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PAPER

Development of a lipase-based optical assay for detection of DNA†

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A lipase-based assay for detection of specific DNA sequences has been developed. Lipase from *Candida antarctica* was conjugated to DNA and captured on magnetic beads in a sandwich assay, in which the binding was dependent on the presence of a specific target DNA. For amplification and to generate a detectable readout the captured lipase was applied to an optical assay that takes advantage of the enzymatic activity of lipase. The assay applies *p*-nitrophenol octanoate (NPO) as the substrate and in the presence of lipase the ester is hydrolyzed to *p*-nitrophenolate which has a strong absorbance at 405 nm. The method provides detection a detection limit of 200 fmol target DNA and it was able to distinguish single base mismatches from the fully complementary target.

Specific and reliable assays for DNA detection are becoming increasingly important for applications in clinical diagnosis, food safety and environments. This has spurred an intensive search for new sensitive, selective, simple and rapid methods. During the past decade, different approaches to DNA detection using various readout systems have been discussed. These methods have mainly included optical/colorimetric techniques,[1–6] surface plasmon resonance,[7,8] microcantilever,[9,10] and electrochemical detection.[11–14]

Enzymes have successfully been used for colorimetric detection of analytes providing both recognition, and amplification of the binding event with a detectable readout. In DNA analysis, enzyme-labelling is widely used for the development, of *e.g.* electrochemical[15,16] and optical[17] assays. Commonly used enzymes include horseradish peroxidase,[18] glucose oxidase,[19] galactosidase,[20] alkaline phosphatase.[16] They are usually coupled to target or reporter DNA strand by avidin–biotin binding technology or chemical coupling.[19]

Lipases are important enzymes that catalyze the hydrolysis and formation of a wide variety of esters. They are pivotal in nature and have industrial applications in dairy, fat and oil, food, pharmaceutical, cosmetic, detergents, and textile industries, and in the manufacture of fine chemicals synthesis and polymeric materials.[21,22] Because of their great application and economic importance, numerous lipases are available. In one prior example lipase was applied as the enzymatic label in an assay used for the electrochemical detection of DNA.[23]

Here we have developed a different lipase-based assay for detection of DNA providing an optical readout (Fig. 1 and 2). For the capture of lipase on magnetic beads (MBs)[24–27] in the presence of target DNA, we applied a magnetic bead (MB)-DNA-lipase sandwich assay familiar with the assay described above. For signal amplification and optical readout we have employed the lipase induced cleavage of *p*-nitrophenyl esters (NP esters). The combination of this lipase NP ester assay with the MB-DNA-lipase capture sandwich assay has enabled us to develop a new and versatile detection method for DNA.

p-Nitrophenyl octanoate (NPO), is an ester which is hydrolyzed by the lipase enzyme to form octanoate and *p*-nitrophenolate ($pK_{aH} = 7.08$) (Fig. 1a). The yellow colour of the *p*-nitrophenolate formed in the reaction allows for the detection of the lipase activity. The target DNA is detected in a sandwich assay where it hybridizes with a DNA strand on magnetic beads and to a DNA strand conjugated to lipase. Application of the MB-DNA-lipase to a NPO solution results in enzymatic hydrolysis of the ester. The rate of hydrolysis can be directly correlated with the amount of lipase captured on the beads and thereby also with the amount of target DNA present in the assay.

The esterase activities of lipase can be assayed by UV-Vis spectroscopy by monitoring the absorption of *p*-nitrophenolate (NP) which is produced during the enzymatic ester bond hydrolysis in the NP esters. The catalytic activity of lipases increases with increasing hydrophobicity and in particular with the chain length of the alcohol moiety.[28,29] However, the solubility of the substrate decreases with increasing hydrophobicity. Thus, to avoid turbidity and maintain a high lipase activity, *p*-nitrophenyl octanoate (NPO) was chosen as the substrate. The hydrolysis reaction of NPO was first investigated at different concentrations of NPO to reveal a linear correlation between the NPO concentration and the absorbance (See ESI, Fig. S1†).

