High-speed AFM imaging of the Entropic Disassembly of DNA Origami for Single-Molecule Biosensing

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Abstract

Circulating microRNAs (miRNAs) are promising biomarkers for disease diagnosis, but their small size and instability hinder direct detection. The detection of small RNA, such as miRNA, using solid-state nanopores typically involves the binding of miRNA to a larger carrier molecule to generate signals. However, this approach is entropically unfavourable, and it is prone to RNAse degradation in the environment leading to accidental digestion of miRNA prior to analysis. Here, we present an alternative approach based on DNA origami disassembly driven by toehold mediated strand displacement (TMSD). Symmetrical DNA origami dimers are separated into monomers via TMSD using miRNAs as invading strands. We visualized the real-time dynamics of dimer separation at high resolution using high-speed atomic force microscopy (HS-AFM), providing direct insight into the kinetics of the TMSD process. The entropically driven disassembly process can be followed using single molecule nanopore sensing leading to a quantitative readout of the ratio of dimers to monomers. This direct read-out method enabled the multiplexed detection of miRNAs. Due to the near irreversible process of the TMSD, we performed the detection of miRNA in crude RNA tissue extracts under the presence of RNAse and showed that our approach allowed the robust detection of small RNA that is unaffected by the complex degrading environment.

Keywords: Single molecule, Nanopore, DNA origami, RNA, toehold mediated strand displacement, High speed AFM

Introduction

The programmability of base pairing between nucleic acids has driven the advancement of DNA nanotechnology, enabling the construction of DNA nanostructures with precise sizes and shapes[1]. One notable example is DNA origami, which uses hundreds of short DNA oligonucleotide staples combined with a sequence-defined single-stranded DNA (ssDNA) scaffold to rationally design nanoscale structures [2-5]. Each DNA origami unit can be further assembled into higher-order supramolecular structures[6,7] through blunt-end stacking[8] or DNA strand hybridization[9-18].

The connection between DNA origami units can also be severed through methods such as toehold mediated strand displacement (TMSD) [10,19]. TMSD is an enzyme-free oligo strand exchange process, where the starting hybridised dsDNA is composed of the original strand and a protector strand. The original strand includes an overhang region, or toehold, which is complementary to a third, invading strand. This invading strand is fully complementary to the original strand. The process initiates when the invading strand partially hybridise to the toehold and, through branch migration, displaces the protector strand, so that the resulting dsDNA no longer contains the toehold [20,21].

The programmability of DNA origami also enables the formation of DNA:RNA heteroduplexes, which, when paired with an appropriate readout strategy, can be used for the specific detection of RNA molecules, including microRNAs (miRNAs)[14,22-27]. MiRNAs are a class of small, non-coding RNAs with average length of 19-25 nucleotides [28,29]. They are highly conserved and play an important role in post-transcriptional gene regulation through translational repression and mRNA degradation [30]. As the expression profile of miRNA is intimately related to the cell status [31], bodily fluid circulating miRNAs are clinical biomarkers for diseases including infectious diseases, cancers and physical injuries like traumatic brain injury (TBI) [32-35]. Direct detection of miRNA is difficult, as they present in sub nanomolar concentrations in bodily fluids and are easily degraded when exposed to ribonuclease (RNAse) in the environment [36,37]. MiRNA levels are routinely quantified using hybridization-based microarrays and RT-qPCR[38]. More recently, innovative approaches such as nanopore sequencing have been successfully employed for miRNA detection [39].

Nanopore sensing is a label-free biophysical technique allowing single molecule analysis [40-51] that can provide a quantitative readout [52], and it can be been successfully employed for the fingerprinting of DNA origami [16,53,54]. The detection of miRNA with nanopores relies on a functional carrier such as magnetic bead, gold nanoparticle, DNA or DNA origami [45,46,55-58]. The carriers change size or conformation upon miRNA binding, and these changes can be "read"

using a nanopore sensor. However, all the approaches described above are entropically unfavourable as they all rely on the formation of higher order structure. Furthermore, these approaches are vulnerable to RNAse which can cause the instant degradation of the bound miRNA leading to false negative read-outs.

Here, we propose an entropically-driven DNA origami strategy which is compatible with a nanopore readout and allows for the selective detection of miRNA in crude RNA extract. We designed and validated a DNA origami dimer formed through the dimerization of DNA origami tiles.[16-18] The connection between the tiles consists of dsDNA linkers containing toeholds. Upon exposure of the dimer to oligo sequence complementary to the linkers, the dimer is disassembled into two monomers. A nanopore sensor in combination with a polymer electrolyte [16,40-43,50,59] can then quantitatively monitor the relative concentration of the dimer and monomer tiles and infer the miRNA presence and concentration. The polymer electrolyte method allowed us to detect the translocation of DNA origami in its native buffer without the need of high salt conditions, thus avoiding potential instability of the structure. We observed that both DNA and RNA can be used to separate the dimer, and miRNA can also be used when its secondary structure is disrupted. We also demonstrated that detection can be carried out under RNAse contamination at the point of measurement. The presence of RNAse does improve our detection sensitivity as the DNA origami are resistant to its action, and its presence eliminates background signal caused by small RNAs. In our approach, whether the miRNA is bound to the DNA origami does not affect the readout as long as the miRNA has already disassembled the dimer. In addition to the nanopore readout, we directly visualised the TMSD reaction in real time using high speed-atomic force microscopy (HS-AFM)[60,61]. This enabled single molecule mapping of the stepwise separation of the dimer, revealing the dynamic progression of strand displacement and the positional preferences of linker regions. These observations provide unique mechanistic insights into the nanomechanics of TMSD at the DNA origami interface.

Results and discussion

DNA origami-based oligonucleotide sensor

A dimer is generated through the dimerization of two units of DNA origami monomer tiles – the left and the right monomers (Figure 1a). The left and the right monomers are asymmetrical tiles, and their design is based on previously published structures [16-18,59]. For dimerization, the staple sets of the edges of these monomers are modified with additional complementary ssDNA overhangs, such that a dimer can be formed through strands hybridisation [10-15]. Except for the overhang edge's staples, the left and the right monomers are otherwise identical and composed of the same staples.

The formation of the dimer can be carried out with a maximum 11 linkers [16-18]. We screened the formation of dimers with 1, 3, 5, 7 and 9 linkers to identify the minimal number of linkers needed to generate a high yield of dimers (Supporting Figure 1a) and confirmed that dimer can be formed with 5, 7, and 9 linkers at high yield, while 1 and 3 linkers are not sufficient (Figure 1b, Supporting Figure 1b-c). Based on these results, we selected 5 linkers to generate the dimer.

Atomic Force Microscopy (AFM) was used to characterise the formation of the dimer as shown in Figure 1c, the resultant dimer were asymmetric and connected with 5 linkers, both the left and the right monomers could be identified and confirmed the dimerization of the monomers occurred through the designated linkers [8]. The left and the right monomers could self-dimerize to form dimeric structures through blunt end stacking [3,8,18], these unintended stackings happened predominately at the overhang modified edges, as shown in the AFM images in Supporting Figure 2, of less than 10% occurrence. We also employed high-speed AFM (HS-AFM) and localization-AFM (LAFM)[62,63], to enhance the spatial resolution of the topographic features as shown in Figure 1 (d,e). Figure 1d shows the high-resolution localized visual of the DNA for all 5 linkers, on rare occasion, we observed that the DNA linkers of the dimer crossed to form an "X" shaped connection between the 2nd and 3rd linker as shown by the LAFM at high-resolution (Supporting Figure 3a). While this observation is rare, it is not unexpected since all 5 linkers have the same sequences and are complementary. Additional high-resolution LAFM images of the origami can be found in Supporting Figure 3. The DNA linker's length was approximately 7.8 nm long as shown by AFM and LAFM images, agreed with the theoretical length of 8.2 nm (0.34 nm/bp, 24 bp) per DNA linker.

