Synthesis and Evaluation of Methylene Blue Oligonucleotide Conjugates for DNA Interstrand Cross-Linking

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ABSTRACT

Efficient DNA interstrand cross-linking can be achieved with furan containing oligonucleotide probes upon activation by singlet oxygen ($^1O_2$). Previously, we have described how this can be achieved by irradiation of these furan probes with visible light in the presence of photosensitizers. Now, in an effort to explore cross-linking under conditions that are representative for experiments in cellular context, the furan mediated oligonucleotide cross-linking was investigated at low oligonucleotide concentrations, ensuring a sufficiently high local concentration of singlet oxygen by attaching the sensitizing methylene blue moiety to the oligonucleotide complementary to the furan modified strand. Four methylene blue-oligonucleotide conjugates were synthesized, each with a different positioning of methylene blue with respect to the furan unit present on the complementary strand. The conjugates were evaluated for singlet oxygen generation and subsequent cross-linking ability. It was observed that not only the distance of the $^1O_2$ source to the furan unit, but also the specific interaction of methylene blue moiety with the duplex, which is position dependant, influences cross-linking yields.

1. INTRODUCTION

Interstrand cross-linking (ICL) between oligonucleotides has been the subject of interest in multiple interdisciplinary fields. The possibility to induce these cross-links can be used to gain insight in DNA repair mechanisms effective in biological systems. Indeed, a DNA abasic site, which is a well-known form of DNA damage [1,2], can react with a guanine residue of the opposite strand resulting in cross-link formation [3]. To understand the mechanism whereby the repair of these defaults occurs, a need for easy accessibility to cross-linked oligonucleotides is created [4]. In addition to this, the inherent high selectivity of DNA hybridization can be exploited for gene regulation, were an oligonucleotide can selectively block a complementary sequence [5, 6]. Sustained blockage and thus more effective regulation could be reached by introducing a covalent linkage to the oligonucleotide target.

Different chemical procedures have been developed to selectively induce ICLs upon photochemical activation [7, 8, 9, 10 and 11]. Our group previously reported upon the formation of interstrand cross-linking through the use of furan modified oligonucleotides [12, 13, 14]. Originally, N-bromosuccinimide (NBS) was used to ensure oxidation of the furan unit leading to the formation of a very reactive 4-oxo-enal (figure 1). This moiety is prone to react with exocyclic amines present on the bases of the complementary strand with the formation of an interstrand cross-link.

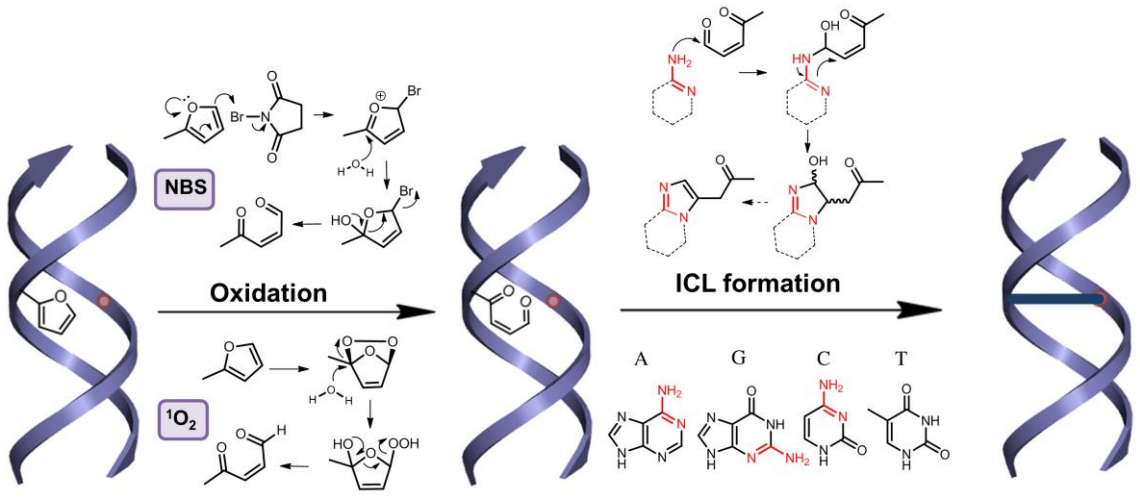

Figure 1: Schematic representation of furan mediated DNA cross-linking. The furan group can be oxidized using NBS or singlet oxygen ($^1O_2$). After oxidation, the formed reactive moiety can react with exocyclic amines of the opposite base on the complementary strand. Both adenine (A) and cytosine (C) contain an exocyclic amine which is located in close proximity of the furan building block, leading to the selective formation of a covalent bond as depicted on the right-hand side of the figure.

To increase the biocompatibility of this cross-linking methodology, a more biologically compatible and sustainable oxidation method was needed. Singlet oxygen ($^1O_2$) was shown to also have the capability to selectively oxidize the furan unit leading to the formation of an interstrand cross-link [15] (figure 1). $^1O_2$-generation can occur in a highly controllable manner, which constitutes an important step towards the biological applicability of the furan cross-linking methodology. The furan unit reacts with singlet oxygen through a [4+2] cycloaddition [16] generating a reactive intermediate. Singlet oxygen is formed by irradiation of a photosensitizer (PS) with a specific wavelength characteristic for each sensitizer [17]. By carefully choosing a sensitizer which can be exited by light from the visible region, a cross-linking method is now available where the use of invasive UV-radiation can be avoided.

