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Signal amplification on a DNA-tile-based biosensor with enhanced sensitivity

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Aims: Herein, we report our work to improve the detection sensitivity of a DNA-tile-based and self-assembled biosensing platform. This was achieved using hybridization chain reaction (HCR) as a signal amplifier on a water-soluble self-assembled DNA nanoarray carrying detection probes. **Materials & methods:** The fluorescence enhancement on the addition of specific detection targets was observed directly by confocal fluorescence microscopy. **Results & discussion:** The versatility of the system was demonstrated by successful detection of SARS viral DNA and ATP. Improvement of sensitivity by two orders of magnitude was achieved for both targets, compared with our previously reported system without signal amplification. **Conclusion:** In summary, this work provides proof-of-concept that HCR can occur efficiently on DNA-tile-based nanoarrays, thus facilitating more sensitive detection. The merging of HCR with combinatorial encoding systems to realize highly sensitive and multiplexed biosensing may provide new tools for nanomedical applications.

Keywords: biosensing, DNA nanotechnology, hybridization chain reaction, self-assembly, signal amplification

DNA nanotechnology has evolved into a highly interdisciplinary field in the past two decades [1]. In particular, DNA-tile-based self-assembly, which uses branched DNA nanostructures with sticky ends as basic building blocks (tiles), has matured with such vigor that it is currently possible to build micro- or even millimeter-sized arrays with desired tile geometry and periodicity [2–5]. The ability to organize biomolecules as well as position functional groups with nanometer precision makes self-assembled DNA arrays extremely useful for constructing biosensing platforms [6–8]. This type of biosensor is composed of two modules:

- DNA array as a supporting and organizing module
- Molecular-recognition probe (that reports the binding event) as a detection module

For example, we have previously reported a self-assembled signaling aptamer array that detects human α-thrombin at a subnanomolar concentration [6]. More recently, we demonstrated the combinatorial self-assembly of DNA-tiles into nanoarrays that bear nucleic-acid probes and unique barcoded fluorescence labels to realize multiplexed biosensing [7]. Although traditional DNA microarrays involve multistep surface-chemistry reactions [9], water-soluble biosensing platforms are easy to construct and provide relatively rapid detection. However, detection sensitivity is limited either by the binding strength between the probe and target or by the difficulty of spotting nanoarrays under the microscope.

One potential solution to enhance the detection sensitivity of self-assembled DNA nanoarrays is to introduce signal amplification to the system. Here, we report the use of hybridization chain reaction (HCR) as a signal amplifier on the DNA-tile-array-based biosensor. HCR is an isothermal, enzyme-free cascade reaction, in which the closed hairpins hybridize sequentially to each other and finally form a long double-stranded DNA (dsDNA) with alternating nicks on both strands [10]. The HCR reaction is initiated by a single-stranded DNA (ssDNA) that opens one of the closed hairpins and starts the cascading hybridization. The ability of a small amount of ssDNA to trigger the assembly of a high molecular-weight complex makes HCR a suitable candidate as a signal amplifier for biomolecular detection. Furthermore, all the components involved in HCR are simple oligonucleotides, which can be grafted easily onto DNA-tile-based arrays. Finally, one or both of the hairpins can be modified with fluorophores, resulting in HCR products that are fluorescence-rich and capable of being read out by simple optical means.

Materials & methods

Materials

All DNA strands used (see Figure A1 for sequences) were purchased from Integrated DNA Technologies [101] and were purified by denaturing polyacrylamide gel electrophoresis (PAGE). The concentrations of the DNA molecules were

10.2217/17435889.3.4.521 © 2008 Future Medicine Ltd ISSN 1743-5889

measured by absorbance at 260 nm. ATP was purchased from Sigma. YOYO-1 was purchased from Invitrogen.