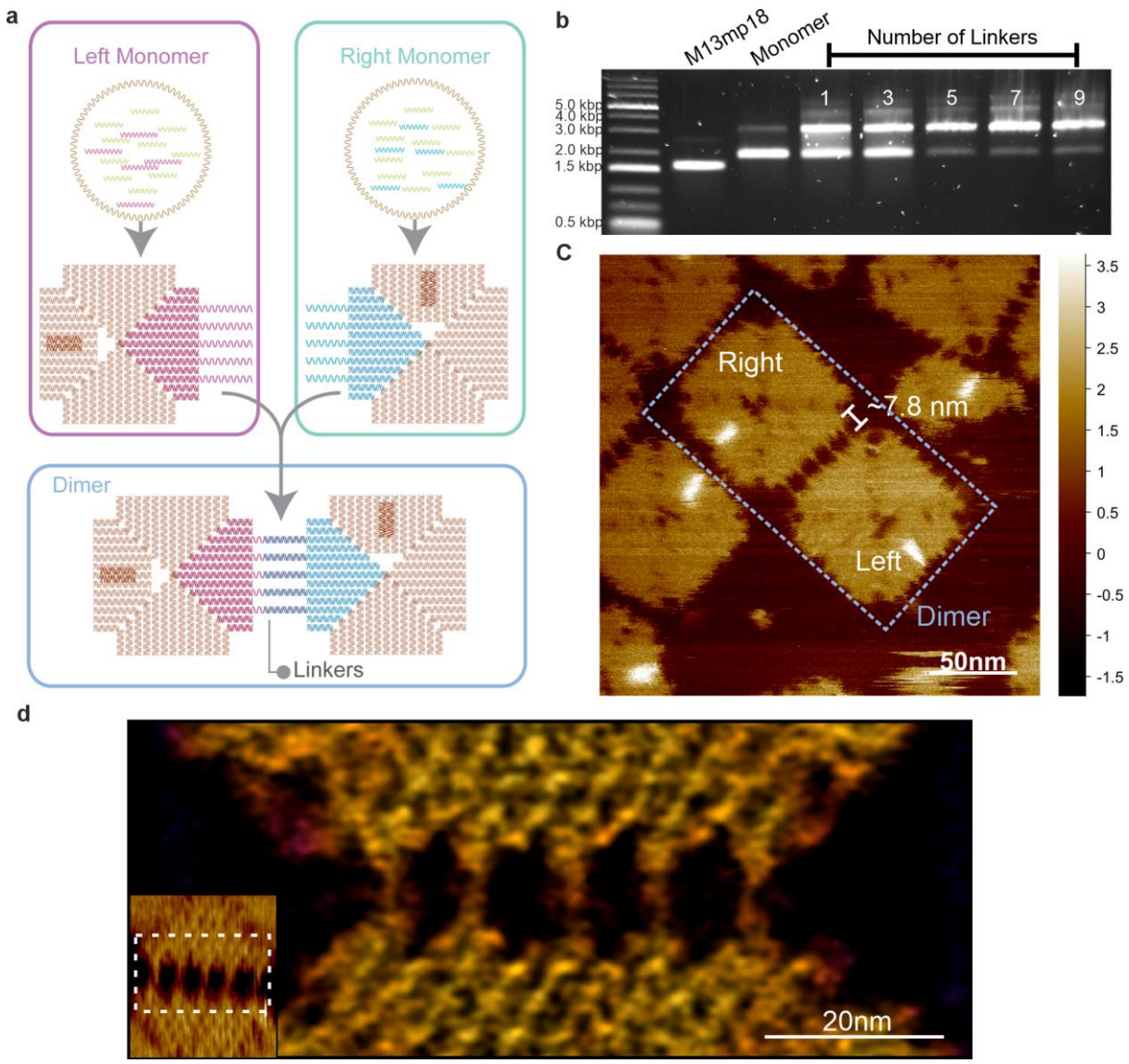

Figure 1. Design and characterisation of the DNA origami dimer. (**a**) Schematic illustration on the formation of the 5 linkers dimer. The process involves two DNA origami monomeric units, the left (red) and the right (blue) DNA origami monomers, the edges of both left and right monomers are 5×ssDNA overhangs that are more than 20 nt long, extending away from the main tile body of the origami. Additionally, the left monomer's edges contain extra 8 nt at the 5' direction to act as toehold after hybridisation. After the synthesis and purification of the monomers, the left and right monomers are mixed at equal portion and undergoes a thermal incubation step to form the asymmetric 5 linkers dimer . (**b**) Agarose gel electrophoresis screening on the number of linkers necessary to form the dimer, 5 linkers and above led to high yield of dimer, as can be observed that the monomer bands are a lot fainter than monomer, 1 linker and 3 linkers. (**c**) AFM imaging of the resultant dimer. The asymmetric property of the dimer allows us to confirm the formation of the dimer is via designated linkers not lateral bond stacking. The length of the linkers is approximately 7.8 nm which is close to the theoretical length of 24 bp dsDNA (8.16 nm; 0.34 nm/bp). (**d**) Combining the HS-AFM (inset) and LAFM approaches to visualise the 5 connecting linker strands.

The ssDNA overhangs of the left monomer contain additional nucleotides (8 nt) at the 5' direction to act as toehold, when the is formed after dimerization, a toehold is generated through the hybridisation between the original strands (left monomers) and the protector strands (right monomers). The toehold in the linkers allows the dimer to be separated through the toehold mediated strand displacement (TMSD) reactions [20,21]. The resultant 8 nt toehold

leads to fast displacement kinetics [64-67]. The TMSD reaction follows a sequence of steps, as shown in Figure 2a. The TMSD reactions at the linker regions will separate the dimer into two monomeric units.

The TMSD approach to separate the dimer was tested via mixing 2 nM of the dimer with 100 nM of the invader strands (50× excess). The samples were incubated at 25°C for 30 mins followed by storage at 4°C until analysis. Gel electrophoresis and AFM were used to characterize the success of the TMSD displacement reaction (Figure 2b-c, Supporting Figure 4). The dimers separated into monomers after the TMSD reactions, and where the toeholds were absent from the linkers, the addition of the invaders failed to separate the dimers. The AFM images showed the dimer (left) and the dimer after TMSD displacement (right).