We here aimed at investigating the furan-mediated cross-link reaction at physiologically relevant concentrations. Indeed, oligonucleotide concentrations above 20 µM have shown to lead to a loss in sequence specificity [18]. However, as the lifetime of singlet oxygen in

63 aqueous medium is limited [19], the distance between the site where it is generated, i.e. the
64 photosensitizer, and the furan moiety to be activated can play an important role, rendering the
65 cross-link reaction inherently concentration dependent. In order to ensure a sufficiently high
66 local concentration of the photosensitizer, we decided to couple it to the strand complementary
67 to the furan modified oligonucleotide. By doing so, the influence of the position of the
68 photosensitizer within the oligonucleotide, on singlet oxygen generation and subsequent cross-
69 linking can be investigated. To determine which photosensitizer is best suited for attachment to
70 the oligonucleotide, results of cross-linking experiments with a variety of photosensitizers
71 added in solution were compared. The PS which showed most promise in these experiments
72 was then chosen for incorporation. Furan building block **X** (Figure 2), chosen based on
73 previous research in our lab in which different building blocks were synthesized and evaluated
74 on their $^1O_2$-cross-linking capacities [15], was incorporated in the oligonucleotide sequence
75 FON1 (Table 1). Methylene blue, rose bengal, ruthenium tris bipyridinium and zinc
76 phthalocyanine were selected for screening of their capacity to induce cross-linking. After
77 optimization of the cross-linking conditions, the chosen photosensitizers were evaluated on the
78 cross-linking yields (as calculated from HPLC chromatograms) against the observed
79 competitive degradation of the oligonucleotide strands. Based on these results, methylene blue
80 was chosen for conjugation to the oligonucleotide. To this end, a reactive form of the sensitizer
81 was synthesized after which it was coupled to the oligonucleotide and cross-linking at low
82 oligonucleotide concentration could be executed.

83 ## 2. MATERIALS AND METHODS

2.1. Chemical synthesis: All chemical reagents and solvents were purchased from Sigma Aldrich.

2.1.1. Synthesis of methylene blue succinimide ester s2. The methylene blue carboxylic acid **s1** was synthesized (supporting information, figure S5) and subsequently transformed into the succinimide ester **s2** as described by Barton et al. [20]. The structure of the synthesized compound was confirmed by ESI-MS (454 g/mol) $[M+H]^+$ in correspondence to [20]. The methylene blue succinimide ester **s2** was subsequently coupled to an oligonucleotide, containing an amino modified thymine base. This methylene blue modified thymine base is presented in Figure 2 (**M**).

2.1.2. Synthesis of acyclic furan phosphoramidite. An acyclic furan building block (Figure 2, **X**) was synthesized containing a furan moiety coupled to a phenyl group. The synthesis was carried out as previously described [13]. After conversion to the phosphoramidite, the furan building block was incorporated in the oligonucleotide through automated DNA synthesis.

2.2 DNA synthesis: DNA reagents and the thymine amino modified phosphoramidite (aminomodifier C6 dT) were obtained from Glen Research. All the oligonucleotides were synthesized DMT-on at 1 µmol scale on an ABI 394 DNA synthesizer. The synthesis proceeded through an automated phosphoramidite coupling cycle and was interrupted for a manual incorporation of the modified phosphoramidites. This coupling involved a repetitive application of a dry 0,05 M solution of the modified phosphoramidite in acetonitrile and a dry 0,1 M dicyanoimidazole solution in acetonitrile. The synthesized oligonucleotides were cleaved of the solid support by treatment with 1 mL of aqueous NH_4OH while shaking overnight at a temperature of 55°C. Purification with concurrent DMT removal of the synthesized oligonucleotides was

carried out using a Sep-Pak C18 cartridge obtained from Waters. The purity of the oligonucleotides was evaluated by RP-HPLC, recorded on an Agilent 1200 system equipped with a Phenomenex Clarity 110 Å C18 column (250 x 4,6 mm, 5 µM) or a Phenomenex Aeris Widepore column (150 x 4,6 mm, 3,6 µM), both used at 50°C. The mobile phases consisted of A/acetonitrile and B/0,1 M TEAA buffer containing 5% acetonitrile. Masses of the oligonucleotides were determined by MALDI-TOF analysis on an ABI Voyager DE-STR MALDI-TOF. The oligonucleotide samples were mixed with a matrix consisting of 3-hydroxypicolinic acid and ammonium citrate present in a 9:1 ratio, in a 1:1 sample:matrix ratio and a 1:2 ratio for the methylene blue conjugated oligonucleotides and the cross-linked species. After mixing with the matrix, the samples were desalted by treatment with DOWEX beads. The samples were spotted on a MALDI plate, together with a commercial oligonucleotide sequence with a known mass (5'-GCA TCT CGT CAG-3'), purchased from Eurogentec, for calibration of the measurement. The concentrations of the oligonucleotides were measured on the Trinean DropSense96 UV/VIS droplet reader. The various sequences which were synthesized are presented in table 1 and will be referred to by their assigned name in what follows.

