Enrichment and fluorogenic labelling of 5-formyluracil in DNA†

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Recently, the detection of natural thymine modified 5-formyluracil has attracted widespread attention. Herein, we introduce a new insight into designing reagents for both the selective biotin enrichment and fluorogenic labelling of 5-formyluracil in DNA. Biotinylated α-phenylenediamine directly tethered to naphthalimide can switch on 5-formyluracil, under physiological conditions, which can then be used in cell imaging after exposure to γ-irradiation. In addition, its labelling property caused the polymerase extension to stop in the 5-formyluracil site, which gave us more information than the fluorescence did itself. The idea of detecting 5-formyluracil might be used in the synthesis of other modified diaminofluoresceins.

Introduction

The recent discovery of formylpyrimidines in genomic DNA has energized the field of epigenetics. 5-Formyl-2′-deoxycytidine (5fC) and 5-formyl-2′-deoxyuridine (5fU) have been identified as crucially important forms of canonical nucleoside modifications that play significant roles in gene expression1 or are regarded as oxidative lesions that lead to gene regulation, such as introducing mispairing, causing genotoxic lesions, inducing perturbations of DNA function and altering DNA structures.2

Methods to sensitively and selectively detect formylpyrimidines have the potential to facilitate an improved understanding of epigenetics. 5fC can be effectively labelled by amine,3 hydrazine,4 aminooxy,5 indantrione6 and indole derivatives.7 However, research on highly tagging 5fU, where 5fC and the abasic sites (AP) cannot disturb the detection of 5fU, has been sparse. Matsuda and co-workers first realized a breakthrough in the highly selective fluorescence “switch-on” of 5fU in a 100 mM NaOH solution with a high signal-to-noise ratio after reacting it with the reagent bis[4,5-dimethoxyaniline-2-yl]disulfide.8 However, this reagent is not suitable for the enrichment of 5fU because it can also react with AP, though it cannot disturb the fluorogenic detection of 5fU. In 2015, Höbartner and co-workers reported a remarkable and significant fluorogenic labelling method towards 5-formyluracil in both DNA and RNA at pH 6.0 and 45 °C, for 6 h, by the indole reagent.9 However, this also could not be used in imaging 5fU in cells and is not easily modified to enrich 5fU in the genome. Balasubramanian and co-workers explored a biotinylated α-phenylenediamine linker that can selectively enrich fragments containing 5fU in DNA by exploiting the chemoselective reactivity of the aldehyde present in 5fU.9 The research was timely, systematic and noteworthy. However, when α-phenylenediamine reacted with 5fU to form a benzimidazole, there was no fluorescence, except under acidic conditions, according to our previous report.10 5-Hydroxymethyl-2′-deoxyuridine (5hmU) is easily oxidized to form 5fU by KRuO4,11 and recent research revealed that 5hmU can be an important epigenetic mark because thymidine (T) can be enzymatically oxidized by TET enzymes to generate the 5hmU form during mouse embryonic stem cell differentiation.12 Thus, we wanted to find a reagent that can not only realize the fluorescence “switch-on” of 5fU but also enrich 5fU through linking with a biotin tag that can be used in the streptavidin-coated magnetic bead system. Reagents that can fluorescently label target nucleosides and enrich them have potential applied value in mapping target nucleosides through the nanochannels.12 To image 5fU in the cell, the reaction should be under physiological conditions (37 °C, PS buffer and neutral pH value), which provides even further requirements in order for us to design a reagent to meet all of these demands.

Herein, we explored a strategy to solve this problem. In designing a reagent to selectively fluorescently tag 5fU, we observed a series of compounds in which α-phenylenediamine was linked to the fluorophore (diaminofluoresceins).13 Due to the photoinduced electron transfer (PET) effect, α-phenylenediamine turns off the fluorescence of the fluorophores naphthalimide,14 BODIPY15 and cyanine.16 α-Phenylenediamine is also an effective trapper of 5fU.17,18 Thus, in Scheme 1, α-phenylenediamine is designed not only as an electron donor (a}

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fluorescence quencher) for naphthalimide but also as a 5fU
trapper that is directly tethered to the imide position of naph-
thalimide. The polyethylene glycol linker both makes the overall
reagent more hydrophilic and allows a greater distance between
the biotin and the reactive site.