Self-assembly of detection platforms & detection of analytes

All purified DNA strands involved in the DNA nanoarrays, including the on-tile hairpin strand for DNA detection or HCR initiator strand for ATP detection, were mixed at equal molar ratios (60 pmol each). To assemble the ATP-sensing nanoarray, an additional 12 pmol of inhibitor was added to ensure protection. The volume of the mixture was adjusted to 60 µl containing 1×TAE-Mg buffer (40 mM Tris-acetic acid buffer, pH 8, 12.5 mM magnesium acetate). The hairpin strands (H1 and H2) were diluted in 1×TAE-Mg buffer in separate tubes at 10 µM concentration. The DNA nanoarrays and the individual hairpins were then annealed separately from 94–25°C for approximately 36 h. The desired amount of target molecule (SARS viral DNA or ATP) was then added to 10 µl of the annealed DNA nanoarrays (200 nM or 1 µM), followed by the sequential addition of 1 µl of the annealed H1 and H2 solution (10 µM). The detection samples were incubated at room temperature for approximately 12 h in the dark before imaging. A total of 0.5 µl of 10 µM YOYO-1 solution was added to the mixture as a 'green' fluorescent label of the DNA nanoarrays for SARS virus DNA detection. A confocal fluorescent microscope was used to read out the detection result.

Nondenaturing polyacrylamide gel electrophoresis

Four samples (10 µl each) were prepared for a nondenaturing PAGE assay:

- 1 µM hairpins (H1 and H2 for ATP detection)
- 1 µM initiator plus 1 µM hairpins
- 1 µM initiator protected by 2 µM inhibitor, plus 1 µM hairpins
- Sample three plus 2 mM ATP

The samples were incubated in the dark at room temperature overnight and then resolved by gel electrophoresis on a 10% nondenaturing polyacrylamide gel in 1×TAE-Mg^{2+} buffer. The gel was run at a constant voltage of 200 V for 5 h and scanned by a Typhoon™ Trio multifunction imager (Amersham Biosciences, Buckinghamshire, UK) using a manufacturer set-up designated for Alexa fluor 488.

Confocal fluorescent microscopy

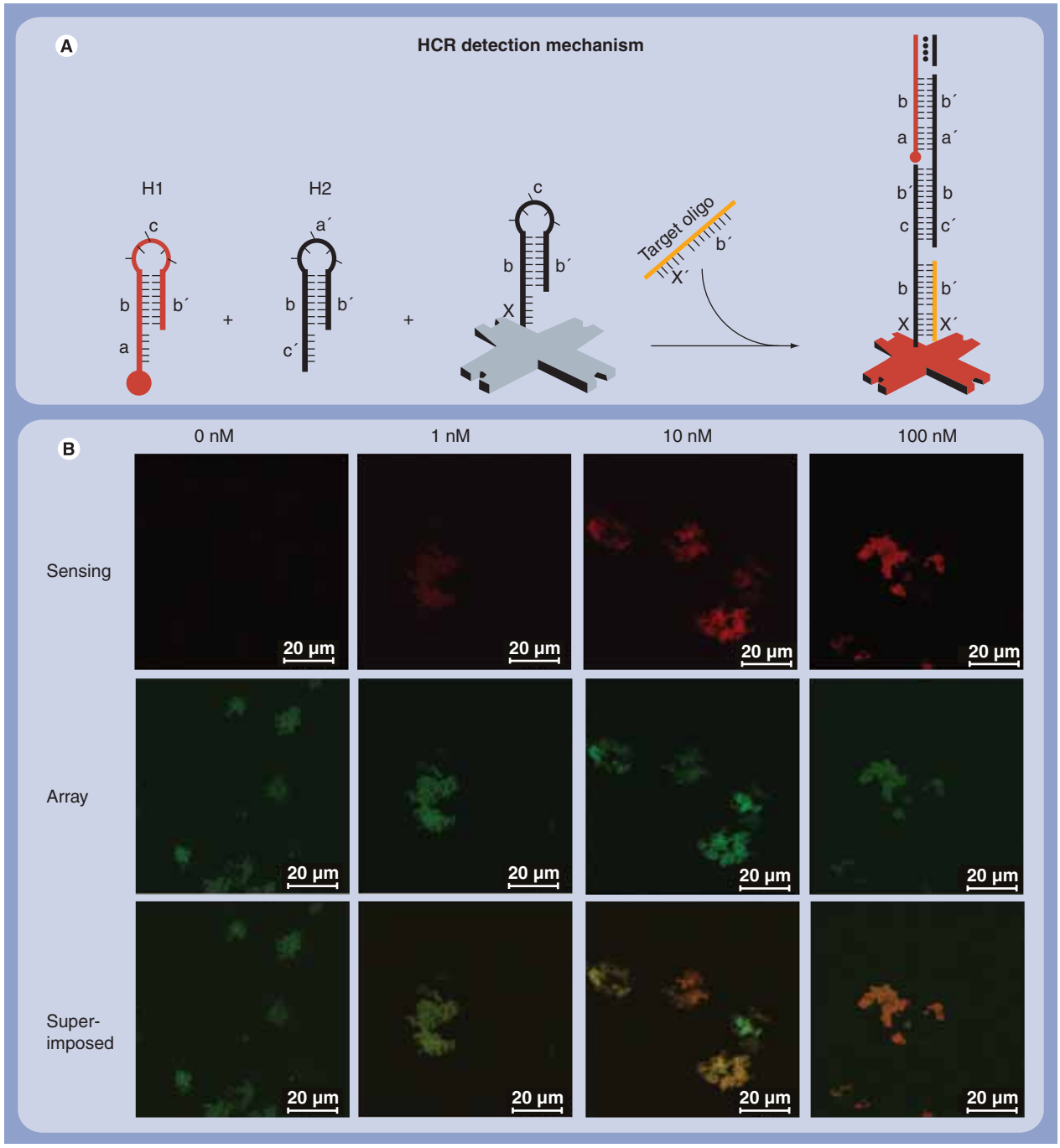
A total of 2.5 µl of the premixed and incubated sample was deposited on a glass slide and covered immediately by an 18 × 18 mm^2 cover slip (the solution was spread over the entire covered area) for imaging. All the fluorescence microscope images were taken using a Zeiss® LSM 5 DUO (Carl Zeiss) laser-scanning confocal microscope. The sample was scanned at a selected confocal plane and sequentially through the 'green' and 'red' channels. The color of the channels and the superimposed color are assigned by a program associated with Zeiss® LSM 5 and may not reflect the true emission color of the fluorophores. Unless otherwise noted, a 90 × 90 μm^2 framed image was taken with 512 × 512 pixel resolution.

