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Singlet Oxygen in DNA Nanotechnology

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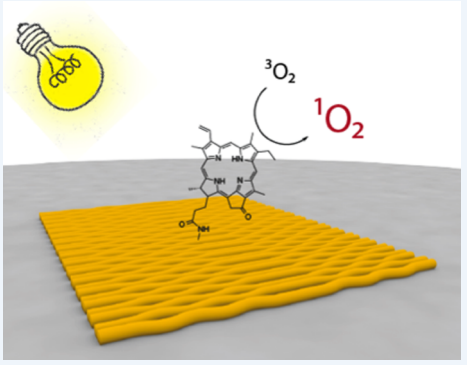
CONSPECTUS: Singlet oxygen ($^1O_2$), the first excited electronic state of molecular oxygen, is a significant molecule, despite its minute size. For more than half a century, the molecule has been widely used and studied in organic synthesis, due to its characteristic oxygenation reactions. Furthermore, $^1O_2$ plays a key role in mechanisms of cell death, which has led to its use in therapies for several types of cancer and other diseases. The high abundance of oxygen in air provides a wonderful source of molecules that can be excited to the reactive singlet state, for example, by UV/vis irradiation of a photosensitizer molecule. Although convenient, this oxygen abundance also presents some challenges for purposes that require $^1O_2$ to be generated in a controlled manner.



In the past decade, we and others have employed DNA nanostructures to selectively control and investigate the generation, lifetime, and reactions of $^1O_2$. DNA-based structures are one of the most powerful tools for controlling distances between molecules on the nanometer length scale, in particular for systems that closely resemble biological settings, due to their inherent ability to specifically form duplex structures with well-defined and predictable geometries. Here, we present some examples of how simple DNA structures can be employed to regulate $^1O_2$ production by controlling the behavior of $^1O_2$-producing photosensitizers through their interactions with independent quencher molecules. We have developed different DNA-based systems in which $^1O_2$ production can be switched ON or OFF in the presence of specific DNA sequences or by changing the pH of the solution.

To further illustrate the interplay between DNA structures and $^1O_2$, we present three pieces of research, in which $^1O_2$ is used to activate or deactivate DNA-based systems based on the reaction between $^1O_2$ and cleavable linkers. In one example, it is demonstrated how a blocked oligonucleotide can be released upon irradiation with light of a specific wavelength. In more complex systems, DNA origami structures composed of more than 200 individual oligonucleotides were employed to study $^1O_2$ reactions in spatially resolved experiments on the nanoscale.

Three decades ago, Ned Seeman proposed the concept of building synthetic nanostructures with DNA.[1] He envisioned that the information carried by DNA oligonucleotides and the canonical rules for base-pairing set forth by Watson and Crick could allow researchers to design and self-assemble complex structures composed entirely of DNA. During the 1990s, molecular beacons were introduced.[2] These are DNA structures that, upon hybridization, change the distance between two photoactive molecules and thereby change the fluorescent output. In the past decade, major advances in the development of both static and dynamic DNA nanostructures have converged and now offer a powerful toolbox for studying biological as well as physical phenomena on the nanometer length scale.[3] Others have focused on studying the plasmonic properties of metal nanoparticles when DNA controls their arrangement[4−6] or on studying the kinetics of enzymatic cascades assembled on DNA templates.[7−9] We and others have explored the use of DNA to study and control the formation of $^1O_2$.

The ground electronic state of molecular oxygen is a spin triplet ($^3O_2$), while $^1O_2$ is the first excited electronic state of molecular oxygen.[10] The chemical properties of these two species are different, and this can be particularly important in a biological context. For example, as a consequence of its unique chemical reactivity, $^1O_2$ is known to be involved in mechanisms of cell signaling and, at the limit, in mechanisms of cell death.[11]

The transition from $^3O_2$ to $^1O_2$ is highly forbidden as an electric dipole process and, therefore, is a rare event. The transition can be facilitated, however, by so-called photosensitizers, which are light-harvesting molecules that can absorb energy from photons and, through a collision, transfer this excitation energy to another molecule.[12] In this manner, ground state oxygen can be excited to $^1O_2$, as illustrated in Figure 1. Although $^1O_2$ is a reactive species that can engage in chemical reactions, such as addition to alkenes, it can also emit near-infrared light (1275 nm), thereby returning the oxygen