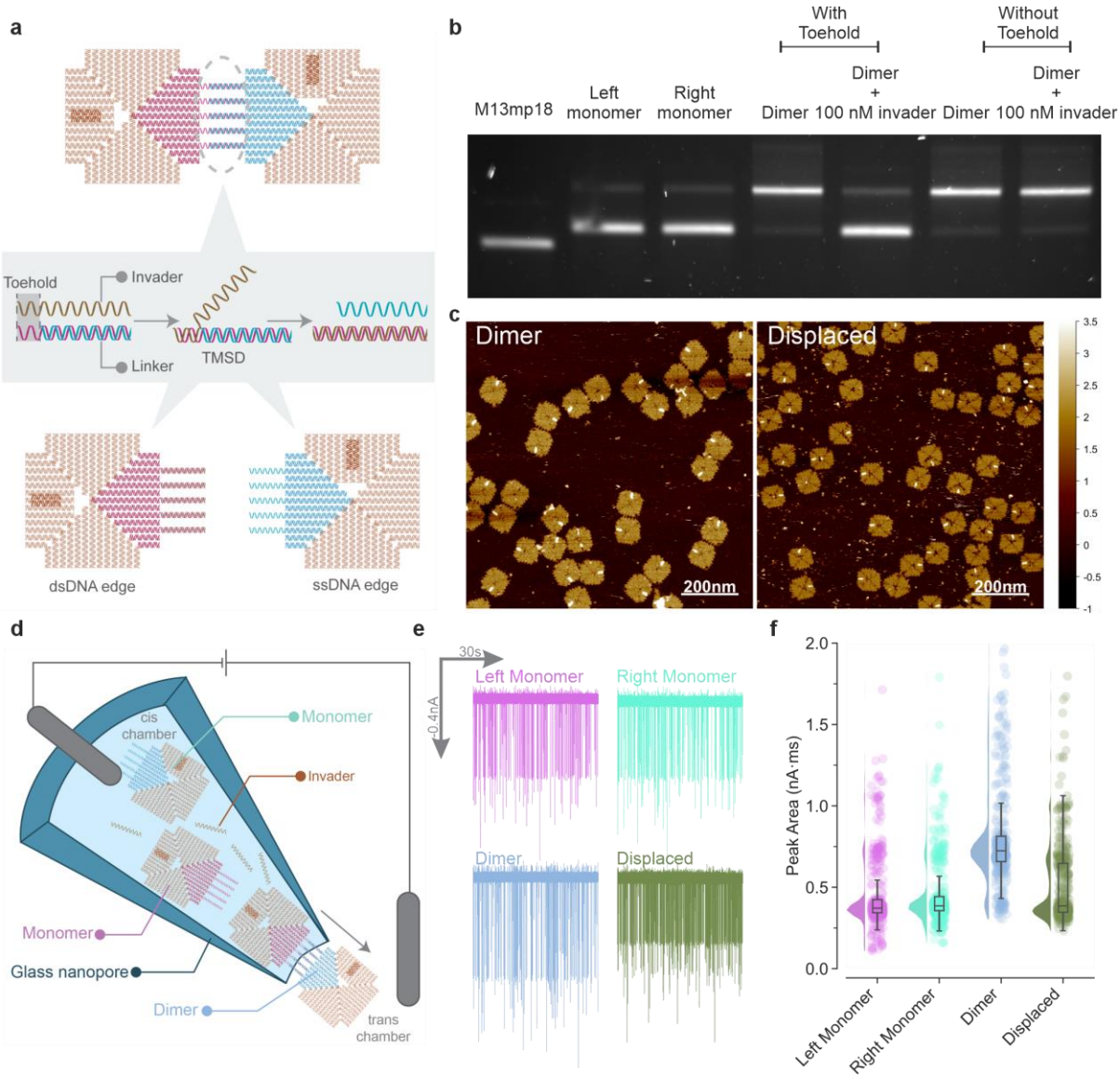

Figure 2. The short oligo responsive dimer. (**a**) Schematic on the displacing mechanism through TMSD. The 5 linkers dimer after dimerization, each linker contains 8 nt long toehold, these toeholds are partially complementary to the ssDNA invader strand, the invader strands are full complementary to the original strand. The invader strand partially hybridises onto the toehold region of the linker, resulting in a 3 strands complex, branch migration of the

invader strand causes the displacement of the initially hybridised protector strand. The TMSD happens in all 5 linkers and separate the dimer into monomers again. After the displacement, the left monomer contains dsDNA overhangs, and the right monomer contains multiple ssDNA overhangs. (**b**) Gel electrophoresis analysis on the displacement of the dimer. The dimers were displaced by the introduction of invader at 100 nM, as evidenced by the band migrates to the monomer position. A dimer without toehold could not be displaced by the invader at 100 nM, indicating that displacement was not spontaneous at room temperature. (**c**) AFM imaging of the dimer and the dimer after displacement. (**d**) The schematic illustration of the translocation of the monomers and dimer. The DNA origami were diluted in the origami buffer (10 mM Tris-acetate, 15 mM Mg-acetate and 1 mM EDTA, pH 8.0) to 9.4 ng/µl (2 nM for dimer, 4 nM for monomers). The DNA origami were loaded into the cis chamber of the nanopore, a voltage of -500 mV was used to cause the translocation of DNA origami from the cis to trans chamber. The trans chamber is composed of PEG polymer electrolyte (0.1 M KCl, 50% (w/v) PEG 35K). (**e**) The translocation trace of the left monomer (top left), right monomer (top right), dimer (bottom left) and the displaced dimer (bottom right). (**f**) The translocation event peak area comparison. The left, right monomers and displaced dimer had a peak area median of approximately 0.4 nA·ms and the dimer had a peak area median of 0.7 nA·ms, the dotted line draws across the position of 0.4 nA·ms. The distribution was estimated by kernel density estimation.

Previously, we employed nanopores to analyse the structures of DNA origami tiles, showing that the nanopore could differentiate and fingerprint supramolecular DNA origami structures [16]. Here, we utilised glass nanopores to quantify and differentiate monomers and dimers under native buffer conditions (10 mM Tris-Acetate, 15 mM Mg-Acetate, 1 mM EDTA, pH 8.0) (Figure 2d). Our nanopore sensing approach uses a poly(ethylene) glycol (PEG) polymer electrolyte [40-43], which allows us to perform single molecule detection high signal-to-noise ratio(Figure 2e) with different analytes at physiological relevant salt condition (~0.1 M monovalent salt) in contrast to all other studies[42,44,51] requiring monovalent salt within the range 0.1-4 M [44,57,68]. This capability is enabled solely due to the properties of the PEG polymer electrolyte [40-43,50].

The nanopores used in this study had a diameter of approximately 50 nm, and their fabrication was highly reproducible, as confirmed by the voltammogram analysis (Supporting Figure 5a–b). No translocations were observed when the measurements were carried out in pure buffer (no DNA origami present) (Supporting Figure 5c). Following folding and purification, the DNA origami structures were diluted to 2 nM for monomers and 4 nM for dimers. These samples were then analysed using the nanopore system under a range of applied voltages, from –100 mV to –700 mV. The applied voltage drove the negatively charged DNA origami from the cis to the trans chamber (Figure 2, Supporting Figure 5d). A voltage bias of –500 mV was selected as the standard for further translocation experiments. This setting provided a high capture rate of exceeding 300 events per minute while minimizing the risk of nanopore clogging. The translocation of the dimer elicited a single molecule event with a larger current amplitude magnitude and slightly longer dwell time when compared with the monomer (Supporting Figure 5e). The most effective method for distinguishing between monomer and dimer events was by analysing the peak area of the translocation signals, consistent with previous findings from our group[16]. The dimer population exhibited a peak area of approximately 0.7 nA·ms, while the monomer population centred around 0.4 nA·ms (Figure 2f).