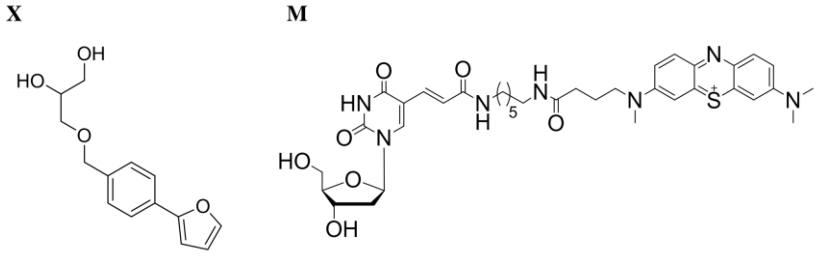

Figure 2: Structure of the furan and PS modified building blocks incorporated in the oligonucleotide sequences. (**X**): furan building block, (**M**) methylene blue building block

Table 1: Overview of the synthesized oligonucleotide sequences. The furan building block incorporated in the sequences is presented in Figure 2, **X**. The methylene blue building block is presented in figure 2, **M**. Both the non-modified sequences and the methylene blue modified sequences are presented from 5' to 3'. The furan modified sequences are presented from 3' to 5' to highlight the complementarity.

Non-modified sequences (**5'-3'**)	
ON1	5'-GCA CCC CGT CAG-3'
ON2	5'-GTA CCC TGT CTG-3'
Furan (**X**) modified sequences (**3'-5'**)	
FON1	3'-CGT G**X**G GCA GTC-5'
FON2	3'-CAT G**X**G ACA GAC-5'
Methylene blue (**M**) modified sequences (**5'-3'**)	
MON1	5'-GTA CCC **M**GT CTG-3'
MON2	5'-G**M**A CCC TGT CTG-3'
MON3	5'-GTA CCC TG**M** CTG-3'
MON4	5'-GTA CCC TGT C**M**G-3'

2.3. General procedure for conjugation of methylene blue to an oligonucleotide: The coupling procedure was based on the work of the group of K. Weisz [21] after which the methylene blue conjugated oligonucleotide was obtained by purification via RP-HPLC (linear gradient: 0 – 20% ACN in 30 min) (Supporting information, figure S6). The peak belonging to the product, which absorbed both at 260 nm and 600 nm, was collected and analyzed by MALDI-MS, indicating the correct product: exact mass = 4102,87 Da.; observed mass (MON1) = 4101,76 Da.; observed mass (MON2) = 4103,86 Da.; observed mass (MON3) = 4103,73 Da.; observed mass (MON4) = 4103,01 Da. HPLC and MALDI TOF-MS data can be found in the supporting information (Fig S8, S9, S10 and S11).

2.4. Cross-link protocol: Cross-link reactions were carried out at 20 µM oligonucleotide concentration (standard conditions in all previous experiments) and at an oligonucleotide concentration of 2 µM (and 1 µM described in the supporting information, section 5). The

oligonucleotides were dissolved in a 10 mM phosphate buffer (pH 7) containing 10 mM of sodium chloride. All cross-link experiments were repeated three times to ensure reliable and reproducible results. When cross-linking with the photosensitizer in solution, the sensitizer was added to the sample just before starting the reaction. With the diluted samples, cross-linking was preceded by an annealing procedure. This implied heating the samples to 95°C and keeping them at this temperature for 30 minutes while shaking. After this, the samples were allowed to slowly cool to room temperature over a time span of 3 hours, ensuring correct duplex hybridization. During the cross-link reaction the temperature was kept constant at 25°C, in an Eppendorf Thermomixer comfort with constant shaking at 950 rpm. The cross-link temperature is kept under the melting temperatures of the used oligonucleotide duplexes ensuring duplex formation at these conditions (Supporting information fig. S19). Irradiation of the samples was done using a Euromex fiber optic light source Ek-1 equipped with a color filter dependent on the used photosensitizer and placed 1 cm of the sample. For methylene blue and zinc phthalocyanine, the samples were irradiated with red light. For rose bengal, green light and for ruthenium tris bipyridinium blue light was used.

2.5. Cross-link analysis: Samples were taken at different sampling times. At every sampling point a sample was taken for RP-HPLC analysis and gel electrophoresis.

RP-HPLC analysis: Cross-link samples with the photosensitizer added in solution were measured with the RP-HPLC equipped with a Clarity C18 (linear gradient: 0 – 20% ACN in 30 min). Cross-link samples with the photosensitizer conjugated to the oligonucleotide were measured with the RP-HPLC equipped with an Aeris C18 column (linear gradient: 0-20% ACN in 45 min). Cross-link yields were determined by integration of the corresponding peaks in the HPLC-chromatogram and by comparing the area of the peak of the cross-linked species with the peak of the limiting

single oligonucleotide strand, both corrected for their extinction coefficient. The extinction coefficient of the duplex was calculated based on the method described by R. Owczarzy *et al*. [22]. *Gel electrophoresis:* Samples were analyzed by denaturing gel electrophoresis. The gels were prepared by dissolving 4,2 g urea in 5 mL acrylamide:bisacrylamide (19:1) and 1 mL 10x TBE buffer. 100 µL of a 0,5 M solution of ammonium persulphate was added and the solution was diluted to 10 mL with milliQ water. After cooling of the solution, N,N,N',N'-tetramethylethylenediamine was added and the sample was poured between glass plates after which it was allowed to polymerize for one hour. The gels were subjected to a pre-run in the consort EV202 at a voltage power of 225V for half an hour. After mixing the samples with formamide in 9:1 formamide:sample ratio, they were loaded on the gel. During a run of approximately one hour, the temperature was kept at 25°C with a Julabo F12. The gels were stained with GelRed^{TM} Nucleic acid gel stain (VWR) and photographed using an Autochemi imaging system.

