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On-bead fluorescent DNA nanoprobes to analyze base excision repair activities

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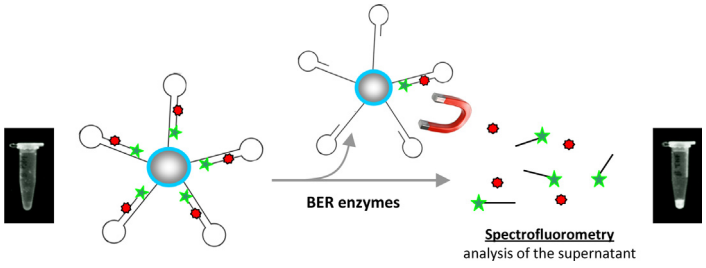
HIGHLIGHTS

- On magnetic beads fluorescent enzymatic assays.
- Simple, easy, non-radioactive and electrophoresis-free functional assay.
- Lesion-containing hairpin DNA probes are selective for repair enzymes.
- The biosensing platform allows the measurement of DNA repair activities from purified enzymes or within cell free extracts.

GRAPHICAL ABSTRACT



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ABSTRACT

DNA integrity is constantly threatened by endogenous and exogenous agents that can modify its physical and chemical structure. Changes in DNA sequence can cause mutations sparked by some genetic diseases or cancers. Organisms have developed efficient defense mechanisms able to specifically repair each kind of lesion (alkylation, oxidation, single or double strand break, mismatch, *etc*). Here we report the adjustment of an original assay to detect enzymes' activity of base excision repair (BER), that supports a set of lesions including abasic sites, alkylation, oxidation or deamination products of bases. The biosensor is characterized by a set of fluorescent hairpin-shaped nucleic acid probes supported on magnetic beads, each containing a selective lesion targeting a specific BER enzyme. We have studied the DNA glycosylase alkyl-adenine glycosylase (AAG) and the human AP-endonuclease (APE1) by incorporating within the DNA probe a hypoxanthine lesion or an abasic site analog (tetrahydrofuran), respectively. Enzymatic repair activity induces the formation of a nick in the damaged strand, leading to probe's break, that is detected in the supernatant by fluorescence. The functional assay allows the measurement of DNA repair activities from purified enzymes or in cell-free extracts in a fast, specific, quantitative and sensitive way, using only 1 pmol of probe for a test. We recorded a detection limit of 1 $\mu g\,mL^{-1}$ and 50 $\mu g\,mL^{-1}$ of HeLa nuclear extracts for APE1 and AAG enzymes, respectively. Finally, the on-bead assay should be useful to screen inhibitors of DNA repair activities.

1. Introduction

Our genome, composed of 3.10^9 base pairs, contains all the instructions necessary for development, maintenance and reproduction of our cells. However, endogenous and exogenous agents constantly threaten DNA integrity, modifying its structure [1–3]. Resulting damaged nucleobases can cause genetic mutations, leading to some genetic diseases and carcinogenesis. Fortunately, DNA lesions may be specifically recognized and eliminated by several DNA repair pathways [3,4]. Base modifications, abasic sites and single strand breaks are repaired by the base excision repair (BER) pathway [5,6]. Briefly, a DNA-glycosylase first recognizes the damaged base and cleaves the *N*-glycosidic bond, forming an abasic site (AP site). Subsequently, an AP-endonuclease incises

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the phosphodiester bond 5′ of the AP site generating a nick in the strand with a 3′-OH extremity and a 5′-phosphate 2-deoxyribose. It is noteworthy that several bifunctional DNA-glycosylases also exist that possess an additional AP-lyase activity. The predominant short path BER involves the excision of the sugar moiety and the introduction of the correct nucleotide triphosphate by the polymerase β followed by the restoration of the strand continuity by a ligase.

