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Fast thiol–maleamic methyl ester addition for facile covalent cross-linking of oligonucleotides

Liqiang Cao, Zhen Xi *

State Key Laboratory of Elemento-Organic Chemistry and Department of Chemical Biology, Nankai University, Tianjin 300071, China

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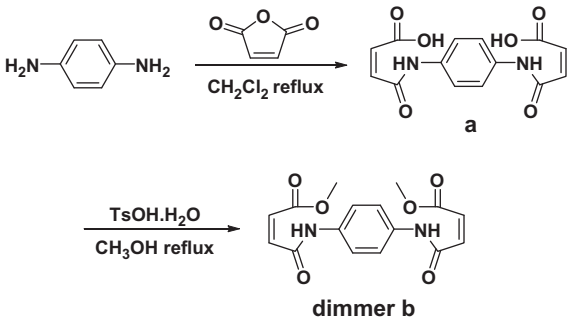
ABSTRACT

Thiol-Michael-cross-linked oligodeoxynucleotides were synthesized through the thiol–maleamic acid methyl ester addition reaction. As a metal-free 'click' reaction, the cross-linked method was fast responsive and highly sensitive. The newly synthesized oligodeoxynucleotides were thermally stable and their global structures retained those of non-cross-linked oligodeoxynucleotides and showed responsive reaction to different DNA restriction enzymes.

Synthetic oligonucleotides (ODN) are one of the most important molecular tools for genomic research and biotechnology.[1] Recently much effort has been dedicated to the application of the relatively short double-stranded oligodeoxynucleotides (ODN) in developing therapeutic agents as *cis*-elements, referred to as decoy ODN, to trap *trans*-activators, and transcription factors.[2] Many bioconjugation methods for ODN modifications were reported in the literature.[3] And the 'click' reaction concept has become important in efficient ODN modification. These reactions, using two complementary strands to provide a quantitative yielding reaction, should ideally be able to be applied under a wide range of mild conditions and be tolerant to many other functional groups.[4] The most commonly studied reaction of this family is Huisgen 1,3-dipolar cyclo-addition of azide and terminal alkyne functionalities, usually catalyzed by Cu(I) that may show potential nucleic acid damage.[5,6] Other reported approaches include Diels–Alder reaction[7] or the oxidation of thiols to form a disulfide.[8]

The thiol-Michael addition, as a kind of metal-free 'click' reaction with fast reaction to form a stable thioether bond and mild aqueous reaction condition, should be useful for ODN modification.[9,10] Dumbbell ODN, a circular ODN consisting of a double-stranded stem region and nucleotide loops at both of their termini, possessed increased exonuclease resistance.[11,12] We have recently developed a very fast thiol-reactive probe based on thiol-Michael addition.[10] This fast reaction may be beneficial for nucleic acid modification. In this Letter, the thiol–maleamic acid methyl ester addition was successfully applied to cross-linking oligodeoxynucleotides. And the capped ODN still showed an excellent response to different DNA restriction enzymes.

The artificial dumbbell ODN was constructed by using thiol-containing oligodeoxynucleotides making Michael addition to the dimmer **b** (Scheme 1). Dimmer **b** can be easily synthesized from available starting materials (see Supplementary data) and is stable both in the solid state and in DMSO or methanol solution for several months. The β-mercaptoethanol addition results supported that dimmer **b** reacts with thiols immediately (see Supplementary data), and the product is stable. The thiol–maleamic acid methyl ester reaction showed an excellent cross-linking function in the modifications of ODN, which was employed as a strategy to synthesize decoy ODN (Fig. 1a). The fast reaction between the thiol



Scheme 1.

* Corresponding author. Tel./fax: +86 22 23504782.
E-mail address: zhenxi@nankai.edu.cn (Z. Xi).

