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PAPER

A DNAzyme based label-free detection system for miniaturized assays

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Sensitive detection assays are a prerequisite for the analysis of small amounts of samples derived from biological material. There is a great demand for highly sensitive and robust detection techniques to analyze biomolecules. The combination of catalytic active DNA (DNAzyme) with a peroxidase activity with rolling circle amplification (RCA) is a promising alternative to common detection systems. The rolling circle amplification leads to a product with tandemly linked copies of DNAzymes. The continuous signal generation of the amplified DNAzymes results in an increased sensitivity. The combination of two amplification reactions, namely RCA and DNAzymes, results in increased signal intensity by a factor of 10^6. With this approach the labeling of samples can be avoided. The advantage of the introduced assay is the usage of nucleic acids as biosensors for the detection of biomolecules. Coupling of the analyte molecule to the detection molecules allows the direct detection of the analyte molecule. The described label-free hotpot assay has a broad potential field of applications. The hotpot assay can be adapted to detect and analyze RNA, DNA and proteins down to femtomolar concentrations in a miniaturized platform with a total reaction solution of 50 nl. The applicability of the assay for diagnostics and research will be shown with a focus on high throughput systems using a nano-well platform.

Introduction

Detection of small amounts of a sample plays a critical role in biomedical research and clinical diagnostics.[1] Standard array formats have the disadvantages of labeling or modifying the sample, which leads to high costs and can change the biochemical and structural character of the analyte.[2] During the last few years big effort was made to extend the sensitivity of detection assays.[3] The enhancement of the sensitivity of miniaturized assays is required for the reliable detection of small amounts of the sample (RNA, DNA and proteins) derived from limited material like biopsies and embryonic stem cell cultures. The presented approach is a promising alternative to common methods. Two amplification reactions are combined to detect a sample in a miniaturized nano-well platform, resulting in increased signal intensity. The involved processes are the rolling circle amplification (RCA) of DNAzyme sequences on one side and the catalytic activity of the synthesized DNAzyme itself on the other side. This approach avoids time consuming and expensive labeling of the sample.

The mechanism of catalytic active DNAzymes as sensors can be used for different kinds of detection systems due to their multi-faceted applicability and adaptability.[5–8] The used DNA sequence forms a four stranded structure the so called G-quadruplex. The complex obtains peroxidase activity by intercalation of hemin and is therefore also called hemin binding aptamer.[9,10]

RCA is a powerful tool for signal enhancement.[4] The general principle of the combination of RCA and DNAzymes is depicted in Fig. 1. The requirement for the RCA is a circular oligonucleotide, the starting molecule. It consists of an analyte hybridization site and a sequence complementary to a DNAzyme. Base pairing of the analyte with the starting molecule forms a double stranded DNA stretch that serves as the starting point for the Phi29 polymerase (Fig. 1a).

DNA is continuously synthesized due to the strand displacement activity of the polymerase. Thereby rolling circle products (RCP) of tandemly linked DNAzyme sequences are synthesized (Fig. 1b–d). This is a first amplification step. The amplified DNA forms in the presence of potassium a G-quadruplex. The intercalation of hemin results in catalytic active DNAzymes (Fig. 1e). The formation of the G-quadruplex and the intercalation of hemin into this structure is called conformation and results in a measurable substrate turnover of the DNA (Fig. 1f). Each DNAzyme can continuously catalyze the substrate turnover itself and represents the second amplification step. Cheglakov *et al.* have described a detection system for viruses

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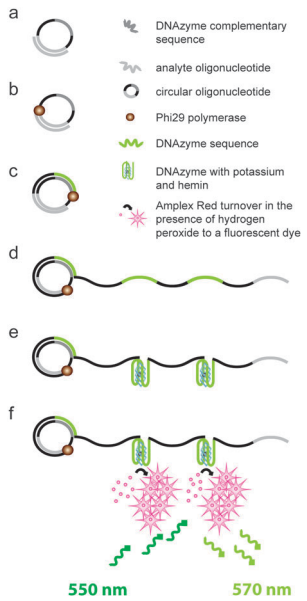

Fig. 1 Combination of DNAzyme and rolling circle amplification. (a) An analyte oligonucleotide is hybridized to a circular template for the RCA (b) creating the starting point for the Phi29 polymerase. (c) After the first amplification round the Phi29 polymerase starts to displace the analyte oligonucleotide and continues synthesizing the rolling circle product (RCP). (d) The RCP can reach a length of up to 30 000 nucleotides (e) containing multiple copies of the DNAzyme sequence. By adding potassium to the RCP the G-quadruplex is formed and hemin intercalates in the four stranded structure. (f) After conformation of the DNAzymes the peroxidase substrate Amplex Red is oxidized in the presence of hydrogen peroxide to a fluorescence signal and the signal intensity is measured. The fluorescence signal is detected at λ_{abs} 550 nm and λ_{em} 570 nm.