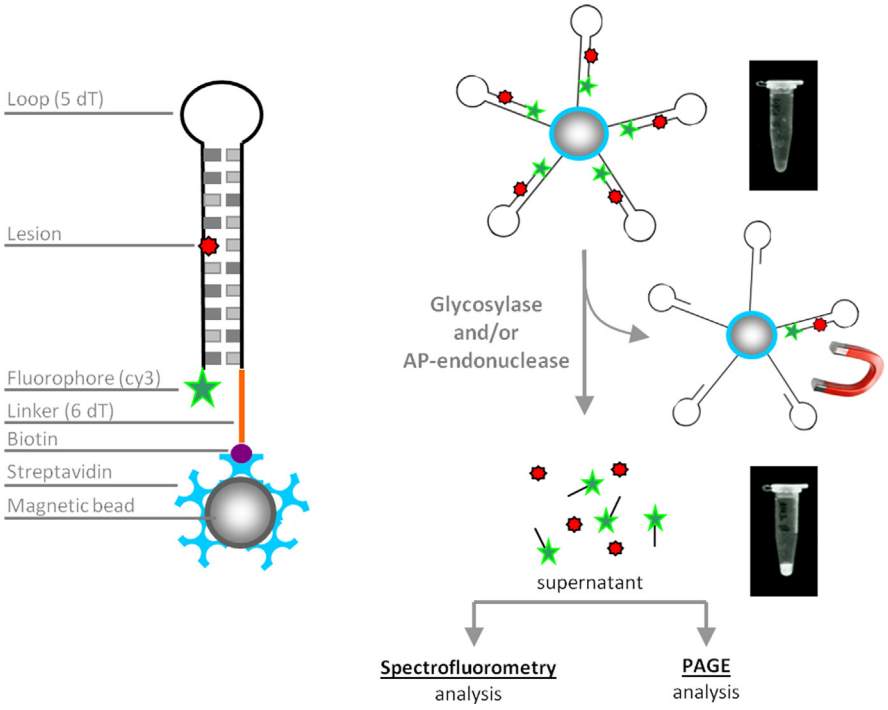
Numerous studies have reported the development of molecular tools to detect BER activities, more precisely the single strand break produced by a bifunctional DNA-glycosylase activity or by the joint activity of a glycosylase and an AP-endonuclease. Classically, activity and specificity of these enzymes are characterized by polyacrylamide gel electrophoresis of substrate oligonucleotides, labeled by radioactivity or fluorescence [7–13]; although sensitive, this method is discontinued and time-consuming. The use of molecular beacons, initially developed by Tyagi and Kramer for nucleic acid detection by hybridization [14,15] has been extended to the study of various DNA repair activities since the early 2000s [16–23]. Molecular beacon is characterized by a hairpin-shaped structure provided by the self-complementarity of the sequence. Both extremities of this particular oligonucleotide are labeled by a quencher and a fluorophore so that the close proximity of the pair of chromophores prevents fluorescence emission. Probe scission, induced by targeted enzymatic activity leads to the dehybridization of the probe and separation of the quencher from the fluorophore, causing an increase in fluorescence. Although these methods are efficient to measure DNA repair activities with purified enzymes, molecular beacons are rapidly degraded by cellular extracts. This limitation has been addressed by the grafting of (pro)fluorescent nucleic probes on solid support (mostly glass slides), however the latter technologies require expensive equipment and are time-consuming [24,25]. Other studies have shown that DNA substrates grafted on magnetic beads could be used as a platform to detect repair activities, but require radioactively labeled oligonucleotide probes [26,27]. Recently, Kool and co-workers have reported the development of new DNA probes using the quenching properties of the uracil base toward some fluorescent reporters in order to detect uracil DNA glycosylase activity [28,29]. Although original and robust, such constructions are limited to the functional detection of deoxyuridine repair.

Here, we report the conception of a new tool, characterized by a set of hairpin-shaped oligonucleotide probes immobilized on micrometric magnetic beads [30]. Each probe contains a selective targeting DNA lesion in the stem and is labeled by a 5′-terminal Cyanine 3. This device is associated to a simple fluorometric assay which allows detection of BER activities, namely DNA-glycosylase and AP-endonuclease. When the lesion is recognized by BER enzymes, single strand cleavage of the probe induced the release of a short fluorescent fragment in the supernatant at the incubation temperature. Unreacted probes can be separated by a magnetic rack. The fluorescence of the dissociated fragment is quickly quantified by spectrofluorometry in a reduced volume (Scheme 1). The activities of AAG and APE1 enzymes and of several restriction enzymes have been successfully monitored using a droplet fluorometer. The functional assay allows the detection of AAG and APE1 enzymes within cell-free extracts. Finally, as a proof of concept, we have applied this bioanalytical device to highlight the inhibitor effect of methoxyamine toward the APE1 enzyme.

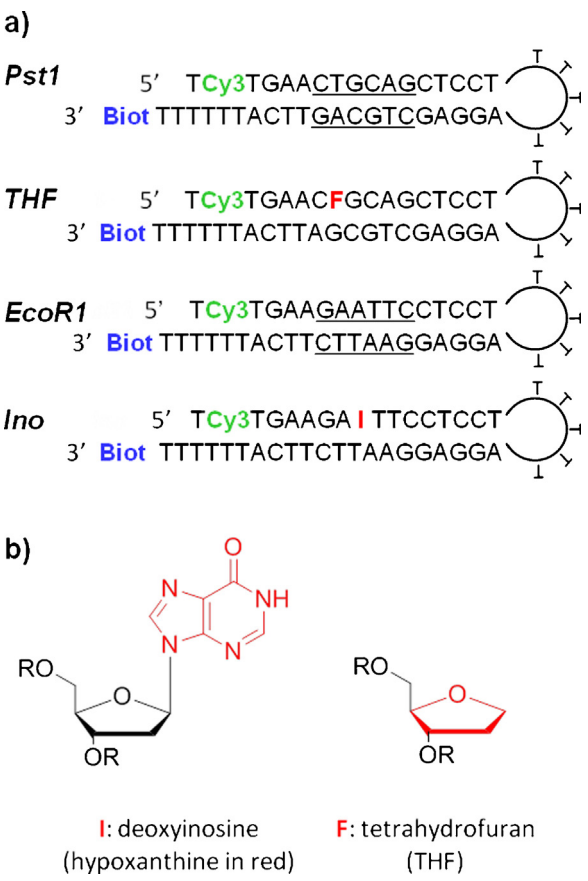
2. Material and methods

2.1. Synthesis of hairpin-shaped DNA probes

All oligonucleotides (see Scheme 2 for sequences) were prepared with an automatic nucleic acid synthesizer (Applied Biosystems 392 DNA/RNA Synthetizer) on a 1 μmol scale, following the standard protocols given by the supplier. All reagents (activator, capping reagent, trichloroacetic acid, dry solvents), normal phosphoramidite monomers (Pac-dA-CE, dT-CE, Ac-dC-CE, Pac-dG-3′-CE phosphoramidites), modified derivatives (dInosine-CE, Tetrahydrofuran-CE and Cyanine3-CE monomers) and 3′-biotin TEG CPG columns) were purchased from Glen Research (Sterling,VA). At the end of the solid phase chemical assembly,



Scheme 1. Schematic representation of the functionalized DNA probe and principle of the fluorescent detection of the cleavage by BER enzymes.



