Quinone Methides

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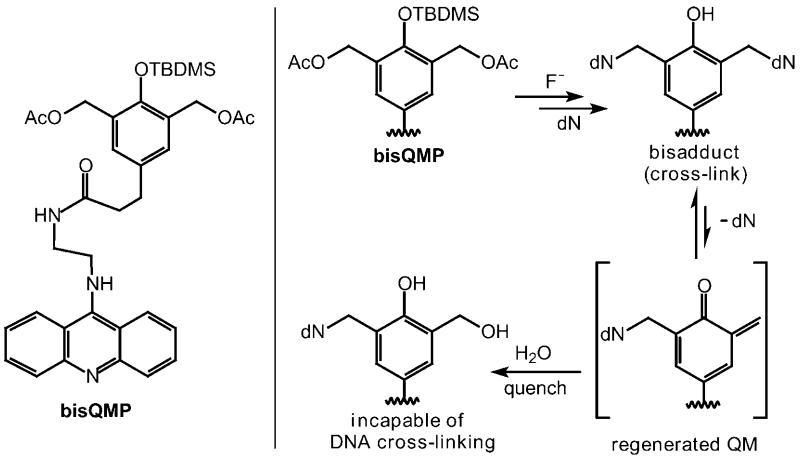
Dynamic Cross-Linking Is Retained in Duplex DNA after Multiple Exchange of Strands**

*Huan Wang and Steven E. Rokita**

The utility of a cross-linking agent is typically evaluated by nothing more than its selectivity and efficiency, and this is quite reasonable for processes involving irreversible reactions. Reversible reactions, in contrast, offer another dimension to consider. Dynamic and covalent chemistry supports iterative pathways that essentially interrogate many possible targets but ultimately accumulate only the products most favored by thermodynamics. Such reversible chemistry has been applied to combinatorial systems with much success and provides many opportunities in biology, chemistry, and material sciences.[1] The ability to adapt to a changing environment is a key property of these systems. For example, assembly of macrocyclic ligands can respond to a changing array of added metal ions[2] and assembly of components forming a monolayer can redistribute according to a shifting pH value.[3] Nucleic acids and their analogues can similarly be assembled and coupled reversibly in response to added templates.[4] However, the fate and consequences of reversible cross-linking once established within duplex DNA has not been sufficiently explored despite its impact on a wide range of topics from biomaterials to DNA repair.

Alkylation reactions are not often considered in the design of dynamic combinatorial systems,[1] but a few examples involving DNA have been discovered. The anticancer drug candidate ecteinascidin 743 alkylates duplex DNA reversibly to allow its migration from a site of greatest kinetic reactivity to an alternative site of greatest thermodynamic stability.[5] Of course, this reversibility may also allow for regeneration and subsequent return of the drug to DNA after initial excision during cellular repair. The cytotoxicity of another series of drug candidates based on cyclopropylpyrroloindole even correlates with their reversibility of alkylation.[6] Compounds related to malondialdehyde also form adducts reversibly, and these equilibrate between intra- and interstrand derivatives as a function of DNA structure.[7]

Quinone methide (QM) intermediates can additionally alkylate reversibly when their nascent adducts undergo benzylic elimination and QM regeneration.[8] A variety of QMs form in vivo from metabolism of alkylphenols and other xenobiotics, and their reaction with DNA has been well characterized.[9] Numerous synthetic quinone methide precursors have also been developed for DNA reaction and rely on a variety of strategies for initiation including most notably UV irradiation.[10] Reversibility both extends the effective lifetime of the transient QM reagents from minutes to days and supports a method of QM delivery that is promoted by the chosen target itself (Scheme 1).[11,12] The extent to which the dynamics of QM alkylation could influence cross-linking has not previously been determined nor is it easily predicted because this process depends on two alkylations in tandem.[13] Covalent attachment to a target is consequently retained during all subsequent QM regeneration. Still, the reversibility of QM reaction can persist in cross-linking and adapt to changing stimuli as now demonstrated in a strand-exchange model described below.



Scheme 1. Formation of reversible nucleotide adducts with a bisfunctionalized QM–acridine conjugate. TBDMS = *tert*-butyldimethylsilyl.