Both short ssDNA and RNA oligos can trigger a TMSD [65,69-72] reaction by forming either a dsDNA duplex (denoted as ssDNA:ssDNA) or a RNA:DNA heteroduplex (denoted as RNA:ssDNA). We conducted a screening experiment to assess the displacement of the dimer using varying concentrations (2–25 nM) of ssDNA and RNA invaders. The dimer population was quantified via nanopore measurements using a recently developed analytical pipeline. [50] . The ssDNA and RNA oligos were identical in sequence, except for the substitution of thymine (T) with uracil (U) in the RNA. The percentage of dimer remaining varied slightly depending on whether the invader was ssDNA or RNA (Supporting Figure 6). In all cases, RNA invaders displaced fewer dimers compared to ssDNA. However, at a concentration of 15 nM, both types of invaders achieved complete dimer displacement. To further monitor TMSD kinetics, we employed a fluorophore-quencher pair covalently attached to the linkers. Upon displacement, the fluorophore was separated from the quencher, enabling real-time tracking of the reaction using a fluorescence plate reader over several hours (Supporting Figure 7). In agreement with a previous study [65], the ssDNA:ssDNA reaction reached equilibrium slightly faster than the RNA:ssDNA reaction at all tested concentrations (5, 15, and 25 nM), as indicated by the rate constants. Approximately 50% of the reaction occurred within the first 10 minutes, with the majority completing within 30 minutes.

Visualising TMSD

The separation of the dimer relies on TMSD [73-76]. The molecular details of this process are typically probed through fluorescent based bulk assays (Supporting Figure 7), single molecule fluorescence, force spectroscopy or computational simulation[77,78]. However, directly observing TMSD in action remains challenging due to the difficulty of imaging oligonucleotide dynamics. Here, leveraging the dimer origami's design and the high spatial-temporal resolution of HS-AFM we can directly visualize the TMSD process that leads to the separation of the dimer. This allows us to capture its kinetic behaviour and reveal positional preferences of the linkers involved in displacement.

High-Speed AFM imaging of the dimer, connected by 5 DNA linkers (L1-L5), showed these linkers remained stable and connected during repeated AFM imaging for more than 840 frames (Supporting Figure 8, Supporting Movie 1, 2). Invader DNA strands were added in situ to the HS-AFM imaging buffer at a final concentration of 10 nM without interrupting imaging, enabling real-time capture of the TMSD process. Following the addition of invader strands, the linkers of the dimer were observed to disconnect and separate at different time points to their neighbouring linkers (Figure 3a-c, Supporting Movie 3, 4).

HS-AFM imaging revealed that separation can initiate from any of the five DNA linkers between the dimer 's monomeric subunits (Supporting Figure 9, 10, Supporting Movie 4, 5). Interestingly, in some cases, the severed linkers reconnected for a fraction of time, this could be due to an incomplete TMSD reaction and/or unintended lateral stacking effects. We also observed that the reconnection could be formed between linker at different positions (e.g. between L1 with L3)(Supporting Figure 10, Supporting Movie 5). Overall, these short-lasting reconnections of the linker do not halt the TMSD initiated separation process and eventually lead to the complete separation of the dimer into two monomers on the mica. After the full separation of all 5 linkers, the initial monomers could not reconnect and form the dimer again. Free monomers could randomly weakly interact with another free monomer for a very small fraction of time, these interactions were unstable and could be identified due to the asymmetric design of the dimer.

To measure the TMSD molecular process and dynamics, we produce kymographs [79] of aligned HS-AFM movies to profile the topographic height over time of the linker regions during separation after addition of the invader strands to the imaging buffer (Figure 3a-c). The kymograph profile (Figure 3b) is obtained by measuring the height values along the dashed vertical line marked in Figure 3a. In this kymograph, each vertical line represents a different frame of the HS-AFM at different point of time. Therefore, when the linkers are connected, the kymograph will show a higher height response at a particular point, and when the linkers are disconnected, the linker region height will decrease.

To track the TMSD process, the height profiles of all five linkers from the kymograph (Figure 3c) were analysed as binary states (0 = disconnected, 1 = connected), using a threshold of 0.75 nm in height to distinguish between them. Figure 3c shows 10 separation events that could be observed at different time intervals as marked in the plot and inset images (1-10). The 1st separation event was observed at t = 8s (state-0, linker-2 position), when the linker-2 became disconnected (Figure 3b, inset image 1), however, this linker reconnected back and subsequently became disconnected again at t = 187s. Similarly, other separation events at different linker positions were identified at different time points that led to the separation of all 5 linkers (t=853s). Figure 3d summarised the number of connected linkers over the HS-AFM tracking time, showing how the final 4 linkers separated in quick succession for this particular dimer .

We monitored 4 additional dimers' full separation after the addition of the invader strands and acquired their separation events' on/off states in the height profile and associated kymographs as shown in Supporting Figures 9, 10. Analysis of the reconnection time between individual linkers at different positions (Figure 3e) showed that after the initial TMSD mediated

separation, stochastic reconnection events occurred within less than 20 seconds for each individual linker, but the full separation of the dimer required longer time (Figure 3b). We then summarised the linker position that separated first (between L1-L5) after the addition of the invader strands throughout these HS-AFM videos, our data showed that the outer linkers (L1/L5) are more prone to be the first linker to be separated from TMSD reaction (Figure 3f). Although this could be an unintended effect from 2D surface diffusion of invaders on the mica, since the outer linkers would be most exposed, this observation still highlights the importance of the strand position and placement within the nanostructure that can affect the nanomechanical action of TMSD.

