Measuring and suppressing the oxidative damage to DNA during Cu(I)-catalyzed azide-alkyne cycloaddition

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ABSTRACT: We have used quantitative polymerase chain reaction (qPCR) to measure the extent of oxidative DNA damage under varying reaction conditions used for copper(I)-catalyzed click chemistry. We systematically studied how the damage depends on a number of key reaction parameters, including the amounts of copper, ascorbate, and ligand used, and found that the damage is significant under nearly all conditions tested, including those commonly used for bioconjugation. Furthermore, we discovered that the addition of dimethylsulfoxide, a known radical scavenger, into the aqueous mixture dramatically suppresses DNA damage during the reaction. We also measured the efficiency of crosslinking two short synthetic oligonucleotides via click chemistry, and found that the reaction could proceed reasonably efficiently even with DMSO present. This approach for screening both DNA damage and reactivity under a range of reaction conditions will be valuable for improving the biocompatibility of click chemistry, and should help to extend this powerful synthetic tool for both *in vitro* and *in vivo* applications.

Due to its biocompatibility and facile reaction kinetics, the Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC) has become widely adopted as a robust means of functionalizing biomolecules. [1-3] However, the toxicity of the copper catalyst has been a persistent impediment to carrying out bioconjugation in settings that are susceptible to oxidative damage. [4-8] The most commonly utilized reaction conditions involve generation of the catalytically active Cu(I) species via *in situ* reduction of Cu(II) by ascorbate, which occurs with the concomitant generation of reactive oxygen species (ROS) that are damaging to biomolecules. [9] Significant efforts have been focused on the discovery and optimization of Cu(I)-stabilizing ligands that can improve reactivity and suppress oxidative damage. Finn, [6,10] Wu [7] and Pezacki [11] and coworkers have developed water-soluble ligands that can stabilize the Cu(I) catalyst and also function as sacrificial reagents that intercept ROS. However, even with protective ligands, the damage is often extensive enough to affect cell viability. [6,7,11] More recently, azides that chelate with copper [12] have been found to accelerate the reaction and allow the use of lower copper concentrations to reduce oxidative damage. [8,13] Despite the progress made so far, the concern of toxicity in CuAAC-based bioconjugation has not been eliminated. As a result, copper free alternatives such as strain-promoted azide-alkyne cycloaddition, which have slower kinetics and lower specificity, remain preferred over CuAAC in applications that are more sensitive to oxidative damage. [3,14-16]

Here we focus on a gap in the development of effective CuAAC protocols: the methods used to monitor oxidative damage during CuAAC are not sufficiently sensitive for applications that are highly susceptible to damage. While ROS generated during CuAAC are detrimental to a variety of biological molecules, of particular concern is the oxidative damage to DNA, including base modifications and scission of the phosphodiester backbone in one or both strands. [17-20] Such damage can have deleterious genotoxic and mutagenic consequences for living organisms, [19,21,22] particularly when the high levels of oxidative stress overwhelm cellular DNA repair mechanisms. [23,24] In relation to health, oxidative DNA damage has been implicated in cancer and other aging-related diseases. [17,19,25,26] Anticipating this issue, many CuAAC bioconjugation studies have evaluated cell viability or proliferation. [6,7,11,27] However, these assays do not provide chemical insight into the kinetics of oxidative damage, which is needed for rationally minimizing the cytotoxicity of CuAAC. In addition, a large portion of genetic mutations do not observably impact cell viability, [28] and thus it is likely that much of the damage goes undetected by these methods. Some other studies have measured oxidation kinetics of proteins [29] as well as proxy molecules, such as histidine and short oligonucleotides, using HPLC and gel electrophoresis. [10] However, because of their relatively low sensitivity, these methods cannot detect very low levels of damage that, while sporadic, are detrimental for most *in vivo* chemical biology applications. Such damage may also be a concern for a number of methods that use click chemistry to prepare DNA bioconjugates [30] for sensing, [31,32] diagnostics, [33] sequencing, [34] and gene synthesis. [35-37] Therefore, owing to the biological and technological importance of maintaining genomic integrity, a highly sensitive method for directly measuring the damage to DNA would be valuable for improving the biocompatibility of CuAAC chemistry.

