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COMMUNICATION

A DNA Minimachine for Selective and Sensitive Detection of DNA

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Synthetic molecular machines have been explored to manipulate matter at the molecular level. Here we designed a multifunctional DNA nano-construct, dubbed 'DNA minimachine' (DMM) that (i) tightly binds complementary DNA; (ii) recognizes specific fragment with high selectivity and (iii) amplifies output signal. DMM1 detects lower concentrations of both single stranded DNA and double stranded DNA than a conventional probe. This study sets a direction towards development of molecular machines for selective, sensitive and cost-efficient DNA analysis.

Molecular machines are known in biology[1] and have been synthesized in laboratories.[2] DNA nanotechnology builds functional molecular associations from synthetic DNA strands.[3] One important advantage of DNA-based machines is their ability to organize several functional units in precise orientations on a DNA plaform.[4] In this study, we took advantage of DNA nanotechnology for building a DNA nanomachine for the analysis of double-stranded DNA (dsDNA) amplicons, which potentially can find application in molecular diagnostics of human diseases.

Detection of single nucleotide differences in double stranded DNA (dsDNA) is important in the context of single nucleotide substitutions (SNS) analysis in native biological DNA[5] as well as in dsDNA amplicons obtained by PCR, LAMP, RPA and other DNA amplification techniques.[6] One common approach uses heat denaturing of dsDNA at elevated temperatures, which separates the complementary DNA strand and allows a hybridization probe to get access to the binding site.[7] In an alternative approach, chemically analogies of natural DNA/RNA (e.g. peptide nucleic acids (PNA),[8] locked nucleic acids (LNA))[9] are used to displace one DNA strand in dsDNA due to the elevated affinity to ssDNA. However, PNA and LNA are expensive commercial products. Furthermore, the challenge in dsDNA analysis is reflected by the need for asymmetric PCR,[10] or λ exonuclease treatment,[11] which produce ssDNA accessible by a hybridization probe. The disadvantage of asymmetric PCR is the low yield of single stranded DNA (ssDNA) product, while λ nuclease treatment is an extra time a reagent-consuming step. Here we propose an alternative approach, named DNA minimachine (DMM) for detection of dsDNA amplicons made of all DNA strands with increased affinity for a targeted dsDNA fragment, while retaining high selectivity.

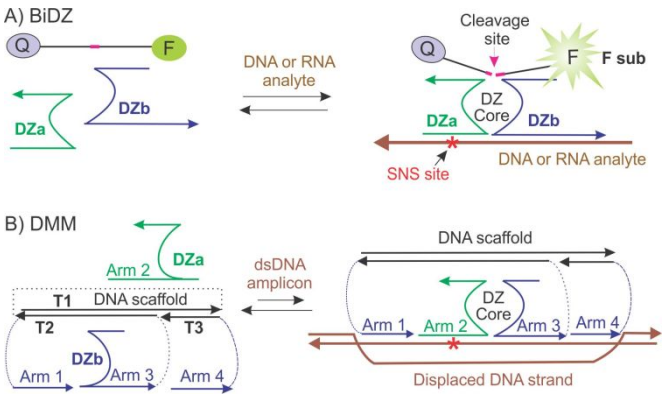

Fig 1. Design of a DNA minimachine (DMM) for double stranded DNA (dsDNA) analysis. A) Binary deoxyribozyme (DZ) probe: DNA strands DZa and DZb bind single stranded DNA and form DZ catalytic core, which is capable of cleaving multiple F sub molecules.[12] B) DMM consists of DZa and an association of T1, T2 and T3 strands, two of which are covalently linked to DZb, Arm 1 and Arm 4 functions. DMM binds dsDNA amplicon by 4 binding arms capable of displacing one strand in dsDNA, which is a reversible process.

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We took advantage of binary deoxyribozyme (BiDZ) probe for nucleic acid analysis (Fig. 1A).[12] In this strategy, strands DZa and DZb hybridize to a targeted fragment and form a DZ catalytic core, which is capable of cleaving a fluorophore and a quencher labelled reporter substrate (F sub in Fig. 1A). A major advantage of BiDZ over other hybridization probes (e.g. molecular beacon,[13] adjacent probes[14]) is its improved sensitivity due to the catalytic amplification of the signal over time.[12] BiDZ uses universal reporter substrate, therefore adjusting the probe to each new analyte requires only two unmodified DZa and DZb strands, which is more cost efficient than that for other fluorescent probes. Another BiDZ advantage is its high selectivity: the DZa analyte-binding arm can be made short to form stable complex only with fully complementary, but not with SNS-containing analyte.[12] Earlier BiDZ was proposed for analysis of PCR products with only moderate efficiency[15] (see discussion below). Here we compare the performance of BiDZ with that of DMM in the ability to detect dsDNA amplicon.