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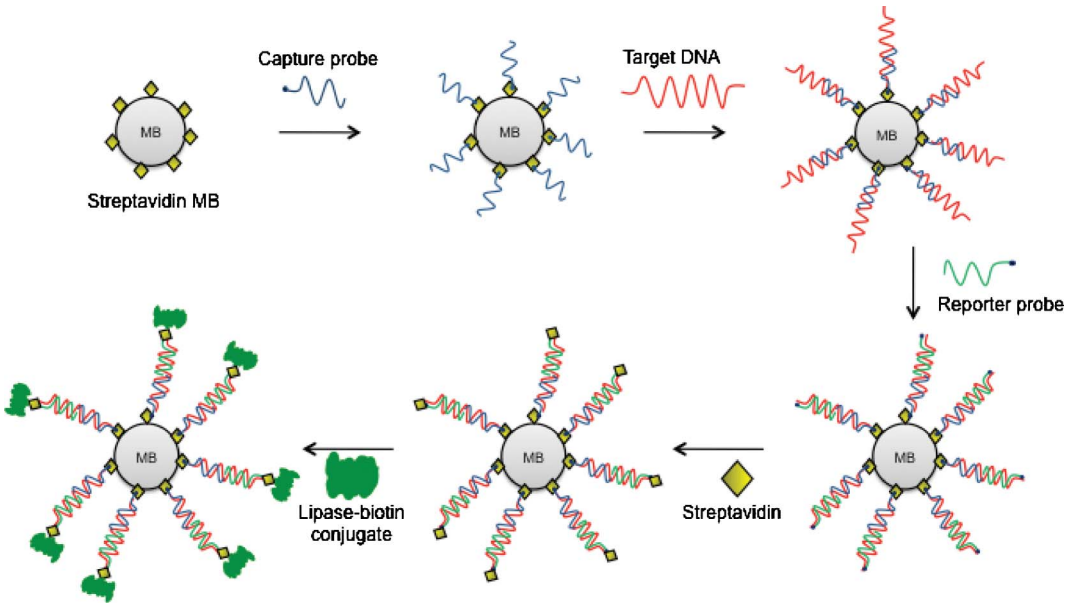

Fig. 1 Capture of the target DNA by the MB-DNA-lipase sandwich hybridization.

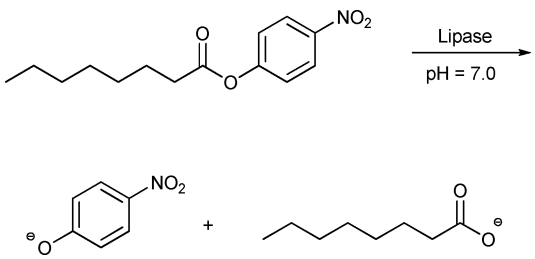

Fig. 2 Lipase-catalysed generation of *p*-nitrophenylate absorbing at 405 nm.

A correlation coefficient of 44.6 μmol^{-1} (or 8.9 mM^{-1}) was derived.

Next we have investigated the activity of different derivatives of the lipase *Candida antarctica* using NPO as the substrate. The concentrations of lipase of the MB were calculated based on the binding capacity of the beads. The assay was followed by incubating the MB-lipase enzyme in the NPO solution. The relation between the varying concentrations of lipase and the absorption at 405 nm is shown in Fig. S2.† However, comparison of the activities of free lipase from *Candida antarctica* and lipase immobilized on MB was not straightforward. As described by Pencreac'h, the lipases used are enzymatically prepared and contain other protein components.[30] To overcome this problem we have compared the activities of lipase conjugated on MBs to lipase conjugated to DNA. It was performed and measured by using the NPO assay as shown in Fig. S2.† The absorbance resulting from of MB-lipase hydrolysis was 1.3 times larger than the absorbance from the lipase-biotin hydrolysis of NPO after 4 h. It is assumed, based on previous work that this difference is caused by enhancement of the surface area-to-volume ratio for the MB immobilized lipase.[23]

Hybridization experiments were carried out in the sandwich type format (Fig. 1). The commercially available MBs are coated with streptavidin and an excess of a biotin-labelled capture sequence was linked to the MBs *via* the streptavidin biotin linking. Then the target DNA in the selected concentration was added to the MB-capture probe conjugate. This was followed by the addition of a biotin-labelled reporter DNA sequence to form the DNA sandwich. The capture and reporter sequences each form an 18 bp duplex with the 42 nt target. The hybridization assay is designed with a 6 nt single stranded gap in the middle of the sandwich to increase the flexibility of the system and to avoid stacking between the two duplexes, since this would decrease the single point mismatch selectivity of the assay.