Scheme 2. (a) Sequences and structures of the five synthesized hairpin-shaped probes; (b) Chemical structures of lesions used in this study.

oligonucleotides were deprotected and cleaved from the support with a 30% ammonium hydroxide solution, at room temperature for 16 h. Ammonia was removed by evaporation under vacuum *via* a Speedvac SPD 111 V. All oligonucleotides were purified by HPLC *via* a Hypersil HS C18 column (250 × 7.5 mm, 5 μm) on a MERK L600 pump. DNA fragments were separated with a linear gradient of acetonitrile (from 5% to 30%) in triethylammonium 10 mM (Aldrich-Sigma). Fragments were detected by a UV detector MERK L400 at 260 nm. Collected fractions were concentrated, freeze-dried and then dissolved in Milli-Q ultrapure water. Purified oligonucleotides (fractions with a purity of more than 95% from RP-HPLC at 260 nm and containing the Cyanine 3 moiety; λex = 546 nm, λex = 563 nm) were finally dosed by measuring the absorbance at 260 nm *via* a Nanodrop ND-1000. The integrity of the sequences was controlled by denaturing PAGE and Maldi-Tof mass spectrometry measurements. Maldi-Tof mass spectra were recorded with a Bruker Microflex spectrometer following previously reported procedures [31]. Finally fluorescent DNA probes were lyophilized and frozen at −20 °C until use.

2.2. Intramolecular hybridization of oligonucleotides

All reagents used for the buffer were purchased from Aldrich-Sigma (Saint Quentin-Fallavier, France). The self-complementary DNA probes were diluted in an aqueous hybridization buffer (10 mM HEPES pH 7.8, 2 mM EGTA, 40 mM KCl, 0.1 mM $ZnCl_2$, 1 mM DTT, 0.5 mg mL^{-1} BSA) at a final concentration of 1 μM. Samples were heated at 90 °C for 2 min after which the heater was turned off to slowly cool the solution to room temperature, promoting annealing.

2.3. Immobilization of DNA probes on beads

Magnetic beads Dynabeads® M280-Strepta were purchased from Life Technologies (Saint-Aubin, France). These microparticules of 2.8 μm in diameter are coated with a monolayer of streptavidin, allowing the immobilization of biotinylated DNA probes. To separate the beads from the supernatant we used a magnetic rack Dynal MPC-E.

100 μg of beads were first washed and prepared according to instructions provided by the manufacturer. Washing and binding buffers were supplied in the Dynal Kilobase Binder kit. After undergoing an hybridization cycle, an aqueous solution of 12 pmol oligonucleotides (30 μL in 2× binding buffer) was added onto the beads and the mixture was incubated for 1 h (final volume of 60 μL), at room temperature under agitation in the dark. The fixation yield was estimated to be 90%, based on the residual fluorescence recorded in the supernatant. Saturated beads were pelleted and washed twice with the washing buffer, once with water, and were finally resuspended in the desired buffer before the enzymatic assay (1 pmol of immobilized DNA probe in 5 μL buffer). Finally, the suspension was stored at 4 °C until use. No modification of assay performance was noticed after one month of storage.

2.4. Enzymatic cleavage by purified enzyme

Human alkyl-adenine glycosylase, AP-endonuclease 1 and corresponding buffers were purchased from New England Biolabs (Evry, France). Restriction enzymes EcoR1 and Pst1 and their corresponding buffers were purchased from Trevigen (Gaithersburg, Maryland). Each enzymatic digestion was led with 1 pmol of fluorescent self-complementary DNA probe in solution or immobilized on beads. In the latter case, 5 μL of the functionalized bead stock was decanted in a test tube, beads were pelleted, and the supernatant was removed. Enzymatic buffer, ultrapure water, and the enzyme were added for a final volume of 10 μL. Samples were incubated for 1 h at 37 °C under agitation in the dark. Fluorescence of the supernatant was recorded on a droplet spectrofluorometer Nanodrop ND-3300 from Thermo scientific.

Samples were also analyzed by denaturing polyacrylamide gel electrophoresis (PAGE): 3 μL of "loading buffer" (90% formamide, 0.02% xylene cyanol, 0.02% bromophenol) were added to 7 μL of samples. Samples were heated for 3 min at 90 °C and then loaded on a 20% v/v acrylamide gel with 7 M urea. After migration with Tris-Borate-EDTA buffer 1X (TBE 10X from Gibco/Life Technologies), the gel was finally read with a fluorescence scanner Typhoon 9400 from GE Healthcare. Fluorescence intensities of stripes were quantified with the software *ImageJ*.

2.5. Enzymatic cleavage by nuclear extracts

Nuclear extracts from HeLa cells were purchased from Cilbiotech, aliquoted at 9 μg $μL^{-1}$ and kept frozen at −80 °C. The stock digestion buffer 4× (40 mM HEPES pH 7.8, 8 mM EGTA, 160 mM KCl, 0.4 mM $ZnCl_2$, 4 mM DTT, 2 mg mL^{-1} BSA), water milli-Q and nuclear extract were added to 1 pmol of ODN for a final volume of 10 μL. Samples were incubated for 1 h at 37 °C under agitation in the dark. The solution or the supernatant was analyzed first by spectrofluorometry with ND-3300 and then by denaturing PAGE as described above. For the APE1 inhibition study, methoxyamine (Mx, Aldrich-Sigma) is diluted in ultrapure water and 1 μL (ranging from 0.5 to 4 M) is added to the sample just prior to the nuclear extract.