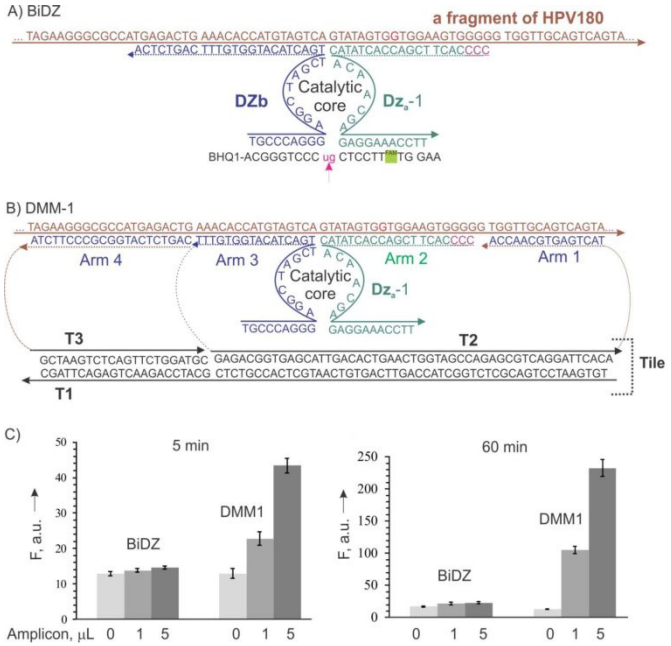

Fig 2. Design BiDz and DMM-1 and detection of a plasmid-derived dsDNA amplicon. A) HPV 16 ssDNA analyte is recognized by binary deoxyribozyme (BiDZ) probe followed by cleavage of fluorogenic F sub (AAG GTT^{FAM} TCC TCg uCCC TGG GCA-BHQ1)^{12c} B) The design of DMM-1 for recognition of HPV16 analyte. C) Detection of HPV 16 after 5 (left) and 60 (right) min of incubation. BiDZ1 or DMM1 were incubated in the presence of 0, 1 μL (6 nM PCR product) or 5 μL (~32 nM) of dsDNA amplicon from plasmid at 50°C for 5 or 60 min, panels A and B, respectively. The concentration of DNA amplicons was estimated as described in SI (Fig. S5 and S6). The data of 3 independent experiments are presented.

Major challenge in the analysis of folded ssDNA and dsDNA by hybridization probe is the thermodynamic stability of dsDNA, which prevents access of the probe to the analyzed sequence. For example, MB probe fails in detecting analytes folded in stable secondary structure sequences.[16] Increased affinity of BiDZ to dsDNA would increase the concentration of probe-amplicon complex and thus improve limit of detection and/or shorten the assay time. One strategy to increase affinity is to elongate analyte binding arms of DZa and DZb. However, elongation of analyte-binding arms would render the probes insensitive to SNS thus making it less selective.[17] Moreover, long DNA constructs tend to fold in secondary structures having reduced affinity to targeted sequences. We, therefore, followed the biology-inspired approach in which tight binding together with selective recognition is achieved by using multiple weak interactions. DMM was designed to have 4 DNA-binding arms: Arms 1, 3 and 4 attached to the common DNA scaffold made of complementary fragments of strands T1, T2 and T3, while Arm 2, a part of DZa stand, was detached from the DNA scaffold (Fig. 1B). Arms 1, 3 and 4 are responsible for binding the amplicon tight, while Arm 2 is complementary to an SNS site and is responsible for high selectivity of target recognition.