In this work we have used a qPCR-based analytical method to study the oxidative damage to a long double-stranded DNA molecule (3.5 kbp) under varying conditions used for CuAAC

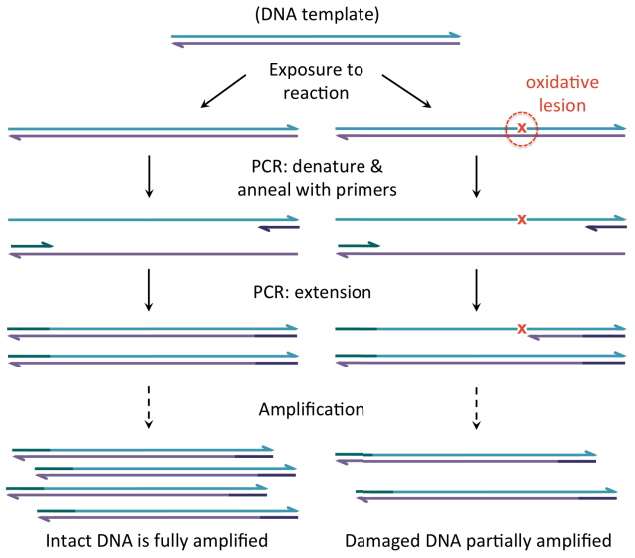

Figure 1. Illustration of the use of qPCR to determine the fraction of DNA damaged during a reaction. First, the DNA template strand is exposed to the reaction for a predetermined amount of time before being quenched in TAE (Tris-Acetate-EDTA) buffer on ice. Next, the DNA is subjected to PCR amplification. During the extension step of PCR, oxidative lesions in either strand of the template obstruct the progress of the polymerase enzyme, preventing it from generating a full complement of that strand. Thus, as the cycle is repeated only intact strands contribute to the exponential amplification of the DNA template. By monitoring the fluorescence of an intercalating dye and comparing to an untreated control sample, the relative fraction of intact DNA present in the initial sample can be quantitatively determined.

bioconjugation. This method is capable of measuring the frequency of oxidatively induced strand lesions (base modifications or strand scission) in double-stranded DNA as low as 1 per 10^5 nucleotides. The order-of-magnitude higher sensitivity has afforded us new insight into how the damage rate is influenced by key reaction parameters, including the type of Cu(I)-stabilizing ligand used and the concentrations of copper, ascorbate, and the ligand, at a relevant time scale. This improved measurement capability has led us to discover that the addition to the aqueous reaction of up to 10% dimethylsulfoxide (DMSO), a commonly used component for bioconjugation solvent mixtures, reduces the rate of oxidative damage by as much as two orders of magnitude. Moreover, our measurements of the efficiency of CuAAC in crosslinking two short synthetic oligonucleotides showed only a relatively modest reduction in reaction rate attributed to the inclusion of DMSO. The strategy presented here is complementary to the existing efforts in improving the biocompatibility of CuAAC through engineering the ligand and reactants, [6-8,10,11] and allows for rationally minimizing the oxidative damage of CuAAC for both *in vitro* and *in vivo* bioconjugation.

The qPCR technique is a facile, parallel and highly sensitive method for quantifying the frequency of lesions in DNA. [38,39] As illustrated in Figure 1, the 3.5 kbp DNA strand is first exposed to the CuAAC reaction mixture for a predetermined amount of time before being quenched by dilution in TAE buffer on ice. Next, the DNA is used as a template for PCR amplification. During the extension step of PCR, lesions in either strand of the template inhibit the polymerase from generating a full complement of that strand, and thus only intact strands contribute to the exponential amplification of the product DNA. By monitoring the total amount of DNA using an intercalating dye and comparing to an untreated control, the relative fraction of intact DNA present in the sample can be quantitatively determined. In order to calculate the DNA damage frequency (lesions per base) from the intact DNA fraction φ, we assumed that the damage occurs in a random and sequence-independent fashion, an approximation that is often used for long genomic DNA. [39] Then the lesions can be described by a Poisson distribution f(k, λ), such that the probability P of a DNA strand containing k lesions is given by the following equation:

$$P(k) = f(k,\lambda) = \frac{\lambda^k e^{-\lambda}}{k!}$$

where λ is the mean number of lesions per single strand. Then, equating the probability P(0) of k = 0 lesions with the intact DNA fraction φ gives the following:

$$\varphi = P(0) = \frac{\lambda^0 e^{-\lambda}}{0!} = e^{-\lambda}$$

which can be rearranged to give the mean number of lesions per DNA strand:

$$\lambda = -\ln(\varphi)$$

Finally, dividing λ by the number of bases n in each strand gives the mean number of lesions per base:

$$\text{damage frequency (lesions per base)} = \frac{\lambda}{n} = \frac{-\ln(\varphi)}{n}$$