based on this principle with ABTS.[11] A calorimetric readout was also used by Tian and colleagues for the signal amplification.[13] In both cases nucleic acids were detected. In this publication the detection principle was refined in the newly developed hotpot assay (Fig. 5) combining both amplification reactions in a homogeneous reaction mix. The assay set-up has several advantages. First of all the fluorescence readout allows the miniaturization. The miniaturization in a nano well system opens new ways for the detection of rare analytes in multiplexed detection systems. The assay was miniaturized from 70 µl down to 50 nl. The analyte is directly connected to the detection molecule and doesn't undergo adulteration like during common amplification (*e.g.* PCR) or modification steps (*e.g.* labeling with signal molecules). Therefore true signals are detected and an effective direct detection of even low concentrated samples spotlights with this approach in the near future.

Result and discussion

DNAzyme characterization

The catalytic activity of DNAzymes can be influenced by several factors like the hemin to DNAzyme ratio, sequence composition of the DNAzyme and temperature. To address these questions an assay in a 96 well format was established and the different parameter tested. Oligonucleotide A (Table 1) with a characterized DNAzyme was chosen to establish the assay.[11] It is known that the surrounding sequence has an influence on the conformation efficiency of the DNAzyme.[16]

Table 1 Sequences. DNAzymes are highlighted in bold. Oligonucleotides A, B and C have first generation DNAzymes. Mimicking DNAzymes within the RCP oligonucleotides have 2 or 3 DNAzyme sequences. Oligonucleotide D is a second generation DNAzyme sequence. Oligonucleotides E and H were used for the RCA and have a first (E) and second (H) generation DNAzyme complementary sequence (underlined) and a hybridization site for the analyte oligonucleotide F (italic). Oligonucleotide G is binding within the RCP to generate a double stranded DNA stretch serving as recognition site for the restrictions enzyme *Dde*I

ID	Sequence 5′–3′	Reference
A	CTC AGA AAA TCT TTT CCA TCG GTA CGG GTC TTT **TGG GTA GGG CGG GTT GGG** TTT TCC ATC GGT ACG GCT GGA ATA CGC C	11
B	CTC AGA AAA TCT TTT **GGG TAG GGC GGG TTG GGT** TTT CCA TCG GTA TTT T**GG GTA GGG CGG GTT GGG** TTT TGA ATA CGC C	11
C	CTC AGA AAA TC**G GGT AGG GCG GGT TGG** GTT TT**G GGT AGG GCG GGT TGG** GTT TT**G GGT AGG GCG GGT TGG** GGA ATA CGC C	11
D	**AGT CCG TGG GTA GGG CGG GTT GGG GGT GAC T**	12
E	*GGC GTA TTC* CAG CCG TAC CGA TGG AAA ACC CAA CCC GCC CTA CCC AAA AGA CCC GTA CCG ATG GAA AA*G ATT TTC TGA G*	11
F	GAA TAC GCC CTC AGA AAA TC	
G	GAT TTT CTG AGG GCG TAT T	
H	*GGC GTA TTC* CAG CCT ACA TGA AAA GTC ACC CCC AAC CCG CCC TAC CCA CG**G ACT** AAA CCT CCA TGA AAA *GAT TTT CTG AG*	12

Hemin is the prosthetic group of the catalytic active DNAzyme. The hemin itself can spontaneously catalyze reactions in the absence of a DNAzyme sequence. Therefore the influence of excess hemin concentrations on the signal intensity was analyzed by different hemin to DNAzyme ratios. Although the fluorescence intensity increases with increasing amounts of hemin, the signal-to-background ratio decreased. A hemin to DNAzyme ratio of 2 : 1 showed the highest signal-to-background ratio at 21 °C. A sample with hemin but without DNAzyme was used for the normalization to subtract background signals.

Different platforms and assay conditions require an adaption of the temperature. G-quadruplex formation and stability depends on the temperature. The influence of different conformation temperatures and repeated heating steps on the DNAzyme activity was tested. After the general heating step during the conformation (Experimental) and the direct addition of hemin and conformation buffer, the samples were incubated for three hours at the respective temperature (21 °C or 4 °C) before determining the enzymatic activity. The DNAzyme samples showed a similar catalytic activity at both temperatures (Fig. 2a).

All DNAzyme samples showed a clear signal above the negative control without DNA. The signal of the negative control was traced back to catalytic activity of the hemin itself. This means that neither the different conformation temperature nor a denaturation step had an influence on the activity of the DNAzymes.