Results and discussion
Evaluating the reactivity of 5-formyluracil with the compound
Lyso-NINO
To explore the feasibility of this approach, we first evaluated the
reactivity of 5fU with the compound Lyso-NINO, a two-photon
fluorescent probe known to detect endogenous NO in cells,14
that had the same o-phenylenediamine linked to the naph-
thalimide structure (Fig. 1a). Lyso-NINO reacted with 5fU in
methanol to generate a fluorescent nucleotide named LysU
(Fig. S1†). The absorbance and fluorescence emission properties
of LysU were investigated in various buffer solutions. The
absorbance was detected at 439 nm, and the fluorescence
emission maxima were found at 530 nm. (Fig. S15a and S15c†). We
then used a 15-mer oligodeoxynucleotide ODN-5fU containing
one 5fU site as a model reaction test, which was
synthesized using an efficiently protected 5-formyluracil phos-
phoramidite reported recently.34–37 We incubated Lyso-NINO
with ODN-5fU under optimized conditions (50 mM PS buffer, pH 7.0, 37 °C, and 4 h). The RP-HPLC (monitored at 260 nm and 439 nm) analysis showed complete conversion to the new
product ODN-LysU (Fig. 1b). Lyso-NINO labelled DNA was
identified by MALDI-TOF (Fig. S4 and S5†) to ensure the reaction
integrity. As for the selectivity, we also treated ODN-T (with the
5fU site replaced by T), ODN-5fC (with the 5fU site replaced by
5fC), and ODN-AP (with the 5fU site replaced by AP) as the
negative controls. The RP-HPLC (monitored at 260 nm and 439 nm) analysis showed no reaction in the DNA controls.

Evaluating the reactivity of 5-formyluracil with the
biotinylated reagent Biotin-Lys
With these encouraging findings, we commenced synthesizing
the biotinylated reagent Biotin-lys (Fig. S2†). The compound can
also react with 5fU to generate a fluorescent nucleotide named
Biotin-lysU (Fig. S3†). The absorbance and fluorescence emission
analysis (Fig. S15b and S15d†) showed that the fluorophore
naphthalimide was PET-quenched by the electron rich amino
substituents of o-phenylenediamine; however, the conversion to
the benzimidazole functionality resulted in a turn-on emission
because of the blocking of the PET quenching. Next, we treated
ODN-5fU with the reagent Biotin-lys under the same conditions
as with Lyso-NINO for different times (from 0 to 4 h). From the
PAGE analysis results (Fig. S16d†), 4 h is long enough to
produce a complete reaction. The RP-HPLC (monitored at 260 nm and 439 nm) analysis showed complete conversion into the
new product ODN-biotinlysU (Fig. 3a). The biotin-lys labelled DNA was identified by MALDI-TOF (Fig. S6 and S7†) to
ensure the reaction integrity. As for the selectivity, we also
treated ODN-T (with the 5fU site replaced by T), ODN-5fC (with
the 5fU site replaced by 5fC), and ODN-AP (with the 5fU site
replaced by AP) as the negative controls. The RP-HPLC (moni-
tored at 260 nm and 439 nm) analysis showed no reaction in the
DNA controls (Fig. S11–S13†). We also attempted to detect
different ODNs through PAGE analysis. Due to the good fluo-
rescence properties of ODN-LysU, the fluorogenic labelling DNA band can be directly detected using the Pharo
FX Molecular imager (Bio-Rad, USA) (λex: 488 nm), while the
others cannot be seen. The gel was then stained with Gel Red
to obtain the other DNA bands (λex: 532 nm). The slower
migration of ODN-LysU was due to its larger molecular weight
(Fig. 1c). The exciting selectivity of the fluorogenic labelling of
5-formyluracil in DNA can also be verified by the fluorescence
readout. A dramatic fluorescence enhancement (λex: 439 nm,
λem: 530 nm) for 5fU can be observed compared to that of
other oligodeoxynucleotides (ODN-T, ODN-5fC, ODN-AP) (Fig. 1d).

Examining the status of 5-formyluracil at a particular position
on the target DNA
To further explore whether the strategy can be used to examine
the status of 5fU modification at a particular position on the
target DNA, we used ODN2-5fU as a model test using a similar
method described by Höbartner et al.7 The 5fU site of ODN2-T
was replaced by T, whereas the other sites were the same as
that in the ODN2-fU sequence; ODN2-LysU and ODN2-fU were
then incubated with the reagent Lyso-NINO. The site-specific
analysis of ODN2-fU in primer-extension assays showed that
the strategy-labelled nucleosides may act as a “roadblock”
to abort the primer extension by the Bsu DNA polymerase (NEB) in
a reaction time of 1 min or 3 min and enable the detection of
5fU (Fig. 2).
the reagent could be used to obtain 5fU quantitative information on the specific site in the DNA sample, although the fluorescence detection could only supply the overall 5fU quantitative information of the DNA sample via fluorescence intensity detection by a fluorescence detector device. Firstly, we verified the possibility of Biotin-lysU aborting the primer extension. ODN2-5fU was incubated with Biotin-lys as described before. T and 5fU were bypassed by Bsu DNA polymerases, while Biotin-lysU acted as a “roadblock” to abort the primer extension in the reaction times of either 1 min or 3 min (Fig. S16a†). We then generated a model mixture by spiking known amounts (0–100%) of ODN2-5fU into the samples of the unmodified DNA analogue ODN2-T, followed by labelling with Biotin-lysU. The Bsu DNA polymerase was used in the next primer extension assay, and the larger amounts of Biotin-lysU labelled 5fU sites acted as a stronger pause in the polymerase extension process (Fig. 3d). These results demonstrated that the reagent can have immediate application in identifying the status of the 5fU modification at a particular position on the target DNA.