We used this equation to determine the frequency of damage to a 3.5 kbp DNA strand after exposure to varying catalytic conditions, with the goal of understanding the factors affecting the extent of damage. For most bioconjugation applications, the catalyst consists of three key components: the copper source, typically a Cu(II) salt; a reducing agent such as ascorbate, which is needed to generate and maintain the catalytically active Cu(I) species; and finally a protective ligand, which is used to stabilize copper in the +1 oxidation state. For this study we chose to use Cu(II) sulfate and sodium ascorbate, along with the commonly used tris-(3-hydroxypropyltriazolylmethyl)amine (THPTA) ligand, [10] to serve as a model catalytic system. In order to investigate the effects of the catalyst composition on the DNA damage, we systematically varied the concentration of copper, the ascorbate:copper ratio (Asc:Cu), and the ligand:copper ratio (L:Cu) during the reaction, and measured both the fraction of DNA remaining intact and the corresponding DNA damage frequency after a timed exposure to the catalyst, as compared to an untreated control.

The results of our DNA damage measurements are shown in Figure 2. When the Cu concentration was increased while the Asc:Cu and L:Cu ratios were held constant, we observed a decrease in the fraction of DNA remaining intact, which indicates an increasing frequency of damage (Figure 2a). The degradation was extremely rapid for Cu concentrations of 50μM or higher, with less than 1 in 5,000 DNA molecules remaining intact after only 2 minutes of reaction time. Transition metals are known to generate ROS in the presence of ascorbate via Fenton chemistry:

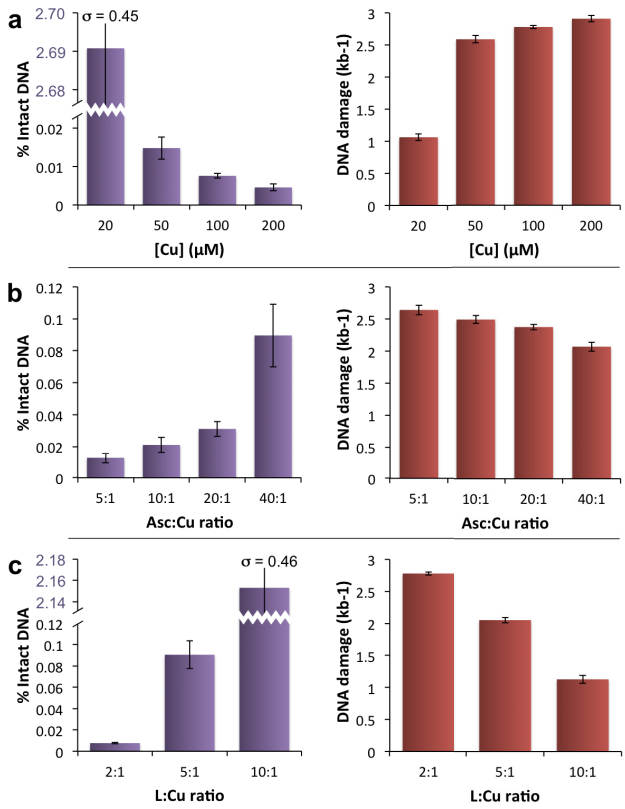

Figure 2. Results from systematically varying the reaction conditions and measuring the percentage of intact DNA and the DNA damage frequency (lesions per kilobase) after a 2-minute reaction time. (a) Percentage of intact DNA (purple) and corresponding DNA damage frequency (red) as a function of copper concentration ([Cu]). The ratios of ascorbate to copper (Asc:Cu) and THPTA ligand to copper (L:Cu) were held constant at 10:1 and 2:1, respectively. (b) Intact DNA percentage and damage frequency as a function of Asc:Cu, for constant [Cu] = 100μM and L:Cu = 2:1. (c) Intact DNA percentage and damage frequency as a function of L:Cu, for constant [Cu] = 100μM and Asc:Cu = 10:1. The error bars represent ± one standard deviation (σ) calculated from at least four identical qPCR replicates.