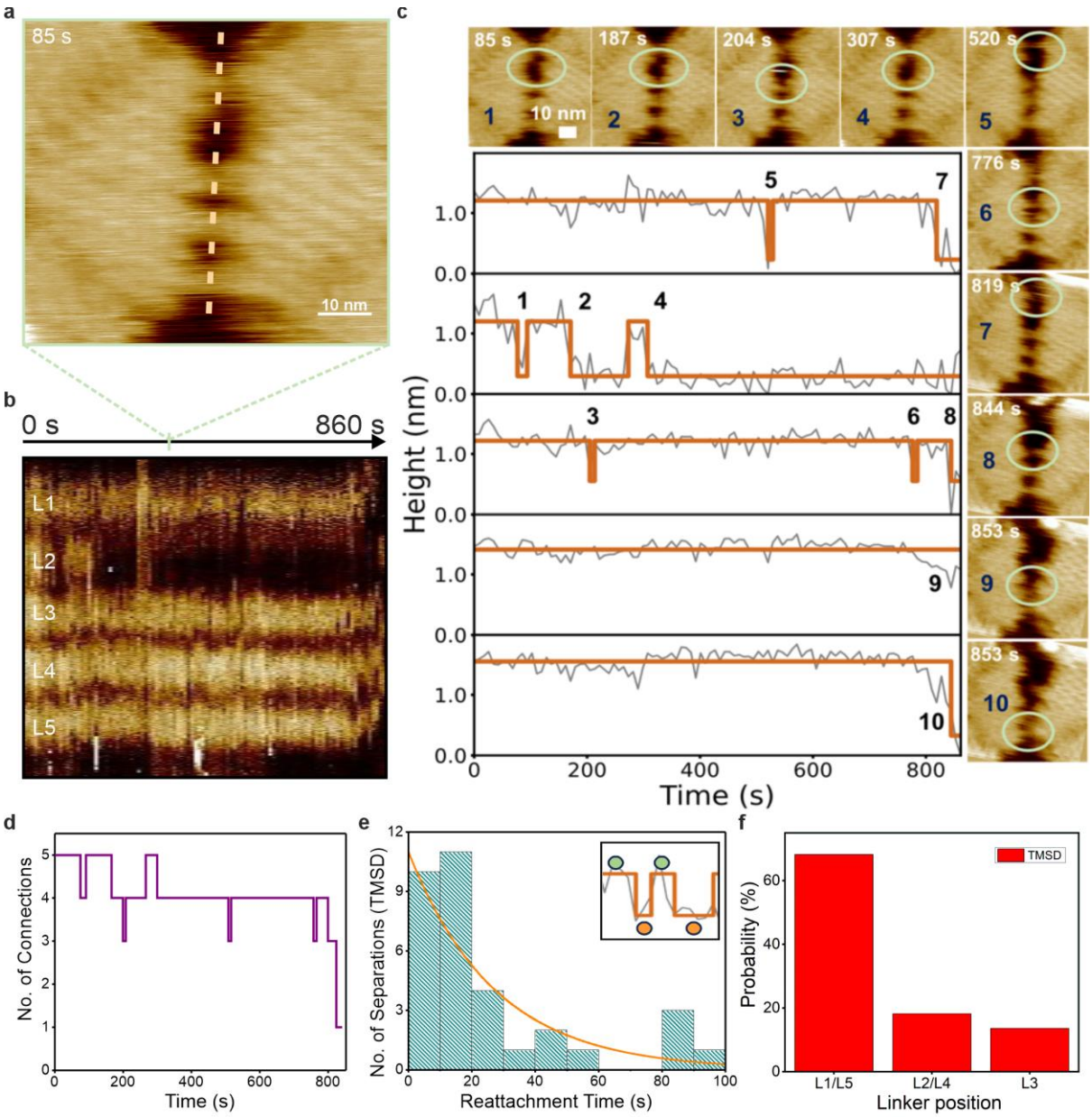

Figure 3. HS-AFM tracking of the separation of the dimer. To track the TMSD mediated separation of the dimer, a single dimer was tracked in HS-AFM, the separation was initiated by directly adding a final concentration of 10 nM of invader strand, into the imaging buffer. (**a**) A snapshot of the dimer at 307 seconds, after start of the separation event

on kymograph timeline. (**b**) Kymograph profile of HS-AFM video to visualize the separation of each linker (L1, L2, L3, L4, L5) for the dimer. All 5 linkers were monitored over 860 seconds. (**c**) Kymograph height profiles along each individual linker over time with HS-AFM images selected at time points corresponding to on/off events (1-10). (**d**) Summarised data for all the linker separation with time. (e) The time required for the TMSD to separate each linker, the histogram showed that majority of times, the TMSD mediated separation required less than 20 seconds. An exponential fitting was used to fit the histogram data. (**f**) Probability of separation of each linker (between L1-L5) during the TMSD process. The bar graph shows that the outer linkers (L1/L5) are more prone for separation after TMSD is initiated.

A multiplexable miRNA sensor

We modified the dimer linker sequences to create a miRNA-responsive dimer for detecting miRNA in solution (Figure 4a). As a proof of concept, we selected miR-532-5p, a microRNA associated with traumatic brain injury (TBI) [32] , as our target. The DNA sequence of the linker was engineered so that miR-532 could act as an invader, triggering the separation of the dimer via strand displacement. The successful construction of the miR-532-responsive dimer and its displacement mechanism were validated using gel electrophoresis and atomic force microscopy (AFM) (Supporting Figure 11a).

Due to sequence differences between miR-532 and the original artificial invader, we observed that miR-532 was less efficient at displacing the dimer. While the artificial RNA sequence achieved complete dimer displacement at 15 nM and 25°C (Figure 2), miR-532 only displaced about 50% of the dimer under the same conditions (Figure 4c). We hypothesize that the secondary structure of miR-532 may hinder the toehold-mediated strand displacement (TMSD) reaction in this context. Using the ViennaRNA package [80], we calculated the minimum free energy (MFE) and the secondary structure of the at under lower salt condition (Figure 4b). The simulation indicated that at 25°C miR-532 adopted a hairpin secondary structure with only 4 nt accessible to the linker's toehold. This condition was less thermodynamically favourable for the TMSD reaction to proceed efficiently. We also analysed the secondary structure of the artificial sequence (Supporting Figure 11b) and found no stable or defined folding, suggesting it could fully hybridize with the linker's toehold and thereby promote efficient TMSD. Indeed, previous studies have shown that RNA invaders with a 5' toehold binding region tend to form RNA:RNA duplexes at the 3' end of the target, which significantly slows the TMSD reaction and reduces its overall efficiency [71].

By increasing the incubation temperature from 25°C to 37°C, the secondary hairpin structures of miR-532 were disrupted (Figure 3b, Supporting Table 1). We confirmed that the miR-532-responsive dimers remained stable at 37°C for at least 30 minutes (Supporting Figure 11c,d). TMSD reactions were then performed at 37°C for 30 minutes, resulting in complete separation of 1 nM miR-532 dimer with as little as 5 nM miR-532, as determined by nanopore analysis (Figure 4c). These results contrast sharply with the results at 25°C, where nearly all dimers remained intact at the same miR-532 concentration. These findings suggest that disrupting the

secondary structure of the invader strand through temperature modulation significantly enhances the reactivity and efficiency of the dimer displacement reaction.