The set-up parameters for each fluorescent label are listed in Table A1. For example, to image YOYO-1, an excitation wavelength of 488 nm (Ar^+ laser) was used, which is reflected by a DD 488/543 dichroic mirror and focused by an oil-immersed PL APO 100.0×/1.40 DIC objective lens to irradiate the sample. The emitted photons were collected by the same objective, transmitted through the same dichroic mirror, filtered by a spectra-photometer (bandpass: 500–550 nm) and focused onto a 182 µm pinhole before reaching the detector in the green channel. For the Alexa fluor 488, rhodamine red and Cy5, the same set-up was used with changing of the excitation light source, the dichroic mirror and the spectra-photometer bandpass.

Results & discussion

We first tested the detection of SARS viral DNA by using the HCR reaction on DNA-tile-based nanoarrays. The design is illustrated in Figure 1A. Here, the HCR initiator is protected initially by a self-folded hairpin that is anchored on a cross-shape DNA-tile (detection tile). The sequence of this hairpin is designed precisely so that it will hybridize partially with SARS viral DNA and switch from a closed state to an open state to free the initiator (sequence cb'). With this design, the on-tile HCR can be triggered by the addition of SARS viral DNA. In the presence of another cross-shape DNA-tile (linker tile, not shown in the figure) with complementary sticky ends to the detection tile, the on-tile hairpins can be assembled into 2D arrays with approximately 28 nm spacing between adjacent hairpins [11]. Two additional hairpins (H1 and H2) are designed to facilitate cross-hybridization on the deprotection of initiator.

Figure 1. Detection of SARS viral DNA using HCR reaction on DNA-tile-based arrays.



