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Specific Detection of the Cleavage Activity of Mycobacterial Enzymes using a Quantum Dots based DNA Nanosensor

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We present a quantum dot based DNA nanosensor specifically targeting the cleavage step in the reaction cycle of the essential DNA-modifying enzyme, mycobacterial topoisomerase I. The design takes advantages of the unique photophysical properties of quantum dots to generate visible fluorescence recovery upon specific cleavage by mycobacterial topoisomerase I. This report, for the first time, demonstrates the possibility to quantify the cleavage activity of the mycobacterial enzyme without the pre-processing sample purification or post-processing signal amplification. The cleavage induced signal response has also proven reliable in biological matrices, such as whole cell extracts prepared from *Escherichia coli* and human Caco-2 cells. It is expected that the assay may contribute to the clinical diagnostics of bacterial diseases, as well as the evaluation of treatment outcomes.

Introduction

Rapid and quantitative detection of pathogenic bacteria, such as *Mycobacterium tuberculosis*, the etiological agent of tuberculosis (TB), is critical for effective antibiotic treatment and prevention of the disease spreading at an early infection stage. While an array of diagnostic approaches has been developed for this purpose, challenges remain particularly when accessibility and time-to-results are considered collectively. For instance, although polymerase chain reaction (PCR)^{1, 2} and sequencing are promising for highly sensitive identification of bacteria, both techniques are expensive and require extensive resources. For diagnosis of bacterial diseases, including TB, culturing the bacteria from the specimen/samples followed by microscopic examinations of colonies is the most commonly adapted clinical gold standard^{3, 4}. However, the long culture time of bacteria (up to 8 weeks in the case of mycobacteria⁵) often delays the reporting. A handful of technological innovations have been recently introduced for identifying the presence of bacteria in general, including quartz crystal microbalance (QCM)^{6, 7}, surface plasmon resonance (SPR)^{8, 9}, electrochemical impedance spectroscopy (EIS)¹⁰, surface enhanced Raman scattering

(SERS)^{11, 12}, and fluorescence spectroscopy¹³. However, the performance of these techniques rely heavily on complex purification procedures to isolate bacteria from the biological matrices, hampering their applications in the field settings. Therefore, there is a critical need of a generic, accurate, and more importantly, user-friendly assay that allows fast and quantitative detection of the bacterial pathogens in biological samples.

To this end, we report the development of a novel use of quantum dots (QDs) based DNA nanosensors for the rapid detection of bacterial enzymes, which are essential for bacterial cell survival. More specifically, the proposed assay directly measures the cleavage activity of mycobacterial topoisomerase I (TopoI) as a proof-of-concept quantifiable indication of viable mycobacterial cells. Topoisomerases are a ubiquitous class of enzymes, which maintain topological homeostasis during a variety of DNA transaction processes such as transcription, replication and chromosome segregation. The reaction mechanism is a multi-step process consisting of DNA binding, cleavage, strand passage and religation. The mycobacterial TopoI shows properties which are markedly distinct from its *E. coli* counterpart¹⁴⁻¹⁷. For instance, the mycobacterial enzyme can bind to and cleave double-, as well as single-stranded DNA with comparable affinity. The binding and cleavage are sequence specific and the hexameric sequence is referred to as Strong Topoisomerase Site (STS)^{16, 17}. The site specific cleavage property is used in the proposed nanosensor to distinguish the activity of the mycobacterial TopoI, and hence the presence of mycobacteria, from the activity of TopoI from other species. The visual detection is made possible by the unique

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† Footnotes relating to the title and/or authors should appear here. Electronic Supplementary Information (ESI) available: Characterization of the QD-based DNA Nanosensor. See DOI: 10.1039/x0xx00000x

