

Supplementary information

A Large size-selective DNA nanopore with sensing applications

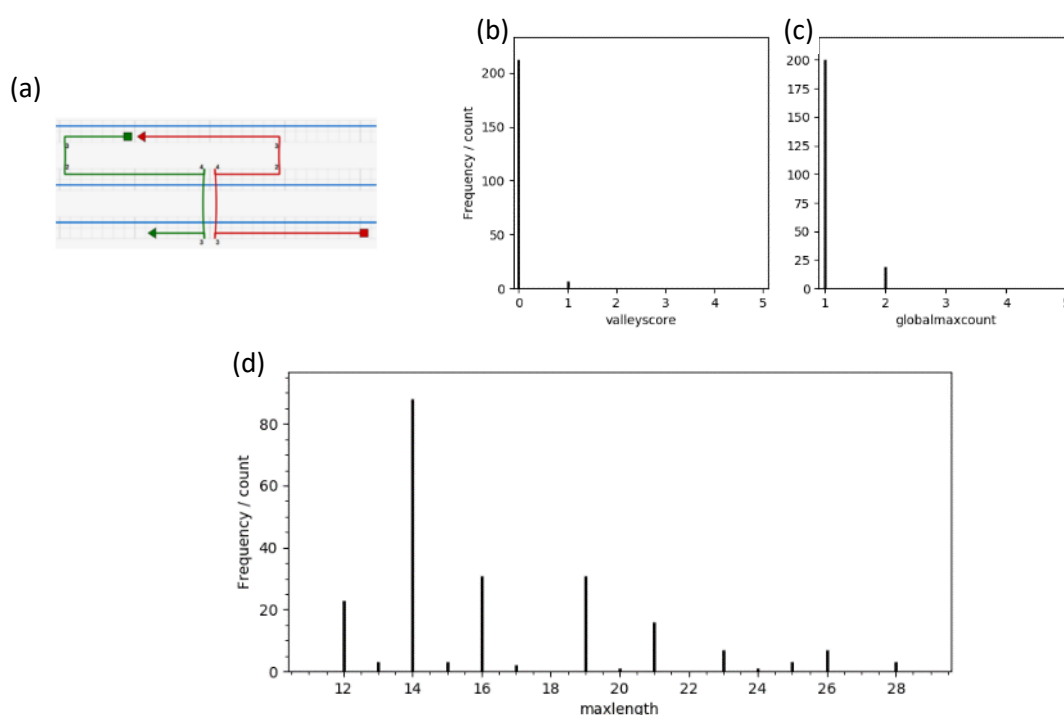
R.P. Thomsen *et al.*

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Supplementary Note 1 – Analysis of structural design.

As several features was desired for the design of the nanopore, including flaps, inner channel functionality and crossovers, it was important for us to ensure that the designed staples were incorporated according to known important parameters to avoid apparent kinetic and thermodynamic folding traps. As a result, R.S.S. wrote a CaDNA¹ plugin termed “Staplestat” available as GitHub repository (<https://github.com/scholer/staplestat>). Usage allow fast overview of staple patterns and stretches which further can be used to get an overview of melting temperatures etc.



Supplementary Figure 1 – Design optimization of DNA nanopore staples.

(a) Example of 2 strands where the green strand has a desirable long nucleation site of 16-nts with two shorter 8-nt sequences flanking. Meanwhile, the red staple contains an undesired structural “valley” - a short segment flanked by two long stretches. (b) The number of strands with “valley” was kept to a minimum and analyzed by a CaDNA¹ plugin written by R.S.S. The lower the score the better to avoid kinetic folding traps. (c) Analysis of the number of global max lengths of the staples. Optimally a single site is desired since it indicates a single “nucleation” site. (d) Analysis of the longest continues segment of individual staple strands to ensure all strands have sufficiently long nucleation site. With the plugin the T_M of segments can also be calculated by input of the scaffold sequence.

Supplementary Note 2 – Free energy of insertion.