$$AH_2 + O_2 \rightarrow A + H_2O_2$$

$$Cu^{+} + H_2O_2 \rightarrow {}^{\bullet}OH + OH^{-} + Cu^{2+}$$

where AH_2 is ascorbic acid, A is dehydroascorbic acid, and $^{\bullet}OH$ is the hydroxyl radical. [40] Thus increasing the copper concentration increases the rate of ROS production. Our results corroborate previous *in vitro* and *in vivo* studies that showed more extensive oxidative damage with increasing copper concentration during CuAAC. [6,7] The role of ascorbate, however, is more complex. From the above chemical equations, it is apparent that increasing the ascorbate concentration also accelerates ROS generation, and this has been observed experimentally. [41] However, ascorbate is also known to scavenge oxygen radicals, meaning that it can function as both an antioxidant and a pro-oxidant in the same system. [9,42] Note that when the reaction is performed under ambient conditions that oxidize Cu(I), excess ascorbate is needed to maintain the copper in the +1 oxidation state. When we varied the ratio of ascorbate to copper in the catalyst, we generally observed a decrease in the damage with increasing Asc:Cu, particularly for very high concentrations of ascorbate (Figure 2b). This data suggests that for the conditions used here, the antioxidant activity of ascorbate more than compensates for its ability to generate ROS. A similar trend was observed in plasmid relaxation studies of DNA damage by ascorbate and copper. [9] Finally, we tested the effect of varying the ratio of the THPTA ligand to copper. In addition to stabilizing the catalytically active Cu(I) species, such ligands can also act as sacrificial agents that intercept and react with the ROS generated, [43] and previous reports have found that using excess ligand relative to copper can have a protective effect on molecules both *in vitro* [10] and inside cells. [6] Indeed, we observed that increasing the L:Cu ratio from 2:1 to 10:1 reduces the damage frequency by more than a factor of two (Figure 2c). However, even in the presence of tenfold excess ligand, damage to the relatively long DNA is substantial— after 2 minutes, roughly 98% of the DNA has been damaged. The extensive DNA damage observed in our qPCR experiments is consistent with previous studies that found a decrease in cell viability or proliferation rate after exposure to the Cu/Asc/THPTA catalyst. [7,11] This problem is not specific to the THPTA ligand, as we observed similarly high levels of damage when we tested alternative Cu-binding ligands (Figure S4). More generally, we found that the frequency of damage is a complex function of both the catalyst composition (copper, ascorbate and ligand) and the reaction time, and cannot be easily predicted. This highlights the importance of directly measuring the oxidative damage under the conditions being used, in order to optimize the reactivity while minimizing the damage.