(A) Detection mechanism: a unique hairpin designed for the specific oligonucleotide target (SARS viral DNA) is incorporated on a DNA tile that can self-assemble into 2D arrays (not illustrated here). Two hairpins (H1 and H2), one of which (H1) is labeled by a red fluorescent dye (rhodamine red), are in the mixture with the DNA array. In the absence of the target, the hairpins, either on-tile or in-solution, retain their close states. The addition of the oligonucleotide target opens the on-tile hairpins, triggering HCR reaction between H1 and H2, leading to the elongation of nicked DNA double helices on tiles and resulting ultimately in the accumulation of red fluorescence signals on DNA nanoarrays. Unique stretches of DNA sequences are denoted by lower-case letters, in such a way that a' is the Watson–Crick complement to a'. **(B)** Detection by confocal fluorescent microscope. The DNA array is at 200 nM concentration (DNA tiles) and prestained by YOYO-1 (green fluorescence) before imaging. The concentrations of SARS viral DNA are labeled above the corresponding images. Each sample is scanned through 'green (array)' and 'red (sensing)' channels and the superimposed image is displayed in the bottom row. The detection limit is estimated to be 1 nM for the target.
HCR: Hybridization chain reaction.

When HCR occurs, it leads to the elongation of nicked double helices on the array. H1 is labeled by a red fluorescent dye (rhodamine red). The net effect of the addition of SARS viral DNA is the accumulation of a red fluorescent signal on the array, which can be visualized directly under fluorescent microscope.

A series of samples containing 200 nM sensing arrays and 0–100 nM SARS viral DNA was first stained by YOYO-1, a green fluorescent dye that intercalates into dsDNA, and then imaged using a confocal fluorescent microscope. In the absence of SARS viral DNA, no red fluorescence signal was observed. On the addition of SARS viral DNA with a concentration as low as 1 nM, the arrays start to appear red. The detection signal grew stronger with the addition of more targets, suggesting that the fluorescence color change is indeed owing to the presence of target. It is interesting to note that, at relatively low target concentrations (e.g., 10 nM or 1:20 ratio of target-to-probe), the HCR does not occur uniformly on every array, resulting in some arrays appearing a deeper red than the others. This is probably a result of the uneven opening of initiator hairpins on different arrays owing to insufficient target amount. Nevertheless, the HCR happens exclusively on the self-assembled DNA arrays on the addition of target. The detection limit here is estimated to be approximately 1 nM, which is approximately 100-fold more sensitive compared with detection using a similar design at the same array concentration without signal amplification.