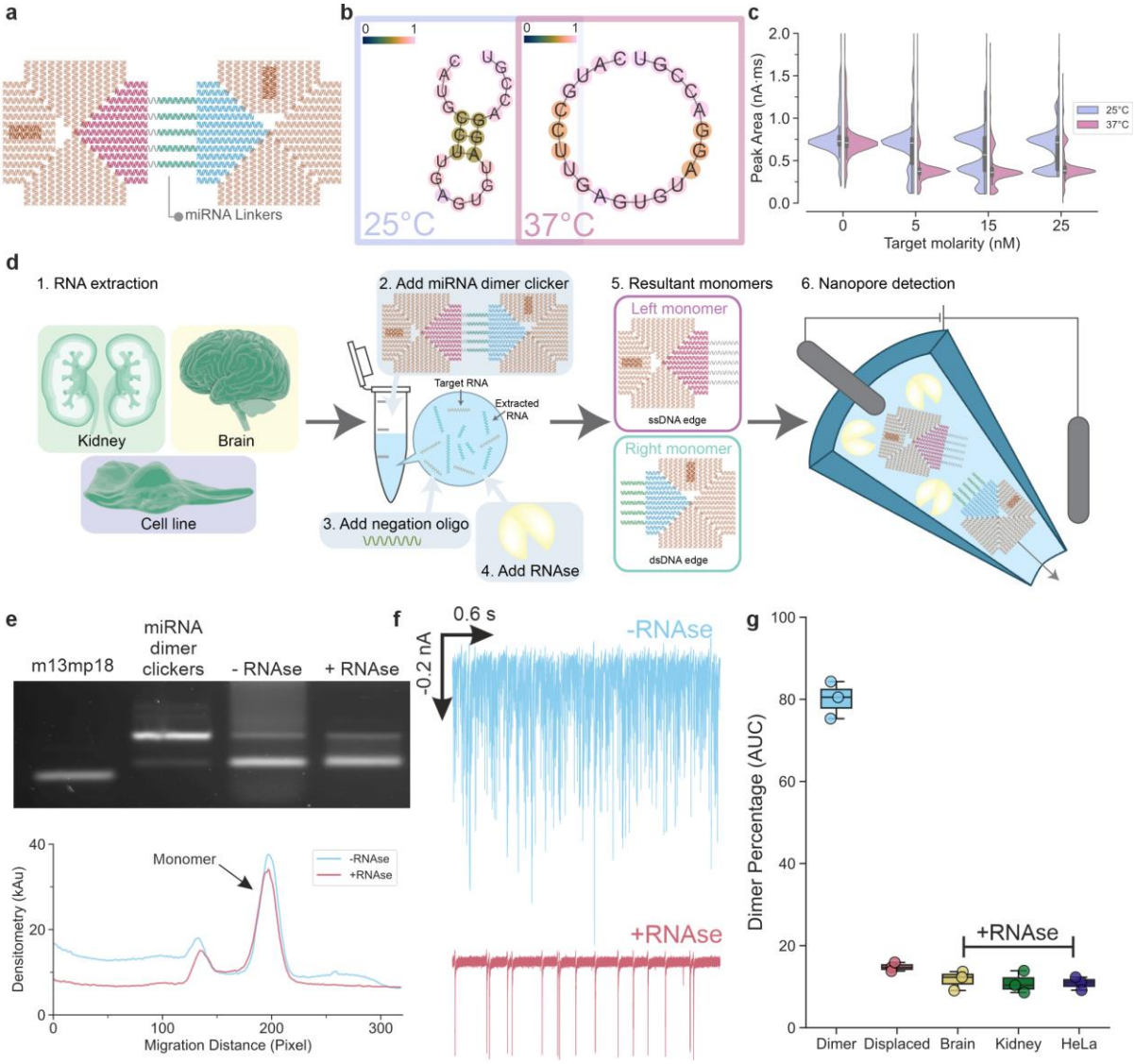

Figure 4. Dimer for the detection of miR-532. (**a**) Schematic illustration of the dimer specific for miR-532. The miR-532-5p (5' arm of the miRNA) sequence was used to construct the miRNA linker dimer. (**b**) The minimum free energy (MFE) prediction of the miR-532-5p secondary structure at 10 mM Na^+ condition under either 25°C or 37°C with ViennaRNA 2.0 package. The colour gradient showed the probability of either pairing (if paired) or unpairing (if unpaired) with 0 being the lowest probability and 1 being the highest probability. The miR-532 adopted an open conformation (no secondary structure) at 37°C contrasting its hairpin secondary structure at 25°C. (**c**) The peak area of the nanopore translocation events of the miR-532 dimer at 1 nM. The data showed that the displacement carried out at 25°C leads to inefficient separation of the miR-532 dimer. The displacement was more efficient when carried out at 37°C where 5 nM was sufficient to separate the dimer into monomers, in contrast to 5 nM at 25°C where majority of the dimer remained as dimer. (**d**) Schematic illustration of the selective detection under RNAse contaminated environment. Tissue or cell line extracted RNAs are used as RNA background, the RNA background was spiked with miR-532 (target RNA) at final concentration of 5 nM. The miR-532 responsive dimer (1 nM) was added to the mixture and followed by the addition of negation ssDNA oligo, and then RNAse. The resultant RNAse treated samples were quantified with nanopore. (**e**) The agarose gel analysis on the dimer treated with or without RNAse after separation. Larger smearing background could be observed in the densitometry when not treated with RNAse. (**f**) The nanopore translocation trace of the samples with or without the RNAse treatment, due to the high quantity of the background RNAs translocation through the nanopore, the displaced origami monomer signals were hidden. The RNAse treated samples revealed a clean trace showing the translocation of the displaced origami monomers. (**g**) The detection of the displaced dimer at normal pure buffer condition and RNA background with RNAse contaminated environment.

Previous studies using nanopore-based miRNA detection from fluid have typically relied on the miRNA itself serves as a linker between two larger nano-entities like nanoparticles or multi-armed DNA nanostars[45,57]. This concept can be replicated using DNA origami tile monomers, with the miR-532 oligo acting as a bridge to hybridize and connect two tiles into a dimer (Supporting Figure 12a,c). This approach has several disadvantages. First, it is entropically unfavourable, second the miR-532 linked dimer must be kept in RNAse free or inactivated environment, because of the fast degradation of RNA when exposed to RNAse[36], and third it could lead to the formation of unintended higher order structures that require removal before nanopore detection. As demonstrated, the miR-532-linked dimer is highly sensitive to RNase exposure and can be readily dissociated into monomers (Supporting Figure 12b). This introduces a significant source of uncertainty in assays aiming to quantify miRNA concentrations, as RNase contamination could lead to false negatives. Consequently, this method requires specialized training and stringent storage conditions, including -80°C storage for RNA, to ensure reliable detection.

In contrast, our dimer approach is designed to be irreversible after the TMSD reaction and can be stored at 4°C (and potentially room temperature) by simply incorporating the addition of DNA negation strands to hybridise with the right monomers after TMSD reaction. This step prevents the re-hybridisation between the left and right monomers after the RNAse digested the RNA:ssDNA duplex (Figure 4d). We introduced a counter intuitive step to add RNAse prior to the analysis, to simulate the quantification of miRNA assay in an RNAse environment, and to remove any background RNA (including the target miRNAs) that would interfere with the nanopore readout[41,47]. At this stage, the TMSD reactions had already proceeded irreversibly, meaning that the removal of RNA, including miRNA, no longer affecting the overall dimer population.

To demonstrate the effectiveness of our approach, we supplemented total RNA extracted from tissues or cell lines with 5 nM of synthetic miR-532 (Figure 4d). A 1 nM concentration of the miR-532-responsive dimer was then added to the RNA mixture and incubated at 37°C for 30 minutes. This was followed by the addition of negation oligos (short DNA strands complementary to the ssDNA linker) of the right monomer, then a second 30-minute incubation at 37°C. Finally, the samples were treated with RNase (50 U/mL RNase A and 2,000 U/mL RNase T1) at 25°C for 30 minutes. Gel electrophoresis and densitometry confirmed that the miR-532 dimers were successfully separated into monomers, which migrated to the expected positions on the gel (Figure 4e, Supporting Figure 13). In contrast, samples that did not undergo RNase treatment showed significant background smearing, indicating the presence of high RNA content (~100 ng). This background RNA interfered with nanopore analysis, producing

numerous translocation peaks from both DNA origami structures (monomers and dimers) and the background RNA. As a result, the distinct peak signatures of the DNA origami were obscured (Figure 3f). However, RNase digestion effectively removed this interference, allowing clear detection of the displaced monomers via nanopore analysis (Figure 3g). This confirmed the presence of at least 5 nM of miR-532-5p in all RNA extract samples.