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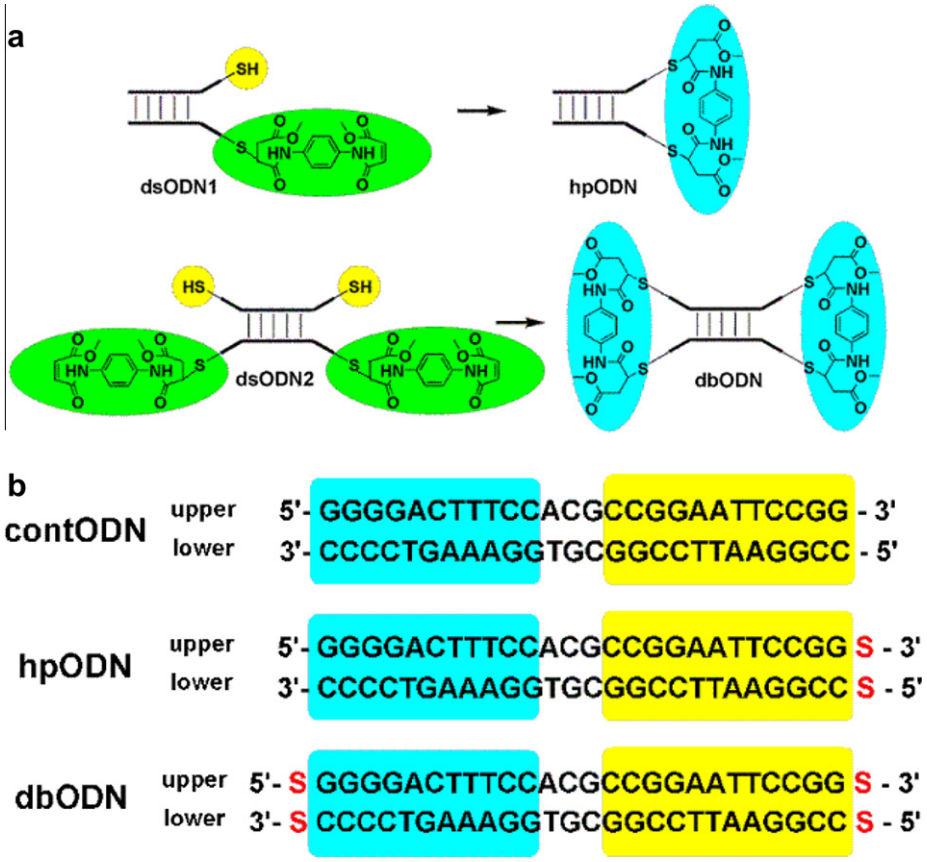


Figure 1. Formation of the thiol–maleamic acid methyl ester cross-linked dsODN by the metal-free 'click' reaction: (a) cross-linking of hairpin ODN (hpODN) at a single site and cross-linking of dumbbell ODN (dbODN) at both termini. (b) Sequences of the ODN used in this study. S: Thiol-modified C6 S–S. NF-κB recognition regions are marked with a blue box, and EcoRI recognition regions are marked with a yellow box.

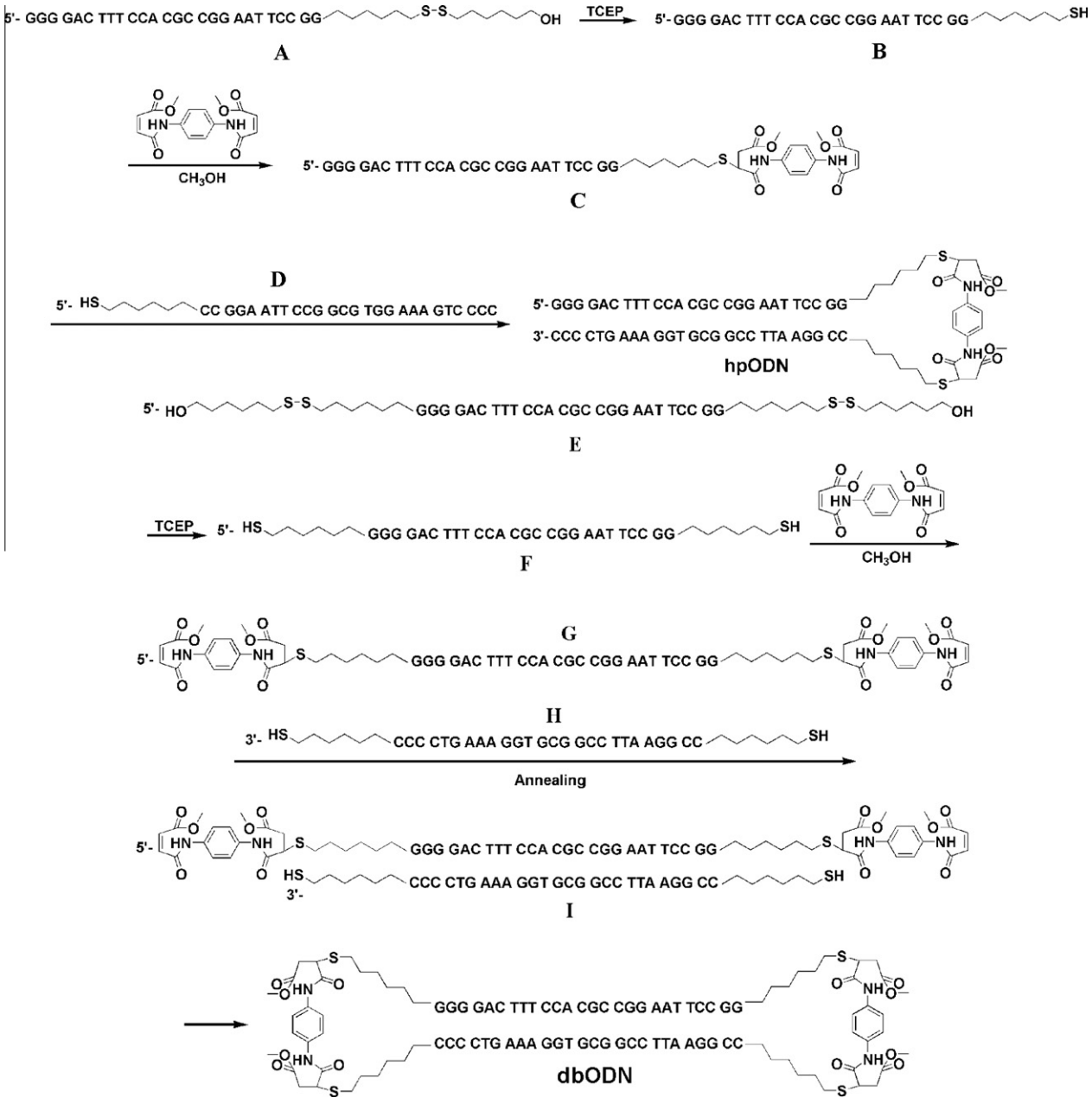
and maleamic acid methyl ester permits the formation of an intermolecular bridge.