photophysical properties of the QDs, monodisperse semiconductor nanocrystals, that emit strong fluorescence under UV excitation^{18,19}. The designed DNA substrate consists of a single-stranded oligonucleotide, with the STS recognition sequence and dual-labelled with biotin and Cy5 on the 5' and 3' end, respectively. Upon pre-assembly with the streptavidin functionalized QD, forming the DNA nanosensor, energy transfer due to the mechanism of Förster Resonance Energy Transfer (FRET)^{20,21} from the QD (emission peak at 605 nm) to Cy5²²⁻²⁵ would lead to quenching of the QD fluorescence, shown as "the initial stage" in Fig. 1. Similar with the sensing principle of proteolytic activity using QD^{26, 27}, upon specific recognition of mycobacterial Topol, the cleavage would then turn the FRET "off", leading to a recovery of QD fluorescence. The increase of QD emission thus represents the presence of "active" mycobacterial Topol, and may serve as a direct indication of viable mycobacterial cells. Purified *Mycobacterium smegmatis* topoisomerase I (MsTopol), from non-pathogenic *M. smegmatis*, is selected a model enzyme, for its similar biochemical characteristics with the Topol from its pathogenic relative, *M. tuberculosis*¹⁷. This report, for the first time, demonstrates the possibility to quantify the cleavage activities of mycobacterial enzyme *via* the response from the QD-based DNA nanosensors without the need of separation or amplification. The cleavage induced signal response has also proven reliable in biological crude samples containing human cell extracts of Caco-2 and *Escherichia coli* (*E. coli*). Besides the clear clinical value in diagnosing bacterial diseases, and evaluating treatment outcome, the developed methodology may be promoted for monitoring different target

enzymes situated in various kinds of microorganisms. It is also therefore expected to generate benefits in other sectors, such as food industries, homeland security and agriculture.

Experimental

Preparation of DNA Nanosensors

The MsTopol specific DNA substrates (sequence, labelling and the cleavage site listed in **Table 1**) were synthesized, dual labeled, and HPLC purified by Integrated DNA Technologies, Coralville, Iowa, USA. This substrate included a previously validated cleavage site of MsTopol (CGCT[^]TG, [^] cleavage site)²⁸.

Streptavidin-functionalized CdSe-ZnS QDs conjugates (Qdot® 605 ITK™ Streptavidin Conjugate) were purchased from Life Technologies. The DNA nanosensors were prepared by conjugating the MsTopol DNA substrates onto QD *via* biotin-streptavidin interaction in the conjugation buffer (40 mM Tris-HCl, 200 mM NaCl and 1 mM EDTA, pH=8). The molar ratio of MsTopol DNA substrates to QD was kept at a ratio of 25 : 1 (1.25 pmol of MsTopol DNA substrate and 50 fmol of QD). The ratio was empirically determined at where the optimal quenching of QD fluorescence occurred (Supplementary Information).

Table 1

5'-biotin- CAG TGA GCG AGC TTC CGC T [^] TG ACA TCC CAA TA -Cy5-3' ([^] cleavage site)
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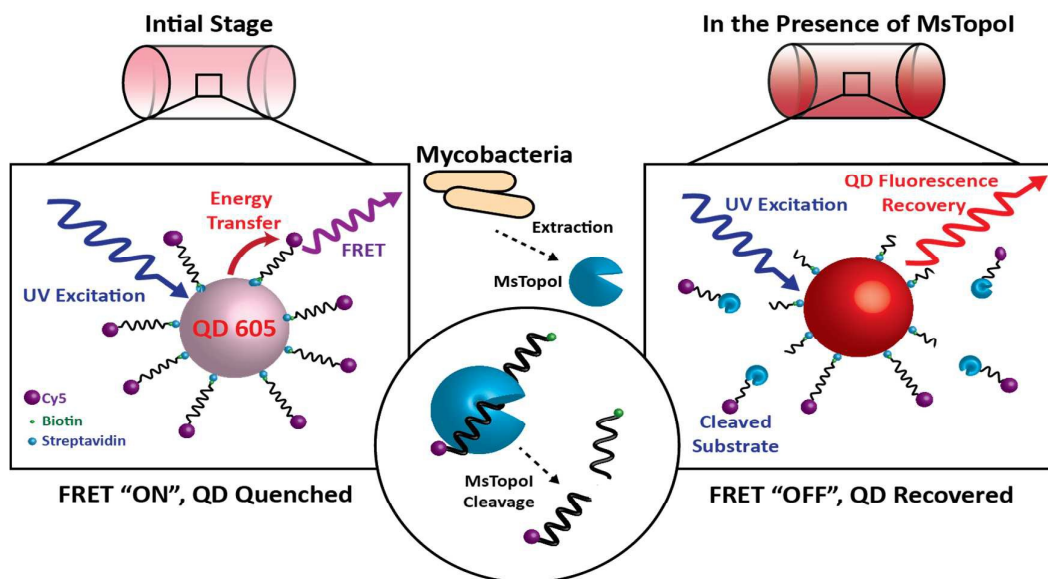