After removal of the sandwich-MBs from the excess of reporter probes streptavidin was added. After another round of purification the biotin labelled lipase was added to the sandwich and a final separation step was performed. During these addition/separation cycles all additives except the target strand were in excess and thus it is the quantity of the target DNA that determines the amount of lipase linked to the magnetic beads. Several addition/separation steps were required, but since the interaction relies on the very efficient and high yielding DNA hybridization and streptavidin–biotin interaction high efficiency and specificity are expected. Furthermore, the separation procedures are easily performed by removal of the solution from the magnetic beads.

We have investigated the relation between the incubation time of the MB-lipase and the absorption at 405 nm. For these initial experiments the target DNA was fixed at 2 pmol. As shown in Fig. 3, absorption was measured as a function of time for every hour from 1 to 26 h.

The absorption increased gradually during the incubation time. At the beginning the activities processed quite fast reaching ~0.9 after 12 h and it is not necessarily needed to extend the detection time further. In our experiments, 4 h was chosen as the optimized incubation time.

We have also investigated the response of the assay to different amounts of target DNA as shown in Fig. 4. Target DNA amounts were varied between 2 fmol to 200 pmol and the background absorption obtained in the absence of target DNA was subtracted.

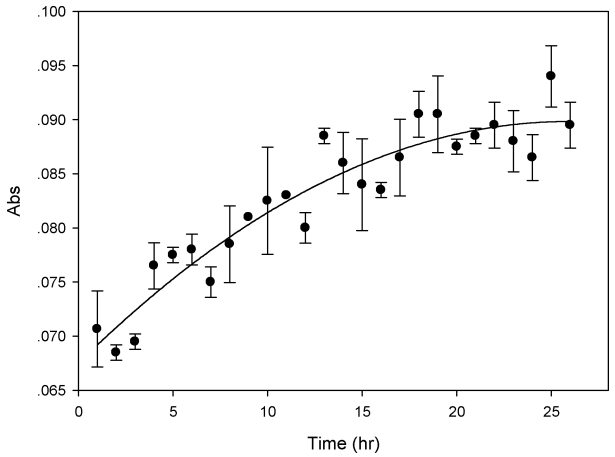

Fig. 3 Time dependency of the ester digestion after incubation with DNA-MB assembled in the presence of 2 pmol target DNA.

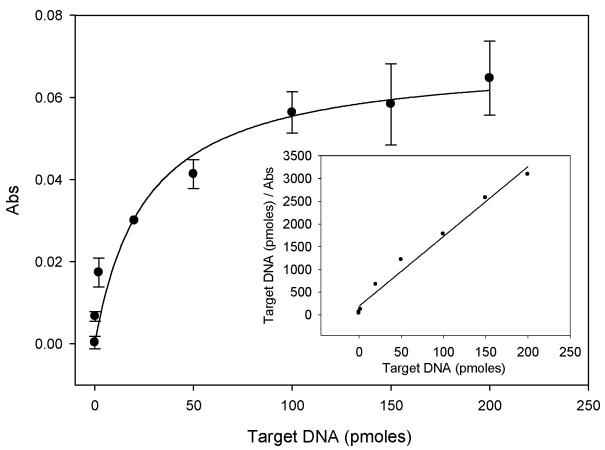

Fig. 4 Calibration of target DNA captured by the MB sandwich assay *versus* the background subtracted absorption in the absence of target DNA; inset: 0.02–200 pmol range shown with linearized data.

At low amounts of target DNA of 2–20 fmol no significant increase in the absorbance was observed, however, at 200 fmol a clear increase of the absorbance was observed. A hyperbolic parabolid curve was used to fit the data (Fig. 4). This hyperbolic graph was depicted in a linear fit in the inset of Fig. 4. Increasing the target DNA from 200 fmol to 200 pmol showed increases in the absorbance that indicated a relationship between the amount of target DNA and the absorption.