The designed double-stranded ODNs are shown in Figure 1b. The left shaded part is the sequence of NF-κB.[13] Activation of NF-κB upregulates genes related to inflammatory and immunological responses, such as TNF-α, adhesion molecules, macrophage colony-stimulating factor (MCSF), granulocyte/macrophage colony-stimulating factor (GMCSF), and monocyte chemoattractant protein (MCP). Therefore, transfection of NF-κB decoy ODN leads to anti-inflammatory and immuno-suppressive activities.[14] The right shaded part is the target sequence of EcoRI. EcoRI is an endonuclease enzyme isolated from strains of *Escherichia coli*, as a part of the restriction modification system[15] to check if the terminal base modifications distort the helical geometry of the duplex.

The cross-linking process for dsODN was shown in Scheme 2. Thiol-modified C6 S–S was first introduced at one side of the ssODN on DNA synthesizer using a standard phosphoramidite elongation cycle for coupling of commercial nucleoside phosphoramidites.[16] Then the thiol protecting group of oligonucleotide **A** was removed by tris(2-carboxy-ethyl)phosphine (TCEP) to afford the oligonucleotide **B**, which was subsequently treated with saturated solution of dimmer **b** in methanol at 37 °C for 5 h and purified by OMEGA MICROSEP 3K to give the oligonucleotide **C** (Scheme 2). The cross-linked hpODN was obtained by annealing from equal equivalent of oligonucleotides **C** and **D** (10 μM) in 0.1 M MOPS buffer (pH 8.0) containing 1 M NaCl. After the incubation of dsODN at 37 °C for 10 h, the reaction product was monitored by denaturing polyacrylamide gel electrophoresis (PAGE) and further analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) after PAGE purification. The MALDI-TOF MS of the PAGE purified cross-linking gel band showed a single major peak at 16,726 Da, which is consistent with the hpODN formula weight, 16,709 Da $[M+Na]^+$.

Encouraged by the above results, the thiol–maleamic acid methyl ester conjugation was used to construct dumbbell ODNs. ssODN labeled by thiol-modifier C6 S–S group at both the 5′-end and the 3′-end (**E**) was synthesized (Scheme 2). Synthesis of cross-linked dsODN2 was conducted by the identical procedure of dsODN1 (Fig. 1). To avoid the intermolecular polymerization, an optimized concentration that the Michael addition reaction of thiol–maleamic acid methyl ester conducted only after annealing was found to be below 1 μM. Thus the equal equivalent of oligonucleotides **G** and **H** (Scheme 2) (0.5 μM) was annealed in 0.1 M MOPS buffer (pH 8.0) containing 1 M NaCl. Then the dsODN solution was incubated at 37 °C for 2 h, and monitored by denaturing PAGE (Fig. 2a). When we incubated the dsODN (0.5 μM) for some time before annealing, there was no apparent cross-linked product without annealing (Lane 11 in Fig. 2a). These results implied that the thiol-Michael addition was carried out during the annealing process. The MALDI-TOF MS of the PAGE purified cross-linking gel band showed a single major signal at 17,442 Da, which is consistent with the calculated dbODN formula weight, 17,418 Da $[M+Na]^+$.