Fig. 1. The Principle of the Proposed DNA Nanosensor: Left: Initial Stage (FRET "ON", QD Quenched): The MsTopol DNA substrates, consisting of the previously validated recognition sequence (Ref 31), are dual-functionalized with biotin and Cy5 on the 5' and 3' end, respectively. The DNA nanosensor is assembled by conjugating the MsTopol DNA substrates onto the QD through biotin-streptavidin interactions in an optimized ratio. The QD fluorescence is quenched by the FRET-mediated energy transfer. Right: In the Presence of MsTopol (FRET "OFF", QD Recovered): MsTopol binds and cleaves on the recognition site, which lead to the diffuse-away of Cy5 fluorophore. The cleavage activity of MsTopol is quantitatively determined by the level of recovered QD fluorescence.

Reaction of MsTopol and QD DNA Nanosensors

Recombinant MsTopol was purified by transforming pPVN123 encoding Mstopol in *E. coli* BL21 expression strain. The protein was purified by two column chromatographic steps using heparin Sepharose followed by SP Sepharose as previously described²⁸. The validation of cleavage with the QD DNA nanosensors was conducted by mixing the DNA nanosensors (prepared as above described) with titrated amount of purified MsTopol. Titration of MsTopol was prepared by diluting the purified MsTopol in a dilution buffer (50 mM KCl, 1 mM EDTA, and 20 mM Tris-HCl, pH=8). After the dilution, MsTopol was then mixed with the DNA nanosensors and incubated at 37 °C for 60 minutes. EDTA was present in the reaction to deplete the divalent cations and thus inhibiting DNA nucleases from unspecific cleavage.

Measurement of Relaxation Activity of MsTopol

The relaxation assay was done with 200 fmol PAD negatively supercoiled plasmid in the relaxation buffer (150 mM NaCl, 1 mM EDTA, 5 mM MgCl₂, 5 mM CaCl₂, and 10 mM Tris-HCl, pH=7.5). Subsequently after MsTopol was added to the mixture, the reaction was incubated at 37 °C for 60 minutes. After the incubation, the relaxation reaction was stopped by adding SDS (0.25 % final) and proteinase K (20 µg) to digest MsTopol at 37 °C for 60 minutes. DNA relaxation was assessed by gel electrophoresis in 1% agarose in TBE with ethidium bromide. Gels were subjected to electrophoresis at 10 V overnight, stained with ethidium bromide and visualized under UV light (Versadoc 5000, Bio-Rad, Hercules, CA).

Preparation of Human Cell Nuclear Extract

Caco-2 cells (heterogeneous human epithelial colorectal adenocarcinoma cells) were cultured in complete medium (Minimum Essential Media, MEM-GlutaMAX, supplemented with non-essential amino acid, 20% fetal bovine serum (FBS), 1% penicillin, and 1% streptomycin). Cells were harvested with 0.5% Trypsin-EDTA (Gibco) and media was then discarded. Cells were subsequently washed in phosphate-buffered saline (PBS) prior to nuclear extraction. Extraction was executed following the previously established protocol²⁹. Briefly, 5x10⁶ cells/mL of cells were incubated in 1 mL of lysis buffer (0.1 % Igepal CA-630, 10 mM Tris-HCl (pH=7.9), 10 mM MgCl₂, 15 mM NaCl, and 1 % PMSF (phenylmethyl sulfonyl fluoride)) on ice for 10 minutes. The lysate was then spun at 400 g for 5 min and the supernatant containing cell debris was subsequently removed. The nuclear extraction was prepared by adding 50 µL of extraction buffer (0.5 M NaCl, 20 mM HEPES (pH=7.9), 20 % glycerol, and 1 % PMSF) into the precipitation and incubated at 4°C for 1 hr. Followed by a final spin at 11,200 g for 10 min, the supernatant was collected as the nuclear cell extract and used for subsequent tests.

