-NH₂ groups, which permit immobilization on the NHS-activated silicon surface by a microarrayer generating typical 75-micron-size spots.^[22] We have successfully demonstrated the proof of principle of this RNA microchip technology by using biotin-labeled dNTPs (Figure 1).

In principle, RNAs of any sequence can be specifically detected by this RNA-microchip strategy by designing the corresponding detection probes. We demonstrated selective and simultaneous detection of bacteria and pathogen RNAs (Figure 1), including RNAs from the *E. coli lacZ* mRNA (lacZ), *Bacillus anthracis* RNA (BA), bird flu RNA (BF), and swine flu RNA (SF). We showed that RNAs can be detected by incorporating biotin-labeled dNTPs (dATP, dCTP, dGTP and/or dUTP analogues). For instance, we showed the specific detection of swine flu RNA by RNase H digestion and DNA polymerase extension of the SF RNA with biotin-labeled dGTP and dATP on the underlined template of the swine flu probe (Figure 1 A). As all four types of biotin-labeled dNTPs are commercially available, our detection system is generally applicable to any RNA sequence. Moreover, we demonstrated that multiple RNA targets can be simultaneously and selectively detected on the same microchip even with different RNAs present in the sample (Figure 1 D–F). No negative control signals were detected, even when a higher quantity of the RNAs (50 or 100 fmol) was used. Therefore, the RNA microchip is well suited for multiple pathogen detection on a single microchip.

As expected, hybridization of the RNA targets on the detection probes provides high specificity and discrimination. Moreover, the RNase H digestion is 5'-DNA-guide dependent, and the polymerase extension is 5'-DNA-template dependent. Thus, the 5'-DNA region, which offers discrimination by both hybridization and enzymatic reactions, should generate more selectivity and mismatch discrimination than the 2'-Me-RNA region, which offers discrimination primarily through hybridization. We performed specificity and comparison studies (Figure 2), and indeed observed mismatch discrimination by both the 5'-DNA and 2'-Me-RNA regions. Consistent with our analysis, our experimental results revealed that the target RNA double mismatches within the 5'-DNA region resulted in much more discrimination (ratio of BA3 vs. BA5 =>200; BA5 single was very weak) than the

double mismatches within the 2'-Me-RNA region (approximate ratio of BA1/BA2 = 3). Similarly, strong discrimination against the single-nucleotide mismatch in the 5'-DNA region was also observed (approximate ratio of BA3/BA4 = 10), while the single-mismatch discrimination in the 2'-Me-RNA region was not significant (data not shown). It is expected that single-nucleotide mismatches are more tolerated in the center of target RNA sequence than in its 3'-region owing to the fact that both RNase H and Klenow need a perfectly matched sequence for their catalytic hydrolysis and extension reactions. This finding helps better designing the detection probes by placing the mismatches in the 5'-DNA region of the probes.

In addition, our previous DNA polymerase study shows that a dinucleotide overhang facilitates dNTP incorporation.^[25] Thus, we incorporated 5'-GA as the overhang (Figure 2) and found that the incorporation and signal increased drastically (BA3 vs. BA1). Interestingly, mismatch discrimination of the probes with the overhang is also strong. The signal ratio of the perfect match and single-nucleotide mismatch is approximately 10:1 (BA3 vs. BA4). Furthermore, the signal of the double-nucleotide mismatch (BA5) is very weak. As expected, the high specificity of the RNA microchip was enhanced at an elevated temperature. Fortunately, RNase H and Klenow are still active during the short reaction period at 60 °C. The single-nucleotide discrimination can most likely be attributed to a combination of the 5'-DNA/RNA duplex destabilization and RNase H/Klenow reaction disruption, which are further enhanced by the effects of elevated temperature.

In general, hybridization is the major rate-limiting step among DNA microchip technologies,^[4, 6, 23, 24] and usually has to take place overnight. Shorter oligonucleotides can hybridize to their complementary sequences much faster than longer ones. Since short target RNAs (normally 15–25 nt.) are used in our RNA microchip detection, we expect fast kinetics of the target RNA/probe hybridization. Our normal fragmentation of mRNA substrates into smaller pieces not only speeds up the binding kinetics, but avoids the problem of competition between RNA hybridization and self-folding. Furthermore, the formation of an RNA–RNA duplex between the target RNA and the chimeric probe can better compete with folded RNA. Thus, by synthesizing a fluorescent-dye-labeled RNA, we are able to perform a series of time-course experiments to investigate the formation and kinetics of the target RNA/probe hybridization (a 24 bp duplex; Figure 3). Excitingly, our experimental results show that the hybridization process on solid surfaces is fast for target RNA binding to the chimeric probe. The formation of the short target/probe duplex is rapid, reaching 50% hybridization in approximately 5 min, and almost reaching a plateau after 20 min. These fast hybridization kinetics lay a solid foundation for the speed of this RNA microchip technology. In order to achieve rapid detection, we can keep a short hybridization time (5 min), though it might sacrifice approximately 50% of the sensitivity.