CD spectroscopy was used to study the helical geometry of the chemically modified hpODN and dbODN, together with the natural double-stranded control ODN (contODN). The natural double-stranded 26-mer ODN (contODN) displayed a typically characteristic B-DNA spectrum with a positive ellipticity at 273 nm, a negative ellipticity at 248 nm, and a crossover between 252 and 258 nm (Fig. 3).[17] hpODN and dbODN possessing the thiol–maleamic acid



Scheme 2. Synthesis of hpODN and dbODN.

methyl ester cross-linking at one or both ends of the helix displayed an increase in both positive amplitude at 273 nm and negative one, while there was no other significant change in the overall CD spectrum. A similar CD characteristic was also observed in double-stranded ODN possessing CuAAC bridges at the terminis.[2] The slight differences in the CD spectra between a natural ODN and the cross-linked ODN may simply arise from inherent differences of the sequences, or the increase in ellipticity at 273 nm may be related to an increased winding of the helix induced by the presence of the dimmer **b**. The general consistency of CD spectra suggested that the modifications on DNA terminis did not significantly distort the helical geometry of the DNA duplex.

UV melting experiments were also conducted to evaluate the thermal stability of the modified ODN via the thiol–maleamic acid methyl ester cross-linking (Table 1). The T_m values for hpODN and dbODN were 72, and 85 °C, respectively, demonstrating significantly enhanced thermal stability by the thiol–maleamic acid methyl ester cross-linking of double-stranded DNA, in comparison with the double-stranded ODN control (62 °C). These results of enhanced thermal stability by the thiol–maleamic acid methyl ester cross-linking of double-stranded DNA were similar to those of a CuAAC cross-linked 17-mer ODN (ΔT_m 26.0 °C), although the sequence and length of the ODN were different.[2]

The class II restriction endonuclease *Eco*RI recognizes the sequence 5′-GAATTC and cleaves it between the G-4 and A-5.[15] *Eco*RI is sensitive to slight conformational changes and has been used to probe for perturbations in the B-DNA structure; a reduction in the rate of cleavage can be directly correlated to a change in helical geometry. Therefore, analysis of the cleavage rates of hpODN and dbODN can provide another way to determine whether our modifications affect the double-strand structure of contODN. After