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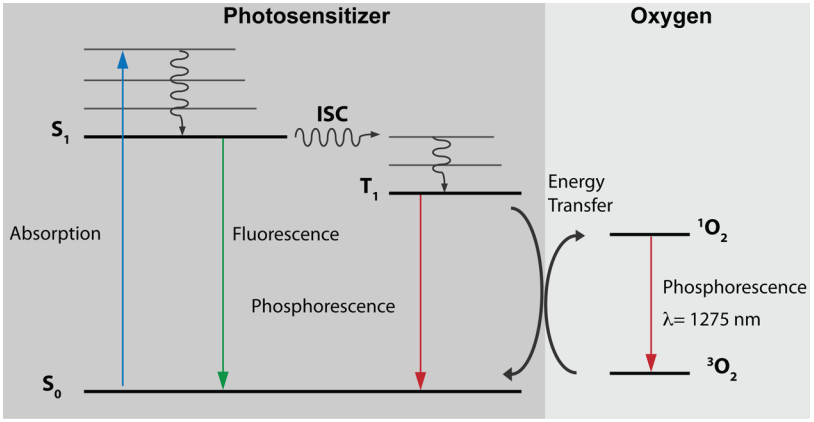

Figure 1. Jablonskii diagram depicting the photosensitized excitation of triplet oxygen ($^3O_2$) to $^1O_2$. First the photosensitizer is excited by absorption of a photon (blue arrow). This excited state can decay to the ground state through fluorescence (green arrow) or undergo intersystem crossing (ISC) to a triplet state. The resulting triplet state can decay to the ground state through phosphorescence (red arrow) or collide with ground state $^3O_2$ and, through a process of energy transfer, make $^1O_2$. The subsequent phosphorescent decay of $^1O_2$ can be monitored at 1275 nm.

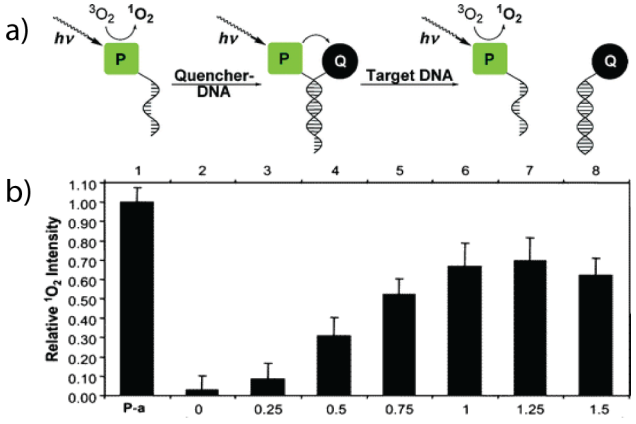

Figure 2. Controlling the production of $^1O_2$ by DNA strand displacement. (a) A DNA oligonucleotide attached to a porphyrin-based photosensitizer, P, converted $^3O_2$ to $^1O_2$ upon irradiation. When a complementary oligonucleotide, functionalized with a quencher, Q, was added to the solution, this formed a complex with the photosensitizer–DNA and effectively quenched the production of $^1O_2$. The production of $^1O_2$ could be restored by displacement of the photosensitizer–DNA by a predesigned target oligonucleotide. (b) Column 1 shows the relative phosphorescence intensity of $^1O_2$ sensitized by the DNA-functionalized porphyrin (i.e., ON state), column 2 shows the quenched complex (i.e., OFF state), and columns 3–8 show the effects of titration with increasing amounts of the target DNA designed to restore $^1O_2$ production. The decrease in intensity at high titration ratios reflects the quenching of $^1O_2$ by the added DNA. Reprinted with permission from ref 22. Copyright 2006 American Chemical Society.

molecule to its triplet ground state.[12] This latter phosphorescence serves as a useful direct probe of $^1O_2$.

Because $^1O_2$ can initiate cell death, photosensitizers have attracted a great deal of interest as therapeutic agents against cancers, skin diseases, and macular degeneration.[13] In this regard, several issues have arisen, including delivery of the photosensitizer to diseased and not healthy cells, excitation of the photosensitizer with light at a wavelength that does not harm the surrounding tissue, and ensuring that the photosensitizer itself is not toxic, for example, has no dark toxicity.[14]

Approaches to overcome these limitations have focused on incorporating regulatory functions in $^1O_2$ sensitizers that make them responsive to the environment. For some photosensitizers, it has been demonstrated that $^1O_2$ production can be controlled by intercalating the sensitizer in DNA.[15−18] By incorporating an amine in the sensitizer chromophore, McDonnell et al. developed a pH responsive $^1O_2$ sensitizer.[19] In 2004, Chen et al. developed an enzyme-controlled $^1O_2$ sensitizer.[20] This was achieved by tethering a $^1O_2$ sensitizer to a molecule that can deactivate (i.e., quench) the sensitizer. The tether used was a nine amino acid long peptide that is the substrate for the cancer specific caspase-3 protease (Casp3). The peptide is cleaved only in the presence of Casp3, and the increased spatial separation of the $^1O_2$ sensitizer and the quencher led to a significant increase in $^1O_2$ production. This was later extended by Zhang et al. to a matrix metalloproteinase-7 (MMP7)-triggered protein beacon that showed *in vivo* MMP7-mediated photodynamic cytotoxicity in cancer cells.[21]