### Fluorescence images of γ-irradiated Hela cells

Pouget and Wang et al. reported that different cells exposed to γ-rays would generate 5fU in detectable amounts.18 We next exposed Hela cells to 60Co irradiation at 1044 Gy (17.4 Gy min⁻¹, 60 min) and at room temperature. The unexposed cells were maintained under the same conditions as the negative controls. We then incubated Biotin-lysU (10 μM, containing 1% DMSO in DMEM) with the cells at 37 °C for 4 h. Cell imaging was
performed on a confocal laser scanning microscope (Nikon C1-si TE2000, Japan) after washing the cells with PBS (0.01 M) three times. The remarkable sensitivity of Biotin-lys was demonstrated by the cell imaging (Fig. 4). Only the cells that were treated with \( \gamma \)-rays, generating 5fU, yielded a notable green fluorescence. To further verify the accuracy of the Biotin-lys reagent reacting with 5fU, we next digested the DNA from the \( \gamma \)-irradiated Hela cells using Degradase Plus (Zymo Research, USA). The LC-MS data showed the exact generation of the Biotin-lysU nucleotide in the DNA from the \( \gamma \)-irradiated Hela cells (Fig. S14†).

Thymidine derivatives like 5hmU and 5fU generally display poor sensitivity when measured by LC-MS/MS, which is likely attributed to their relatively poor proton affinity. Conjugation with the probe may enable the sensitive detection of the modified pyrimidine nucleoside by LC-MS/MS in the future. As a more rigorous consideration, ionizing radiation may produce other reactive aldehydes in DNA that may also conjugate with the probe (e.g. certain deoxyribose breakdown products or some DNA adducts arising from by-products of lipid peroxidation). Thus, the fluorescence signal increase observed in Fig. 4 might not be attributed entirely to 5fU in the DNA. More sensitive methods for the in situ detection of low abundant 5fU in normal cells or tissues are also needed.
Enriching 5-formyluracil in DNA fragments

Finally, we applied the reagent in enriching DNA fragments that contain 5fU. Firstly, we exploited an 80-mer single-stranded ODN bearing two 5fU modifications (ODN-SS-fU) and its canonical analogous ODN sequence (ODN-SS-fC) and its canonical analogous ODN sequence (ODN-SS-T) as a control. After incubation with Biotin-lys (50 mM PS buffer, pH 7.0, 37 °C, 6 h), these DNA fragments were enriched by streptavidin-coated magnetic beads. The following qPCR analysis showed a 101-fold enrichment of ODN-SS-fU over ODN-SS-T (Fig. 5 and S17†), and the PAGE analysis also showed the desirable property of the reagent tagging the 5fU. Only the DNA containing 5fU can react with this reagent (Fig. S16b and S16c†). These data demonstrated the effective selective enrichment of 5fU towards its C modification analogue 5fC. We then made a mixture of double-stranded DNA bearing two specific sites per strand (ds-DNA-fU, ds-DNA-fC, and ds-DNA-T), as described by Balasubramanian et al.6 We treated it using an optimized pull-down procedure (Fig. S17†). The qPCR results showed a 73-fold enrichment of ds-DNA-fU over ds-DNA-T and a 1.1-fold enrichment of ds-DNA-fC towards ds-DNA-T (Fig. 5 and S17c-e†). The enrichment fold verified the selective capture of 5fU through this reagent. However, the consequence is moderately poorer than that of a biotinylated ω-phenylenediamine linker (~150-fold).8 In the future, the design of a compound to selectively enrich 5fU in the genome with less steric hindrance and more space between the reactive site and the biotin substitution that can easily be eluted from the streptavidin coated magnetic beads could be considered.

Conclusions

In conclusion, we created a biotinylated ω-phenylenediamine directly tethered to naphthalimide that can not only fluorescently tag 5-formyluracil under physiological conditions but also enrich it in DNA. Its remarkable fluorogenic properties made it possible to obtain 5fU quantitative information on the specific site in the DNA sample and imaging cells after their exposure to γ-irradiation. In addition, the strategy for designing a compound to detect 5-formyluracil might be suitable for other diaminofluoresceins.

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