One of the crucial aspects of DNA assays is their ability to detect single point mutations in DNA and therefore the specificity of our technique was examined. The response from 100 pmol of fully complementary target was compared with the following: (1) 100 pmol of complementary target DNA, (2) 100 pmol of a target with a single base mismatch in the part of the sequence which is complementary to the capture DNA attached to the MBs, (3) 100 pmol of a target with a single base mismatch at the part complementary to the reported (lipase bound) sequence, and (4) 100 pmol of a target sequence with mismatches at both sides. As shown in Fig. 5, the fully complementary target provides a response that is larger than any of the sequences containing mismatches. The sequences containing a single point

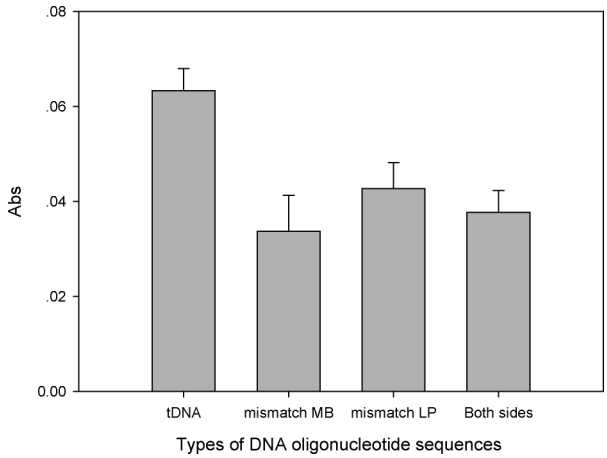

Fig. 5 Histogram shows peak absorbance using 100 pmol complementary target, 100 pmol single base mismatch at MB side, 100 pmol single base mismatch at lipase side, 100 pmol of mismatches at both sides, after subtraction of the background absorption obtained without target DNA.

mismatch provided significantly lower signals. Surprisingly, the double mismatch target resulted in a signal of similar magnitude.

Conclusion

A lipase-based colorimetric assay for detection of DNA was developed. The target DNA was captured on MBs in a sandwich assay that also binds a reporter DNA strand, linked to lipase from *Candida antarctica*. Application of magnetic beads enables fast and efficient removal of nonspecific reagents after the washing steps.[26] The binding of target DNA in the sandwich is amplified by the enzymatic hydrolysis of *p*-nitrophenol octanoate (NPO) by the lipase. Hydrolysis of NPO leads to the formation of *p*-nitrophenolate which has a strong absorption at 405 nm to provide a colorimetric readout. The method allowed detection of down to 200 fmol (or ~0.97 nM) of target DNA in the 205 μL detection solution. The method is also highly selective and could distinguish between fully matched target DNA and target DNA containing single point mutations. This is the first example of the employment of lipase for optical detection of DNA. Lipases are readily available and furthermore no "fuel" is required for the enzymatic hydrolysis to take place, unlike the commonly used redox enzymes. Future development of the lipase-based assay may enable fast and direct visual detection of low concentrations DNA.

Experimental

Materials

Lipase from *Candida antarctica*, BiotinTag™ Micro Biotinylation Kit, Streptavidin from *Streptomyces avidinii* and *p*-nitrophenyl octanoate were supplied by Sigma–Aldrich. Streptavidin-coated magnetic beads Dynabeads® MyOne™ Streptavidin T1 was purchased from Invitrogen, USA.

An oligonucleotide capture probe with a biotin-modified 5′-position, target oligonucleotide and 3′-position biotin-modified reporter probe were synthesized by DNA Technology A S^{-1}, Risskov, Denmark. The DNA strands had the following sequence:

DNA capture probe: 5′-biotin-$(CH_2)_6$-TTT TTT TTT TAA GTC GAA CGA GCT TCC-3′

DNA target: 5′-AAC TCA CCA GTT CGC CAC TGA CGT GGA AGC TCG TTC GAC TTA-3′

DNA reporter probe: 5′-GTG GCG AAC TGG TGA GTT TTT TTT TTT (TEG)-biotin-3′

(TEG = triethyleneglycol)

DNA mismatch at MB side: AAC TCA CCA GTT CGC CAC TGA CGT GGA AGC TCC TTC GAC TTA

DNA mismatch at lipase side: AAC TCA CCA CTT CGC CAC TGA CGT GGA AGC TCG TTC GAC TTA

DNA mismatch at both sides: AAC TCA CCA CTT CGC CAC TGA CGT GGA AGC TCC TTC GAC TTA

All the chemicals were purchased from Sigma–Aldrich and prepared with 18.2 MΩ Millipore water (MilliQ) throughout the work.

Apparatus

UV-visible spectra were recorded using a Bio-TEK Instruments model μQuant spectrophotometer for wells plate. Micro well plate (clear color/sterile) was from NUNC™ (Nunclon™ Δ Surface).