We originally set out to develop an ON/OFF switch for the production of $^1O_2$ that specifically exploited sequence dependent DNA interactions that, in turn, better controlled the distance between a photosensitizer and a molecule that could quench the photosensitizer excited state. The first system was designed to arrange two functional molecules in close proximity based on sequence complementarity between DNA oligonucleotides.[22] The first component was an analogue of the photosensitizer pyropheophorbide a (PPa) modified with a chemical linker allowing it to be incorporated via automated DNA synthesis. Control experiments established that the DNA did not affect the photosensitizer's ability to excite $^3O_2$ to $^1O_2$ (Figure 2). For the second component, the so-called black hole quencher (BHQ) was attached to a DNA oligonucleotide capable of hybridizing to the PPa–DNA. Annealing the two components and measuring the $^1O_2$ phosphorescence at 1275 nm demonstrated a dramatic decrease in the relative amount of $^1O_2$ produced as a consequence of the quenching of excited state PPa by BHQ. Most importantly, the production of $^1O_2$ was restored by adding a third and independent DNA oligonucleotide designed to competitively bind the BHQ–DNA and thereby liberate the PPa–DNA (Figure 2).

This proof-of-principle experiment established that the photosensitized production of $^1O_2$ could be controlled by designed DNA systems and thereby turned on by the presence of specific nucleic acid species. We hypothesized that such a

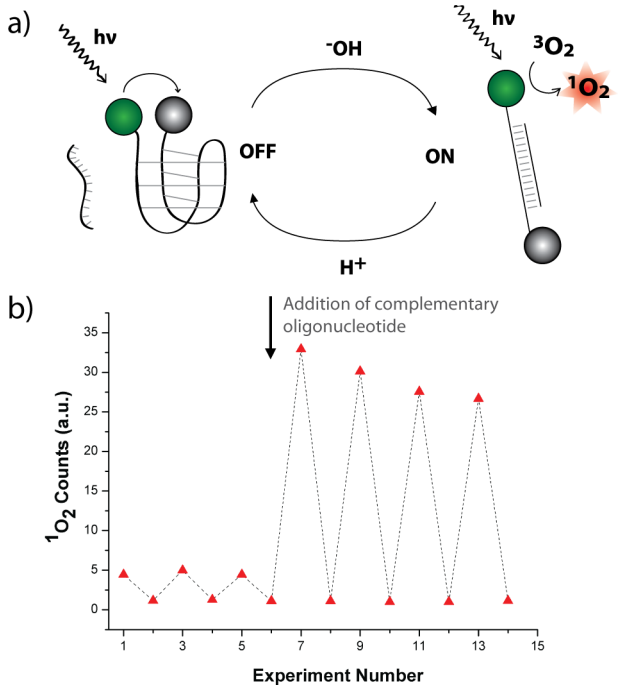

Figure 3. Controlling the photosensitized production of $^1O_2$ by changing the pH. (a) When the solution containing the i-motif beacon was acidified (pH 4−5), the cytosine-rich domains became partially protonated allowing the formation of a tetrameric i-motif. In this configuration, the photosensitizer (green sphere) and the quencher (black sphere) came into close proximity, which precludes the photosensitized production of $^1O_2$ (i.e., the OFF state). If the solution was brought to neutral or slightly alkaline conditions (pH 7−8.5), the beacons opened up, liberating the photosensitizer from the quencher and allowing for photosensitized $^1O_2$ production (i.e., the ON state). This effect became even more pronounced in the presence of a partially complementary oligonucleotide capable of rigidifying the otherwise "floppy" single stranded segment between the photosensitizer and quencher in the open state; a rigid DNA segment ensured an effective intramolecular separation of the sensitizer from the quencher. (b) Intensity of $^1O_2$ phosphorescence upon switching the system between acidic (even numbers) and alkaline (odd numbers) pH. The complementary oligonucleotide that ensures a more rigid DNA segment was added after the first five measurements. Adapted with permission from ref 28. Copyright 2010 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