3. Results

In order to detect the specific enzymatic activity of BER we designed a set of hairpin-shaped oligonucleotide probes. All probes contain the pattern biotin-triethyleneglycol (noted "Biot" in Scheme 2) at the 3′-end, followed by a six unpaired thymidine spacer to maximize the immobilization onto streptavidin-coated beads. At the 5′-end of the DNA probes we introduced a fluorescent dye, namely Cyanine3 (Cy3) to detect strand cleavage. The single-strand loop of the self-complementary DNA nanoprobe is composed of five thymidine residues (see Scheme 2 for detailed sequences and structures). *EcoR1* and *Pst1* probes are undamaged sequences that contain restriction sites of EcoR1 and Pst1 enzymes, respectively. The *Ino* probe results from the substitution of a 2′-deoxyadenosine into the restriction site of EcoR1 by a 2′-deoxyinosine monomer (Scheme 2). This lesion, stemming from the deamination of adenine (hypoxanthine), is recognized by a monofunctionnal enzyme, alkyl-adenine glycosylase, which excises the hypoxanthine base. AP-endonuclease 1 is used to reveal the newly-formed AP site by cleaving the phosphodiester bond next 5′ to the damaged site, releasing a fluorescent fragment of 8 nucleotides into the supernatant. Another fluorescent hairpin-shaped DNA substrate, namely the *THF* probe, which contains a tetrahydrofuran residue instead of a thymidine in the restriction site of Pst1 enzyme, was also designed. THF is a stable analog of an AP site and is efficiently recognized and cleaved by AP-endonucleases [32,33]. The latter enzymatic activity generates a strand scission releasing a fluorescent 7-mer.

3.1. Immobilization of fluorescent DNA nanoprobes on streptavidin coated magnetic beads

The assay reported here was based on the selective detection of an excision repair activity (strand cleavage assay) acting upon on-bead fluorescent DNA nanoprobes. To immobilize probes on a solid support we used micrometric magnetic beads (2.8 μm in diameter). These beads were coated with a monolayer of streptavidin allowing fast and easy immobilization of biotinylated oligonucleotide probes, which can be broken off only in highly denaturing conditions [34,35]. The PEG-linker constitutive of the biotin monomer together with the insertion of a (T_6)-spacer should facilitate the accessibility of the substrate by the targeted enzymes. Microspheres are composed of a ferromagnetic core allowing for rapid, and specific sedimentation with a magnet, without aggregation in zero magnetic field. First of all, we have optimized the immobilization of probes onto the beads. 10 pmol of the fluorescent *Pst1* and *EcoR1* nanoprobes were incubated with an increased amount of beads for 1 h. Amount of unfixed oligonucleotides was quantified by an end-point fluorescence measurement of the supernatant on the Nanodrop ND-3300 fluorometer (see supplementary data, Fig. S1). The optimal amount of beads used to fix 10 pmol of biotinylated probes was determined to be 90 μg. In those conditions, we estimated the number of DNA probes to 10^6 per bead. The stability of the biotin/streptavidin interaction was investigated over time (Fig. S2). After 24 h of storage, only 2% of grafted oligonucleotides were released and less than 5% after 72 h.

3.2. Functionality of on-bead fluorescent DNA nanoprobes

To validate this molecular device, it was necessary to confirm that supported DNA probes acted as functional substrates (annealing and accessibility). 1 pmol of *Pst1* and *Ecor1* probes, in solution or immobilized on beads, were digested by 10 units (U) of the corresponding restriction enzyme for 1 h. Then the DNA fragments cleaved and liberated in the supernatant were first analyzed by the rapid non-destructive fluorometric technique, followed by an additional PAGE analysis (Fig. 1). A similar experiment was also carried out with the probe containing the inosine moiety in the restriction site of EcoR1 (Fig. 2), since it has been previously shown that this modified nucleobase did not prevent the DNA cleavage by EcoR1 [36].

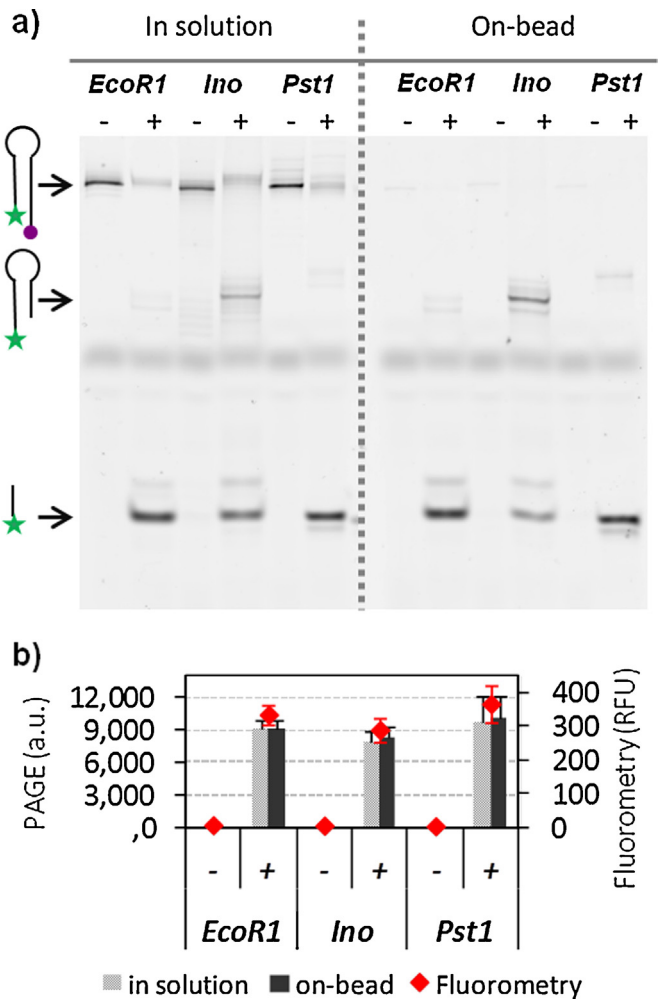


Fig. 1. Cleavage of 1 pmol of in solution or on-bead probes by restriction enzymes: *EcoR1* and *Ino* probes are digested with EcoR1 since *Pst1* probe is cleaved with Pst1 enzyme (– = without enzyme, + = 10 U of enzyme). (a) PAGE analysis; (b) compared quantification of cut product after gel electrophoresis (histogram: left scale) and with the fluorometric assay on the Nanodrop ND-3300 (diamonds: right scale).