Interstrand cross-links formed in DNA by a bisquinone methide precursor conjugated to acridine (**bisQMP**, Scheme 1) are sufficiently stable for detection after denaturing gel electrophoresis despite the estimated half-life of about 2.5 hours for the dG N7-QM adduct representing the major site of alkylation and cross-linking in this system.[13,14] Nevertheless, the QM adducts are also sufficiently labile over days to support at least a single transfer from intra- to interstrand cross-linking.[12] This process has now been used to prepare

[*] Dr. H. Wang, Prof. S. E. Rokita
Department of Chemistry and Biochemistry
University of Maryland
College Park, MD 20742 (USA)
Fax: (+1) 301-405-9376
E-mail: Rokita@umd.edu
Homepage: http://www.chem.umd.edu/directory/facultydirectory/stevenrokita/

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cross-links in **OD1**/**OD2** indirectly to avoid the simultaneous presence of **OD2** and **bisQMP** that might otherwise permit subsequent transfer of a QM independent of **OD1**. Initial treatment of **OD1** with **bisQMP** followed 24 hours later by addition of **OD2** produced the expected interstrand cross-link product as evident from denaturing gel electrophoresis (Figure 1). The observed low mobility species is consistent with the product's high molecular weight, and an equivalent

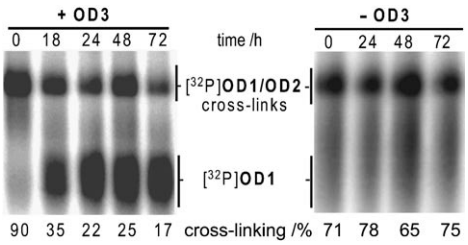


Figure 1. Cross-linking of **OD1**/**OD2** by bisQMP dissipated in the presence, but not absence, of **OD3**. The initial [^{32}P]**OD1**/**OD2** cross-linking was formed indirectly by first treating [^{32}P]**OD1** (3 μM) with **bisQMP** (60 μM) for 24 hours. **OD2** (3 μM) was then added to the mixture, and incubation was continued for another 24 hours. Finally, **OD3** (3 μM) was added to one portion of the mixture, and aliquots were removed for analysis after further incubation as indicated. Products were separated by denaturing polyacrylamide (15%) gel electrophoresis, quantified by phosphoimagery of the indicated area and reported (%) relative to total DNA. **OD1** = 5′-d(TGGTGTGGTGGTTGGGTGTGTGGTGGGTGG), **OD2** = 5′-d(TTTACCTCTTCAACCGCCACCCACCACACACCCAACCACCACACCA), **OD3** = 5′-d(CTACTGACGTCGTGGTGTGGTGGTTGGGTGTGTGGTGGGTGGCGGTTGAAGAGGTAAA).

species is not observed for oligonucleotide duplexes in the presence of **bisQMP** prior to formation of the quinone methide intermediate.[12,13] The potential dynamics of cross-linking within **OD1**/**OD2** was next challenged by adding one equivalent of a competitor strand **OD3** (Scheme 2). Strand exchange of **OD3** for **OD1** in the duplex is both thermodynamically favored and kinetically promoted by the presence of the 5′ extension, or so-called toehold, of **OD2** (Table S1 in the Supporting Information).[15,16]

Even a single equivalent of **OD3** was sufficient to drive substantial release of **OD1** (80%) from the reversibly cross-linked **OD1**/**OD2** over 3 days (Figure 1). In the absence of **OD3**, no such release was observed, and the original **OD1**/**OD2** species persisted throughout this same period. Concomitant with the **OD3**-dependent loss of **OD1**/**OD2** cross-linking was the formation of **OD3**/**OD2** cross-linking (Figure 2). This result provided the first evidence that covalent and reversible cross-linking does not prohibit strand exchange within duplex DNA. Common to other dynamic chemistry, cross-linking by QM also responds to a change in the system's thermodynamics as signaled by addition of **OD3**.