As a target we chose a sequence of human papillomavirus type 16 (HPV16), which is known to cause cancer and is the subject for clinical analysis by PCR-based test systems.[18] For initial experiments, we used short synthetic single-stranded DNA analytes HPV-45 (5'-TGA GAC TGA AAC ACC ATG TAG TCA GTA TAG TGG TGG AAG TGG GGG) and HPV-180 (see Table S1 for sequences), 45 and 180 nucleotide-long, respectively. HPV-45 analyte folded in unstable ($\Delta G = -1.62$ kcal/mol), while HPV-180 folded in a more stable ($\Delta G = -5.86$ kcal/mol) secondary structures (Fig. S1A) and the assay conditions.

Table 1. Limits of Detection for the DNA – minimachine (DMM1) and BiDZ1 with ssDNA analytes

Sensor	Analyte	LoD, pM	
		Incubation time	
		60 min	180 min
BiDZ1	HPV-45	59	69
	HPV-180	47	13
DMM1	HPV-45	89	31
	HPV-180	15	0.96

BiDZ1 and DMM1 constructions were designed to bind the targeted sequences of HPV-45 and HPV-180 (Fig. 2A and B and S1B). The correct assembling of DMM1 was confirmed by analysis the DNA associations in agarose gel (Fig. S2). It was found that HPV-45 was detected by BiDZ1 with roughly the same limit of detection (LOD) of 60-90 pM (after 60 min assay) by both BiDZ1 and DMM1 (Table 1). At the same time HPV-180 was detected by DMM1 with lower LODs (Fig. S3 and S4, Table 1). Specifically, BiDZ1 detected folded HPV-180 at 13 pM, while DMM1 achieved 0.96 pM LOD after 180 min of incubation (Table 1). Based on this data we estimate the DMM1 improves LOD by about 13 times over conventional BiDZ. This data indicates that Arm 1 and 4 in DMM1 improve LOD only for longer analyte, which proves the hypothesis that the arms unwind long folded analytes. Importantly, the LOD for longer analyte was lower than for the short unstructured HPV-45 (15 and 0.96 pM after 180 min, respectively), which is opposite to that expected from the performance of conventional

hybridization probes. However, this inverse sensitivity is expected if we take in account that Arm 1 and 4 contribute to tight bind of HPV-180, but not HPV-45. The tighter binding of HPV-180 resulted in increased sensitivity. To further asses if Arms 1 and 4 contribute to binding dsDNA amplicon, we tested the three variations of DMM1 lacking Arm 1 or Arm 4 or both in ability to detect dsDNA amplicon (Fig. S7). Removing one arm reduced the fluorescent signal by about 2 times, while removing both arms lowered fluorescence response ~ 4 times. This data additionally verifying our hypothesis that Arms 1 and 4 help tighter binding of the amplicon thus increasing the concentration of the signal-producing complex (Fig. 1B, right).

We then explored the ability of BiDZ1 and DMM1 in detection of dsDNA amplicon. A plasmid containing HPV16 genome was PCR amplified using a plasmid containing HPV-16 genome. Molar concentrations of dsDNA amplicon was estimated as described in SI (Fig. S6). DMM1 reliably detected both 5 μL (~32 nM final concentration) and 1 μL (6.4 nM) DNA amplicons after 5 and 60 min of the fluorescent assay, while BiDZ1 failed to produce signal above the background (Fig. 2C). This data indicate that DMM1 design enables detection of dsDNA, which otherwise is a challenging task.

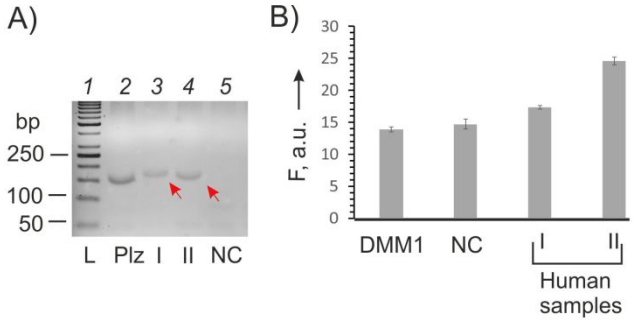

Fig 3. Analysis of dsDNA amplicons from human samples. A) Analysis of human DNA samples in 2.0 % agarose gel after PCR amplification of HPV16 infected human samples I and II in the presence of HPV16 – specific primers (Lines 3 and 4). Lane 2: PCR amplified HPV16 genome-carrying plasmid. Lane 1: 50 bp DNA ladder. B) Data of DMM1 fluorescent assay. All samples contained DMM1 and F sub in reaction buffer as described in SI. In addition, samples NC (negative control), I and II contained 15 μl of PCR sample without human DNA or with human DNA from samples I and II, respectively. The samples were incubated 5 min at 50 °C followed by measurements of fluorescent output at 517 nM (λ_{ex} = 485 nm). DMM1 bar: – control DMM1 strand and F-sub only. Average values of 3 independent measurements are presented with one standard deviation.