E. coli Expression of MsTopol and Preparation of Enzyme Extract from *E. coli*

The expression plasmid pPVN123 was transformed into *E. coli* BL21, and induced as previously described²⁸. Briefly, the expression plasmid pPVN123 was transformed into *E. coli* cells and induced at 37°C for 3 hrs with 0.3 mM IPTG. The cells were harvested by centrifugation. To prepare the extract, the pellet was resuspended in ice cold PBS (1 g pellet in 2 mL PBS). The cell suspension was then mixed with equal volume of glass beads (212-300 µm, acid-washed, Sigma-Aldrich) and 2 µL/mL of PMSF. The *E. coli* were lysed by repeated vortexing the cells vigorously with glass beads. In detail, the mixture was vortexed for 1 min and left on ice. This process was repeated 3 times, with 1 min incubation on ice in between each repetition. After extraction, the mixture was centrifuged at 13,000 g (4°C) for 5 min. The supernatant was then collected and used immediately. Untransformed *E. coli* was processed the same way and used as a negative control. To simulate the crude biological sample, extract from the MsTopol expressing *E. coli* was titrated with untransformed *E. coli* in a final concentration of 25 pM in the reaction buffer. The mixed extract was tested for the cleavage activity as described in previous section without further purification.

Measuring Cleavage Activity of MsTopol via Recovery of QD Fluorescence

Fluorescence emission spectra and end-point measurements were achieved by a commercially available plate reader (FlexStation 3, multi-mode microplate reader, Molecular Devices, Sunnyvale, USA). The emissions for QD605 (bandpass 570-590 nm) and FRET-mediated Cy5 (660-680 nm) were measured upon excitation at 360 nm in 380-well plates.

Calculation of Recovery Efficiency

Empirically, the recovery was characterized using the ratiometric FRET^{25,30}. The recovery efficiency (Recovery %) was determined according to the following equation. Briefly, the obtained fluorescence was background subtracted and normalized by the difference of positive control (QD conjugated with unlabelled DNA substrates) and negative control (QD nanosensors in the absence of enzymes).

$$\text{Recovery\%} = \left(\frac{I_{\text{QD-Sample}} - I_{\text{QD-NegativeControl}}}{I_{\text{QD-PositiveControl}} - I_{\text{QD-NegativeControl}}} \right) \times 100\%$$

At least three individual experiments, unless otherwise noted, were conducted for each condition. Error bars were calculated as the standard error of the mean from each independent experiment. The statistical significance was determined by Prism 5.0 (GraphPad Software, La Jolla, CA).

Results and discussion

The Configuration of Proposed DNA Nanosensor and the Model System: *Mycobacterium smegmatis* Topoisomerase I (MsTopol)

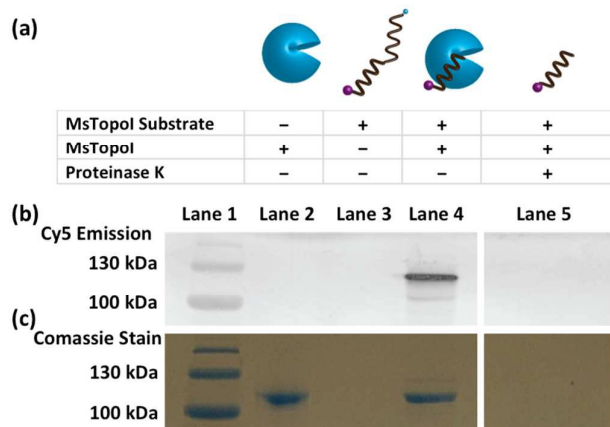


Fig. 2. Recognition and Cleavage of MsTopol. (a) Samples containing different combinations of entities were prepared (+: Presence, -: Absence), and retarded in a SDS polyacrylamide gel. The gel was scanned for (b) Cy5 emission (Excitation: 630 nm, Emission: 670nm), and (c) Coomassie stain. Lane 1: Size marker (kDa); Lane 2: Sample containing MsTopol alone was retarded to the position of around 110 kDa and only visualized upon addition of the Coomassie stain. Lane 3: Sample containing the MsTopol DNA substrate alone was not retarded. Lane 4: Upon interaction of MsTopol and DNA substrates, the Cy5 band was co-localized with the MsTopol band of the Coomassie stain. Lane 5: The DNA substrate bound MsTopol was fully digested by proteinase K.

The site specific nature of mycobacterial Topol is exploited for the proposed DNA sensors in this study. The working principle of the nanosensor is shown in Fig. 1. The MsTopol DNA substrates, consisting of the previously validated recognition sequence³¹, are dual-functionalized with biotin and Cy5 on the 5' and 3' end, respectively. The DNA nanosensor is assembled by conjugating the MsTopol substrates onto the QD through biotin-streptavidin interactions in an optimized ratio. The QD fluorescence is initially quenched (Fig. 1, Left), due to the FRET-mediated energy transferred to Cy5. Upon specific recognition of MsTopol, the cleavage would then lead to a recovery of QD fluorescence (Fig. 1, Right).

