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DNA Origami as a DNA Repair Nanosensor at the Single-Molecule Level**

*Maria Tintoré, Isaac Gállego, Brendan Manning, Ramon Eritja, and Carme Fàbrega**

The development of DNA origami[1] has been one of the most important advances for structural DNA nanotechnology.[2] This method uses hundreds of nucleotide staples to fold a long single-stranded DNA scaffold of 7-kilobase, the M13 phage genome in a rational and desired shape. DNA origami is a versatile method for the self-assembly of other molecular species[3] and is an excellent way to create a variety of new nanoscale devices[4] with great potential and applications.[5] Yan and co-workers have used the DNA origami as an addressable support for label-free detection of RNA hybridization;[6] more recently, Seeman and co-workers have developed a nanosensor to detect single nucleotide polymorphism (SNP).[7] Both strategies are an innovative way to use the DNA origami method to create a nanosensor for biomedical applications at the single-molecule level using atomic force microscopy (AFM).

Advances in the molecular biology of cancer have identified key mechanisms involved in the DNA repair pathways induced by chemotherapeutic drugs, as for example alkylating agents. Adducts formed at the O^6 position of guanine are of major importance in both the initiation of mutations and in the cytotoxic effects of these agents. Human O^6-alkylguanine-DNA alkyltransferase (hAGT) is a DNA-binding protein responsible for the repair of the O^6-methylguanine, contributing to the resistance to chemotherapeutic agents. For these reasons, hAGT is considered relevant as a prognosis marker of cancer and is a potential therapeutic target.[8] Intense research efforts have been devoted to the identification of small molecules capable of inhibiting hAGT activity and enhancing the cytotoxic effect of the alkylating agents in tumor cells.[9] Several methods are available to characterize the mechanism of action of hAGT, its activity, and its inhibition by small molecules. However, most of these methods are based on radioactivity assays, while others are based on multiple-step enzymatic reactions.[10] Our research group developed a new fluorescence method using a DNA G-quadruplex, the thrombin binding aptamer (TBA), as a molecular beacon for the detection of hAGT activity and the development of new inhibitor compounds.[11]

G-quadruplexes are a family of four-stranded DNA structures stabilized by the stacking of guanine tetrads in which four planar guanines form a cyclic array of hydrogen bonds stabilized by the presence of monovalent cations.[12] Modifications in the base composition of the tetrads are poorly tolerated by these structures. As an example, O^6-methylguanine can form a smaller number of hydrogen bonds and consequently destabilize the G-quadruplex, provoking the loss of its conformation (Supporting Information, Figure S1).[13]

Herein, we exploit the spatial addressability of DNA origami in combination with the change of conformation of a DNA G-quadruplex to visually detect by AFM the change in its binding affinity to α-thrombin. As this structural change is caused by a single methylation in the central guanines, it can be utilized to detect the DNA repair activity of hAGT, given that methylguanine is the substrate of this protein. To attain this goal, we have used the specific binding properties of the TBAs (aptamers with anticoagulant properties) to α-thrombin, their natural substrate.[14] The two TBA sequences used in this work (see the Supporting Information, Table S1, for sequences) are known to bind specifically and cooperatively to two specific and almost opposite epitopes of α-thrombin.[15] TBA1 (primarily fibrinogen-recognition exosite binding)[14] is a 15 mer nucleotide composed of two G-tetrads that are connected by three edge-wise loops, forming a well-characterized intramolecular chair-like, antiparallel quadruplex. In contrast, TBA2 (29mer nucleotide, heparin-binding exosite) forms a combined quadruplex/duplex structure.[15]

Previous studies provide evidence that the TBAs are able to bind α-thrombin in the absence of monovalent cations promoting the TBA folding to its 3D structure, following the typical chaperone-macromolecule system.[16]

Previously, to study the interaction between TBA1 and α-thrombin, fluorescence quenching experiments and electrophoresis mobility shift assays were performed (Supporting Information, Figures S2 and S3). Both results confirmed that the introduction of a methylated guanine prevented α-thrombin interaction, and furthermore that a 10-fold concen-