Inactivation of enzymes includes incubation of the sample at elevated temperatures. In contrast to most enzymes (proteins)

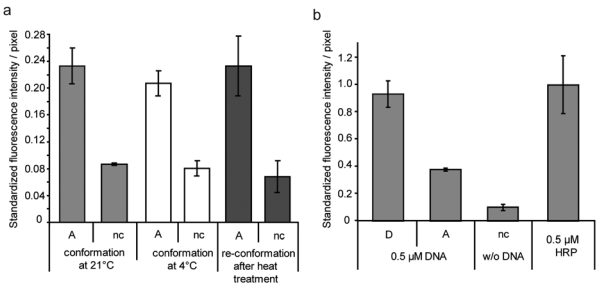

Fig. 2 Characterization of DNAzymes in an Amplex Red 96 well assay. The negative controls (nc) contained no DNA. (a) Thermostability of oligonucleotide A was determined. The DNAzymes were conformated at 21 °C or 4 °C, respectively. Catalytic activity of 0.5 µM DNAzyme was determined. After conformation the samples were incubated at 95 °C a second time followed by an additional incubation step at 21 °C for 3 hours (re-conformation). The activity of the DNAzymes was plotted against each other after standardization against 0.1 units HRP. (b) The catalytic activities of oligonucleotide A (first generation DNAzyme) and oligonucleotide D (second generation DNAzyme) were measured together with the same amount of HRP. The depicted signal intensities were standardized against the signals of the HRP.

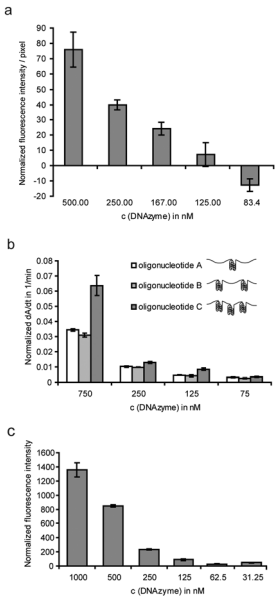

Fig. 3 Sensitivity of the DNAzymes. In each depicted plot the samples of the experiment were normalized by a negative control without DNA (Experimental). (a) Conformation of a dilution series of oligonucleotide A was prepared and the catalytic activity of the DNAzyme was measured with an Amplex Red 96 well assay. The DNAzyme concentration was plotted against the normalized fluorescence signal. (b) Signal intensities from three oligonucleotides with one (A), two (B) and three (C) DNAzymes were compared. The oligonucleotide concentration was adjusted according to the DNAzyme concentration. ABTS turnover was measured every 20 seconds for 30 minutes in a 96 well assay. The slope per minute was determined and the data were normalized as described. (c) Conformation of oligonucleotide A was done and a dilution series was prepared in reaction buffer and spotted together with the Amplex Red reaction mix in the nano-wells with a total volume of 150 nl per well. After 1 hour the catalytic activity was measured in the µPCR-Chip reader. The DNAzyme concentration was plotted against the normalized fluorescence signal.

DNAzymes can overcome heating steps and refold into stable complexes. Oligonucleotide A was incubated for 9 minutes at 95 °C. After heat denaturation the oligonucleotide underwent re-conformation. The original signal intensity of the substrate turnover of the DNAzyme was recovered. This result underlines the thermostability of most nucleic acids. The thermostability makes the DNAzyme an important detection tool compared to proteins like the horseradish peroxidase (HRP) that irreversibly lose their activity after heat treatment.

Novel analyses have described second generation DNAzymes.[12] These sequences form DNAzymes with a higher catalytic activity and higher hemin binding efficiency than the previously reported ones. The activity of oligonucleotide A, carrying the first generation DNAzyme, was compared with a second generation DNAzyme, oligonucleotide D (Table 1) and the protein horseradish peroxidase (Fig. 2b). Oligonucleotide D has a two fold higher catalytic activity compared to oligonucleotide A. The signal intensity of the second generation DNAzyme and the HRP were comparable. Second generation DNAzymes form a structure with an additional stem-loop adjacent to the G-quadruplex conferring higher hemin binding affinity and thereby higher DNAzyme activity.[12] Together with the thermostability this result shows that DNAzymes are an excellent alternative for common assays with protein labels due to their high robustness and a catalytic activity comparable to proteins.

To determine the sensitivity in a 96 well assay oligonucleotide A with a simpler catalytic active structure was chosen. The catalytic activity of oligonucleotide A was analyzed after conformation at 21 °C. A dilution series ranging from 500 nM to 83 nM was analyzed (Experimental). The sensitivity of the formed DNAzyme was determined after normalization (Experimental) to 125 nM (Fig. 3a). This is the lowest tested concentration of DNAzymes with a clear signal above the detection limit after normalization (background subtraction).