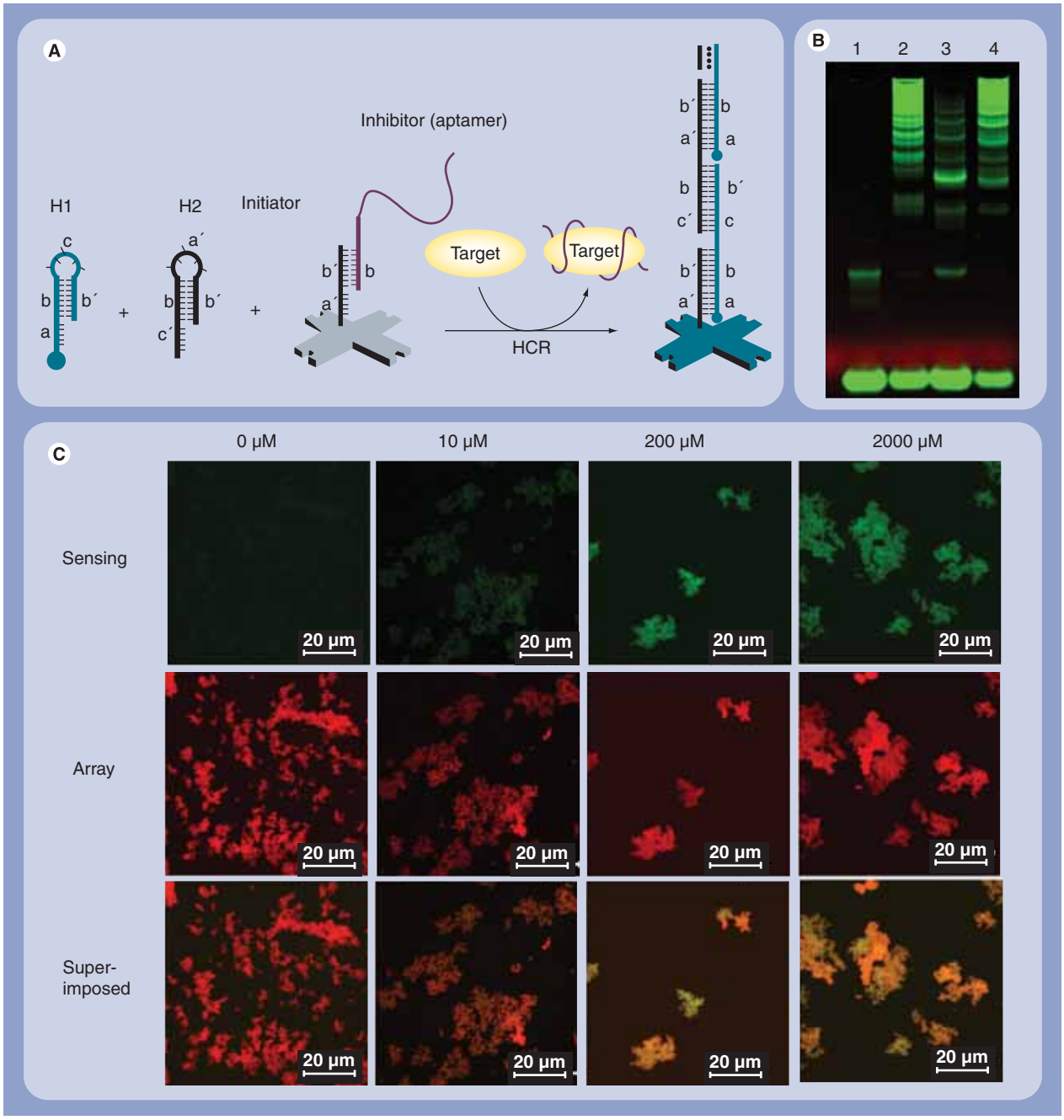
HCR can also be triggered by aptamer-binding molecules (instead of DNA or RNA oligomers) if the initiator is protected properly by the aptamer of the detection target. This offers increased versatility of our sensing system. To investigate the possibility of detecting aptamer-binding molecules, we chose ATP as a model target. The design for ATP detection is similar to the design for SARS virus detection, with two alterations (Figure 2A). First, instead of having the initiator protected as a hairpin, a ssDNA inhibitor comprising the sequence of the ATP aptamer is hybridized partially to the initiator to inhibit the HCR reaction before target addition. Such a design ensures that the free end of the aptamer protrudes out from the tile plane, granting better access for ATP. When ATP is present, it will bind to the aptamer and cause strand displacement. This will expose the bare initiator on the detection tile, which triggers HCR between the hairpins. Second, H1 is labeled by a green fluorescent dye (Alex Fluor 488) rather than red and, as an accommodation, the linker tile (not shown in the figure) is labeled with a red fluorophore (Cy5) to assist with the observation of the DNA arrays without the occurrence of HCR.

To validate the altered design, we first tested the HCR in solution (without tile) by nondenaturing PAGE (Figure 2B). An excessive amount of inhibitor (2×) was added to ensure minimal leakage reactions in the absence of ATP. From the PAGE assay, the HCR reaction was inhibited to a low level before the addition of ATP (Figure 2B, lane 3). In the presence of 2 mM ATP, HCR occurs at the same level as when the hairpins (H1 and H2) are exposed directly to the unprotected initiator, represented by high molecular-weight products with strong fluorescence (Figure 2B, lane 2 and 4). For the fluorescence imaging, increasing amounts of ATP (0–2000 µM) were titrated against 1 µM ATP-sensing arrays (Figure 2C). Although there was background signal without any ATP in the solution (which is in agreement with the findings by PAGE assay), the fluorescence color change in response to 10 µM ATP was significant enough to be clearly distinguished from the leakage. It is worth noting that the dissociation constant (K_d) between ATP and the structure-switching aptamer probe used here, is approximately 600 µM [12]. Therefore, a detection limit approximately two orders of magnitude lower than the probe's K_d value shows the power of signal amplification brought by HCR.