3. RESULTS AND DISCUSSION

3.1 Selection of the most promising system: cross-linking with the photosensitizer in solution

We previously reported on a selective and high-yielding methodology for cross-linking of oligonucleotides using a furan modified oligonucleotide probe [12,13,14,23]. Various furan building blocks have been subjected to an extensive evaluation of their cross-linking properties. The high-yielding furan phenyl acyclic building block (Fig 2, **X**) was chosen out of this set for incorporation in the oligonucleotides in the current study. The observed cross-link

yield was not only dependant on the structure of the furan moiety, but also on the exact nature of the complementary base. As is depicted mechanistically in figure 1, the exocyclic amines present on cytosine (C) and adenine (A) can react with the oxidized furan moiety with the formation of an interstrand cross-link (ICL). Due to the lack of an amino group, thymine cannot act as a reaction partner. Interestingly, it was previously observed that guanine was not able to form cross-links, which can possibly be attributed to the lack of proximity between its exocyclic amino group and the furan moiety. Following this information, cytosine was chosen as complementary base in the study presented in this paper.

Oxidation of furan with singlet oxygen, followed by cross-linking, was already proven successful and reported earlier [15]. Still, a more extensive optimization of this strategy had to be undertaken. Therefore, in first instance, a set of different photosensitizers was tested in solution to select the most promising candidate for conjugation and subsequent cross-linking. Previously, we have extensively investigated the potential occurrence of collateral oxidative damage during the cross-link reactions. It was shown that under the currently used carefully fine-tuned conditions, no 8-oxo dG species were formed [15].

Four photosensitizers (zinc phthalocyanine, ruthenium tris bipyridinium, methylene blue and rose bengal) were tested on their capabilities to induce a cross-link between the furan modified oligonucleotide FON1 and its non-modified complement ON1 (table 1). Both the modified and the complementary sequence were synthesized using automated DNA synthesis. To incorporate the furan phosphoramidite, the synthesis was interrupted by a manual coupling step as described in the experimental section. To evaluate the cross-linking ability of the various photosensitizers, the reaction was followed using both RP-HPLC and denaturing

polyacrylamide gel electrophoresis. Samples consisted of 20 µM of the duplex, while various photosensitizer concentrations were tested.

It was observed that zinc phthalocyanine could not induce a cross-link reaction between FON1 and ON1 during irradiation with red light (figure 3B A1/A2). Furthermore, no degradation of both the furan modified oligonucleotide nor its complement could be observed, demonstrating the absence of singlet oxygen in the reaction mixture. This behaviour can be explained by the lack of solubility of zinc phthalocyanine in the aqueous medium. Ruthenium tris bipyridinium did induce cross-linking between FON1 and ON1 after irradiation with blue light. Concentrations of 1 µM, 2 µM and 5 µM of the photosensitizer were tested, where the latter concentration led to cross-link yields of 16% after 30 minute irradiation (HPLC data presented in figure 3B B1/B2, denaturating PAGE in figure 4A). Rose bengal was examined in concentrations gradually increasing from 0,5 µM until 5 µM, where it was noted that a concentration of 0,5 µM under green light irradiation led to cross-link yields up to 40 %. These high yields were reached after two hours of irradiation and were accompanied by minimal degradation. Increasing the concentration of the photosensitizer went hand in hand with faster reactions. By using only 1 µM of rose bengal, yields up to 35 % were reached after 15 minutes of irradiation (HPLC data presented in figure 3B C1/C2, denaturating PAGE in figure 4B). When using methylene blue, 25 minutes of red light irradiation resulted in a yield of 53% (HPLC data presented in figure 3B D1/D2, denaturating PAGE in figure 4C). These high yields together with its medical relevance, pointed out methylene blue as most promising sensitizer for furan based oligonucleotide cross-linking.