To ease the sample handling at the development stage of the nanosensor, purified MsTopol, from non-pathogenic *M. smegmatis*, was used as a model since it shares similar biochemical characteristics with the Topol from its pathogenic relative, *M. tuberculosis*¹⁷. Therefore, the optimization and development of the sensor for *M. smegmatis* can be easily transformed to the detection of other pathogenic mycobacteria in the future.

Characterization of the Interaction between the Dual-Functionalized DNA Substrates and MsTopol

To validate whether the dual-functionalized substrate can be cleaved by MsTopol, a cleavage assay was performed and the reaction products were analyzed using standard SDS

polyacrylamide gel electrophoresis. To enhance the concentration of cleavage complexes and to avoid unspecific cleavage by e.g. nucleases, the cleavage reaction was performed in an EDTA containing buffer. This prevents religation by MsTopol, due to depletion of divalent cations from the reaction mixture. Samples containing different combinations of entities were prepared (Fig. 2a), and retarded in a SDS polyacrylamide gel. The Cy5 emission and Coomassie stain were scanned for a visualization of the retardations of the Cy5-labeled DNA substrates and MsTopol, respectively (Fig. 2b and Fig. 2c). As evident from the gel scanning, interaction of MsTopol with the Cy5-labeled DNA substrate under these conditions resulted in a Cy5 band (compare lanes 4 and 3, Fig. 2b) to a position corresponding to the approximate mobility of MsTopol (both lanes 2 and 4 showed a mobility of around 110 kDa, the Coomassie stain shown in Fig. 2c). This band disappeared upon proteinase K digestion of the reaction products (lane 5) clearly confirming its identity as a protein coupled product, which is consistent with MsTopol induced cleavage complex. Taken together, these results confirm that the enzyme can bind and cleave the designed

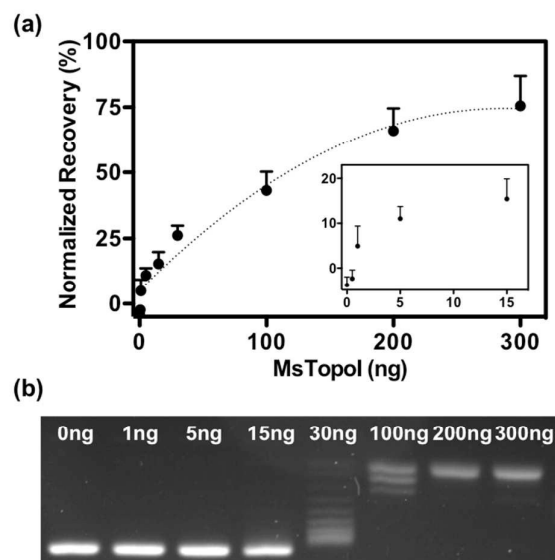


Fig. 3. Validation with Titrated Purified Enzymes: (a) Cleavage activity measured by the QD nanosensor: Titrated concentrations of MsTopol ranging from 0.5 to 300 ng were incubated with the QD nanosensor. Cleavage reaction was carried out at 37 °C for 60 minutes. The measured fluorescence was background subtracted by the negative control and converted to the recovery percentage by comparing to the positive control. The average and standard error of the mean were from five individual experiments. (b) Relaxation activity measured by conventional relaxation assay: Amounts of MsTopol ranging from 1 to 300 ng was incubated with negatively supercoiled DNA at 37 °C for 60 minutes and the reaction stopped with by 0.25 % (final concentration) of SDS. The MsTopol was then digested by proteinase K (20 µg) at 37 °C for 60 min before the samples were analyzed in a 1 % agarose gel and the products were visualized by subsequent staining with ethidium bromide.

cleavage substrate, forming a 5' covalent intermediate as expected²⁸.