Preparation of the lipase-biotin conjugate

Conjugation of biotin to lipase was performed by using BiotinTag™ Micro Biotinylation Kit. Briefly, lipase from *Candida antarctica* was prepared in 20 mM phosphate buffer, pH 7.0 containing 0.15 M NaCl (PBS). The solution was activated with the labelling reagent using freshly prepared solution of biotinamidohexanoic acid 3-sulfo-*N*-hydroxysuccinide ester (BAC-SulfoNHS). It was incubated with gentle stirring for 40 min at room temperature, followed by a fast gel-filtration using G-50 micro-spin columns to separate the conjugate from unreacted or hydrolyzed reagent. The lipase-biotin conjugate was used as labeling for magnetic beads (MB) and DNA hybridization.

Preparation of the MB-lipase conjugate

For this procedure we used 1 μm diameter streptavidin-coated MBs from Dynabeads® MyOne™ Streptavidin T1. First, 100 μL of MB (10 mg mL^{-1}) was prepared in Eppendorf tube. The tube was placed on a strong magnet for 2 min and the supernatant was removed by aspiration with a pipette. Then, the tube was removed from the magnet, the MBs were washed with PBS containing 0.1% Tween 20 and collected by the magnet 3 times and then they were resuspended in PBS buffer. The biotinylated lipase was added to the washed MB, with the binding capacity of 400 pmol of biotin-conjugates per 1 mg of beads, and incubated for 30 min with gentle stirring at room temperature. The mixture tube was placed on string magnet, and the supernatant discharged. The MBs were washed 3 times with PBS and resuspended in PBS buffer.

Preparation of MB-DNA conjugate

The DNA assembly was performed using streptavidin coated MBs, a biotinylated capture DNA probe, target DNA, and a biotinylated reporter probe which can conjugate lipase-biotin with a streptavidin linker. First 100 μL of streptavidin coated MBs (10 mg mL^{-1}) were washed 3 times with PBS containing 0.1% Tween 20 by using vertex system, decanted with a magnet and the MBs resuspended in PBS solution. Then, 4-fold excess of capture DNA probe was added into the MB solution and it was incubated for 40 min. The MB modified capture probe was washed 3 times in PBS, decanted and re-suspended in PBS containing $MgCl_2$ (10 mM). The MB modified capture probe was divided to 4 Eppendorf tubes. Each of the samples was mixed with different concentrations of target DNA from 0.1 to 100 pmol. The solution was incubated for 60 min, vortexing the sample for 2 min every 15 min.. Then the MBs were washed 3 times with PBS and isolated with a magnet and re-suspended in PBS. Next, it was reacted with 0.6 nmoles of the reporter probe to each MB-capture-target DNA complex. It was incubated for 60 min vortexing the sample for 2 min every 15 min. Then it was washed 3 times with PBS and the MBs isolated using a magnet and the beads were re-suspended in PBS. The modified MB was mixed with a streptavidin solution to conjugate with the lipase-biotin conjugate for 45 min (2 min vortexing every 15 min) and the MBs were washed 3 times with PBS and isolated using a magnet. Finally, a 4-fold excess of the lipase-biotin conjugate was added to the MB modified DNA. It was washed 3 times with PBS, decanted with a magnet and re-suspended in PBS.

Detection of lipase activity using *p*-nitrophenyl octanoate, NPO

The assay is based on monitoring the hydrolysis of the ester bond in NPO to form 4-nitrophenlate (pNA), which in contrast to the ester has a strong maximal absorbance at 405 nm.

The initial stock solution was made in DMSO with a concentration of 100 mM of NPO and then it was diluted to 0.1 mM NPO by 20 mM phosphate buffer containing 0.15 M NaCl (PBS), and 0.005% Triton X-100 for substrate solubilization.

The reaction mixture (in a 96 well plate) consisted of 200 μl of NPO solution and 5 μl of lipase or lipase-biotin conjugates of known concentrations. In order to measure the p-nitrophenolate released from the substrate, a control was prepared by adding PBS into the NPO solution.

MB-lipase and MB-DNA conjugates were prepared using the same procedure as for the lipase/lipase-biotin conjugates but they were incubated in Eppendorf tubes The activated solution was then decanted using a magnet to retain the MBs and the solutions were loaded into 96 wells plate. Finally, the absorbance of the solution was monitored at 405 nm on a μQuant microplate spectrophotometer.

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