The assay combining DNAzymes and RCA leads to multiple DNAzymes within the RCP. To mimic multiple DNAzymes within the RCP oligonucleotides B and C with two and three DNAzymes (Table 1) were designed and compared to oligonucleotide A with a single DNAzyme. The catalytic activity of a dilution series from 750 nM to 75 nM DNAzyme was analyzed (Fig. 3b) with ABTS as substrate (Experimental). Even at concentrations of 75 nM all oligonucleotides were detected. The results revealed that linked and single DNAzymes have an almost similar catalytic activity at concentrations below 250 nM DNAzyme. The difference between the sequences declined with lower DNAzyme concentration. Oligonucleotide C with closely connected DNAzyme showed at high concentrations (750 nM) increased signal intensity compared to the other oligonucleotides. There seemed to be a doses effect for the oligonucleotide C at high DNAzyme concentrations. We assume that the DNAzyme sequences can form a supramolecular structure of parallel G-quadruplexes at high concentrations. In this complex of multiple oligonucleotides additional hemin molecules can intercalate resulting in increased enzymatic activity. The data clearly showed that even closely linked DNAzymes form a G-quadruplex and have a measurable substrate turnover. The phenomenon that all DNAzymes

showed a catalytic activity was expected for DNAzymes within the RCP.

Besides a stable fluorescence readout Amplex Red has several advantages over the common peroxidase substrate used for the initial experiments. Luminol has a small time cap of a few seconds until the signal is expiring. This and the necessity of special equipment and transparent sample devices make Luminol for the planned assay useless. ABTS is more stable, but the substrate turnover is very fast and makes it difficult to measure time dependent signal differences. The required pH value below pH 6.0 for the ABTS assay[13] would inactivate the Phi29 polymerase.

Although it was earlier shown to use both substrates to detect DNAzymes in combination with RCA[11,13] we favored the use of Amplex Red for all further assays.

Miniaturization

The Amplex Red assay was established in a 96 well plate and adapted to nano-well chips. The nano-well chips are composed of black polypropylene, are very resistant to thermo treatment and have a low fluorescence background.[14] A chip has a size of 40 × 40 mm with 1024 v-bottom wells with a volume capacity of 150 nl per well. The readout was carried out with an in-house developed μPCR-Chip reader (Experimental).

All solutions were dispensed with a piezo spotter using a PDC70 nozzle (Experimental). One drop has a volume of ~300 pl. This technique allows a contact free and accurate dispensing of solutions.

Sensitivity of the DNAzyme in nano-wells was tested with oligonucleotide A. After conformation a dilution series ranging from 1000 nM to 31.25 nM in the Amplex Red reaction mix was prepared and 150 nl of the mix per well were spotted. Directly after spotting the nano-well chip was sealed to prevent evaporation. After 1 hour of incubation at 21 °C the catalytic activity was measured in the μPCR-Chip reader. Down to 31.25 nM oligonucleotide A in 150 nl (2.7×10^{11} molecules) per well were detected in the nano-well chip (Fig. 3c). By comparing the sensitivity of the Amplex Red 96 well assay (Fig. 3a) with this miniaturized assay a four fold increased sensitivity was obtained. The successful miniaturization opens doors to a label-free detection system for multiplexing and high throughput assays.

RCA, a tool for DNAzyme amplification

In the next step the enzymatic activity of the RCP was analyzed. Per analyte up to 30 000 nucleotide long single-stranded DNA can be synthesized by the Phi29 polymerase within 30 minutes.[15] The starting molecule (Table 1, E) consists of 79 nucleotides and has a sequence complementary to a DNAzyme. The hybridization of the analyte oligonucleotide F to the starting molecule creates the starting point for the RCA. Amplification results in up to 1000 copies of the DNAzyme per RCP. Directly after adding the Phi29 polymerase (0 minutes) and after 30 minutes of incubation aliquots of the sample were taken. The amplification was stopped immediately by incubation for 15 minutes at 75 °C. According to the previous experiment (Fig. 3b) it was expected that all DNAzymes in the amplicon (RCP) would be catalytic active. To test this assumption oligonucleotide G was hybridized to an aliquot of the RCP. The hybridization site was in between two adjacent DNAzymes and created a *Dde*I restriction site. After the *Dde*I digest the fragmented DNA contained single DNAzymes. If all DNAzymes in the RCP had catalytic activity the signal intensity of the fragmented DNA and the full length RCP should be comparable. Aliquots of the RCP and fragmented RCP underwent conformation. Afterwards the samples were added to the Amplex Red reaction mix and spotted with a total reaction volume of 150 nl in the nano-wells of the chip (Experimental). Directly after spotting the chips were sealed to avoid evaporation. The readout of the signal was carried out in the μPCR-Chip reader after 1 hour of incubation at 21 °C (Experimental). A sample without DNA served as negative control.