Detection Limit Determined with Titrated Purified Enzymes

The performance of the sensor was evaluated by a titration experiment with purified MsTopoI in a buffer containing EDTA (as described in the Experimental Section). As shown in Fig. 3a, the measured recovered QD fluorescence was in positive correlation to the amount of purified MsTopoI. The detection limit was determined to be around 1-5 ng (Fig. 3a, inset). Relaxation of negatively supercoiled substrate is typically used to determine the complete catalytic activity of type IA topoisomerases. This technique, however, may not detect activity at low amounts of enzyme. Indeed, as shown in Fig. 3b, the purified MsTopoI was able to relax supercoiled DNA in a concentration dependent manner. However, the detection limit of the relaxation assay of ~15-30 ng is poorer than that of the proposed DNA nanosensor. Hence, in contrast to relaxation assay which relies on specialized equipment for readout and has a higher limit for detection of activity, the fluorescence based assay may present the advantage of enabling the possibility of visualized measurement and detecting low level of enzymatic activity.

The spectra shown in Fig. 4a further confirmed that the QD fluorescence (peak at 605 nm) was recovered by the addition of MsTopoI. As evidenced by the spectra, the ratiometric

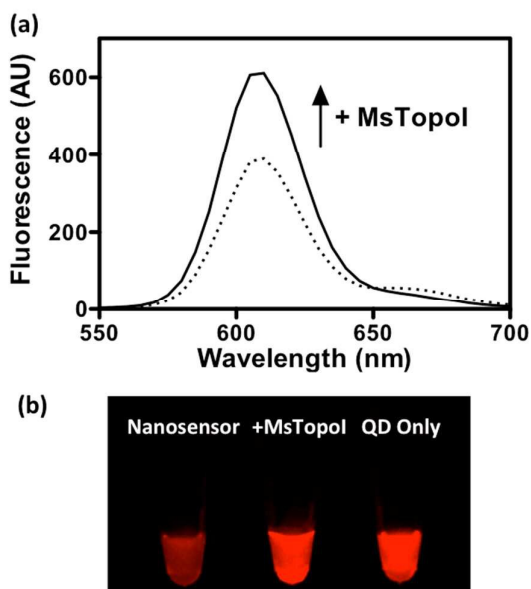


Fig. 4. Detection of the Recovered Fluorescence: (a) The fluorescent spectra at the QD emission (605 nm) were measured by fluorometer prior to and after the addition of MsTopoI. Fluorescence recovery induced by MsTopoI cleavage was clearly observed under 360 nm excitation. (b) The difference in fluorescence was visually differentiable under UV excitation, rendering the assay highly potential for point-of-care.

change depends largely on the QD recovery as compared to the disappearance of FRET-mediated Cy5 emission (See also Supplementary Figure 2). Owing to the unique photophysical properties of QD, the recovered fluorescence was clearly distinguishable under UV (360 nm) excitation in the presence of MsTopoI (Fig. 4b). The possibility to visualize the recovered fluorescence upon cleavage of MsTopoI may open up future possibilities in promoting this assay for tests under the point-of-care setting.

Performance Evaluated in Crude Biological Samples

The subsequent effort investigates whether the nanosensor remains functional in crude biological samples by enabling specific detection of MsTopoI even in the context of human cell content. The nanosensor was tested using the purified MsTopoI spiked-in human nuclear extract from Caco-2