The lag apparent between loss of **OD1**/**OD2** cross-linking and gain of **OD3**/**OD2** cross-linking (Figure 2) likely reflects the numerous intra- and interstrand derivatives of bisQM that may form in the dynamic system. Only the species formed along the path to establishing the **OD3**/**OD2** cross-link are illustrated in Scheme 2, but there is no expectation that release of **OD1** occurs any more frequently than the corresponding release of **OD2** during QM regeneration. Furthermore, transfer of interstrand cross-linking must compete against intrastrand reaction of **OD1** that subsequently releases **OD2** to form a native (noncovalent) duplex with **OD3**. Once this duplex forms, it would likely resist association with, and hence QM transfer from, an **OD1**–QM conjugate. The QM intermediates are also weakly susceptible to irreversible quenching by water, and this may additionally limit the yield of interstrand cross-linking after each transfer (Scheme 1).[12] Ultimately, about 50% of the original cross-link of **OD1**/**OD2** still transferred to **OD3**/**OD2** despite all of the possible obstacles.

Efficient migration of cross-linking requires intimate and complementary association between nucleotide strands. Accordingly, no cross-linking was transferred between two unrelated duplexes (Figure S1 in the Supporting Information).

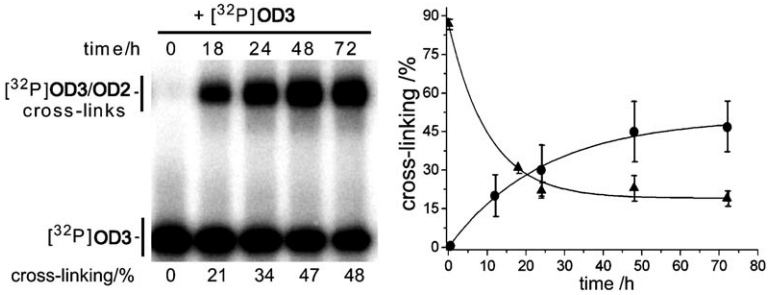
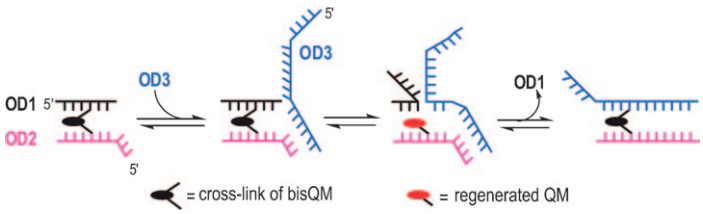
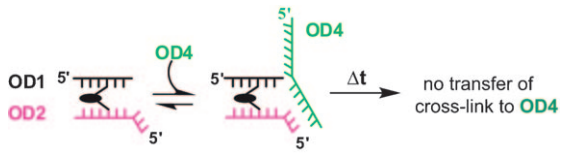


Figure 2. The presence of **OD3** destroys bisQM-dependent cross-linking of **OD1**/**OD2** and generates alternative cross-linking of nascent **OD3**/**OD2**. The experiment described in Figure 1 was repeated using [^{32}P]**OD3** (1 equiv) to quantify the resulting cross-linking of **OD3**/**OD2**. The results of two independent determinations are also summarized graphically ([^{32}P]**OD3**/**OD2** cross-linking (●), [^{32}P]**OD1**/**OD2** cross-linking from Figure 1 (▲)). The range of measurements is indicated by error bars, and the lines are added only to highlight trends in the data.



Scheme 2. Dynamic cross-linking adapts to strand exchange within duplex DNA.

tion).[16] Similarly, no transfer was detected when cross-linked **OD1**/**OD2** was challenged with **OD4** containing a sequence complementary to only the toehold region of **OD2** (Scheme 3 and Figure S2 in the Supporting Information).[16] The lack of cross-linking between **OD2** and **OD4** demonstrates that cross-linking of **OD1**/**OD2** and **OD3**/**OD2** was confined to their duplex regions, and the bisQM cross-link did not migrate to the toehold sequence prior to incubation with **OD4** or, by extrap-



Scheme 3. Mere proximity is not sufficient for migration of DNA strand cross-linking. **OD4** = 5′-d(CTTGAGATACTTTTTTTCTGCGCG-TCGTTGAAGAGGTAAAA).

olation to the previous example, **OD3**. The toehold sequence is not even necessary for cross-link migration and strand exchange. Addition of [^{32}P]**OD1** to cross-linked **OD1/OD2** detected exchange of **OD1** in and out of the cross-linked duplex albeit slowly (Figure S3 in the Supporting Information).[16] Such exchange would have only been possible through the dynamics of the covalent bonding.