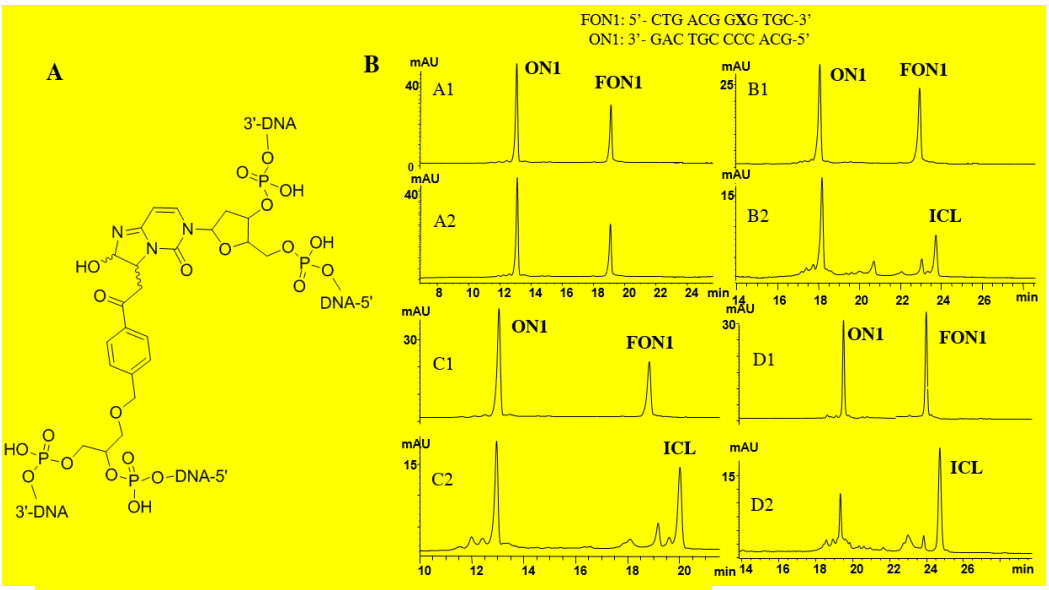

Figure 3: A. Chemical structure of the formed cross-linked species. B. RP-HPLC traces of the cross-link reaction mixtures containing 20 µM of the duplex. (A1, B1, C1, D1): Furan modified **FON1** and complementary sequence **ON1** before oxidation. (A2): After $^1O_2$ oxidation with zinc phthalocyanine (1 µM, 90 min, ICL yield: 0%), (B2): After $^1O_2$ oxidation with ruthenium tris bipyridinium (5 µM, 30 min, ICL yield: 16%), (C2): After $^1O_2$ oxidation with rose bengal (1 µM, 15 min, ICL yield: 35%), (D2): After $^1O_2$ oxidation with and methylene blue (5 µM, 25 min, ICL yield: 53%). Formation of a new peak is observed in B2, C2 and D2 which belongs to the interstrand cross-link (**ICL**).

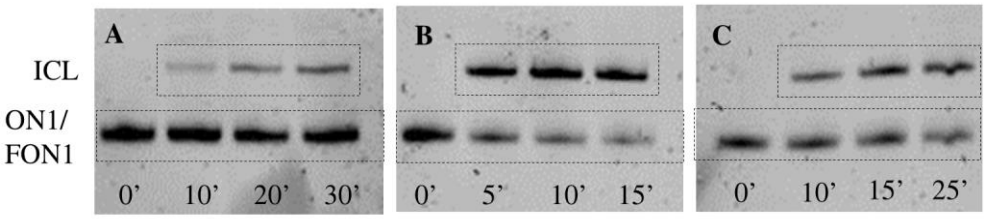

Figure 4: Denaturing PAGE analysis of the cross-link reaction mixtures containing 20 µM of the duplex. All samples contain the furan modified **FON1** and complementary sequence **ON1** before oxidation. All samples were irradiated with a characteristic wavelength for a period as depicted in the figure in presence of the photosensitizer: (A): ruthenium tris bipyridinium (5 µM, blue light, ICL yield: 16%), (B): rose bengal (1 µM, green light, ICL yield: 35%) and (C): methylene blue (5 µM, red light, ICL yield: 53%).

3.1.1. Towards lower oligonucleotide concentrations with methylene blue in solution. In a cellular context, when using high oligonucleotide concentrations, the selectivity for recognition of a specific sequence can substantially decrease (typically, intracellular concentrations above 20 µM have been shown to lead to non-sequence specific binding of oligonucleotides) [18]. Therefore, it has to be verified whether our furan-cross-linking system still functions at higher

dilution. A sample was prepared with FON2 and its complement ON2 (table 1), with the oligonucleotide concentration as well as the methylene blue concentration lowered to 2 μM. A new, thymine-rich sequence (ON2) was chosen to allow the later incorporation of the methylene blue modified thymine at different positions. It was seen that even after 50 minutes of irradiation of the sample with red light only a minimal amount of cross-link was formed (as depicted in fig S4 B1/B2/B3 in the supporting information). This result can be explained by the high dilution, resulting in a low local concentration of singlet oxygen around the furan moiety. Indeed, when methylene blue moves freely through the solution, distances between furan and methylene blue can increase in such a way that they become too large for singlet oxygen to cross. In order to ensure proximity between methylene blue and the furan modified oligonucleotide, methylene blue was conjugated to the strand complementary to the furan modified sequence.

3.2 Cross-linking experiments with methylene blue immobilized on an oligonucleotide strand

Before studying the cross-link formation with diluted oligonucleotide samples, it had to be evaluated whether methylene blue, when attached to an oligonucleotide, is still able to generate a sufficient amount of singlet oxygen to induce ICL formation. To evaluate this, methylene blue was attached to an oligonucleotide sequence by modifying a thymine base. To assess the influence of the position of methylene blue on duplex formation and on the cross-linking process, it was incorporated at four different positions so that within each modified duplex a different distance between the furan building block and methylene blue is ensured. Amino modified oligonucleotides were obtained through automated DNA synthesis, using a

277 commercially available amino modified thymidine phosphoramidite (containing the rather long
278 CHCHCONH$(CH_2)_6$-NH_2-linker, figure 2 (**M**)) was incorporated by manual coupling.
279 Conjugation of the methylene blue derivative **s2** (supporting information, fig S5) with the
280 oligonucleotide could be achieved by reacting the amino modified oligonucleotide with the
281 succinimide ester of methylene blue. Four methylene blue modified sequences were
282 synthesized, MON1, MON2, MON3 and MON4 (table 1), all complementary to the furan
283 modified oligonucleotide FON2. Only one nucleotide is positioned between methylene blue
284 and furan when cross-linking in sequence MON1, two nucleotides in MON2, three in MON3
285 and five in MON4. The composition of the cross-linking samples was identical to the samples
286 discussed in previous paragraphs, all containing 20 μM of the oligonucleotide duplex but
287 lacking the methylene blue added in solution. When comparing the maximum cross-linking
288 yields of MON1 (figure 5 A1/A2), MON2 (figure 5 B1/B2), MON3 (figure 5 C1/C2) and
289 MON4 (figure 5 D1/D2) to FON2, it can be seen that a maximum yield of approximately 20%
290 is reached in all cases at an irradiation of 3,5 minutes to 5 minutes. This result shows that the
291 conjugated methylene blue is able to promote the formation of an ICL. The minor differences
292 in the observed cross-link yields do not show a trend with reference to the varying distance
293 between furan and methylene blue. This lack of correlation can be due to the higher
294 oligonucleotide concentration of the samples. Both singlet oxygen generated by MB on the
295 opposite strand and singlet oxygen generated by a nearby duplex can be expected to induce
296 furan oxidation, as depicted schematically in figure 6.