A ubiquitous strategy used by biological systems to reduce

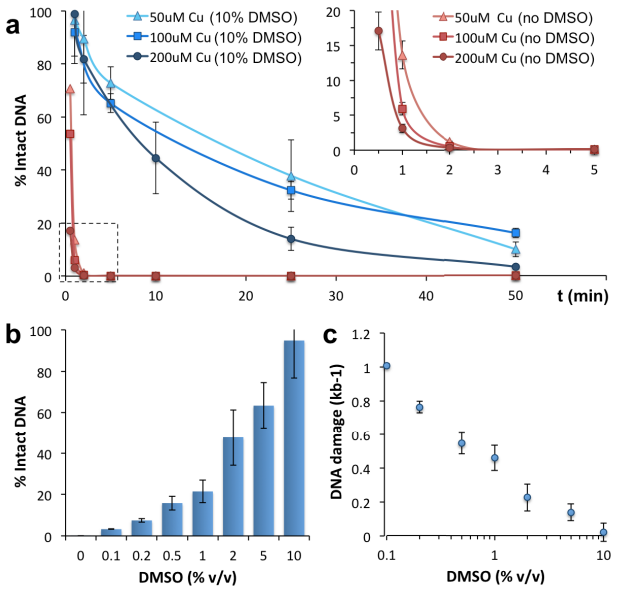

Figure 3. Suppression of oxidative DNA damage by DMSO during CuAAC. (a) Time dependence of the degradation of DNA for a range of Cu concentrations, both with (blue) and without (red) 10% DMSO included in the reaction. Inset shows early time points, scaled for clarity. (b) Percentage of intact DNA remaining after a 5-minute reaction as a function of DMSO volume percentage. (c) DNA damage frequency as a function of DMSO volume percentage, as calculated from the data in (b). For reference, the damage without DMSO is ~2.5 kb^{-1}. For all reactions, Asc:Cu = 10:1 and L:Cu = 2:1. Error bars represent ± one standard deviation.

and mediate oxidative stress is the production of cellular antioxidants. [44] However, a general challenge in adopting this strategy for CuAAC is that many antioxidants, such as TCEP (tris(2-carboxyethyl)phosphine, a sacrificial reducing agent) and catalase (an enzymatic catalyst for H_2O_2 decomposition) are likely to inhibit the reaction by adversely affecting the concentration or coordination environment of the catalytically active Cu(I) ions. [10] Our trials with small molecule ROS scavengers, including trolox [45] and histidine, [11] found that although the damage was suppressed, the inhibition of CuAAC reaction was similarly too excessive for these additives to be useful.

In order to improve the biocompatibility of CuAAC, we sought an alternative antioxidant that could suppress the oxidative damage without significantly inhibiting the reaction. DMSO is a promising candidate, based on its established use as a solvent for CuAAC and its known capacity as an oxygen radical scavenger. [46,47] To test how DMSO would affect the kinetics of DNA damage during CuAAC, we measured the time-dependence of degradation for three different copper concentrations, in both aqueous buffer and 10% DMSO. As shown in Figure 3a, without DMSO the DNA was rapidly damaged, with less than 0.1% of the DNA remaining intact after just 5 minutes for all three copper concentrations. In contrast, with the addition of 10% DMSO, the majority of the DNA was still intact after 5 minutes, and even after 25 minutes as much as one third of the DNA remained undamaged. We also varied the amount of DMSO in the reaction mixture (Figure 3b), and found it to be inversely correlated to the measured damage frequency (Figure 3c). This suggests that DMSO can indeed scavenge ROS that are produced during the reaction. DMSO is known to react with •OH, a key mediator of DNA damage that is generated during CuAAC, to produce the methyl radical, [48,49] which can dimerize to form ethane, thus avoiding the otherwise damaging effects of •OH on DNA. What is notable is that the addition of DMSO leads to a much more pronounced reduction in the damage rate (up to one hundred fold, Figure 3) than varying other reaction parameters, such as copper concentration or the L:Cu ratio, within commonly used ranges (Figure 2).

For an additive to be useful in CuAAC bioconjugation, it must suppress the oxidative damage without strongly inhibiting the reaction itself. To determine the impact of DMSO on CuAAC bioconjugation, we compared the yield of crosslinking two short synthetic oligonucleotides (24 bases) in both water and 10% DMSO. As illustrated in Figure 4a, the 24-base strand bearing an azide group was hybridized with a complimentary 16-base strand modified with an octadiynyl-deoxyuracil base. After exposure to the catalyst mixture, the reactions were quenched and analyzed by denaturing polyacrylamide gel electrophoresis (d-PAGE), which separates the crosslinked products from the unreacted DNA. [50,51] As shown in Figure 4b, we found that DMSO did modestly reduce the rate of crosslinking, possibly due to weak coordination to the copper center; [52] however, even with 10% DMSO, the reaction proceeded reasonably efficiently, reaching nearly the same yield after 50 minutes. From a practical standpoint, it would make sense to compare the amount of damage measured at times that give a comparable reaction yield with and without DMSO. For example, reactions with the same copper concentration reach a comparable yield after 25 minutes with the DMSO/water mixture, as compared to 5 minutes in the aqueous solution, but they have 10^2-10^3 times more intact DNA than their aqueous counterparts. This corresponds to a five- to tenfold decrease in