[*] M. Tintoré,[+] Dr. I. Gállego,[$] [+] Dr. B. Manning, Dr. R. Eritja, Dr. C. Fàbrega
IRB Barcelona, IQAC-CSIC, CIBER-BBN Networking Centre on Bioengineering Biomaterials and Nanomedicine
c/Jordi Girona 18-26, 08034 Barcelona (Spain)
E-mail: carme.fabrega@irbbarcelona.org
Dr. B. Manning
Biokit, S.A.
08186 Lliçà d'Amunt, Barcelona (Spain)
[$] Present address: School of Chemistry and Biochemistry
Georgia Institute of Technology
Atlanta, GA 30332-0400 (USA)
[+] These authors contributed equally to this work.

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tration of α-thrombin can be sufficient to appreciate a different pattern of union of this protein to the TBAs and methyl-TBA-containing origami.

Based on these preliminary studies, we designed a DNA origami in which some of the staple strands were modified by the insertion of TBA1 and TBA2 in the middle, protruding from the DNA origami surface[17] (see the Supporting Information, Table S1, for sequences). The staple strands were arranged asymmetrically along the length of the origami in a way that allowed the differentiation between methylated/non-methylated, to enable the observation and quantification of α-thrombin interaction with the aptamers. For this purpose, we built a dual-aptamer system composed of two lines of five TBA1 and TBA2 doublets placed at a distance of about 5.8 nm from each other, increasing the recognition probabilities by at least 10-fold as reported by Rinker et al.[17] The right double line corresponds to the unmodified dual system, and the left dual-aptamer line consists of 15 mer methylated-TBAs and non-modified 29 mer TBAs (Supporting Information, Scheme S1). The formation of the DNA origami with the modified TBA staple strands was performed successfully, confirming that the addition of a methylated sequence does not affect its assembly (Supporting Information, Figure S4).

Afterwards, the complex formation between α-thrombin and the non-methylated/methylated TBA modified origami was studied. In Figure 1, the asymmetric interaction can be

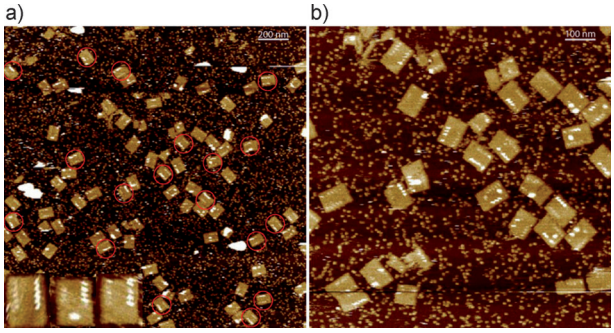
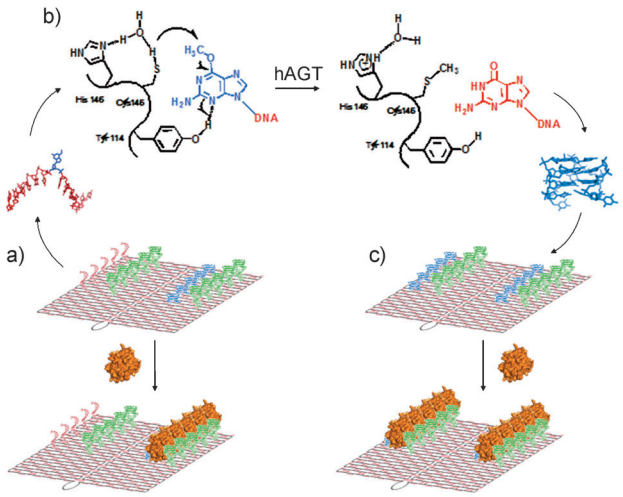


Figure 1. a),b) AFM images (scale bars 200 nm (a), 100 nm (b)) of the interaction of α-thrombin with the origami. The interaction can be observed as white aligned dots deposited over the origami surfaces. The complex was only formed with the native TBAs (right line; see inserts in (a) for more details), whereas in the left line, in which the 15mer TBA carried an O^6-methylguanine, no interaction was observed.