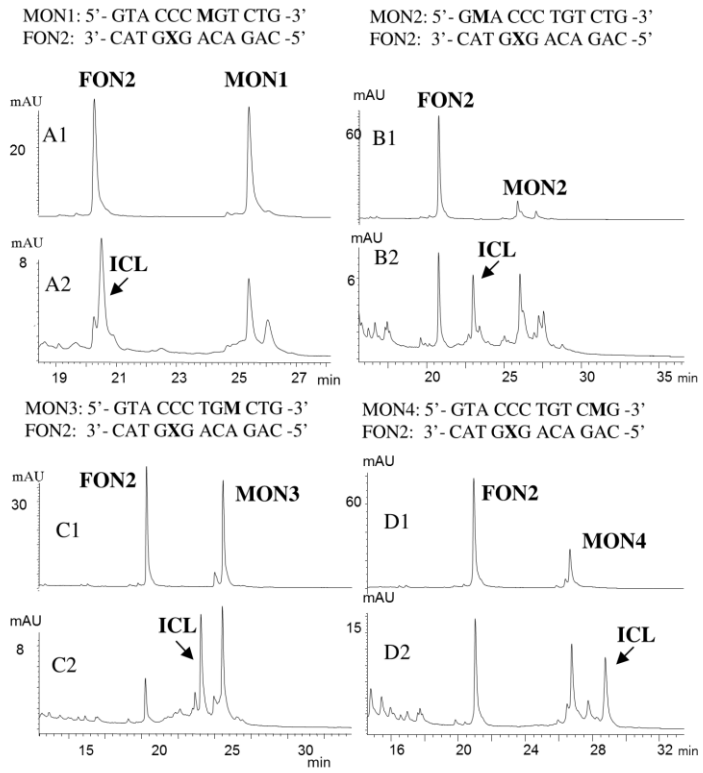

Figure 5: RP-HPLC of the 20 µM cross-link reaction mixtures. (A1): Furan modified **FON2** and the complementary methylene blue modified **MON1** before oxidation, (A2): After $^1O_2$ oxidation (3,5 min, ICL yield: 22%) (B1): Furan modified **FON2** and the complementary **MON2** before oxidation, (B2): After $^1O_2$ oxidation (5 min, ICL yield: 26%), (C1): Furan modified **FON2** and the complementary methylene blue modified **MON3** before oxidation, (C2): After $^1O_2$ oxidation (3,5 min, ICL yield: 22%) (D1): Furan modified **FON2** and the complementary methylene blue modified **MON4** before oxidation, (D2): After $^1O_2$ oxidation (5 min, ICL yield: 23%). A new peak appears in the spectrum which is assigned to the interstrand cross-link (**ICL**). Yields are calculated by comparing the peak area of the ICL with the limiting single strand, both corrected for their extinction factor. Due to the different structures of the formed ICLs, depending on the position of methylene blue on the cross-linked duplex, different retention times of the cross-linked species are observed.

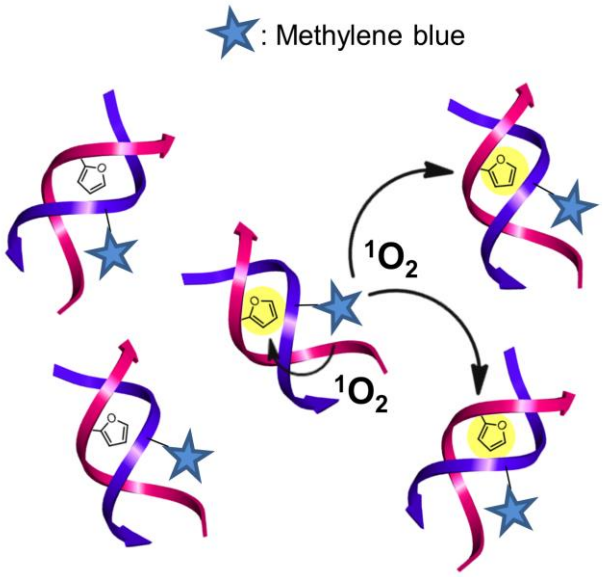

Figure 6: Schematic representation of the oxidation of the furan unit by both singlet oxygen generated by methylene blue present on the complementary strand as well as methylene blue present on other duplexes.

The cross-link reaction was also analyzed by denaturating PAGE experiments (supporting information, figure S13). Even though similar yields are obtained as in case of cross-linking with the photosensitizer in solution, these gels do not show clear bands. Indeed, methylene blue absorbs light in the same region as where the GelRed^{TM} nucleic acid stain emits light. Therefore, visualization of methylene blue conjugated oligonucleotides and their corresponding methylene blue conjugated duplexes required higher sample loading, which results in bands which are not as delineated and clear as in case of their non-methylene blue modified analogues.