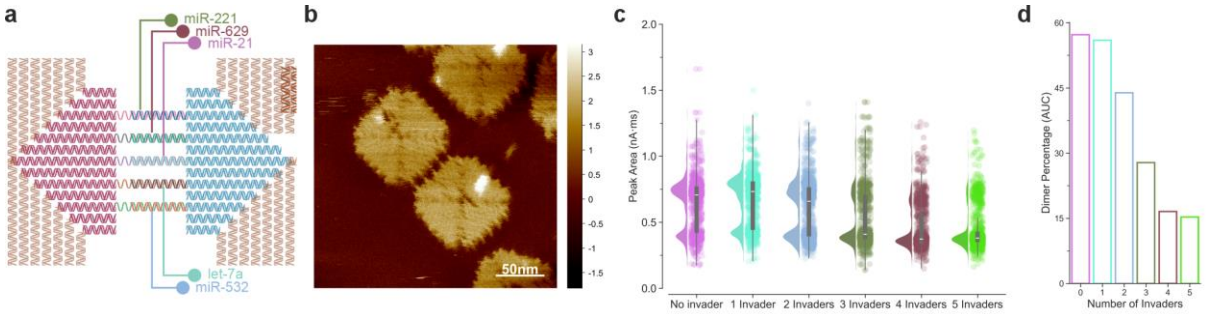

Figure 5. Multiplex miRNA detection. (**a**) The formation of the multiplex miRNA dimer with different sequences of miRNA (miR-221, miR-629, miR-21, let-7a and miR-532) as linkers. (**b**) AFM images of the formation of the multiplex miRNA dimer. (**c**) The displacement of the different linkers' dimer clickers quantified with nanopore (n=1000). The number of invaders correspond to the species of the miRNA, 5 invaders mean a cocktail combination of miR-221, miR-629, miR-21, let-7a and miR-532 while 1 invader means only miR-532 was added at 5 nM. (**d**) Quantification of the dimer percentage.

Our DNA origami design enables high spatial addressability where each linker can target a specific miRNA sequence (Figure 5a). We selected 4 additional miRNAs-5p, the cancer and cardiovascular diseases associated miR-21 [81], viral infection associated let-7a [82], and the TBI associated miR-221 and miR-629 [83]. The formation of this multiplex dimer follows the same procedure as the formation of the miRNA dimer and the original artificial sequence dimer, and the resultant dimer could be seen in Figure 5b. To initiate the dimer disassembly, a mixture of five miRNA invaders (miR-221, miR-629, miR-21, let-7a, and miR-532) was applied at a final concentration of 5 nM and incubated at 37 °C. The dimers were incubated with different mixtures of invaders form by systematically removing an individual miRNA from the mixture to test the minimum number of invaders needed to achieve the disassembly (Figure 5c). The percentage of dimers reduces as the number of invaders increased in the mix until 4 invaders are added (Figure 5d).

This proof-of-concert results that our approach can be employed for multiplexed detection of 3 miRNAs as full disassembly (dimer percentage ~15%) will only occur when only 2 linkers are presented. This indicates that a single linker is not strong enough to hold the dimer (Supporting Figure 1) and this observation was confirmed by HS-AFM, which indicated that a single link can rapidly rotate and break the connection. The progressive reduction of the dimer percentage

from 1 till 4 reflects the stability of the connection between the monomers, revealing the complex dynamic in higher order assembly process and stability of DNA origami.

Crucially, we have already demonstrated that 9 linkers can be introduced in the current DNA origami design (Supporting Figure 1a), indicating that the presence of 7 specific 7 miRNAs could be detected. This approach could allow the digital detection of multiple miRNAs species which will trigger the origami disassembly only when all are presents above a defined concentration.

Discussion

The use of polymer electrolyte as the trans chamber electrolyte allowed us to perform detection at materials native buffer condition, it was widely accepted that for translocation experiment with solid-state nanopore, a minimum of 0.1 M salt (reported up 4 M)[84,85] would be needed for the system to be sensitive to the translocation of materials. Here, against expectations, the detections were carried out with maximum 15 mM of divalent ions (Mg^{2+}). The avoidance of additional high concentration of salt reduced the physical modification on the DNA itself, as concentration of salt modified the properties of the DNA [86,87], which could potentially affect the translocation signals generated [68]. Further development in this could potentially allow solid-state nanopore to be a tool to probe DNA physical properties.

We utilise the established TMSD mechanic to disassemble the dimerised DNA origami with either DNA or RNA and subsequently showed that exposure of RNAse to the DNA origami did not interfere with the detection of the already separated DNA origami, the population differences were readily quantified with our nanopore system as a potential point of care biosensors, bypassing laborious procedures in sample handling in potentially contaminated environment. This approach here only required the initial RNA and DNA origami preparation to be clean, significantly reduced the risk of RNA post extraction degradation due to mishandling [88]. Our approach utilised RNAse to digest irrelevant background RNA after the TMSD step act to reveal the true DNA origami translocation signal. This strategy could be further refined and implemented into the nanopore sequencing device like MinION, providing an alternative strategy for miRNA detection alongside the recently established method [39].

The design of our origami and its strategy relies on a rather simple separation of masses, unlike the recent approach that relies on the detection on the change of conformation [58], our strategy achieved a wider dynamic range and subsequently a more responsive assay. The 2D nature of the origami designs allowed us to trace and visualise the molecular motion of the TMSD in action with HS-AFM in real time. Upon the introduction of the invader strands, the five DNA linkers connecting the dimer separated at different time points, with some linkers temporarily reconnecting before complete dissociation. Kymograph analysis of AFM height profiles enabled state tracking (connected/disconnected) of each linker, revealing detailed dynamic behaviour of the linkers and general separation of the dimer requires less than 20 seconds once initiated by the invaders. The outer linkers (L1 and L5) were the first to separate in most cases, likely due to their greater exposure on the mica surface. Interestingly, we observed transient reconnections between linkers, the full separation was irreversible once initiated. These findings highlighted

the influence of linker position on TMSD efficiency and established HS-AFM as an effective tool for studying dynamic DNA nanomechanical processes.

Conclusion

We developed a TMSD based method for the detection of small RNA such as miRNA with solid-state nanopore. The separation of the dimerised DNA origami dimer is initiated via TMSD when the invading strand of either RNA or DNA is mixed with the dimer. We showed that the detection of DNA origami before and after TMSD displacement could be carried out with even with miRNA biomarkers and multiplexable to enable detection of multiple miRNAs. Our strategy allows nanopore assay to be carried out under the presence of RNAse, providing an alternative strategy to minimise the degradation of target RNA due to sample mishandling and environmental contamination. The molecular motion of TMSD was visualised through HS-AFM imaging, tracing the separation of the origami allowed us to visualise the process at single molecule resolution, revealing the intermediate dynamics states on the dimer initiated by invader strands and the provides a better visual understanding of linker separation dynamics in TMSD.

Methods

For full detailed method, please refer to the supporting information

DNA origami folding and dimerization

All DNA strands used throughout this study were purchased from Integrated DNA Technologies with standard desalting. The caDNAno (2.4.7) software was used to design the DNA origami structures [5]. All origami were folded using the 7249 nt M13mp18 circular single-stranded DNA scaffold (type p7249; tilibit). All folded origami were stored at 4°C or immediately purified. The caDNAno files of the origami designs list of staples could be found in the repository associated with this publication.

For all purposes unless otherwise specified, the origami buffer is composed of 10 mM Tris-Acetate, 15 mM Mg-Acetate, 1 mM EDTA, pH 8.0.