Electrophoresis analyses revealed that after incubation for 1 h, approximately 85% of the DNA probes were cleaved by the corresponding restriction enzymes in solution. It is noteworthy that inosine slightly hinders the enzymatic activity of EcoR1 since the single strand cleavage within the 3′ stem side was more important in the *Ino* probe compared to others probes. In our current probe, the inosine residue was inserted by substitution of an adenosine while in previous work, reported by Doi and coworkers [36], this lesion was incorporated in place of a guanosine. Fluorescent signals measured from the enzymatic cleavage of immobilized DNA probes were not significantly different compared to those obtained in solution. This result proved that the quantity of DNA nanoprobes grafted to the beads is consistent with our calculated value and that the presence of the bead does not affect accessibility of the DNA substrate and the enzymatic cleavage efficiency.

Moreover, the rapid measurement of fluorescence intensity in the supernatant with a Nanodrop ND-3300 apparatus was validated as a well-adapted miniaturized format (*i.e.* fluorescence measurement within a 2 μL sample). The values obtained on the

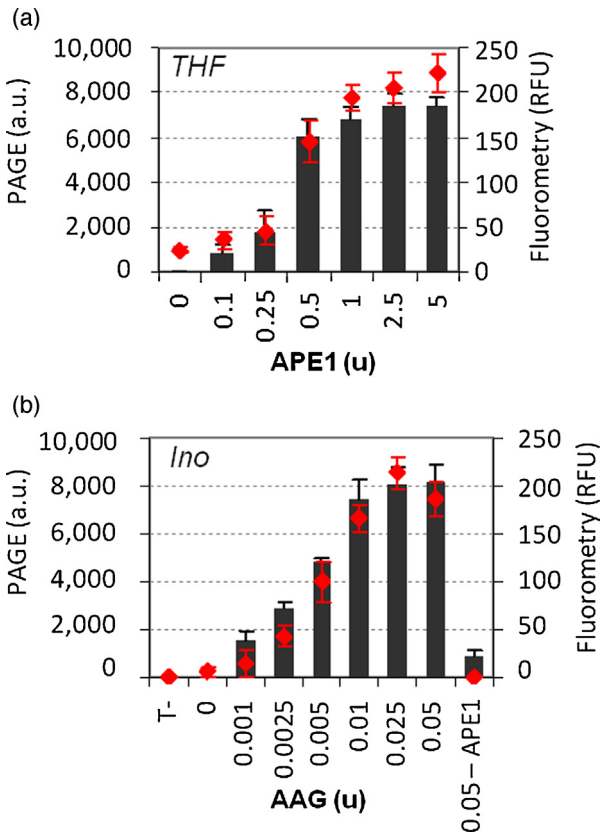


Fig. 2. Digestion of on-bead damaged probes by purified enzymes: 1 pmol of *Ino* probe was incubated with an increasing amount of mouse AAG (from 0 to 0.05 U) and with (2.5 U) or without human APE1. 1 pmol of *THF* probe was incubated with an increasing amount of human APE1 (from 0 to 5 U). Compared analyses of probe cleavage by PAGE technique (histogram: left scale) and with the fluorometric assay on the Nanodrop ND-3300 (diamonds: right scale).

latter fluorescent droplet platform were in total agreement with the quantifications done by PAGE analyses, which were used as references.

3.3. Monitoring the enzymatic activities of Alkyl-adenine DNA-glycosylase and AP-endonuclease

The Ino probe was designed to detect the excision process of the hypoxanthine base by an alkyl-adenine DNA-glycosylase protein (AAG). This initial step was followed by the single strand cleavage at the newly-formed AP-site by an AP-endonuclease (APE1). Accordingly, these two enzymes have to be active in the experimental conditions to allow the detection of the DNA probe site-specific cleavage.

3.3.1. Detection of base excision repair activity with purified enzymes

We carried out the incision of the *THF* probe by APE1 and the joint excision–incision step of the *Ino* probe by both enzymes, namely AAG and APE1 (Fig. 2). 1 pmol of *THF* beads was incubated with an increasing amount of APE1, ranging from 0 to 5 units (U). 1 pmol of *Ino* beads was incubated with an increasing amount of the monofunctional AAG, from 0 to 0.05 U and an excess of APE1, fixed at 2.5 U. Analyses by both fluorometric and electrophoretic techniques (Fig. 2 and S3) show that the fluorescent signals were dependant on the concentration of the corresponding enzyme in the appropriate range (AAG for *Ino* probe and APE1 for *THF* probe). The cleavage level determined from the fluorescent values obtained with the Nanodrop ND-3300 system correlated well with the data obtained by the classical PAGE analysis. Additionally, in the same buffer conditions, the fluorescent signal reached the same maximum value around 200 RFU, for both *Ino* and *THF* probes, underlying the similar functionality of both probes.