The samples at time point 0 minutes showed no signal above the detection limit. At time point 0 minutes no amplification had occurred and as a result no DNAzyme was synthesized. The signals of the RCP after 30 minutes of RCA were clearly above the negative control (Fig. 4). The plotted values of the negative control were influenced by the catalytic activity of the hemin itself in the reaction. Down to 1×10^8 analyte molecules F were detected in 150 nl per nano-well for digested and undigested RCPs. The signals of the fragmented RCPs were higher than those of the full length RCP samples. Although we cannot exclude mass transport limitations within the RCP, we assume that not all DNAzymes within the RCP underwent conformation. The RCA as an amplification step of the DNAzymes leads to a 1000 times higher sensitivity of the assay (Fig. 4) compared to the DNAzyme assay without amplification (Fig. 3c). The nano-well assay showed a time-dependent increased peroxidase activity (Fig. 4). This reaction occurs only in the presence of all components of the assay. The absence of a circular template or a primer providing a free 3′ prime end as the starting point for the polymerase results in

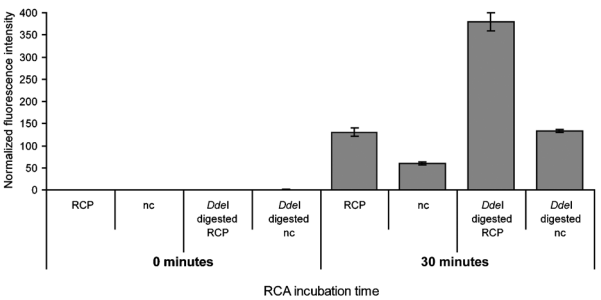

Fig. 4 Catalytic activity of multiple DNAzymes within the rolling circle product. A sample without DNA served as a negative control (nc). The circular oligonucleotide E was hybridized to the analyte oligonucleotide F, this double stranded stretch served as the starting point for the RCA. Samples were taken during RCA after 0 minutes and 30 minutes and an aliquot was digested with *Dde*I. All samples underwent conformation and were diluted in the Amplex Red reaction solution in a 384 well plate. The samples (1×10^8 analyte oligonucleotide per well) were spotted in a nano-well chip with a total volume of 150 nl. Fluorescence signals were detected with the μPCR-Chip reader. Data were normalized as described with blank well values from the nano-well chip as a negative control for the detection limit. The amplicon was detected before and after cutting the RCP in smaller fragments.

no detectable substrate turnover. A circular template with a primer and the presence of a polymerase are essential for the reaction. Based on these data the following hotpot assay was developed.

Hotpot assay

The hotpot reaction describes the simultaneous activity of all involved reactions (rolling circle amplification, conformation and substrate turnover) in one cavity. In Fig. 5 the assay is schematically shown. Hybridization of the analyte to the circularized starting molecule is the precondition for the reaction. The RCA starts and the newly synthesized DNAzyme within the RCP can immediately undergo conformation. The formation of the DNAzymes is followed by the substrate turnover in the same well. In the meanwhile the RCA is going on. The hotpot reaction enables an analysis in a homogeneous solution, thereby avoiding covalent labels and simplifying the assay. The assay is based on the assumption that the incubation at 21 °C of the RCP will ensure a conformation of the amplified DNAzymes. Experiments confirmed that a heating step was not necessarily required for the conformation. Therefore the general heating step of the sample for the conformation was omitted for the hotpot assay to maintain the Phi29 polymerase activity.

All involved substances of the hotpot assay were tested individually to analyze their influence on the RCA reaction and the DNAzyme activity. The hemin solvent DMSO led to a reduced amount of RCP. The influence was limited by using higher concentrated hemin stock solution. No further negative impact by any other component was seen. The assay was established in a 96 well format with a volume of 70 µl. The data showed that the first signal or the enzymatic activity of the amplified DNAzymes was detected after a delay of 55 minutes (Fig. 6a). This delay can be easily explained by the fact that the RCP first had to be synthesized and the amplified DNAzymes had to undergo conformation before a substrate turnover could be measured. Based on earlier experiments it was known that the first signals of the enzymatic activity could be measured after 30 minutes of conformation.

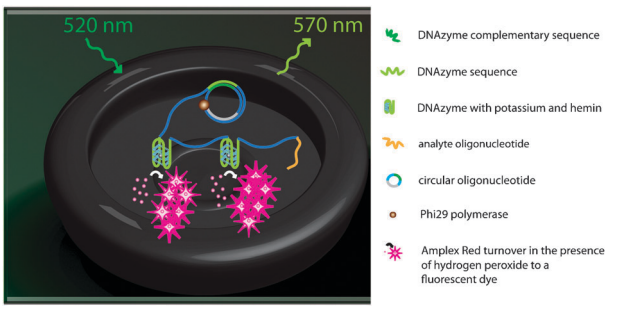

Fig. 5 Hotpot assay. During the hotpot assay all reactions occur simultaneously. For the assay a v-bottom nano-well chip with 1024 cavities was chosen. Each cavity represents an independent reaction chamber. All involved substances were dispensed in the nano-wells. By mixing the starting molecule with the hybridized analyte in the reaction mix, the RCA started and the RCP was synthesized. The synthesized DNAzymes within the RCP undergo conformation and the formed active DNAzymes catalyzed the Amplex Red turnover. The generated fluorescence signal was detected in the µPCR-Chip reader.