Next, we PCR amplified DNA isolated from 2 patients infected by of HPV-16 (Fig. 3A). PCR sample II had higher DNA concertation. The samples were then analyzed by DMM1 with the ultimate goal to minimize the time of fluorescent assay. Both samples produced signal above the negative control (NC) background after 5 min of incubation (Fig 3B). This result strongly suggests that DMM approach can be used for express analysis of PCR samples containing only dsDNA amplicons without the need for asymmetric PCR or λ nuclease treatment.

Tight association of a hybridization probe with analyzed nucleic acids was reported to reduce selectivity for hybridization probes making them incapable of detecting SNS.[17] On the other hands, SNS have been used for genotyping viral and bacterial strains, which is of importance for the accurate diagnosis of infectious diseases.[15,19] We, therefore, tested the ability of DMM1 to differentiate amplicons containing SNS. A DNA amplicon contain G->A substitution was obtained by PCR amplification of a synthetic ssDNA (**HPV-180-mm**, Table S1). The SNS site was localized within the binding fragment of DZa strand as shown in Figure 2B. The fluorescent assay revealed acceptable selectivity with a moderate selectivity factor (SF)[§] ~ 73% (Fig. S8). Importantly, shortening Arm 2 by 2 nucleotides produced DMM2 variation (Fig. S1), which demonstrated exceptional selectivity (SF ~ 99.9%, Fig. 4). DMM2_mm version with DZa strand complementary to **HPV-180-mm** detected corresponding matched analyte with SF ~ 98.9% (Fig. 4). Similar high discrimination ability was found for other types of mismatches (Fig. S9).

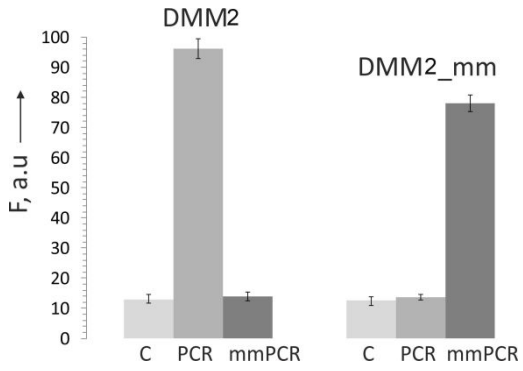

Fig 4. DMM2 detects dsDNA amplicon with high selectivity. Time of incubation was 60 min at 50 °C. Selectivity factor (SF)[§] was 99.9% and 98.9% for DMM2 and DMM2_mm, respectively.

Earlier we used a series of BiDZ probes for the analysis of dsDNA amplicons of different Mycobacterium tuberculosis strains.[15b] The amplicons were typically detected after 45 min assay using 3.5 μL of PCR amplicons. This detection was possible only after λ nuclease treatment of the amplicons to produce ssDNA. In this study, the amplicon was detectable after 5 min using 1 μL PCR amplicon (Fig. 2A). This was achieved by designing the DMM nanostructure (Fig 1B) in which 3 short DNA binding arms were attached to a DNA scaffold and acted cooperatively in binding dsDNA together with strand DZa, which was not attached to the DNA scaffold. This last feature is essential for maintaining high selectivity of DNA recognition, since DZa concertation or the sequence can be conveniently adjusted to form stable complex only with a fully matched, but not with an SNS-containing DNA analyte.

Recently, we designed a DNA nanomachine for detection of biological RNA.[3d] The machine consisted of a DNA platform equipped with the following functions: (i) RNA-binding; (ii) recognizing targeted RNA fragment with high selectivity, (iii) concentrating of F sub near the catalytic core; (iv) amplifying the fluorescent signal by catalysis. The novelty of the work

presented here is the following: (1) The design of the DNA nanorobot is less complex and more cost efficient than that used by us earlier.[3d] (2) For the 1st time the DMM was able to detect dsDNA, while our earlier designs detected only biological ssRNA.[3d] (3) The approach was used for detection amplicons of human clinical samples for the first time, which brings this technology closer to practical needs. DMM is one of the few developments of DNA nanotechnology that can find immediate practical application. DMM is advantageous over state of the art fluorescent probes, e.g. MB probe in its ability to analyze dsDNA as well as in cost efficiency if analysis of several different analytes is needed. Moreover, the ability to catalytically amplify the signal results in reduced incubation time and/or higher S/B level.