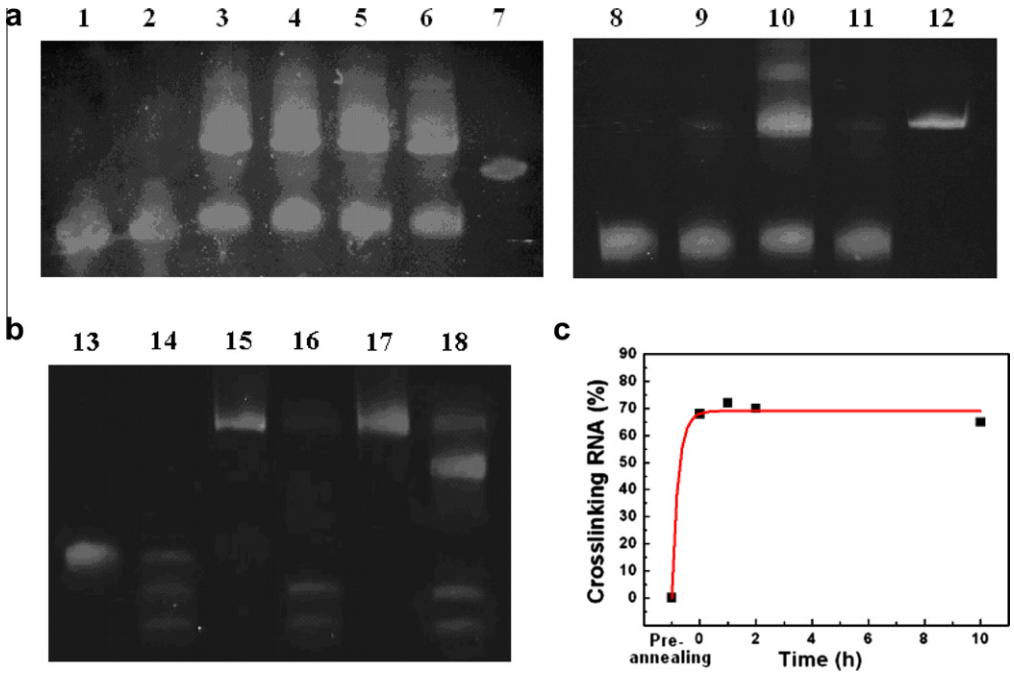


Figure 2. (a) Denaturing PAGE of the cross-linking reaction of hpODN and dbODN. Lanes 1, 2 showed ODN **D**, **C**, respectively (Scheme 1). Lanes 3, 4, 5, and 6 showed the cross-linked reaction mixture of ODN **D** and **C** incubating for 0, 1, 2, and 10 h after annealing. Lanes 8, 9 showed the modified ODN **H**, **G**, respectively. Lane 10 is the reaction mixture for dbODN after annealing. Lane 11 is the reaction mixture incubating at 37 °C for 5 h without annealing. Lane 7, 12 are DNA markers (50nt) with same sequence: GTACG TACGT ACGTA CGTAC GTACG TACGT ACGTA CGTAC GTACG TACGT. (b) Denaturing PAGE of the *Eco*RI restriction enzyme digest. Lanes 13, 15, and 17 denoted contODN, hpODN, and dbODN. Lanes 14, 16, and 18 showed the *Eco*RI restriction enzyme digest results of contODN, hpODN, and dbODN, respectively. (c) The time course curve for the cross-linking reaction between ODN **D** and **C**. The yield of cross-linking at each time point was calculated from the gel scanning results by a BioRad Molecular Imager FX after SYBR Gold staining on the gel.

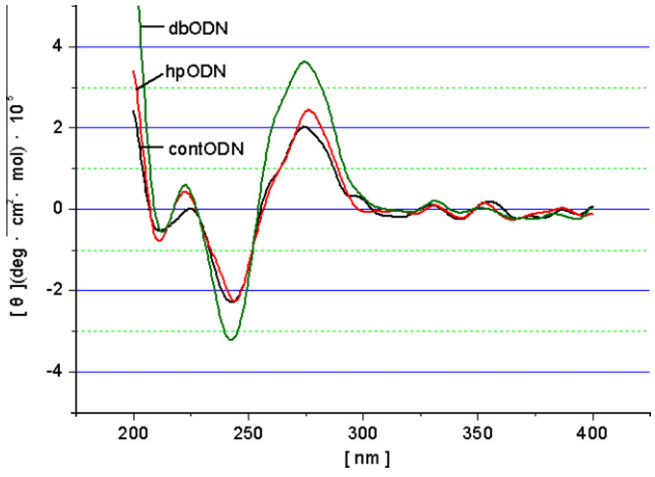


Figure 3. Circular dichroism spectra (conditions: 5 μM of ODN in sodium cacodylate–HCl buffer (1 mM, pH 7.0) containing 10 mM NaCl) of contODN, hpODN and dsODN.

Table 1
Thermal stability of the thiol–maleamic acid methyl ester cross-linked ODN

	T_m[a] (°C)	ΔT_m[b] (°C)
contODN	62	
hpODN	72	10
dbODN	85	23

[a] Conditions: 1 μM each ODN, 1 mM sodium cacodylate–HCl (pH 7.0), 1 mM NaC1.
[b] T_m(modified) − T_m(control).

180 min at 37 °C, hpODN and dbODN were almost completely cleaved to produce digestion patterns unique for each sequence (Fig. 2b). The fact that there is no loss in enzymatic activity for side modification was consistent with the CD data presented above. It can be concluded that thiol–maleamic acid methyl ester modification on DNA ends does not significantly distort the B-DNA helical geometry of the stem of the modified DNA.

In conclusion, a metal-free 'click' reaction: fast thiol–maleamic acid methyl ester Michael addition, can be used not only in organic synthesis, but also in oligonucleotide modification. A dimmer **b** with two maleamic acid methyl ester groups can form facile covalent ligation with thiol modified ODN at the 3′- and 5′-terminis during annealing. The synthesized ODN were thermally stable and the modification on DNA ends did not significantly disturb the B-DNA helical geometry. This simple and fast intermolecular end-capping method for cross-linking double stranded oligonucleotides with minimum modified stem may provide some advantages for bioactive application of nucleic acid, such as minimum modified siRNA for RNAi,[18] which will be reported in due course.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tetlet.2013.01.112.

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