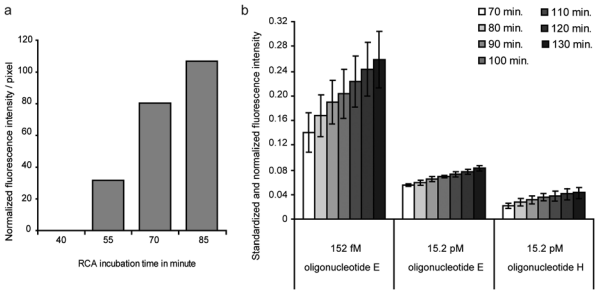

Fig. 6 Hotpot assay. For each experiment a negative control without DNA was analyzed and used for normalization (Experimental). (a) In a total reaction volume of 70 µl an Amplex Red hotpot assay was performed with 7×10^{12} analyte (oligonucleotides F) and starting molecules (oligonucleotides E) per well. The starting material was added to the RCA mix. The fluorescence intensity of the samples was measured at 21 °C over the time. Only one RCA sample was measured in this experiment. Data were normalized against the detection limit and plotted against the incubation time. (b) A dilution series of oligonucleotides E and H with the hybridized analyte oligonucleotide F was analyzed with the hotpot assay. During spotting the nano-well chip was cooled to 17 °C and 50 nl were spotted per well. Data were first normalized and afterwards standardized against the signals of 12.5 µM oligonucleotide A (DNAzyme) in the same chip. After 70 minutes of incubation the fluorescence signal was measured for one hour.

An interesting aspect is the scalability of the assay. Working with smaller volumes opens new ways of multiplexing and high throughput approaches. The reaction volume of 70 µl in the 96 well assay was adapted to the nano-well chips. Best results were obtained with a total volume of 50 nl (Fig. 6b).

The fluorescence intensity was measured every 10 minutes for one hour in the µPCR-Chip reader. Signals were detected after a delay of 55 minutes. This is in line with the data derived from the 96 well assay. Fluorescence intensity of the sample increased proportional with incubation time. This is due to the continuous synthesis and conformation of catalytic active DNAzymes within the RCP. Surprisingly the signal intensities increased with less starting molecules. One possible explanation therefore is a doses effect. The RCP is less dense packed and therefore the substrate can diffuse more easily to the DNAzyme sequences. The substrate turnover was enhanced by a continuous supply of nearby substrate. With the hotpot assay the templates for a first generation and second generation DNAzyme (oligonucleotides E and H) were compared. The detection limit for oligonucleotide E was experimentally determined to be 152 fM (4600 molecules) per well and for the circular oligonucleotide H 15.2 pM. The concentration was calculated based on the input of a dilution series of commercially synthesized primer stock solution. Circularization efficiency was tested by agarose gel electrophoresis and was estimated to be above 90%. At all concentrations the oligonucleotide H (second generation DNAzyme) had lower signal intensities than the oligonucleotide E (first generation DNAzyme). Although second generation DNAzymes have a much higher catalytic activity (Fig. 2b) the first generation DNAzyme showed the best results after RCA (Fig. 6b) for the established hotpot assay. It is likely that the formation of the DNAzyme structure of the second generation was sterically hindered in linked DNAzymes within the RCP.

Another explanation would be that for conformation of DNAzymes of the second generation a heating step is required. The data favored the use of first generation DNAzymes for RCA in the hotpot assay. In summary by combining the RCA with DNAzymes and further on miniaturizing the assay to the hotpot assay an enhanced sensitivity was achieved. From originally 125 nM single DNAzyme detected in a 96 well plate (Fig. 3a) the hotpot assay detected 152 fM analyte molecules in 50 nl in the nano-well chip (Fig. 6b). This is an increase of sensitivity of 10^6.

Experimental

Enzymes were purchased from New England Biolabs unless otherwise mentioned. Nucleotides were received from peqlab Biotechnologie GmbH. Oligonucleotides were obtained from Invitrogen and the concentration was adjusted with aqua bidest to 100 μM. Chemicals were obtained from Merck KGaA unless otherwise mentioned. The reagents were of analytical grade.

Conformation

DNAzyme sequences (Table 1) were incubated at 95 °C for 9 minutes and cooled down to 21 °C. The same volume of 2× conformation buffer (50 mM Hepes, 40 mM KCl, 400 mM NaCl, pH 7.4) with hemin was added. 8.6 mM stock solution hemin was solved in DMSO. A DNAzyme to hemin ratio of 1 : 2 was chosen for all experiments. The DNAzyme–hemin mix was incubated for 3 hours at 21 °C to allow conformation of the DNAzymes.