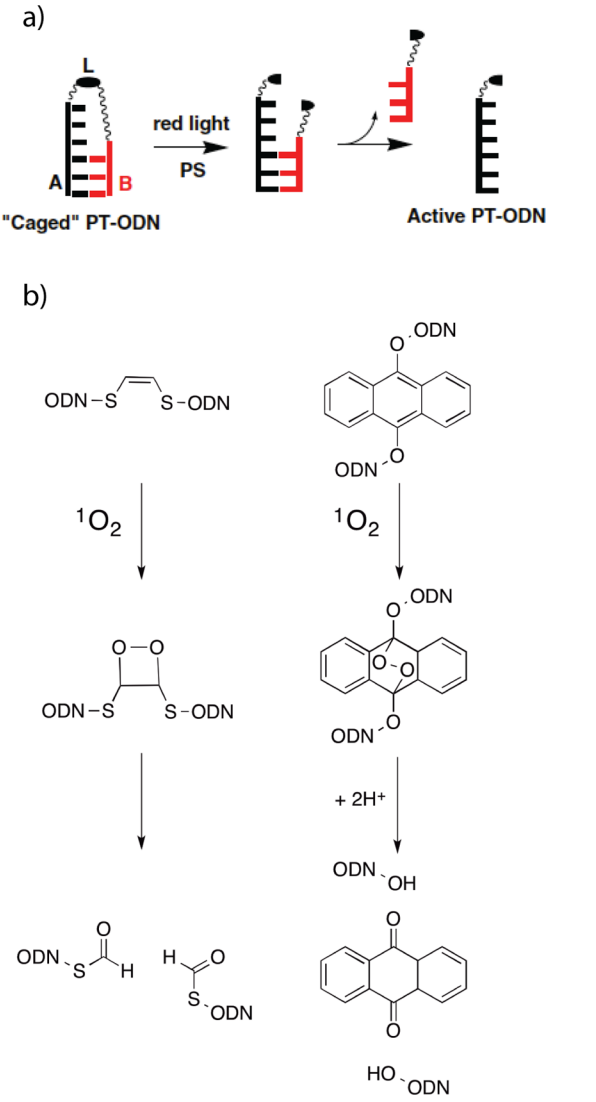

Figure 4. Caged oligonucleotides (ODN). (a) An oligonucleotide **A** was caged by the short oligonucleotide **B** by tethering with a linker **L** that can be cleaved by $^1O_2$. In the presence of a photosensitizer, the linker was cleaved upon red light irradiation and the subsequent production of $^1O_2$, thereby releasing the caged oligonucleotide. (b) Examples of $^1O_2$ cleavable linkers that can be incorporated into automated DNA syntheses, and their reactions with singlet $^1O_2$, (left) 1,2-bis(alkylthio)ethene (BATE); (right) dialkoxyanthracene (DA). Adapted from ref 38. Copyright 2008 Elsevier.

system could be designed to switch ON only in the presence of cancer-specific mRNA, thus minimizing the problem of damaging healthy cells in photodynamic therapy. Later, the Zheng laboratory reported on a similar system where the PPa photosensitizer and a carotene quencher were incorporated into the ends of a hairpin-forming nucleic acid.[23] In its initial state, the ends of the molecular beacon were very close, but in the presence of a specific mRNA, upregulated in certain cancer cells, the beacon was unfolded, which greatly increased the intramolecular distance between the photosensitizer and quencher. Because the quenching of the photosensitizer relied on either contact quenching or the distance-dependent *Förster resonance energy transfer*, the unfolding effectively initiated the production of $^1O_2$ when the sensitizer was irradiated with light of the correct wavelength. Moreover, Zheng and colleagues[23] demonstrated that the beacon was taken up by cancer cells and the cell viability dropped significantly when the irradiated beacon matched the cancer specific mRNA. More recently, Tan and co-workers designed an ON/OFF DNA switch that detected the presence of proteins or a small metabolite by employing a so-called aptamer switch.[24] Aptamers are nucleic acid sequences that have been selected to bind a specific target through a process known as SELEX,[25,26] and aptamer switches are aptamers that have been designed to form a duplex in the absence of such specific targets. As a proof-of-principle, Tan and co-workers demonstrated that a photosensitizer could be controlled by a quencher in a aptamer switch until a target, in these specific cases, the protein thrombin or ATP, bound the aptamer switch and activated the photosensitizer.

As described in the previous examples, dynamic DNA structures, and thereby functional molecules attached to them, can be controlled by exploiting standard Watson and Crick base-pairing. However, nonclassical nucleotide interactions can also control the three-dimensional configuration of DNA. One example is the interaction between cytosine and protonated