In the frame of this proof-of-concept study, we validated the DMM performance using one human papilloma virus sequence. Further validation of this approach with a series of practically significant analytes is needed for establishing a general applicability of the proposed technology. DMM as designed in this study, cannot be used for dsDNA analysis in live cells, since it requires 50°C and a buffer with high Mg^+ concentration. Instead, it can be adopted for in vitro dsDNA analysis obtained by isothermal amplification formats including LAMP, RPA.[6]

Conclusions

A DNA machine for fluorescent analysis of folded ssDNA and dsDNA amplicons was designed and tested. The strategy enabled detection of 1 µL of dsDNA amplicon with signal to background ratio of ~2 after 5 min of fluorescent assay, which outperforms state-of-the art binary deoxyribozyme sensors. The approach is sensitive to a single nucleotide difference in analytes. One could envision further improvements in LOD or reduction the assay time by implementing DMM variations having more analyte-binding arms. We designed unique simply assembled DMM1 which advantage is in ability to detect synthetic dsDNA amplicons and amplicons of human samples thereby this DNA nanotechnology can find immediate clinical application. Here, we present the first attempt to detect viral DNA by the example of human papilloma virus. We hope that this study sets up a direction for the future evolution of hybridization probes towards smart DNA machines for nucleic acid analysis.

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Conflicts of interest

The authors declare no conflicts of interests.

Notes and references

Written Informed consent was obtained from all participants for their tissues be used for this work

§SF, Selectivity factor was calculated using a formula SF= (1-(F_{ns}-F_o)/(F_s-F_o))×100%, where F_0, F_s, F_{ns} are fluorescence intensities of the probe in the absence or in the presence of specific or non-specific analyte, respectively.

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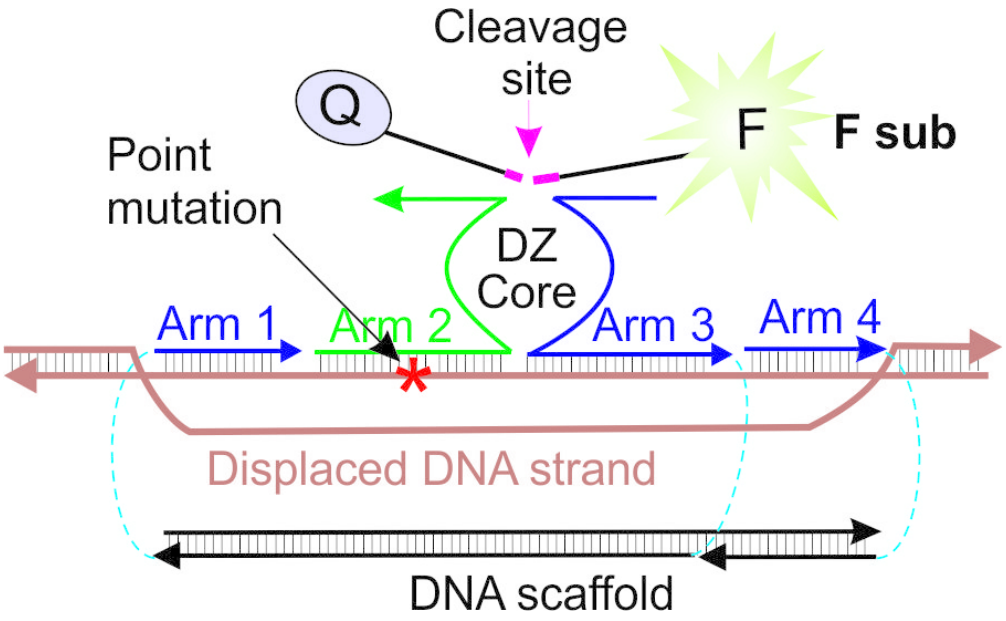

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