observed. As expected (Scheme 1 a), the complex was only formed with the native TBAs, whereas in the left line no interaction was reported, confirming that α-thrombin is not able to bind the disrupted quadruplex. The study of the profiles and pixel distribution (Figure 3 b) confirms that the height of the dots in the dual-aptamer is in agreement with the expected size of α-thrombin (ca. 4 nm in diameter) in comparison with the height of the origami control (Figure 3 a). We observed that more than 95 % of the chemically modified origami tiles faced pointing towards the solution, in agreement with data reported by Voigt et al.[4c]

This result is a clear confirmation that the complex between the dual-aptamer system and α-thrombin is only formed with the non-methylated TBA, and confirms the



Scheme 1. a) Representation of the asymmetric binding of α-thrombin to TBA aptamers of methylated DNA origami. b) Methyl-TBA repair by hAGT, thus allowing G-quadruplex formation. c) Representation of the symmetric binding of α-thrombin to the repaired DNA origami quadruplexes.

ability of our design to discern between the methylated and non-methylated state.

To explore the efficiency of our design, we performed a quantitative study of the binding location of α-thrombin. From the 160 well-formed DNA origami studied, around 20 % of them contained all five α-thrombin molecules in positions coinciding with unmodified TBA and almost none in the methylated line. In all, more than 93 % of the DNA arrays contained at least 1 α-thrombin attached to the unmodified TBAs (see the Supporting Information).

We then intended to repair the O^6-methylguanine of the TBA-containing staple strands by hAGT. For this purpose, the methyl-TBA-staple strands were incubated with hAGT. hAGT was removed and the resulting strands were used to assemble the DNA origami (see Materials and Methods in the Supporting Information). The recovery of the chair-like structure of the now demethylated 15mer was expected, leading to the binding of α-thrombin to both dual-aptamers, as the two of them contain the native 15 and 29mer TBAs.

The binding of α-thrombin in both dual-aptamers is shown in Figure 2, confirming the repair of the alkylated guanine by hAGT. Upon quantitative exploration of the binding, we can conclude that α-thrombin binds with equal contingency in both lines of the origami, with no significant tendency ($p < 0.5$) for any of the dual systems composed by TBA1 and TBA2. The study of the height profiles corroborated the theoretical height of the α-thrombin on both dual-aptamers (Figure 3 c). Furthermore, we titrated hAGT (0 to 10-fold origami:hAGT; see the Supporting Information) against methyl-TBA staples and incorporated these staples into the DNA origami. The results showed that α-thrombin binding to the methylated/repair side was clearly dependent on hAGT concentration (Supporting Information, Figures S10 and S11).

In summary, we have developed a new method to study the DNA repair activity of hAGT. To the best of our

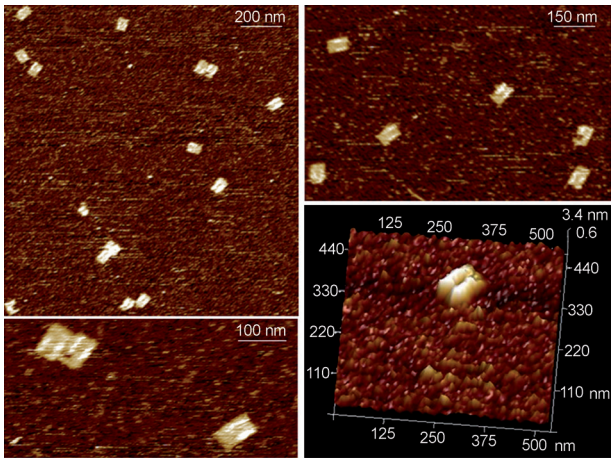


Figure 2. Symmetric binding of α-thrombin to the origami after the repair of the methylation in the TBA1 (left line). The bottom-right panel shows the 3D profile of an origami with all its binding positions occupied by α-thrombin.