3.2.2 ***Towards lower oligonucleotide concentrations with a conjugated methylene blue moiety.*** It was shown that cross-linking of oligonucleotides became very low-yielding when diluting the samples (vide supra). Since this observation probably can be explained by the low local photosensitizer concentration resulting from the large distance between methylene blue and furan, it was expected that by attaching methylene blue to the duplex, higher yields could

be obtained. Indeed, methylene blue and furan are now forced to remain in close proximity. MON1, MON2, MON3, MON4 and the furan modified FON2 were mixed in a 2 μM concentration, retaining the same sample composition as previously described. Maximum yields were obtained after two to five minutes of irradiation with red light. Here, in contrast to the experiments carried out at low concentration with an externally added photosensitizer, quite efficient cross-linking could be achieved. Also, different yields were obtained depending on the exact position of the conjugated methylene blue. A yield of 33% was reached when one base pair is located between MB and furan (figure 7 A1/A2). With two base pairs, yields up to 24% were observed (figure 7 B1/B2) and with three base pairs only 12% cross-link is formed (figure 7 C1/C2). When five base pairs are located between the furan unit and methylene blue, again a yield of 28% is reached (figure 7 D1/D2).

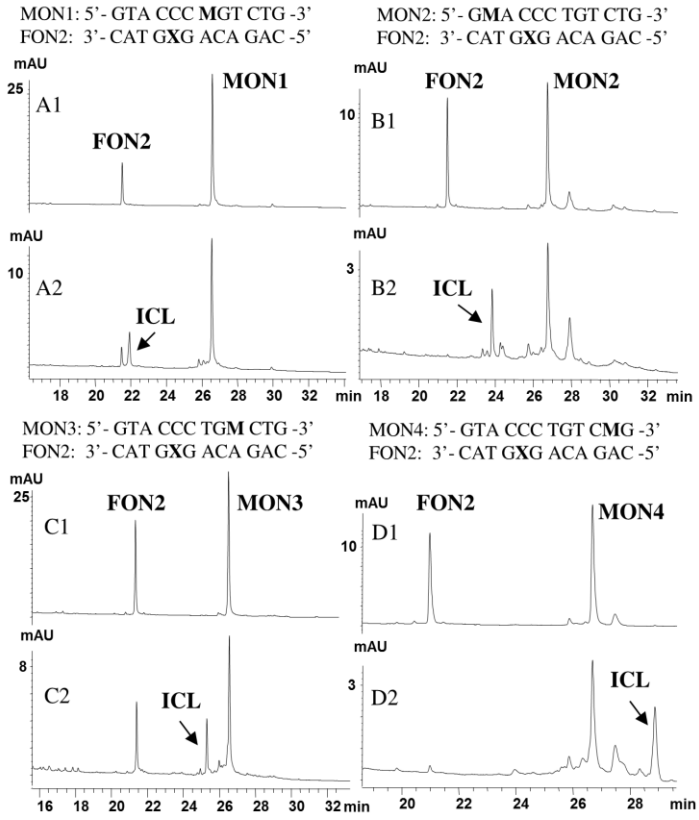

Figure 7: RP-HPLC of the diluted 2 µM cross-link reaction mixtures. (A1): Furan modified **FON2** and the complementary methylene blue modified **MON1** before oxidation, (A2): After $^1O_2$ oxidation (5 min, ICL yield: 33%),(B1): Furan modified **FON2** and the complementary **MON2** before oxidation, (B2): After $^1O_2$ oxidation (2 min, ICL yield: 24%) (C1): Furan modified **FON2** and the complementary methylene blue modified **MON3** before oxidation, (C2): After $^1O_2$ oxidation (5 min, ICL yield: 12%) (D1): Furan modified **FON2** and the complementary methylene blue modified **MON4** before oxidation, (D2): After $^1O_2$ oxidation (3,5 min, ICL yield: 28%). The new peak appearing in the spectrum belongs to the interstrand cross-link (**ICL**). Yields are calculated by comparing the peak area of the ICL with the limiting single strand, both corrected for their extinction factor.

This fluctuation of cross-link yield shows that not only the distance between methylene blue and furan influences the yield, but other factors are of importance. When one base pair is located between furan and methylene blue, the highest cross-link yields are obtained. This can be accounted to the high proximity between furan and methylene blue and thus the short distance singlet oxygen has to travel to oxidize the furan unit, leading to ICL formation. Increasing the number of base pairs between the furan moiety and the sensitizer gradually decreases the cross-link yield. Whereas for both the MON1-FON2 and MON2-FON2 duplexes, T_m analysis shows a clear stabilization of the duplex compared to ON2-FON2, indicating methylene blue intercalation, this is clearly not the case for the MON3-FON2 and MON4-FON2 duplexes (supporting information, figure S19, table S3). As both these duplexes feature a longer distance between the furan and methylene blue moiety, as well as the absence of methylene blue intercalation, it is surprising to see that the observed ICL yield increases again (MON4-FON2: 28% yield versus MON3-FON2: 13% yield), a trend which is consistently observed (as also shown for 1 µM experiments described in the supporting information section 5, figure S20). The explanation of this unexpected increase in yield can perhaps be found in an alternative way of binding between the methylene blue unit and the duplex. Methylene blue is known to also electrostatically interact with oligonucleotide backbones[24], which does not influence the duplex stability and thus the T_m behavior. The larger non-disturbed sequence between methylene blue and furan in MON4-FON2 potentially

promotes electrostatic methylene blue-duplex association, thus increasing again proximity between furan and the $^1O_2$-generating moiety, potentially explaining the observed trends.