A sustained reversibility of cross-linking was monitored by its persistent ability to transfer from **OD1/OD2** to **OD3/OD2** after a variable (0–7 days) delay in adding one equivalent of **OD3** (Figure 3). The yield of **OD3/OD2** cross-

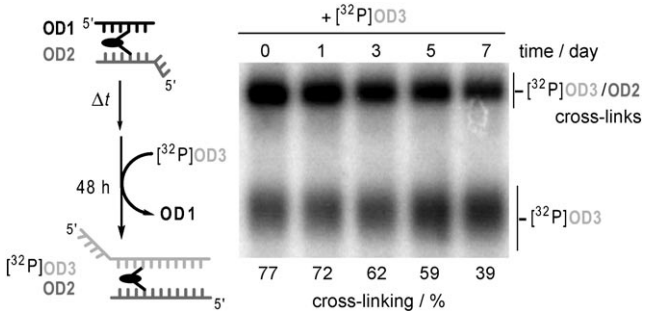


Figure 3. Dynamic cross-linking persists for days in duplex DNA. Cross-linked **OD1**/**OD2** (3 μM) was generated as described in Figure 1, and incubation was continued for the indicated period before addition of an equivalent of 5′-[^{32}P]**OD3** (3 μM). Incubation was again continued for 48 hours to detect the extent of quinone methide equivalent that remained reversible for strand exchange and cross-linking. Products were separated by denaturing polyacrylamide (15%) gel electrophoresis, quantified by phosphoimagery of the indicated area and reported (%) relative to total DNA.

linking resulting from such transfer decreased during this period from 77% to 39%, thus suggesting a half-life of about one week for the QM to remain dynamic in the DNA duplex. This result likely reflects the slow and irreversible trapping of QM by water and weak nucleophiles of DNA including the exocyclic amines of dG and dA.[14,17]

As long as both linkages to bisQM remain reversible (Scheme 1), DNA cross-linking should maintain its ability to respond and adapt to additional stimuli such as that provided by yet another strand of DNA. As anticipated, addition of stoichiometric amounts of [^{32}P]**OD5**“ to the cross-linked **OD3/OD2** promoted release of **OD2** and generation of a new covalent link between **OD3**/[^{32}P]**OD5** in a process analogous to that illustrated in Scheme 2 (Figure S4 in the Supporting Information).[16] Although the net yield of cross-linking between **OD3** and **OD5** is only about 30%, it still represents a transfer of approximately 60% of the QM from **OD3/OD2** (Figure 1) that in turn derived from **OD1/OD2**, which originated from reaction of **bisQMP** and **OD1**. Thus, coupling between **OD3** and **OD5** was the culmination of three net exchanges of cross-linking involving both intra- and inter-strand reactions that can extend over 11 days. Efficiency of each QM transfer was never less than 50%, and the final cross-linked strands were never exposed to the original cross-linking agent **bisQMP** or its first target **OD1**.

The preservation of cross-linking but concurrent exchange of strands in duplex DNA demonstrates a sustained dynamics of cross-linking not previously detected. The reversibility of QM exchange relies on the presence of the strong nucleophiles of DNA that serendipitously also act as stable leaving groups as well. The resulting system shares many of the same properties described in previous examples of dynamic combinatorial chemistry.[1] Most notably, product distribution adapts to changing signals and is ultimately controlled by thermodynamics. Equivalent cross-linking within cells has the potential to defy or even damage repair mechanisms necessary for maintaining the integrity of genomic DNA. Regeneration of QM intermediates over hours should allow cross-links to resist rapid DNA denaturation and yet maintain an ability to adjust to stimuli over longer periods in analogy to the differential response of a dilatant liquid (e.g. an aqueous suspension of cornstarch) to rapid and slow changes in applied pressure. Such behavior may find interesting application in the field of DNA nanotechnology by increasing structural integrity without compromising programmed self-assembly.[18]

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