Conclusion

In summary, we have successfully constructed a self-assembled DNA-tile-array-based biosensor with improved sensitivity compared with its ancestor. The introduction of HCR as a signal amplifier enables a small amount of target to trigger a significantly increased fluorescence signal that is confined locally and observed by fluorescence microscopy. The signal amplification is achieved through HCR reaction by forming a long linear molecule at the target-binding site located on a DNA array, which concentrates the fluorescent dye-labeled DNA hairpins that diffuse freely in solution onto a DNA array immobilized on a surface, making the signal change visible easily under a microscope. Efficient detection can be achieved at low target-to-probe ratios (e.g., 1:200). The on-tile HCR assay presented here is highly reproducible. Quantitative data can be acquired from fluorescence microscope images, as plotted in Figure A2.

Figure 2. Detection of ATP using HCR reaction on DNA-tile-based nanoarrays.



(A) Detection mechanism: the initiator of the HCR reaction, protected by an aptamer specific to ATP, is incorporated on a DNA tile that can self-assemble into 2D arrays (not illustrated here). Two hairpins (H1 and H2), one of which (H1) is labeled by a green fluorescent dye (Alexa fluor 488), are in the mixture together with the DNA arrays. Minus the target, H1 and H2 retain their closed hair-pin structure. The addition of the aptamer-binding target (ATP) frees the initiators on the DNA tiles, triggering the HCR reaction and resulting ultimately in the accumulation of green fluorescence signals on DNA arrays. Unique DNA sequences are denoted by lower-case letters, in such a way that a' is the Watson–Crick complementary to a. **(B)** Validation of the detection mechanism by nondenaturing PAGE assay. Lane 1: H1 + H2; lane 2: initiator + H1 + H2; lane 3: initiator + inhibitor + H1 + H2; lane 4: initiator + 2×inhibitor + H1 + H2 + ATP. DNA strands were mixed sequentially as above. All strands are at 1 μM in 10 μl reaction volume, except for the inhibitor (ATP aptamer), which is in onefold excess (2 μM) to promote better inhibition. ATP concentration: 2 mM. **(C)** Detection by confocal fluorescent microscope. The sensing array is at 1 μM concentration (DNA tile) and is fluorescently labeled by Cy5 (red fluorescence). The concentrations of ATP are labeled above the corresponding images. Each sample is scanned through 'green (sensing)' and 'red (array)' channels and the superimposed image is displayed in the bottom row. The detection limit is estimated to be 10 μM.
HCR: Hybridization chain reaction.

Future perspective

In principle, by only altering the probe and hairpin sequences, this design can be adapted easily to detect other oligonucleotides with varying lengths and sequences, as well as other aptamer-binding molecules, such as proteins. When altering the design, it is crucial for the hairpin probes to have proper length stems and loops so that they are stable at room temperature in the absence of target, but can facilitate polymerization efficiently in the presence of analyte. Hairpins with loop length of six nucleotides and stem length of 18 base pairs meet this criteria perfectly [10]. Therefore, when other nucleic-acid targets are encountered, it is reasonable to maintain the same secondary structure of all hairpins and change only the anchored hairpin on-tile so that it can be opened by the target strand and free the initiator. For example, if HIV virus DNA (39 nucleotides) needs to be detected, the anchored hairpin must be redesigned with its stem (18 base pairs)-loop (six nucleotides) structure maintained and single-stranded anchor elongated (11 nucleotides). Obviously, the sequences of all hairpins (H1 and H2) must be changed accordingly. The work presented here used assorted fluorophores with well-separated spectra (Alexa fluor 488 and rhodamine red) to signal various targets (ATP and SARS viral DNA). This leads us to believe that this signal-amplification strategy can be used in multiplexed sensing systems to realize highly sensitive detection of multiple biomolecules simultaneously. Using a combinatorial encoding strategy, the multiplexing capability of such a system is expected to be incredibly high, limited only by the ability of the fluorescence microscope to differentiate the relative fluorescence intensity levels. In the future, this system can be further improved by using quantum dots with narrower emission spectra and brighter photoluminescence relative to organic dyes as fluorescent labels.

Acknowledgement

This research has been supported by grants CTS-0545652, CCF-0453685, CTS-0609362, DMR-0709938, CCF-0727100 from the NSF, N00014–07–1-0194 from ONR and FA9550–07–1-0080 from AFSOR, 1R21 EB0005844–01 from the NIH to HY and funding from Arizona State University to HY and YL.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, royalties.