4. CONCLUSIONS

Whereas at 20 μM of oligonucleotides and photosensitizer, furan oxidation based cross-linking leads to efficient formation of interstrand cross-linked species, for reactions at 2 μM concentration, only a minimal amount of cross-link could be observed with methylene blue in solution. To further investigate furan mediated cross-linking of oligonucleotides in highly diluted samples, methylene blue was attached to an oligonucleotide complementary to the furan modified strand. Irradiation of methylene blue with red light resulted in the formation of singlet oxygen in near proximity of the furan group to be oxidized. This led to a considerable increase in the formation of interstrand cross-links in comparison to the system where methylene blue was present in a 2 μM solution. Apparently, the distance for singlet oxygen to travel in order to oxidize the furan unit and cause cross-linking became too large when methylene blue was present in solution in low concentration. Connecting the methylene blue moiety to the duplex restored the cross-linking capacity. It was further observed that the position of methylene blue on the oligonucleotide strand had a significant influence on the cross-link yields.

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SUPPLEMENTARY MATERIALS

Figure S1 to S18 can be found at DOI: to be inserted

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FIGURE CAPTIONS

Figure 1: Schematic representation of furan mediated DNA cross-linking. The furan group can be oxidized using NBS or singlet oxygen (1O2). After oxidation, the formed reactive moiety can react with exocyclic amines of the opposite base on the complementary strand. Both adenine (A) and cytosine (C) contain an exocyclic amine which is located in close proximity of the furan building block, leading to the formation of a covalent bond as depicted on the right-hand side of the figure.

Figure 2 Figure 2: Structure of the furan and PS modified building blocks incorporated in the oligonucleotide sequences. (**X**): furan building block, (**M**) methylene blue building block

Figure 3 A. Chemical structure of the formed cross-linked species. B. RP-HPLC traces of the cross-link reaction mixtures containing 20 μM of the duplex. (A1, B1, C1, D1): Furan modified

459 **FON1** and complementary sequence **ON1** before oxidation. (A2): After $^1O_2$ oxidation with
460 zinc phthalocyanine (1 μM, 90 min, ICL yield: 0%), (B2): After $^1O_2$ oxidation with ruthenium
461 tris bipyridinium (5 μM, 30 min, ICL yield: 16%), (C2): After $^1O_2$ oxidation with rose bengal
462 (1 μM, 15 min, ICL yield: 35%), (D2): After $^1O_2$ oxidation with and methylene blue (5 μM, 25
463 min, ICL yield: 53%). Formation of a new peak is observed in B2, C2 and D2 which belongs
464 to the interstrand cross-link (**ICL**).

465 **Figure 4** Denaturing PAGE analysis of the cross-link reaction mixtures containing 20 μM of the
466 duplex. All samples contain the furan modified **FON1** and complementary sequence **ON1**
467 before oxidation. All samples were irradiated with a characteristic wavelength for a period as
468 depicted in the figure in presence of the photosensitizer: (A): ruthenium tris bipyridinium (5
469 μM, blue light, ICL yield: 16%), (B): rose bengal (1 μM, green light, ICL yield: 35%) and (C):
470 methylene blue (5 μM, red light, ICL yield: 53%).

471 **Figure 5**. RP-HPLC of the 20 μM cross-link reaction mixtures. (A1): Furan modified **FON2**
472 and the complementary methylene blue modified **MON1** before oxidation, (A2): After $^1O_2$
473 oxidation (3,5 min, ICL yield: 22%) (B1): Furan modified **FON2** and the complementary
474 **MON2** before oxidation, (B2): After $^1O_2$ oxidation (5 min, ICL yield: 26%), (C1): Furan
475 modified **FON2** and the complementary methylene blue modified **MON3** before oxidation,
476 (C2): After $^1O_2$ oxidation (3,5 min, ICL yield: 22%) (D1): Furan modified **FON2** and the
477 complementary methylene blue modified **MON4** before oxidation, (D2): After $^1O_2$ oxidation
478 (5 min, ICL yield: 23%). A new peak appears in the spectrum which is assigned to the
479 interstrand cross-link (**ICL**). Yields are calculated by comparing the peak area of the ICL with
480 the limiting single strand, both corrected for their extinction factor. Due to the different

structures of the formed ICLs, depending on the position of methylene blue, different retention times of the cross-linked species are observed.

Figure 8: Schematic representation of the oxidation of the furan unit by both singlet oxygen generated by methylene blue present on the opposite strand as well as methylene blue present on other duplexes.

Figure 7. RP- HPLC of the diluted 2 µM cross-link reaction mixtures. (A1): Furan modified **FON2** and the complementary methylene blue modified **MON1** before oxidation, (A2): After $^1O_2$ oxidation (5 min, ICL yield: 33%), (B1): Furan modified **FON2** and the complementary **MON2** before oxidation, (B2): After $^1O_2$ oxidation (2 min, ICL yield: 24%) (C1): Furan modified **FON2** and the complementary methylene blue modified **MON3** before oxidation, (C2): After $^1O_2$ oxidation (5 min, ICL yield: 12%) (D1): Furan modified **FON2** and the complementary methylene blue modified **MON4** before oxidation, (D2): After $^1O_2$ oxidation (3,5 min, ICL yield: 28%). The new peak appearing in the spectrum belongs to the interstrand cross-link (**ICL**). Yields are calculated by comparing the peak area of the ICL with the limiting single strand, both corrected for their extinction factor.