3.3.2. Detection of base excision repair activity in nuclear extracts

It is well-known that most of DNA repair proteins are located in the nucleus to operate their biological function toward damaged DNA. From this fact, first tests were carried out from nuclear extracts of HeLa cells. We have determined the optimal buffer to maximize the lesion excision/incision rate in minimizing the non-specific degradation of DNA nanoprobes. Preliminary studies were carried out in order to evaluate different enzymatic buffers (data not shown). As previously shown [37,38], the presence of a divalent cation is essential for the catalytic activity of AP-endonuclease even for the turnover of the enzyme. However, it seems better to use Zn^{2+} rather than Mg^{2+}, the latter being also a cofactor for most non-specific nucleases. It was also found that the presence of Bovine Serum Albumin stabilizes AAG, allowing an increase of the number of catalytic cycles [39]. Thanks to these first investigations, an optimal digestion buffer was determined, composed of 10 mM HEPES/KOH, 2 mM EGTA/KOH, 80 mM KCl, 0.1 mM $ZnCl_2$, 1 mM DTT and 0.5 mg mL^{-1} BSA.

The benefit of the on-bead format versus the in solution one toward non-specific degradation of the DNA probes was investigated. *EcoR1* probe in solution or on beads was incubated with an increasing amount of HeLa nuclear extracts. PAGE analysis (Fig. 3) confirms the degradation of hairpin-shaped probe in solution, with 50 μg mL^{-1} of extract (30% degradation). The decomposition amount of the same probe was estimated at 70% with 500 μg mL^{-1} and reached 90% at 3000 μg mL^{-1}. The non-specific digestion was mainly mediated by cellular 3'-exonucleases, as evidenced by sequential degradation of the free 3'-biotin extremity. In comparison, the on-bead DNA nanoprobes underwent only minor deterioration, even at the highest concentrations of nuclear extracts. The current experiment unambiguously demonstrates the ability of microbeads to efficiently protect the substrate DNA nanoprobes toward non-specific degradation by cellular nucleases, increasing the signal-to-noise ratio of our current functional assay.

Thereafter, we searched for the detection of BER activities in the same HeLa cell-free extracts. Supported *Ino* and *THF* damaged probes, together with the negative control probe *EcoR1* were incubated with an increasing amount of nuclear extracts. Supernatants were analyzed by fluorometric and PAGE (Fig. 4). The detection limit for APE1 protein was lower than 1 μg mL^{-1} of total proteins. In contrast, concentrations above 50 μg mL^{-1} of cell-free extracts were necessary to detect AAG activity. This difference can be explained first by the need of a concomitant activity of the two enzymes, AAG and APE1, to cleave the hypoxanthine containing probes; In contrast the incision of the *THF* probe requires only the enzymatic activity of the AP-endonuclease protein. Moreover, AAG protein is weakly expressed in HeLa cells in comparison to APE1, which is involved in various metabolic and regulating pathways [40,41]. Altogether these results showed that a direct fluorescence measurement, linked to a specific enzymatic cleavage of immobilized DNA nanoprobes on magnetic beads, was suitable for a rapid and sensitive analysis of complex media such as cell nuclear extracts and constitutes an alternative to the time-consuming PAGE technique.

3.4. A new tool to detect inhibition of BER enzymes by drugs: the methoxyamine-mediated inhibition of APE1 as a proof of concept

Methoxyamine (Mx) is a small organic molecule able to indirectly inhibit the action of AP-endonuclease by reacting with the

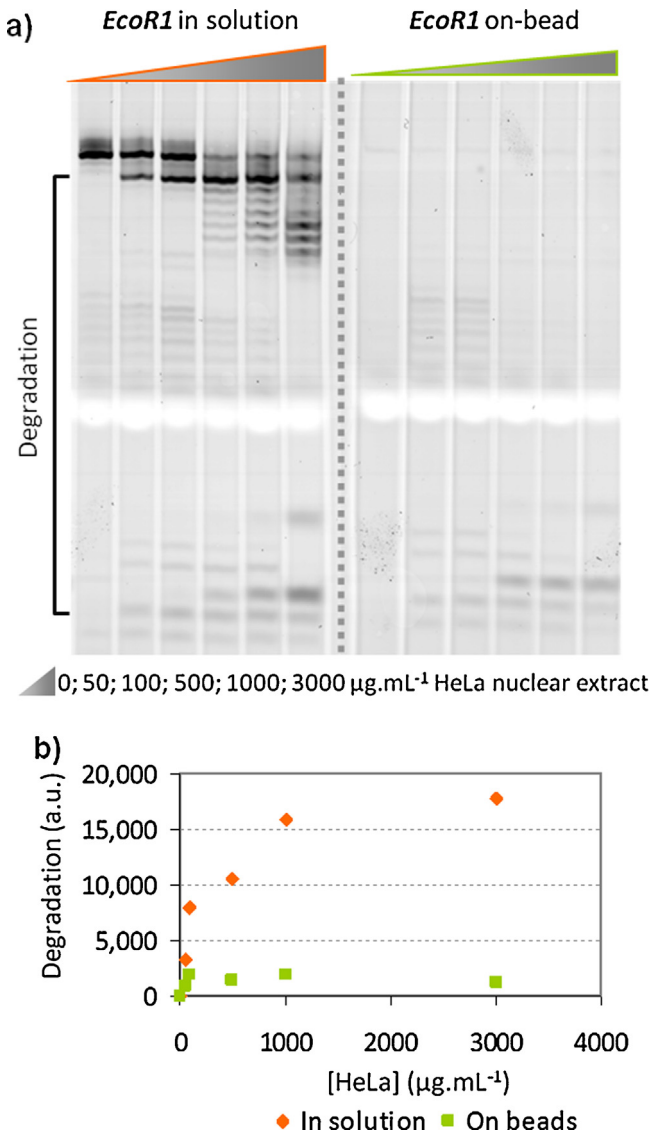


Fig. 3. Protection of DNA probes by the beads toward non specific cleavage. 1 pmol of *EcoR1* probe in solution or on-bead was incubated with an increasing amount of HeLa nuclear extract (µg mL^{-1}). (a) Electrophoresis analysis of probes degradation; (b) quantification of the stripes fluorescence corresponding to degradation.