To overcome the penalty of forming a pore in a lipid bilayer a compensatory energetic gain is required. Using DNA nanopores modified by lipids have demonstrated to be a feasible approach as they provide their solvation energies provide the required energetic gain if adapted to size. In this paper we use lipid moieties of cholesterol and palmitoyl. In previous papers this consideration has been addressed using continuum models² or coarse grained molecular dynamic simulations³.

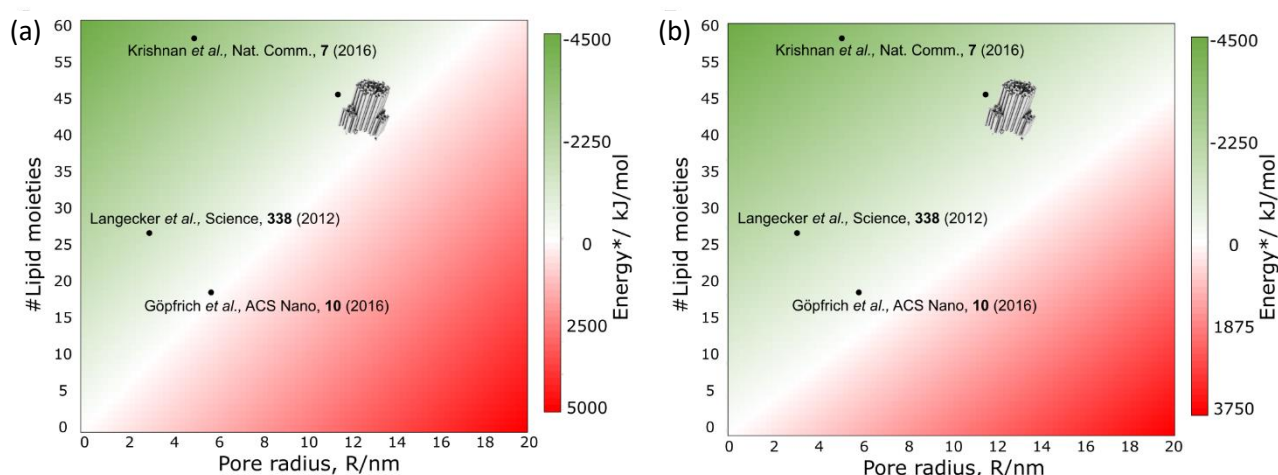
The energy required in the formation of a cylindrical membrane pore is dependent on the pore radius (R) and the bilayer tensions (γ). Using continuum models⁴, the energy requirement can be assumed to be linearly dependent on the pore size and the line tension (γ_L) which describes the cost of the exposed bilayer circumference. Importantly, the exact γ_L values are tied to the specific lipid composition and pore structure, with typical values in the range of 5–40 pN⁴. The surface tension (γ_S) on the other hand describe the gain from minimizing the membrane area however this can be neglected for small pores due to the radius squared. Using the continuum model, we get a linear relationship between required energy and pore radius at the nanoscale:

$$E(R) = (2\pi\gamma_L R) - (\pi\gamma_S R^2) \approx (2\pi\gamma_L R), \text{ for } R \text{ in nm range}^4 \quad (1)$$

Similarly, coarse grained molecular dynamic simulations have previously also provided a linear relationship between pore radius and the energy requirement³.

As the solvation energy of lipids can be determined, the amount of hydrophobic tags needed can be estimated from equation 4.2, or from coarse grained molecular dynamics simulations. While we have not been able to find any good measurements of the solvation energy regarding palmitoyl, cholesterol contributes with a free energy gain of up to -75 kJ/mol upon bilayer insertion³. Based on the assumption of a similar gain for each palmitoyl, an estimated 2.5-3 moieties are necessary each radial nm increment. While we have not done a systematic study to establish the best mixture between palmitoyl and cholesterol for most optimal insertion, we experience the most efficient insertion with all lipids together.