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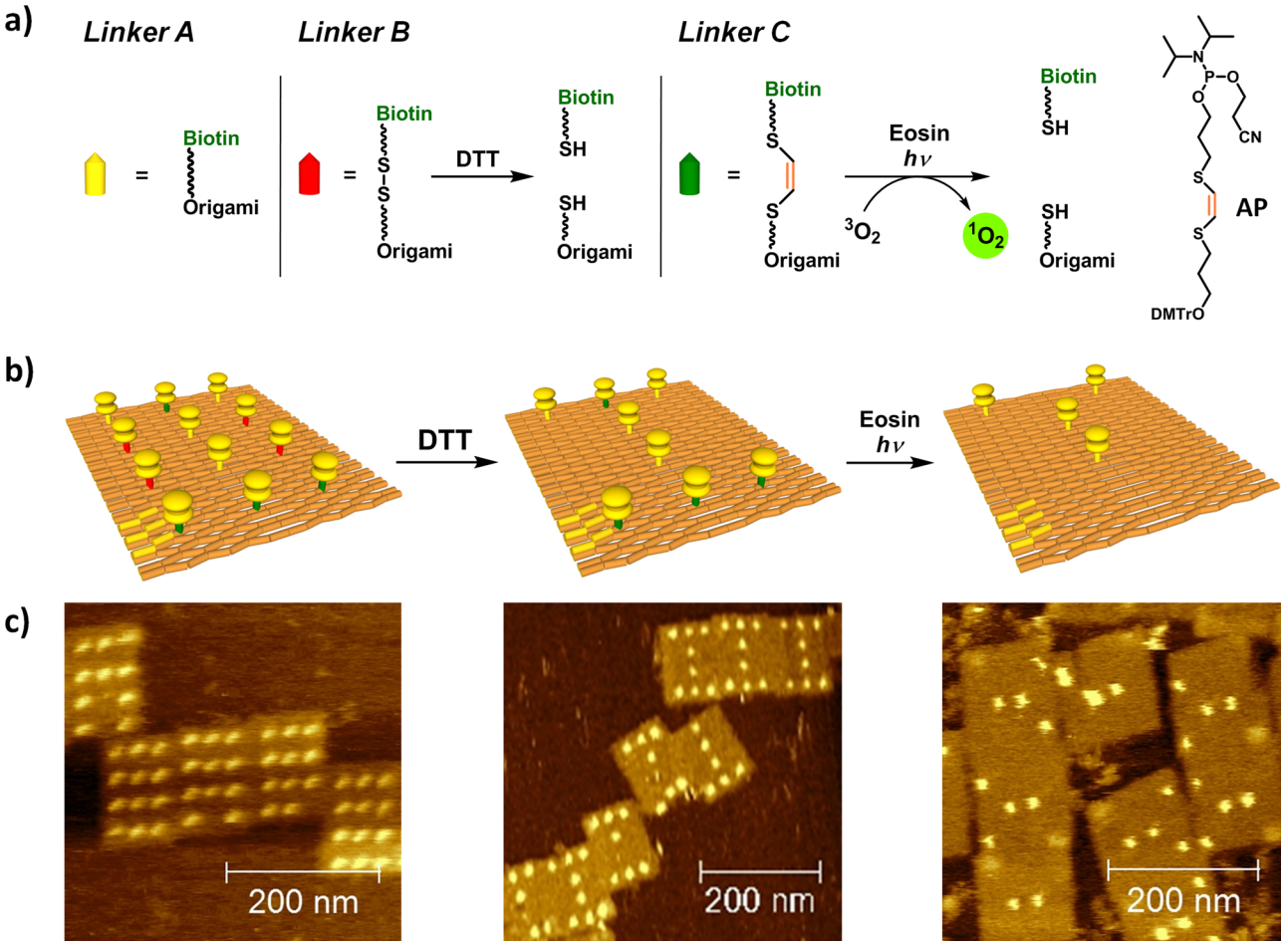

Figure 5. Monitoring single molecule reactions on a DNA origami template. (a) Linkers A, B, and C used to immobilize biotin to the DNA origami. The noncleavable linker A served as a reference, linker B contained a disulfide cleavable by DTT, and linker C was susceptible to $^1O_2$. (b) Schematic representation of the positions of the different modified staple strands and the expected streptavidin pattern after treatment with the relevant cleaving reagents. (c) AFM images showing the observed patterns. Reprinted from ref 39. Copyright 2010 Nature Publishing Group, a division of Macmillan Publishers Limited.

cytosine found in the so-called i-motifs, a tetrameric DNA structure first reported by Gehring et al. in 1993.[27] We envisioned that such a DNA structure functionalized with a photosensitizer−quencher pair could be employed to control $^1O_2$ production by varying the pH.[28] The system was designed to arrange the photosensitizer and quencher in the tetrameric i-motif in close proximity at pH < 5 and hence functioned as the OFF state (see Figure 3a).[28] When the pH was increased, the system opened and was fully stretched upon the addition of a partially complementary oligonucleotide. The resultant separation of the sensitizer and quencher yielded a dramatic increase in PPa fluorescence: a factor of 94. To our knowledge, this is, thus far, the largest increase in fluorescence reported for such i-motif beacons. This originated not only from the decreased quenching but also from an inherent pH-dependence of the PPa fluorescence yield. A large ON/OFF response was likewise observed when the phosphorescence was monitored from the $^1O_2$ produced (λ = 1275 nm). The system was reversible within the pH range of 4.0−8.5 and could be cycled between the OFF and ON states multiple times by the sequential addition of hydrochloric acid and sodium hydroxide (see Figure 3b).