No writing assistance was utilized in the production of this manuscript.

Executive summary

- Improved sensitivities were achieved by introducing signal amplification on a DNA-tile-based biosensor.
- The system has been applied to detect both a nucleic acid target and a bio-related small molecule.
- Improvement of sensitivity by two orders of magnitude was achieved for both targets.
- The signal amplification depends exclusively on the hybridization of ssDNA and is enzyme-free.
- We expect this system to be merged with combinatorial encoding system to realize highly sensitive and multiplexed biosensing.

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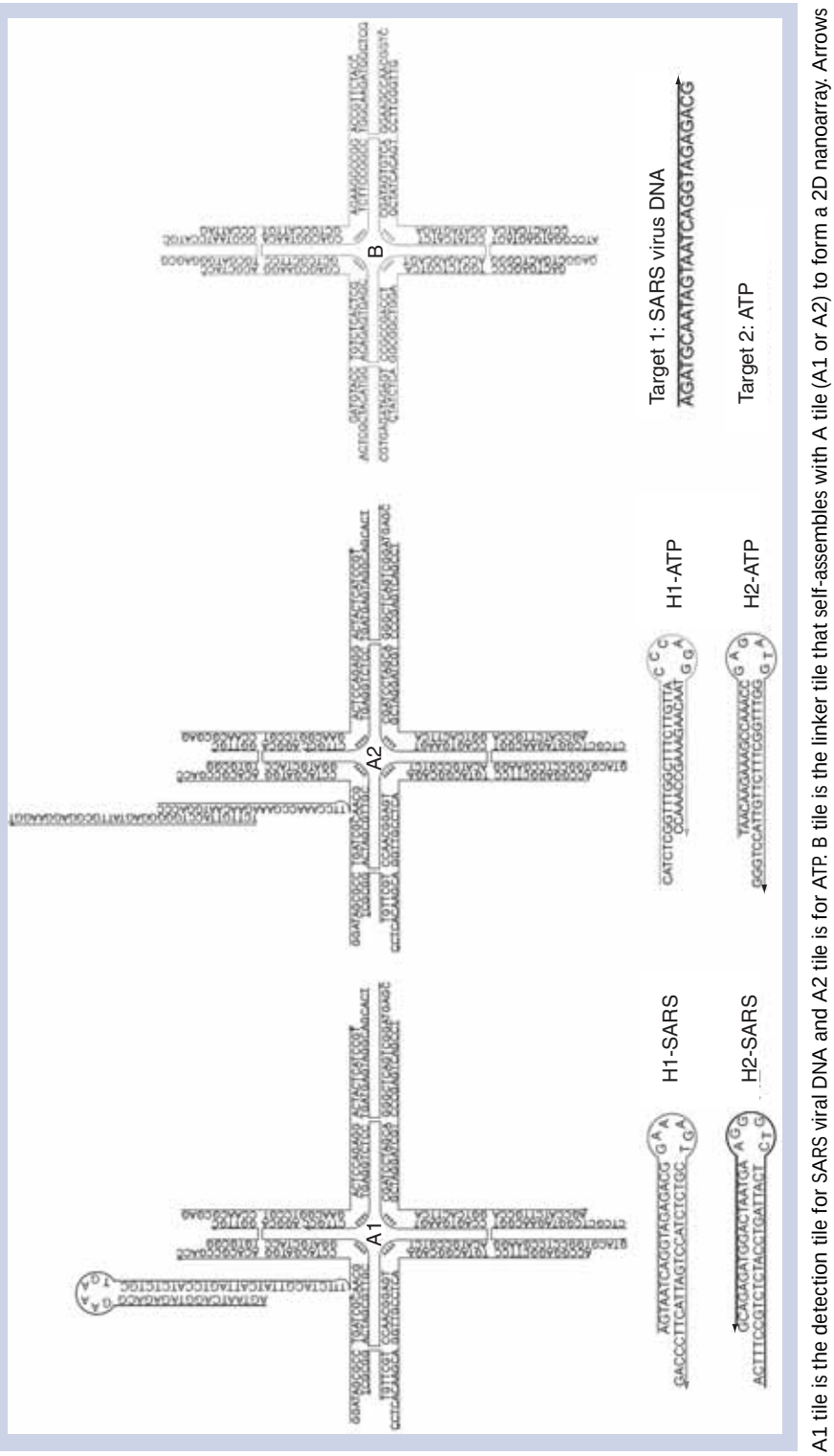
Website