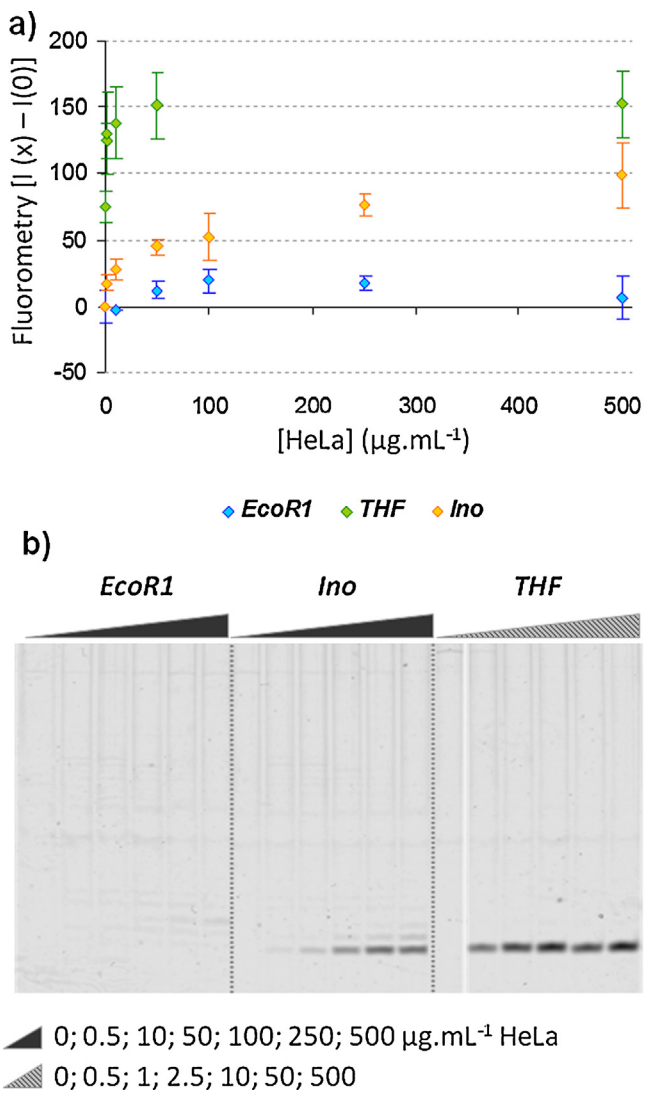


Fig. 4. Digestion of supported probes *EcoR1*, *Ino* and *THF* by HeLa nuclear extract (µg mL^{-1}). (a) the fluorescence of supernantants are analyzed by spectrofluorometry and normalized to the negative Control for each supported probe. (b) PAGE analysis of supported probes' cleavage from supernatants.

aldehydic form of the AP site, forming a stable oxime ether intermediate which deactivates the substrate [42–44]. We checked whether the functional fluorometric assay could highlight this specific inhibition. To address that point, *Ino* and *THF* probes were incubated with 500 µg mL^{-1} of HeLa nuclear extract and an increasing concentration of Mx. The nuclear extract concentration was chosen based on our results mentioned above. Gel electrophoresis and spectrofluorometry analyses (Fig. 5) demonstrate that cleavage of the *Ino* probe was inversely correlated to Mx concentration, while the cleavage rate for the *THF* probe remained unchanged. These results were consistent with the mechanism of action of the alcoxyamine derivative. Indeed, the tetrahydrofuran cycle, which does not proceed through formation of an opened aldehydic form, cannot react with amines to create a stable abortive intermediate like the one that occurs with the real AP site formed by the action AAG on the *Ino* DNA probe. It is noteworthy that high concentrations of Mx have to be used in order to visualize the effect of this compound on APE1 activity. The non-selectivity of the inhibition mechanism induced a non-negligible off-target effect previously described [45].

4. Discussion

The aim of this study was to develop a new sensitive, simple and cost-effective assay to monitor DNA-glycosylases and AP-endonucleases, which have been identified as new promising targets in the frame of anticancer research [46–51]. Magnetic beads have already proved to be well-suited in enzymatic and immunoassays thanks to their ease of handling and reduced cost [52–54]. Such an assay was developed by Xia and co-workers to detect alkyladenine glycosylase activity, using radioisotopes which limits that approach [27]. Our strategy is based on the pre-immobilization of fluorescent probes on streptavidin-coated magnetic beads. The performance of our assay was compared to the classically used polyacrylamide gel electrophoresis (PAGE), which has historically employed radioactively labelled oligonucleotides [11,12,55,56]. PAGE requires low quantities of probes (20 fmol–1 pmol) which confers the great sensitivity of the assay. However, constraints related to the use of radioactivity lead to the substitution of radioisotopes by safe and stable fluorophores, with only slightly lower sensitivity [57]. Nevertheless, this assay still necessitates a long separative step that limits the technique's throughput. In