While the research described so far exploits the ability to design DNA systems that can be reconfigured by adding complementary oligonucleotides or changing global conditions, such as pH, another option is to use the produced $^1O_2$ to irreversibly change the system. In a nanostructured DNA context, this was first reported by Mokhir and co-workers[29] when they employed a singlet oxygen cleavable (SOC) linker to "cage" an oligonucleotide (see Figure 4a). The linker, developed by Breslow and co-workers,[30] is composed of an electron-rich alkene, 1,2-bis(alkylthio)ethene (BATE), that upon reaction with $^1O_2$ creates an unstable dioxetane, which collapses into two carbonyls (see Figure 4b, left), thereby cleaving the linker. Mokhir and co-workers synthesized an analogue of this SOC moiety functionalized for incorporation into DNA by automated DNA synthesis. When the linker was introduced into a hairpin-forming DNA construct, this effectively blocked the DNA from being accessed by other oligonucleotides. Upon exposure to $^1O_2$, the linker was cleaved, which significantly lowered the melting point of the DNA duplex below ambient temperature, thereby "uncaging" the DNA. Mokhir and co-workers demonstrated that this could be done simply by adding a photosensitizer to the solution, but more effectively if the photosensitizer was tethered to the blocking oligonucleotide. Most other light-cleavable functionalities require UV light, which limits the biological applications.[31] Thus, a considerable advantage of the system developed by the Mokhir group is that the wavelength required is

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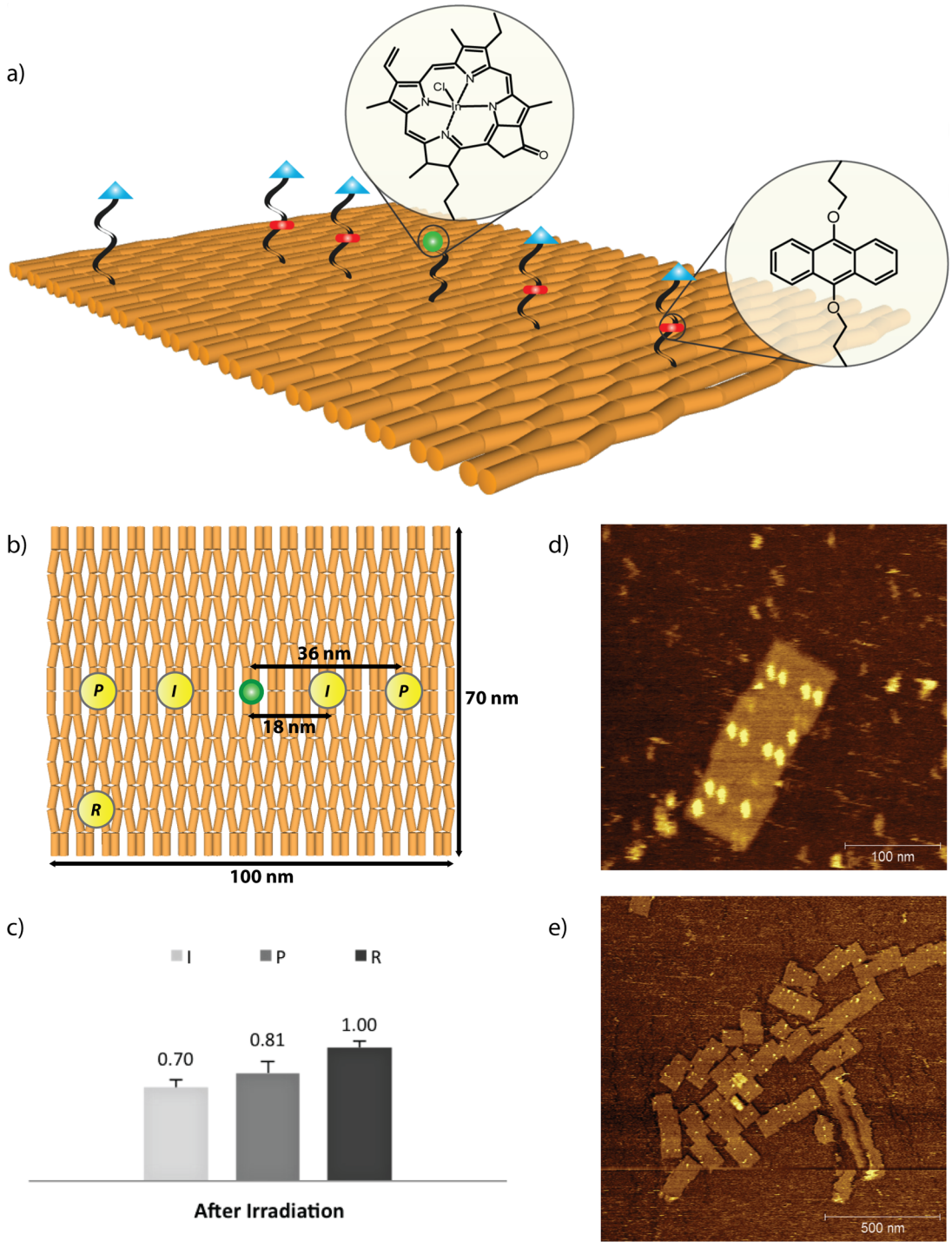