101. Integrated DNA technologies www.idtdna.com

Appendices

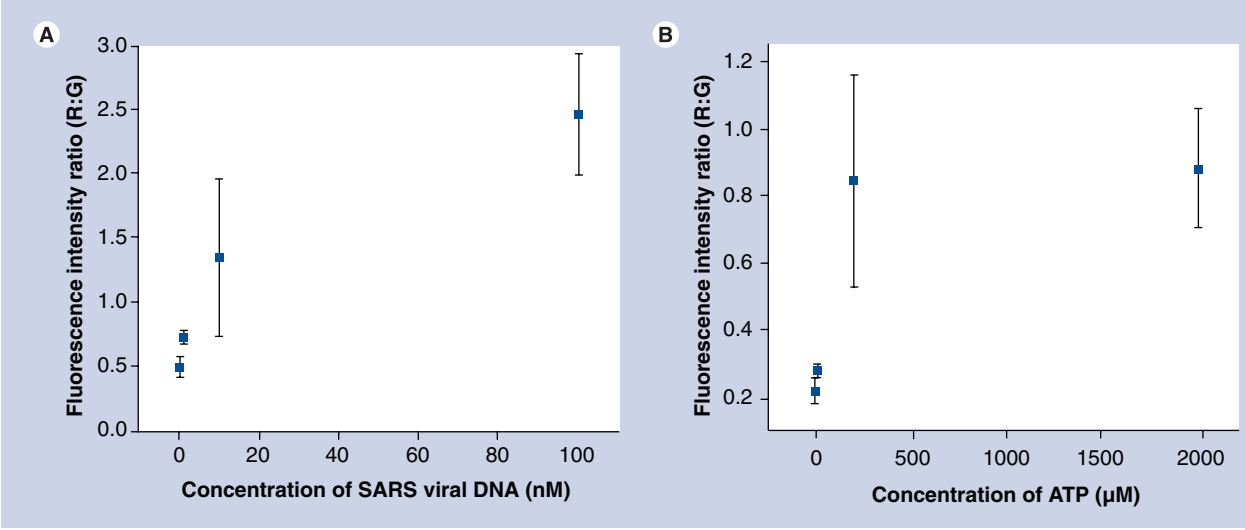
- Figure A1. DNA-tile structures, hairpin sequences and detection targets used in the work.
- Figure A2. Relative intensity of the detection signal as a function of the target concentration, sensing SARS viral DNA and ATP.
- Table A1. Fluorescence microscope setup parameters for fluorescent labels used.

Figure A1. DNA-tile structures, hairpin sequences and detection targets used in the work.



A1 tile is the detection tile for SARS viral DNA and A2 tile is for ATP. B tile is the linker tile that self-assembles with A tile (A1 or A2) to form a 2D nanoarray. Arrows point to the 3′-end of DNA strands. H1-SARS is labeled with rhodamine red and H1-ATP is labeled with Alexa fluor 488.

Figure A2. Relative intensity of the detection signal as a function of the target concentration, sensing (A) SARS viral DNA and (B) ATP.



For each data point, fluorescence intensities in each channel were measured from multiple arrays located at different areas of images using LSM Image Examiner. The sensing-to-marker signal ratio was then calculated for each array and averaged. Error bars are present to show the standard deviation. Note the relatively larger standard deviation for more concentrated targets, which is consistent with the finding that hybridization chain reaction occurs unevenly on different arrays in these cases.

Table A1. Fluorescence microscope setup parameters for fluorescent labels used.

Dye	Pseudo-color	Excitation	Dichroic mirror	Emission peak	Emission bandpass
Alexa fluor 488	Green	488 nm	TD 405/488/543/633	519 nm	500–550 nm
YOYO-1	Green	488 nm	DD 488/543	519 nm	500–550 nm
Rhodamine red	Red	543 nm	DD 488/543	594 nm	580–640 nm
Cy5	Red	633 nm	TD 405/488/543/633	665 nm	650–700 nm