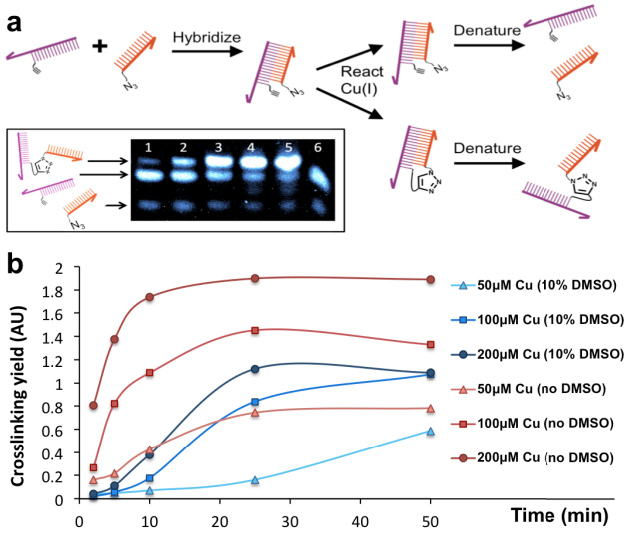

Figure 4. Measuring the time-dependent reaction yield for the crosslinking of two short DNA oligonucleotides by CuAAC. (a) Schematic illustration of the method used to determine the yield of the DNA crosslinking reaction. Inset shows an example denaturing PAGE image, where the uppermost band corresponds to the product. Lanes 1-5 are increasing time points for the reaction, while lane 6 is a control sample that did not contain any Cu catalyst. (b) The time-dependent normalized reaction yield of the DNA-templated CuAAC crosslinking reaction for a range of Cu concentrations, shown both without (red) and with (blue) 10% DMSO. Trend lines are added for clarity.

the frequency of DNA damage at comparable yield. Thus the damage suppression by DMSO more than offsets the reduction in rate of the bioconjugation reaction, and represents a significant improvement over the standard CuAAC bioconjugation protocols.

In addition to solution phase reactions, we have also adapted this approach for CuAAC-based surface immobilization of DNA, [53] during which the extent of oxidative damage is unknown. We previously described a method for sequence-specific and covalent tethering of long DNA to a solid surface using click chemistry. [51] This method uses short DNA 'anchor' strands that are attached to a self-assembled monolayer surface to capture much longer DNA target strands with a complementary sequence; then CuAAC is used to crosslink the anchor strand and the target DNA. In the present work, we used qPCR to measure the amount of oxidative damage to surface-bound DNA during the CuAAC reaction. A major challenge to adapting qPCR analysis to quantify damage on surface-bound DNA is the requirement for a control sample with an identical initial DNA quantity in order to carry out relative quantification. In practice, sample-to-sample variation in surface coverage is difficult to avoid. To account for variation in the total amount of DNA on the surface, we have normalized the DNA quantity to an internal standard, a shorter 200 bp product that is amplified from a segment within the same template strand (see Supporting Information). This allows the shorter product to serve as a reference that, due to its much smaller footprint, is unlikely to be cleaved except in severely damaging conditions. [39,54] To assess the damage sustained by surface-bound DNA, we first hybridized the long DNA template strands with short, single-stranded anchor strands that were attached to an alkanethiol self-assembled monolayer on gold. Next, the sur-