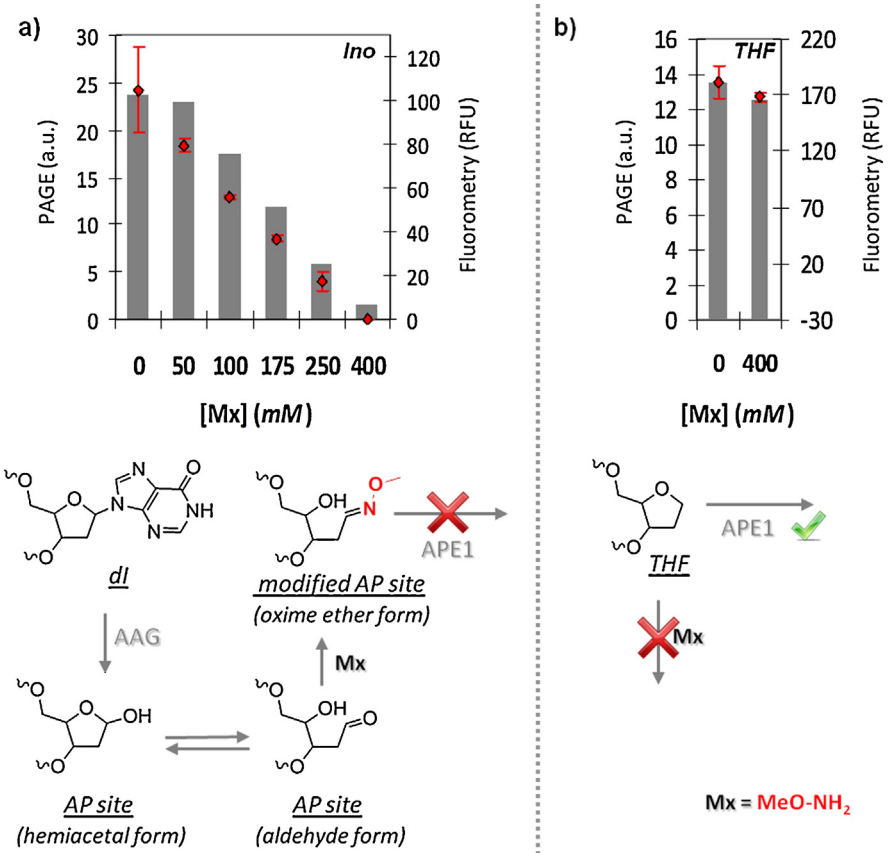


Fig. 5. Inhibition of AP-endonuclease in HeLa nuclear extract by methoxyamine. 1 pmol of on-bead DNA probe *Ino* or *THF* was digested by 500 μg mL^{-1} of HeLa nuclear extract with an increasing concentration of methoxyamine: PAGE analysis (histogram: left scale) compared to fluorometric analysis on the Nanodrop ND-3300 (diamonds: right scale), (a) *Ino* probe; (b) *THF* probe.

our fluorometric assay, approximately 1 pmol of oligonucleotide is used for each test that is close to previously reported studies. Moreover, the preparation of functionalized microbeads is very fast (<1 h for all probes) due to the well-known streptavidin-biotin linkage. Additionally, the magnetic isolation of unreacted probes allows for a single end-point fluorescence reading of the supernatant after the incubation with the sample (purified enzymes, nuclear extracts), still thanks to the immediate and quantitative separation of uncleaved probes.

Cleavage assays with purified proteins suggest that the beads do not altered the accessibility of restriction enzymes Pst1 or EcoR1, nor AAG or APE1. The detection of purified AAG and APE1 with the fluorometric assay presents a detection limit (10^{-3} U AAG and 10^{-2} U APE1) and a dynamic range similar to the PAGE technique (Fig. 2 and S3). The detection limit for purified APE1 is also in accordance with the recently reported study using a THF modified molecular beacon [17]; however, this assay needs 500-fold more substrate per test than our present procedure. In another study, Maksimenko et al. employed molecular beacons to monitor purified base excision repair activities, using 4 pmol of DNA probes in a final volume of 0.4 mL [20]. In this case, the advantage of our assay is that the fluorescence reading is recorded in microvolume on a droplet spectrofluorometer, which allowed the miniaturization of the assay with a minimal amount of substrate.

Preliminary results showed that the conjugation of DNA hairpin probes to microbeads prevents the degradation of oligonucleotides by 3′-exonucleases present in cell-free extracts. This observation guarantees the specificity of the assay in complex media. We found that the *in vitro* assay was very sensitive, requiring less than 10 and 500 ng of total proteins (HeLa nuclear extracts) to detect APE1 and AAG, respectively. In comparison, a previous work using a damaged molecular beacon in solution mentioned a detection limit of 500 ng of proteins (HeLa nuclear extracts) to quantify APE1 activity [17].

5. Conclusion

This work was dedicated to the development of a new functional assay for detecting and quantifying base excision repair activities from purified enzymes or in complex cell extracts with non need of sophisticated equipment. The designed is based on the immobilization of fluorescent DNA nanoprobes on magnetic beads that target DNA-glycosylases, AP-endonuclease as well as restriction enzymes. Fluorescence measurements can be recorded on a microliter format spectrofluorometer which permits the miniaturization and the parallelization of the assay. This device reports on the rate of BER activities and therefore it would be useful to monitor the DNA repair capacities of complex media such as crude cell extracts from cancer cells, to be used for diagnostic or prognostic applications. Another promising application would be the screening of chemical compounds to identify specific enzymatic inhibitors as additional therapeutic agents in chemo- and radiotherapies [47,51,58–61]. Current studies are underway in our laboratory to extend this assay to a multiplexed format to simultaneously quantify different enzymatic activities from a single biological sample.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aca.2013.12.038.

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