To further demonstrate the potential of this RNA microchip strategy in real-life applications, we designed a chimeric probe [5'-d(GTTGTTTTT)-2'-Me-RNA(AUCGCCAAUCCACAU)-d(CTGTGAAAGA-NH₂-3') to target *E. coli lacZ* mRNA (724–748 nt): 5'-AUGUGGAUUGGCGAUAAAAACAA-3']. Using the RNA microchip (Figure 4 A), we demonstrated a high sensitivity for RNA direct detection by detecting target RNA at the low-femtomole level. Though total RNA contains several thousands of different mRNAs, excitingly, no chemiluminescent signal was detected in the total RNA isolated from wild-type *E. coli* grown in the presence of glucose (Figure 4 B), which suppresses *lacZ* mRNA expression. When the synthetic *lacZ* RNA fragment was added to the glucose-suppressed total RNA sample, the signal was detected specifically. Finally, we specifically detected *lacZ* mRNA in the total RNA isolated from wild-type *E. coli* (Figure 4 B) grown in the presence of isopropyl-β-D-thiogalactopyranoside (IPTG), which induces *lacZ* mRNA expression. The reason that the *lacZ* mRNA signal was condensed in a small

area (Figure 4 B 4) is probably due to the mRNA length. Our observation reveals that the presence of the total RNA does not significantly interfere with detection or the sensitivity of the RNA analysis. These results suggest that this RNA microchip can be useful for real-life applications.

In summary, we have demonstrated the proof of principle of a novel and unique RNA microchip strategy that allows direct, rapid and selective detection of individual RNAs without RT-PCR and transcription amplification. Our strategy can be generally applied to the detection of any RNA through the design of complementary probes. We have also found rapid RNA/probe hybridization kinetics on the microchip, which lays a solid foundation for this rapid RNA-detection strategy. Moreover, by taking advantage of the specificity of the probe/target hybridization as well as enzymatic reactions, we discovered that this approach is of high specificity and able to conveniently discriminate a single-nucleotide mismatch by designing the detection probe with the mismatch in its 5'-DNA region. Furthermore, using the RNA microchip (spot size: 75 μm), we have demonstrated high sensitivity (at the low-femtomole level), simultaneous and selective detection of multi-pathogen RNAs, and the direct detection of an individual RNA from a biological sample, without the need for the time-consuming amplification and hybridization steps. The nonspecific background noise, caused by nonspecific binding of the macromolecules (such as the enzyme-binder conjugate) to the microchip surface, compromises detection sensitivity and is a general challenge faced by all microchip technologies, including the DNA GeneChip and RNA analysis on chip.^[15a-c] Surface inactivation and patterning can minimize the background noise.

This RNA microchip (or microarray) strategy is simple and easy to perform. The entire detection can be completed in approximately 20 min, and it is also cost-effective. Our strategy is fundamentally different from the existing technologies, such as real-time PCR and DNA microchips (or micro-arrays), as it does not require target amplification (such as RT-PCR) or fluorescent detection, which requires laser excitation. Our general technology platform can also be used in the detection of siRNA and miRNAs, especially mature ones for function monitoring and studies. Its unique rapidness, specificity, sensitivity, and cost-efficiency are desirable and make it well suited for point-of-care detection with a wide range of potential applications, such as rapid monitoring of epidemics, field detection, clinical diagnosis, and food-processing monitoring.

Experimental Section

Microchip activation

The silicon chips (0.5×0.5 cm) were first degreased in CH_2Cl_2 for 30 min with gentle shaking, then cleaned in concentrated H_2SO_4 for 1 h. The chips were rinsed in RNase-free water several times until the wash was at pH 7.0. The chips were covered with a mixture of 3% 3-aminopropyltrimethoxysilane (Aldrich) in ethanol/water (19:1) for 30 min at room temperature. The chips were washed sequentially with methanol and RNase-free water and were dried at room temperature before activation. Activation was performed by incubation in a solution containing 1,4-phenylene diisothiocyanate (PDITC, 10 mM, Fluka) in CH_2Cl_2 with 1% pyridine for 2 h. The chips were washed with CH_2Cl_2 (3×) and dried at room temperature, then stored under argon at room temperature. A step-wise representation of the activation steps is shown in Scheme 2.