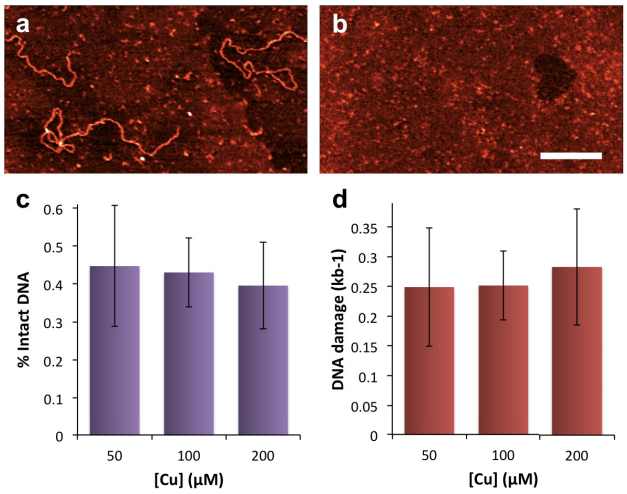

Figure 5. Measuring the oxidative damage to DNA during the CuAAC surface-coupling reaction. (a) Atomic force microscopy (AFM) image of the 3.5 kbp DNA template after hybridization with short anchor DNA strands on a self-assembled monolayer surface and exposure to the catalyst. (b) AFM image of the same surface after denaturation and collection of the DNA for qPCR analysis. Scale bar is 200nm. (c) Measured percentage of DNA remaining intact after the surface reaction as a function of copper concentration. (d) DNA damage frequency as a function of [Cu], as calculated from the data in (c). Reaction times were 5 minutes, and all reactions included 10% DMSO, along with Asc:Cu = 10:1 and L:Cu = 2:1. Error bars represent ± one standard deviation.

face-bound DNA was exposed to the CuAAC reaction mixture, after which the DNA was released from the surface by rinsing with a denaturing alkaline buffer, collected, and analyzed by qPCR. In order to permit collection of the DNA by chemical denaturation, we omitted the azide and alkyne groups that are normally used to crosslink the DNA to the surface. Atomic force microscopy (AFM) imaging was used to monitor hybridization of the DNA template to the surface anchor strands, as well as to confirm the release of over 99% of the DNA after denaturation. AFM images of the surface acquired after the reaction and denaturation steps are shown in Figures 5a and 5b, respectively. Dual amplification qPCR was then used to determine the fraction of intact DNA (Figure 5c) and corresponding damage frequency (Figure 5d) after a 5-minute reaction with varying concentrations of copper in 10% DMSO. Notably, the damage frequency on the surface is two to three times higher than that measured for the same reaction conditions in solution. These results show that the kinetics of oxidative damage may diverge from that in the solution phase, which may be due to a possible increase in the local concentration of copper ions near the surface carboxylate groups. Additionally, the weak dependence of the damage on copper concentration also suggests that the copper ions binding to the surface may mediate the damage, and that the surface coverage of copper ions depends only weakly on bulk concentration; however, further studies are needed to elucidate this surface effect. Unfortunately the damage rate of the surface DNA in the absence of DMSO was too high to permit accurate quantification using this method, which requires that the 200 bp reference be mostly intact. Despite this difficulty, the results confirm that the damage during the surface reaction is also considerably suppressed by the inclusion of DMSO.

We have used qPCR to measure the extent of oxidative damage to a 3.5 kbp DNA molecule under varying catalytic reaction conditions for CuAAC. Combining high-throughput and direct quantification of DNA damage with measurement of coupling kinetics, our approach will broaden the utility of CuAAC for bioconjugation in both solution phase and on solid surfaces. [51,55] For example, CuAAC bioconjugation has been utilized in a number of sensing and diagnostic applications, [31-33,53] and the results presented here may lead to increased specificity by reducing the extent of unintended modification of the probe molecules. Because the qPCR-based assay is also applicable to live cells and organisms, [38,39] our approach to optimizing CuAAC, which is informed by quantitative information concerning DNA damage, may help to improve the biocompatibility of CuAAC for *in vivo* applications. Our discovery that DMSO can suppress the rate of oxidative damage by two orders of magnitude has practical implications for *in vitro* bioconjugation with CuAAC, given that DMSO/water mixtures are popular solvents for *in vitro* bioconjugation. In addition, as DMSO has previously been shown to have low toxicity [49,56] and protect cells from radical-mediated damage, [57-59] it may prove to be a viable strategy for protecting live cells during CuAAC bioconjugation as well.

ASSOCIATED CONTENT

Supporting Information

Additional qPCR results, details on experimental procedures, and a list of DNA sequences used. The Supporting Information is available free of charge on the ACS Publications website.

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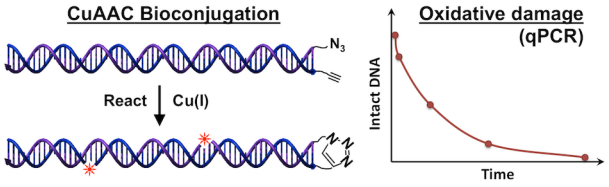
TOC graph


CuAAC Bioconjugation
N3
React
Cu(I)
Oxidative damage
(qPCR)
Intact DNA
Time