Figure 6. Investigating the reactivity and production of $^1O_2$ from a single photosensitizer on a DNA origami template. (a) A schematic representation of the DNA origami template with the staple strands modified with the following functional molecules: blue triangle represents biotin, the green sphere is a photosensitizer, and the red sphere is a $^1O_2$ cleavable linker. (b) The DNA origami template seen from above indicating the distances between the photosensitizer and the interior (I) and peripheral (P) cleavable groups. R is a noncleavable reference. (c) Histogram showing amount of cleavage observed by AFM from the interior (I), peripheral (P), and reference (R) groups. (d) AFM image of origami structures before irradiation. The streptavidins appeared as bright protrusions. (e) AFM image of origami structures after irradiation. The number of streptavidins in the cleavable positions on the origami was significantly reduced, whereas the noncleavable reference remained unchanged. Reprinted with permission from ref 41. Copyright 2010 American Chemical Society.

determined by the photosensitizer and not the linker. This enables the use of red light that is significantly more biologically benign and capable of penetrating tissue. In a series of related studies, Mokhir and co-workers demonstrated that SOC linkers, BATE and 1,8-dialkoxyanthracene (DA)[32] (see Figure 4b, right), could be employed in systems for DNA detection.[33,34] The same group also used strand displacement reactions (as shown in Figure 4a) for detection of oligonucleotides.[35] In one example, this approach was used to detect specific oligonucleotides in live human cells.[36] Mokhir et

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al. also demonstrated autocatalytic amplification of the detection of oligonucleotides by employment of a caged $^1O_2$ sensitizer that is activated by the $^1O_2$ initially produced in the strand displacement reaction.[37]

DNA ORIGAMI AND SINGLE MOLECULE REACTIONS

In collaboration with the Mokhir group, we used the BATE SOC linker (Figure 5a) to control the positioning and release of functional moieties from a DNA origami template.[39] The DNA origami technique was developed in 2006 by Paul Rothemund and immediately made complex DNA structures available to a broader scientific audience.[40] Briefly, the technique employs a long single-stranded scaffold, typically the genome of the bacteriophage M13mp18 (approximately 7000 bases), and a set of ∼200 so-called staple strands, that are specifically designed to fold the scaffold strand into a desired structure. Since each of the staple strands is synthetic in origin, the incorporation of non-natural functionalities is trivial. More importantly, because each staple strand is unique in sequence, its final position in the origami structure is also a design parameter. The origami structure can thus be chemically modified by extending the staple strands located in the desired positions with a few nucleotides and incorporating the desired modification during the staple strand synthesis.

Taking advantage of these features, we designed the origami structure depicted in Figure 5a. We employed three different linkers: a noncleavable linker, a dithiothreitol (DTT)-cleavable linker, and finally the $^1O_2$-cleavable linker described above. All of the modified staple strands carried a biotin moiety capable of binding the protein streptavidin, thus providing a single-molecule read-out system. DNA origami can be imaged using atomic force microscopy (AFM), but small molecules, such as biotin, cannot be detected in this way. Therefore, we utilized the strong affinity between biotin and the much larger streptavidin to visualize the changes caused by single-molecule reactions taking place on the surface of the origami structure. Employing this system, we demonstrated that small molecules and the proteins binding them could be removed selectively from the origami, as demonstrated in Figure 5b,c.

Recognizing that the use of streptavidin enabled a reliable read-out of single molecule reactions induced by $^1O_2$ and the fact that $^1O_2$ generated by a single photosensitizer would have a limited range of effect due to the short lifetime of $^1O_2$ and hence limited diffusion distance, we envisioned that the DNA origami could be used to study the reactivity of $^1O_2$ generated from a single $^1O_2$ sensitizer.[41] The system we designed for this study is depicted in Figure 6a. It is a single-molecule version of the classic Kautsky experiment[42] from 1931, in which two organic dyes were immobilized onto spatially separated solid supports. Kautsky then demonstrated that irradiation of one dye resulted in the oxygenation of the spatially separated second dye. This clearly had to happen through a diffusible "airborne" reactive species, later identified as $^1O_2$. Our nanoscale analogue consisted of an origami template modified with a single photosensitizer in the center, four biotins tethered via SOC linkers, and a noncleavable biotin reference. The four SOC linkers were divided into two subsets: the first *interior* subset was placed 18 nm from the photosensitizer, and the second *peripheral* subset was placed 36 nm from the photosensitizer (see Figure 6b). As previously stated, irradiating the photosensitizer in a controlled fashion and subsequently imaging the extent of streptavidin binding to the structures by AFM, would constitute a unique single-molecule setup for studying $^1O_2$ reactions. The electron-rich alkene used in the previous study decomposes to thiols, and thus has a chance of recombining as non-SOC disulfides. We therefore employed the DA linker (see Figure 4b, right) instead of the BATE linker.[32] The photosensitizer attached to the origami was an indium chelating PPa, which exhibited greater photostability than the free base PPa.[35,36]