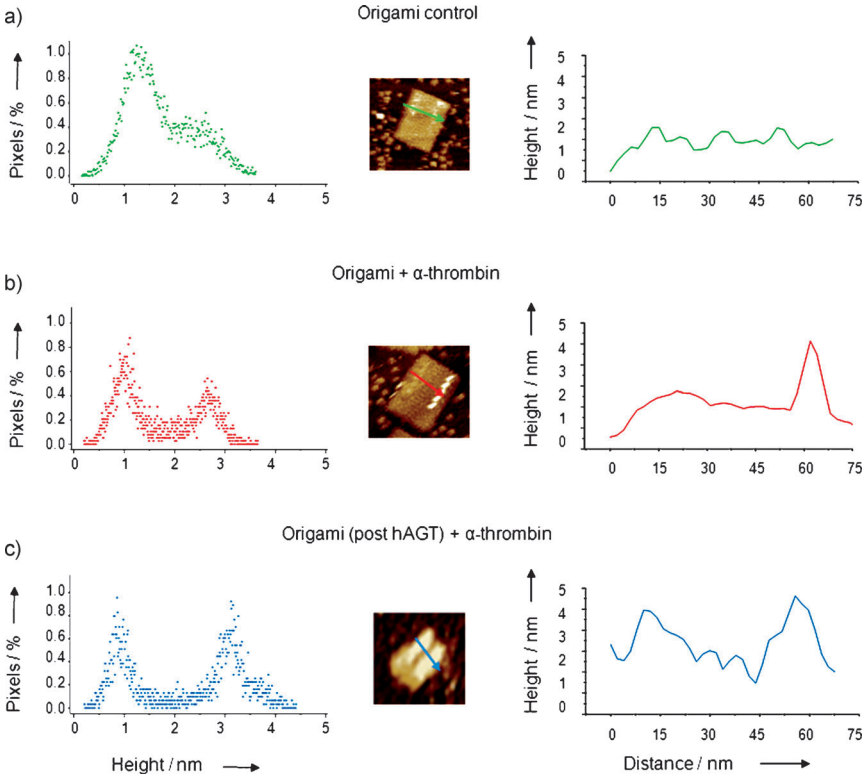


Figure 3. Distribution of heights, corresponding AFM images, and their cross-sections. a) TBA-origami. b) α-thrombin methyl-TBA origami complex. c) α-thrombin complex with demethylated TBA origami.

knowledge, this is the first time the enzymatic activity of hAGT has been visualized on an origami platform. This study combines the capabilities of the α-thrombin recognition/binding to TBA and the single-molecule features of the DNA origami applied to the detection of DNA repair. The system appears to be extremely effective and reliable, and the results are clearly visualized by AFM. Their consistency suggests that our system could be further evolved to design hAGT activity assays for the identification of potential inhibitors as chemotherapy enhancers and for the study of other DNA repair enzymes. The application of the DNA origami as a platform for single-molecule recognition opens the door for the development of new biosensors for the detection of a variety of complexes and the activity of other proteins. Finally, it can also contribute to the study of other DNA lesions that affect G-quadruplexes. This in turn would increase our knowledge on the effect of DNA damage in biologically relevant G-quadruplex structures.[18]

Experimental Section

Standard oligonucleotides were purchased from Sigma. Modified staple strands were synthesized on a DNA synthesizer following standard methods. All of the oligonucleotides sequences are detailed in the Supporting Information. Full-length hAGT was overexpressed and purified as previously described.[10e] A mixture of the modified staple strands containing the methylated 15-mer TBA sequence were left to react with hAGT. DNA origami tiles were assembled following the method developed by Rothemund.[1] A sufficient amount of α-thrombin was added and left to equilibrate before imaging. Images were acquired in tapping mode in liquid environment using triangular-shaped AFM probes and their analysis was performed using NanoScope Analysis Version 1.40. All of the experiments were performed in triplicate. Statistical comparisons of the binding performance were done according to Student's *t* distribution.

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