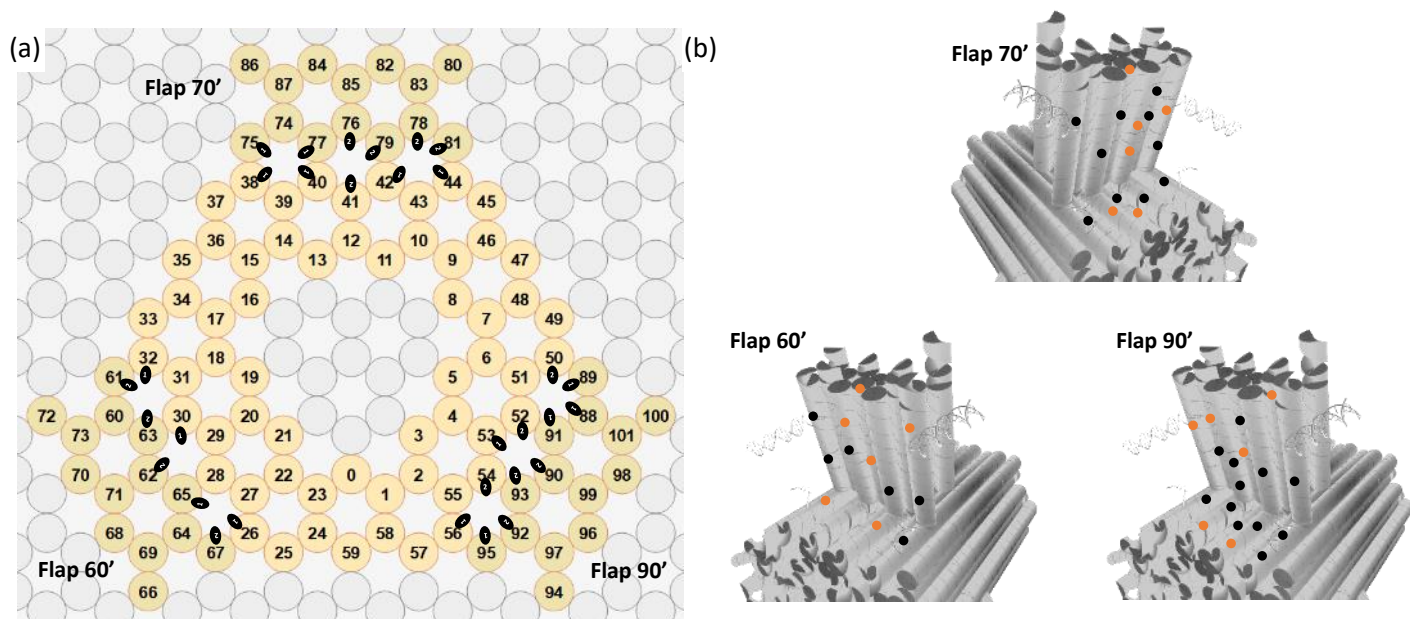
The plot of previous and our DNA origami pores based on the continuum model and coarse grained molecular dynamic simulations have been plotted in Supplementary Figure 2. They show how all DNA origami pores reported are positioned in the energetically favorable region. Importantly these considerations only focus on the stability of the final state of a formed cylindrical pore and does not consider initial nucleation barriers or other related penalties.



Supplementary Figure 2 – Energy considerations.