ABTS 96 well assay

10 mg ABTS (Sigma Aldrich, A9941) was dissolved in 50 mM phosphate–citrate buffer (25 mM Na_2HPO_4, 12.5 mM citric acid pH 5.0) and always freshly prepared. For the immediate readout 1 μl conformation sample was mixed with 49 μl 9 mM ABTS with 0.06% H_2O_2 in a 96 well plate (Nunc, 260895).

Amplex Red 96 well assay

HRP (SIGMA, P8125-5KU) was immediately before usage diluted in reaction buffer (50 mM NaH_2PO_4, pH 7.4). 1 μl conformation sample was added to the 49 μl Amplex Red reaction mix. The Amplex Red reaction mix contained 24 μl working solution (1 mM Amplex Red (Molecular Probes, Inc.) in reaction buffer) and 25 μl 8 μM H_2O_2 (H_2O_2 in reaction buffer). The 96 well plate (Nunc, 260895) was sealed with PCR-film (ABgene, AB-0558) and incubated for 30 minutes in the dark before fluorescence readout.

DNAzyme nano-well assay

20 μl Amplex Red reaction mix were transferred in a 384 well plate, containing 0.4 μl conformation sample, 9.6 μl working solution (1 mM Amplex Red in reaction buffer) and 10 μl 8 μM H_2O_2 (H_2O_2 in reaction buffer). Samples were spotted as described.

Circularization

100 pmol oligonucleotides were added to 10 units T4 polynucleotide kinase and 150 nmol ATP in 1× T4 polynucleotide kinase buffer. The suspension was incubated at 37 °C for 30 minutes, followed by inactivation at 75 °C for 20 minutes. 100 pmol analyte oligonucleotides were added and incubated at 95 °C for 20 minutes. After cooling down to 21 °C samples were incubated over night for hybridization. 3000 units T4 ligase, 80 nmol ATP in 1× T4 ligase buffer were added and the ligation mix was incubated at 16 °C for 2 hours, followed by incubation at 95 °C for 20 minutes. The mixture was incubated with 20 unit's exonuclease VII (EPICENTRE biotechnologies, EN510250) in 1× exonuclease VII buffer (70 mM Tris–HCl, 8 mM EDTA, 10 mM 2-mercaptoethanol) at 37 °C for two hours to remove linear oligonucleotides. Digestion was stopped by incubation at 95 °C for 15 minutes. Circularization was confirmed by agarose gel electrophoresis (3% agarose, 1× TBE buffer). 100 pmol analyte oligonucleotides were added to the circularized oligonucleotides. The hybridization was carried out by incubating the sample at 95 °C for 20 minutes followed by incubation for 3 hours at 21 °C.

RCP in nano-well chips

As negative controls samples without DNA were carried along through the complete assay. 70 μl total RCA mix (1× Phi29 polymerase buffer, 14 μg BSA, 50 units Phi29 polymerase, 200 nmol dNTPs) contained 5 μM circular oligonucleotides with analyte oligonucleotides. The RCA mix was incubated at 30 °C and 22 μl RCP samples were taken after 0 and 30 minutes. These RCP samples were immediately incubated at 75 °C for 15 minutes to stop the amplification. 11 μl of the RCP samples were incubated with 100 pmol oligonucleotide G at 21 °C overnight to create a *Dde*I restriction site. Hybridization occurred after heating up the samples to 95 °C for 20 minutes followed by incubation for 3 hours at 21 °C. The hybridized samples were incubated with 10 units *Dde*I in 1× NEB3 buffer for 3 hours at 37 °C. The enzyme was inactivated by incubation at 75 °C for 15 minutes. Conformation was done as described above with 25 μM hemin. 0.8 μl of the sample in a 384 well plate were mixed with 19.2 μl working solution (1 mM Amplex Red in reaction buffer) and 20 μl 8 μM H_2O_2, followed by spotting.

Hotpot assay

Starting molecules were incubated with analyte oligonucleotides at 95 °C for 20 minutes and afterwards directly diluted in 1× SSC buffer. Samples were incubated at 95 °C for 20 minutes and additional 3 hours at 21 °C. From the starting material 1.4 μl were added to 17.5 μl 2× conformation buffer with 25 μM hemin, followed by addition of the RCA mix (1× Phi29 polymerase buffer, 7 μg BSA, 25 units Phi29 polymerase, 100 nmol dNTPs), 1.75 μl 1 mM Amplex Red and 0.7 μl 200 μM H_2O_2. After mixing the solution was transferred to a 384 well plate and spotted. For the hotpot assay the nano-well chip was cooled down to 17 °C during spotting.