Probe immobilization

The synthesized DNA–RNA–DNA chimeric probe was diluted to 200 μM in sodium phosphate (100 mM, pH 8.5) and was printed on the activated microchip with the OmniGrid Micro, followed by 30 min of incubation in a water bath at 37°C.

Rapid detection of RNA

The prepared microchip immobilized with the chimeric probes was incubated with 5× SSC buffer (20 μ L, 3.0 M NaCl, 3.0 M sodium citrate, pH 7.0) and RNA samples (1–500 fmol) at room temperature for 5 min. Subsequently, the unbound RNAs were removed by quickly washing the chip with 2× SSC buffer (1.2 M NaCl, 1.2 M sodium citrate, pH 7.0). The microchip was then incubated with RNase H buffer (20 μ L, 1×, New England Biolabs) and RNase H (0.5 μ L, 5000 U mL⁻¹, New England Biolabs) at 37°C for 5 min. After the RNase H solution had been drained from the incubation chamber, the microchip surface was quickly rinsed with StartingBlock (TBS) blocking buffer (Pierce). The microchip was then incubated with Klenow buffer (20 μ L, 1×, New England Biolabs), the Klenow fragment (0.5 μ L, 5000 U mL⁻¹, New England Biolabs), and biotin-7-dATP (1.0 μ L, 0.4 mM, Invitrogen) at 37°C for 5 min. Subsequently, the unincorporated biotin-dATP was removed from the microchip by quickly washing with StartingBlock (TBS) blocking buffer. The microchip was then incubated at room temperature for 3 min with poly-HRP-streptavidin conjugate (Thermo Scientific) diluted 5000× in StartingBlock (TBS) blocking buffer. The microchip was quickly rinsed to remove unbound enzymes. Super-Signal ELISA femto maximum sensitivity substrate (Invitrogen) was added, and the chemiluminescent signal was detected within 1 min with an ultra-sensitive CCD camera. The total analysis time for the rapid detection of RNA is less than 20 min. Furthermore, we can consolidate the first three steps (RNA hybridization, RNase H digestion and Klenow extension) into one step, which further simplifies the detection procedure and shortens the analysis time.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Simultaneous and selective detection of multipathogen RNAs on an RNA microchip. The chip was immobilized with the corresponding detecting probes (2×2 spots for each probe) for lacZ mRNA (lacZ), *Bacillus anthracis* RNA (BA), bird flu RNA (BF), and swine flu RNA (SF). A) SF RNA and its detecting probe are shown here as examples; the other RNA sequences and their probes are presented in the Supporting Information. SF RNA was specifically detected by incorporating biotin-labeled dGTP and dATP into the RNA. Selective detection of B) SF RNA or C) BA RNA when only that RNA was present in the sample, while the other three probes worked as controls. Simultaneous and selective detection of D) BA and SF RNAs or E) lacZ and BF RNAs when both were present in the sample, while the immobilized probes for the other two RNAs acted as the negative controls. F) Simultaneous and selective detection of BA, BF, and SF RNAs when all three were present in the sample, while the immobilized lacZ probe was the negative control.

Target RNA:

5' -AUCUUUAGAAGCA-UUAUCUGAAGAUAG-AAAAAAA-3'

Figure 2.

RNA detection on the chip with single-nucleotide discrimination through RNase H digestion and Klenow extension at 60°C.

Figure 3.

Hybridization kinetics and rapid RNA detection. A) The rate of interaction of the target RNA (5'-AUGUGGAUUGGCGAUAAAAACA-A-biotin-Cy3-3') and the chimeric probe [5'-d(GTTGTTTTT)-2'-*O*-Me-RNA(AUCGCCAAUCCACAU)-d(CTGTGAAAGA)-NH₂-3'] on the RNA microchip. B) Rapid detection of *lacZ* RNA on the RNA microchip in the presence of the BA probe as a negative control, while the biotin-labeled DNA was used as a positive control. The target RNA was detected in approximately 20 min.

Figure 4.

Detection sensitivity and selective detection of an individual mRNA. A) RNA detection sensitivity; images 1–5: 0, 1, 10, 25, and 50 fmol RNA, respectively. B) Detection of *lacZ* mRNA in IPTG-induced total RNA; image 1: no RNA, 2: glucose-suppressed total RNA, 3: a mixture of glucosesuppressed total RNA and the *lacZ* mRNA synthetic fragment (50 fmol), and 4: the IPTG-induced total RNA.

Scheme 1.

Flowchart of the direct, rapid, and accurate RNA microchip

Scheme 2.
Activation of the silicon or glass microchip surface.