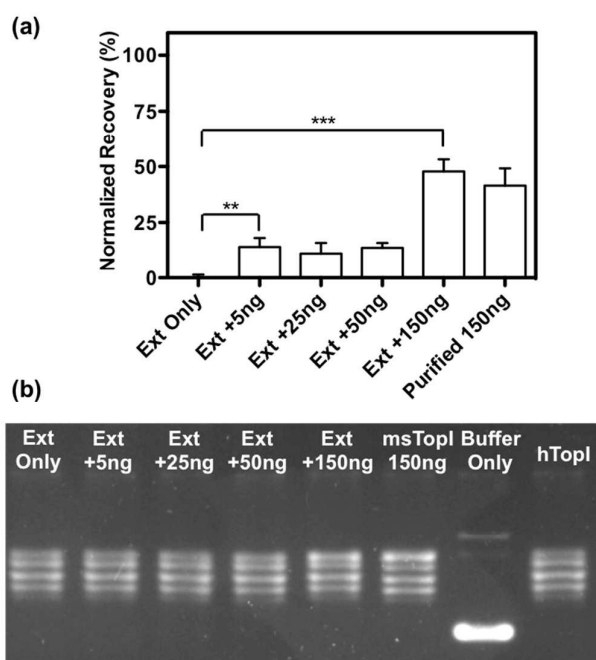


Fig. 5. Validation with Titrated Purified Enzymes Spike-in Human Cell Extract: (a) Cleavage activity measured by the QD nanosensor: Amounts of MsTopoI ranging from 0 to 150 ng was spiked in extract from Caco2 cells and incubated with the QD nanosensor. As a control, a similar experiment using 150 ng of purified MsTopoI alone was included. The average and standard error of the mean were from three individual experiments. Statistics was analysed by unpaired t-test (** $p=0.045$, *** $p<0.0001$). (b) Relaxation activity measured by conventional relaxation assay: Increasing amounts of MsTopoI ranging from 0 to 150 ng spiked in extract from Caco2 cell was incubated with supercoiled DNA in the relaxation buffer (10 mM Tris-HCl pH7.5, 1 mM EDTA, 5 mM CaCl_2 , 5 mM MgCl_2 and 150 mM NaCl) at 37 °C for 60 minutes before the samples were Proteinase K treated (20 μg), analyzed in a 1% agarose gel and the reaction products visualized by ethidium bromide staining. As controls, the plasmid was also incubated with 150 ng of purified MsTopoI, buffer without added enzyme or purified hTopoI alone.

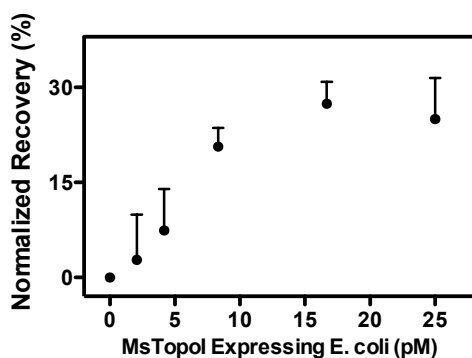


Fig. 6. Validation with Natural Sample: Titrated amount of extract obtained from MsTopol expressing *E. coli* cells was mixed with extract from untransformed *E. coli* in a total *E. coli* concentration of 25 pM. The mixed extract was tested by the DNA nanosensors without further purification. The average and standard error of the mean were from three individual measurements.

(derived from colorectal adenocarcinoma). As shown in Fig. 5a, the detection limit (~5 ng) was minimally affected in the presence of human cell extracts, containing human topoisomerases (hTopI) along with other eukaryotic proteins. Also the sensor was highly specific towards MsTopol and did not detect any of proteins/enzymes present in the human cell extract. Contrasting with the proven specificity of the proposed QD nanosensor, it was not possible to distinguish MsTopol relaxation activity from other relaxation activities present in the human cell extract from Caco-2. As shown in Fig. 5b, almost no difference was observed in the relaxation capability shown in the lanes containing either cell extracts mixed with different amount of purified MsTopol (5-150 ng), purified MsTopol only (150 ng) or purified hTopI only. This is not surprising since it is well established that at least hTopI exhibit relaxation activity in the MsTopol relaxation buffer.

Similar cleavage experiments were also carried out using extracts from *E. coli* cells over-expressing MsTopol while the untransformed *E. coli* was used as a negative control. The samples were prepared by mixing a titrated amount of extract from MsTopol-expressing *E. coli* with extract from the untransformed *E. coli* in a total *E. coli* concentration of 25 pM. The mixed extract was tested for the cleavage activity without further purification. Results shown in Fig. 6 provided a validation that the proposed QD nanosensor remained quantifiable and functional with the biological crude sample in the current setup.

Conclusions

DNA interacting enzymes, such as topoisomerases, are essential for all living organisms, due to their function of maintaining topological homeostasis during vital cellular functions³². Measurement of the activity of topoisomerases

has been reported as a reliable indication to the infectious diseases^{19, 33}. For example, malaria infection has been determined by the level of enzymatic activities based on isothermal conversion of single DNA cleavage–ligation events catalyzed specifically by the topoisomerase I of malaria causing plasmodium species¹⁹. The presented QD-based nanosensor joins the league by providing a facile and rapid measurement through fluorescence generated by the cleavage activity of mycobacterial topoisomerase I, which eliminates washing steps and post-amplification as required in the previously developed platform^{19,33}. Significant clinical value is anticipated by extending this assay for the detection of mycobacteria-associated diseases, such as TB. In particular, an improved species specificity is anticipated when joining the effort with antibodies or peptide based recognition³⁴. Further, the proposed nanosensor is highly transformable for the detection of contaminating mycobacteria in water or food, which may have a broad appeal to the food industries and agricultural sectors.

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