For a single folding reaction of 80 µl, the folding mixture contained: 10 nM M13mp18 scaffold, 12.5 mM magnesium acetate, 75 nM bridge mixture, 75 nM interior mixture, 75 nM edge mixture, and diluted in 10 mM tris-HCl, 1mM Ethylenediaminetetraacetic acid (EDTA) at pH 8.0 (1× TE buffer). The folding of the DNA origami tiles was carried out within a thermal cycler by heating the folding mixture to 90°C for 2 minutes, followed by a gradual temperature decrease from 90°C to 20°C at the rate of 1°C per minute. The temperature was then further decreased to 4°C until use.

The purification of the folded monomers followed our published method using solid-phase reversible immobilization (SPRI) beads [89] with a modification. The HighPrep™ PCR Clean-up System (AC-60050; Magbio) SPRI beads was used, the buffer suspending the SPRI beads were exchanged to 2.5% (w/v) Poly(ethylene) glycol (PEG) 8K, 500 mM $MgCl_2$ via pelleting the SPRI beads down and resuspending the beads in the custom buffer, the concentration of the beads to buffer was at 20 mg/ml. The buffer modification improved the performance of the SPRI beads for the purpose of DNA origami purification.

After the folding reaction, the reaction was diluted to 100 µl with DNA origami buffer, then 40 µl (0.4× beads to solution volume ratio) of the buffer modified SPRI beads were added to the solution and mix via pipetting until homogenous colour and incubated for 5 minutes. Then, the SPRI beads were separated with a 3D-printed magnetic rack, the solution was aspirated and discarded. The pellet was washed with 200 µl of 80% (v/v) ethanol once, after the wash, the pellet was resuspended in 40 µl of the DNA origami buffer, incubated for 5 minutes (40 µl was used to concentrate the DNA origami). The SPRI beads were separated from the solution once

again with the magnetic rack, the purified DNA origami solution was aspirated and collected to a PCR tube, the tube was ready for thermal de-clump step. The PCR tube was heated to 50°C using a thermal cycler for 2 mins, followed by a gradual decrease of the temperature to 20°C at the rate of 3°C/min. The temperature was then further decreased down to 4°C and stored at 4°C until use.

The yield of the DNA origami after the SPRI purification was checked, to generate the asymmetric dimer , the left and right monomer was mixed in the same PCR tube so that the final mixture contains 400 ng of left monomers and 400 ng of right monomers in a total volume of 40 µl per PCR tube (multiple tubes of the same mixture would be used if large volume of dimer is needed). The mixture was then heated to 55°C and the temperature was then gradually decreased to 20°C at a 1°C/min rate. The temperature was then further decreased down to 4°C until use.

Atomic Force Microscopy (AFM)

Freshly cleaved mica discs were pre-treated with 5 µl of 10 mM $NiCl_2$, then immediately 10 µl of the DNA origami samples were deposited onto the mica by pipetting directly onto the $NiCl_2$ droplet, then the samples were topped up with 100 µl of DNA origami buffer and incubated for 5 minutes. The mica was then washed twice with the origami buffer, topped with 100 µl of origami buffer for liquid imaging. The DNA origami samples were imaged using a Bruker Dimension Fastscan Bio (Santa Barbara, CA, USA) with PEAKFORCE-HIRS-F-B probes with a small laser spot on the Fastscan head. The imaging was carried out via PeakForce tapping with ScanAsyst™ liquid imaging mode via the Nanoscope software. All images acquired have a pixel resolution of minimum 512 × 512 to 1024 × 1024, and the images were analysed with NanoLocz AFM image analysis software (v1.20)[63].

High-Speed Atomic Force Microscopy (HS-AFM)

All high speed-atomic force microscopy (HS-AFM) measurements were performed using a NanoRacer HS-AFM (Bruker, Germany) instrument in amplitude modulation mode. All HS-AFM measurements were obtained in liquid and ambient temperature in an acoustic isolation housing on an active antivibration table using short cantilevers (USC-F1.2-k0.15, NanoWorld, Switzerland) with nominal spring constants of 0.15 $N.m^{-1}$, resonance frequencies of ~0.6 MHz and quality factors of ~2. To prepare the sample for HS-AFM imagining of DNA Origami, freshly cleaved mica was treated with 5 µl of 10 mM Ni^{2+}/Mg^{2+} salt solution, to promote the adsorption of DNA origami to the mica. Thereafter, 2-4 µl of DNA Origami sample was incubated on the mica and left for incubation for 2-3 minutes before rinsing, with 5 via fluid exchange with 5 µl of buffer solution. The sample holder was then filled with 1 ml with imaging buffer (5 mM Tris-HCl (pH 8.0), 1 mM

EDTA, 20 mM $MgCl_2$ and 5 mM NaCl). To visualize TMSD, 10 µl of the invader strands at a concentration of 1 µM were added to the imaging buffer. The images and videos were analysed with NanoLocz image analysis software (v1.30)[63].

Nanopore measurement

Quartz capillaries of 1.0 mm outer diameter and 0.5 mm inner diameter (QF100-50-7.5; Sutter Instrument) were used to fabricate the glass nanopores using the SU-P2000 laser puller (World Precision Instruments). A two-line protocol was used: line 1, HEAT 750/FIL 4/VEL 30/DEL 150/PUL 80, followed by line 2, HEAT 700/FIL 3/VEL 40/DEL 135/PUL 180. The pulling protocol was instrument specific, and these would vary between different laser pullers.

The generation of the polymer electrolyte bath followed previously published method [41]. To generate 40 ml of the polymer electrolyte with a composition of 50% (w/v) PEG 35K, 0.1 M KCl, 4 ml of 1 M KCl was mixed with 16 ml of ddH_2O and 20 g of PEG 35K (81310; Sigma) inside a 50 ml centrifuge tube. The tube was then incubated at 85°C for 2 hours and left over night at 37°C, electrolyte was then aliquoted inside multiple 7 ml Bijou tubes, stored on shelves away from sunlight at room temperature (E1412-0710; Starlab), the aliquot would be discarded after 1 month since the first immersion of the nanopore and 6 months after the initial generation.

The translocation experiment followed a similar procedure from the previous publications [16,40-42,89,90] The glass nanopores were all filled with 9.4 ng/µl of origami (4 nM for monomers, 2 nM for dimer), all origami were diluted in the origami buffer without further addition of any salt. In each measurement, the glass nanopore was fitted with an Ag/AgCl working electrode and immersed into the polymer electrolyte bath with an Ag/AgCl reference electrode. The ionic current trace was recorded using a MultiClamp 700B patch-clamp amplifier (Molecular Devices) in voltage-clamp mode. The signal was filtered using a low-pass filter at 20 kHz unless specified, digitized with a Digidata 1550B at a 100 kHz (10 µs) sampling rate, and recorded using the software pClamp 10 (Molecular Devices). Unless otherwise specified, all translocations were carried out at -500 mV.

The python code used to perform the translocation event detection could be accessed at https://github.com/chalmers4c/Nanopore_event_detection. For all events calling, a threshold line of 7 sigmas away from baseline is used.

Data availability

The translocation trace data, DNA origami design, staples list and relevant sequences from this work can be freely accessed via the University of Leeds data repository.

Supporting Information

Supporting information contains additional detailed methods and Supporting Figures.

Author Contributions

C.C.C.C. and V.G. designed and performed the experiments. C.C.C.C. illustrated all schematics. G.R.H., C.W. and P.A. helped with data analysis and acquired the funding. All authors wrote and corrected the manuscript.

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