Data

Analyzed samples were at least measured in technical replicates unless otherwise mentioned. After determining the average and

the standard deviation of the data sets, it was normalized by subtraction of the detection limit.

$$\text{Detection limit} = (3 \times \text{standard deviation of the negative control}) + \text{average of the negative control}$$

Spotting protocol

From a 384 well plate v-bottom (Genetix, X6004) samples were dispensed in the cavities of the nano-well chip with a piezo spotter (Scienion, sciFLEXARRAYER S5). One drop of the PDC70 nozzle has a volume of ~300 pl. During the spotting procedure a washing step was added to ensure the cleanness of the dispensed samples. The washing step contained an ultra sonic bath with the cleaning solution (3× SSC-buffer, 0.1% SDS) for the nozzle. Immediately after spotting the nano-wells were sealed with PCR-film (ABgene, AB-0558). Unless otherwise stated, spotting occurred at 21 °C.

Imaging

ABTS substrate measurements of the 96 well plate were done with the spectrophotometer Spectra Max 250 (Molecular Devices) at 405 nm. Readout of the Amplex Red 96 well assay was done in the FLA-5100 (FUJIFILM) at λ_{abs} 550 nm and λ_{em} 570 nm. For fluorescence readout of the nano-well chips an in-house developed µPCR-Chip reader was used (λ_{abs} 520 nm, λ_{em} 570 nm).

Conclusions

For the first time the combination of RCA and DNAzymes with fluorescence detection in a nano-well chip is described. The assay allows a label-free, sensitive detection and leads to a true detection of unmodified samples in solution. Down to 4600 molecules were detected with the hotpot assay (Fig. 6b). The number of molecules was determined by the input of used oligonucleotide for the circularization based on the assumption of approx. 100% efficiency of circularization, conformation and all other reactions in the assay. All single reactions like circularization and conformation were tested individually and have an efficiency of at least 90%. Assuming a lower efficiency for the template generation it would be possible to detect even lower amounts successfully. Alternative methods for the generation of a circularized DNA template would be the usage of a DNA sequence able to form a stable hairpin structure for an internal ligation or for instance by click chemistry[18,19] avoiding an enzymatic reaction. By combining this detection system with common strategies for detection like that used for padlock probes[20] the hotpot assay has the advantage of being a catalytic protein free detection system. In general the extension of the hotpot assay with the well defined and published padlock probes is one possibility for an application. This combination by the use of the hotpot assay would make the use of a labeled probe needless. The detection of absolute numbers of analytes in a single cell would be eventually possible by the combination of the above mentioned padlock or click chemistry with the here introduced hotpot assay. An attractive option would be the combination of the described readout system with specific aptamers for the detection.

Standard luminescence assays have a detection limit between 30 pM to 1 pM.[21] The here described isothermal hotpot assay has a detection limit of 152 fM and is at least 10 times more sensitive. Methods with a lower detection limit like immuno-PCR (20 aM[21]) go along with excessive heating steps that are not capable for all kinds of samples. Therefore the hotpot assay has the big advantage of being isothermal and can be operable at room temperature.

For the detection of all kinds of agents in research, diagnostics and food industry the established assay offers a simple possibility that can be easily applied. This opens new ways to analyze the content of a few cells or even single cells. The conjugation of oligonucleotides to specific capture reagent like antibodies or aptamers the hotpot assay could be used as a detection tool. This combination will allow a highly specific detection system with improved sensitivity.

As a last example the detection of low abundant protein is given. A single cell contains about 50 000 copies of the transcription factor CREB (cAMP response element-binding protein).[22] Down to 4600 molecules were detected with the hotpot assay (Fig. 6b). For a detection of the CREB with the hotpot assay the nucleic acids for the RCA have to be conjugated to an antibody like used for padlock probes.[17] The conjugation of nucleic acids to proteins leads to highly robust label.[23] We think that the presented study would make tissue analyses of different cell types with different biological functions analyzable and would give a clear insight of overall single cell activities. Especially highlighting is in our opinion the fact that in the established hotpot reaction no immobilization is necessary to detect a protein or DNA. Thereby avoiding the circumstances that accompany immobilization like denaturation or masking of binding sites.

In summary DNA joins proteins for the direct labeling of analyte molecules like presented in this hotpot assay. Analyte molecules are directly connected to the detection molecules (DNAzyme within the RCP). This means that true signal molecules are detected in the hotpot assay. In studies with a PCR amplification step of the sample in front of the analysis a big effort was made to minimize sample artifacts.[24,25] By this assay the problems that accompany the amplification of the sample *via* PCR are avoided. In the hotpot assay the analyte detection *via* hybridization is not influenced by the RCA. In conclusion a label-free, sensitive, high throughput assay is presented that is able to be adapted for any kind of samples and leads to a true detection of unmodified samples in solution.

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