When a large excess of the photosensitizer was added to the solution, the SOC linkers were cleaved with near-quantitative yield, leaving only the noncleavable reference on the origami. On the other hand, when only the origami-bound photosensitizers were present, the *interior* SOC linkers were cleaved more efficiently than the *peripheral* linkers. This is to be expected given the lifetime- and diffusion-dependent concentration gradient of $^1O_2$ centered around the photosensitizer. Further studies demonstrated that there was no singlet-oxygen-mediated cross-talk between the origami structures, thereby leading to the conclusion that the detected linker cleavage was indeed caused by $^1O_2$ originating from the single photosensitizer molecule on each origami structure.

CONCLUSION AND OUTLOOK

The use of DNA nanostructures and exploiting the ease of chemically introducing fluorophores and photosensitizers into such structures have already allowed an impressive control in singlet-oxygen-mediated processes. Indeed, in the authors' opinion, the future of using and studying singlet-oxygen-mediated processes, certainly in biologically relevant systems, depends strongly on developing methods by which more spatial and temporal control can be exerted in the production and deactivation of singlet oxygen. Potentially, such advances may lead to the application of DNA regulated singlet oxygen sensitizers in photodynamic therapy for more selective treatment of diseases. The examples given in this Account serve to indicate that the use of DNA networks and nanostructures in photosensitized $^1O_2$ systems provide many opportunities still to be realized for fundamental mechanistic studies or as a means to achieve practical goals.

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Notes

The authors declare no competing financial interest.

Biographies

Thomas Tørring was born in 1983 in Aalborg, Denmark. He received his B.Sc. and Ph.D. from the interdisciplinary Nanoscience center at Aarhus University working with functionalized DNA nanostructures in the laboratory of Prof. Kurt V. Gothelf. He also worked on new methodologies in DNA-protein conjugation as a postdoctoral

researcher with Prof. Kurt. V. Gothelf before joining the Ass. Prof. Jason Crawford at Yale University as a Carlsberg postdoctoral fellow. His research interests include DNA nanotechnology, biosynthesis and chemical biology.

Sarah Helmig was born in 1986 in Aarhus, Denmark. She received her B.Sc. and M.Sc. from the Department of Chemistry, Aarhus University, working at the Center for DNA Nanotechnology (CDNA). She is currently a Ph.D. student at the Interdisciplinary Nanoscience Center at Aarhus University, working with functionalized 3D DNA nanostructures in the laboratory of Prof. Kurt V. Gothelf.

Peter R. Ogilby was born in 1955 in the Philippines and immigrated to the United States in 1967. He obtained his B.A. (Honors) from the University of Wisconsin—Madison in 1977 and his Ph.D. from UCLA in 1981. After a postdoctoral fellowship at UC-Berkeley, he joined the faculty of the University of New Mexico, rising to the rank of Professor of Chemistry. In 1996, he moved to Aarhus University. Peter Ogilby is the founding Director of the Center for Oxygen Microscopy and Imaging, a consortium funded by the Danish National Research Foundation. Although Ogilby's research interests cover a wide range, they generally involve $^1O_2$ in one way or another.

Kurt Vesterager Gothelf was born in 1968 in Denmark and studied chemistry at Aarhus University, Denmark. He performed his Ph.D. research in organic synthesis and asymmetric catalysis under Professor K. A. Jørgensen, Aarhus University. He did postdoctoral studies in Professor M. C. Pirrung's group at Duke University, USA. From May 2002, he has been an Associate Professor at Aarhus University, and in August 2007, he was appointed Professor in organic nanochemistry. Since 2007, Kurt Gothelf has been the Director of the Danish National Research Foundation: Center for DNA Nanotechnology. His research interests are DNA nanotechnology and organic chemistry including DNA synthesis, DNA conjugation and DNA self-assembly, surface chemistry, and biosensors.

■ ABBREVIATIONS

AFM, atomic force microscopy; BHQ, blackhole quencher; ODN, oligonucleotides; PPa, pyropheophorbide a; SOC, singlet oxygen cleavable; BATE, 1,2-bis(alkylthio)ethene; DA, 1,8-dialkoxyanthracene; ISC, intersystem crossing; DTT, dithiothreitol; Casp3, caspase-3 protease; MMP-7, matrix metalloprotease 7

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