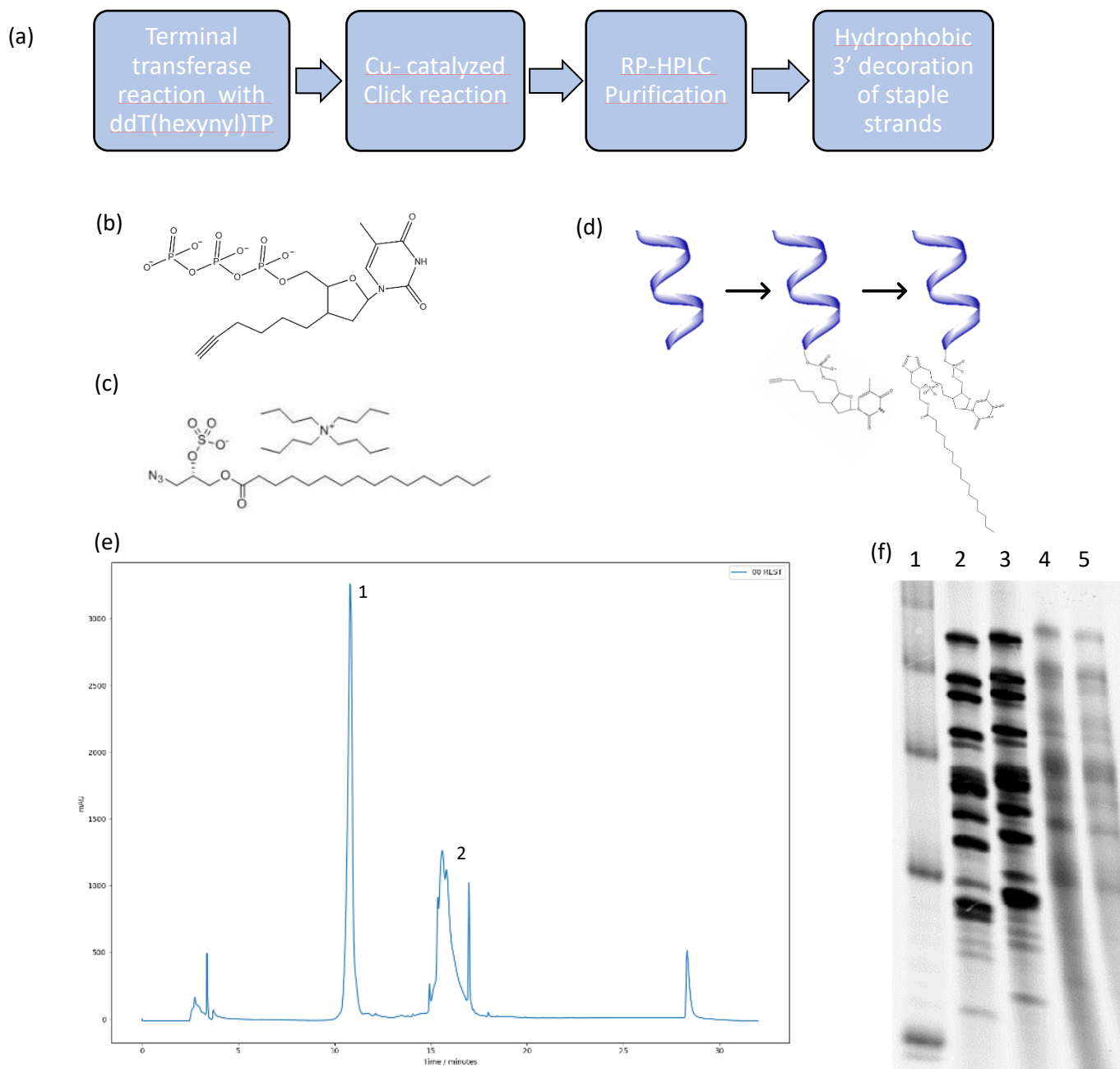
Plot of free energy landscape between pore size and the number of lipid moieties based on a continuum model $E(R) = (2\pi\gamma_L R) - (\pi\gamma_S R^2) \approx (2\pi\gamma_L R)$, for R in nm range. (a) or molecular dynamics simulations (b). (a) Continuum model considerations has been described before by Langecker *et al.*² The plot has adapted a moderate

γ_L -value of 22.5 pN. (b) The MD simulations is adapted from work by Göpfrich *et al.*³. While the DNA nanopores plotted does not use the same lipidation moieties, cholesterol is used as model to estimate the free energy gain per lipid moiety, with a free energy of solvation into bilayers of $\Delta E(n) = -75 \text{ kJ/mol}$. Three additional nanopores have been included to compare the theoretical considerations^{2,3,5}. Theoretically, the 46 hydrophobic moieties are more than enough to stabilize the large 22 nm bilayer pore.



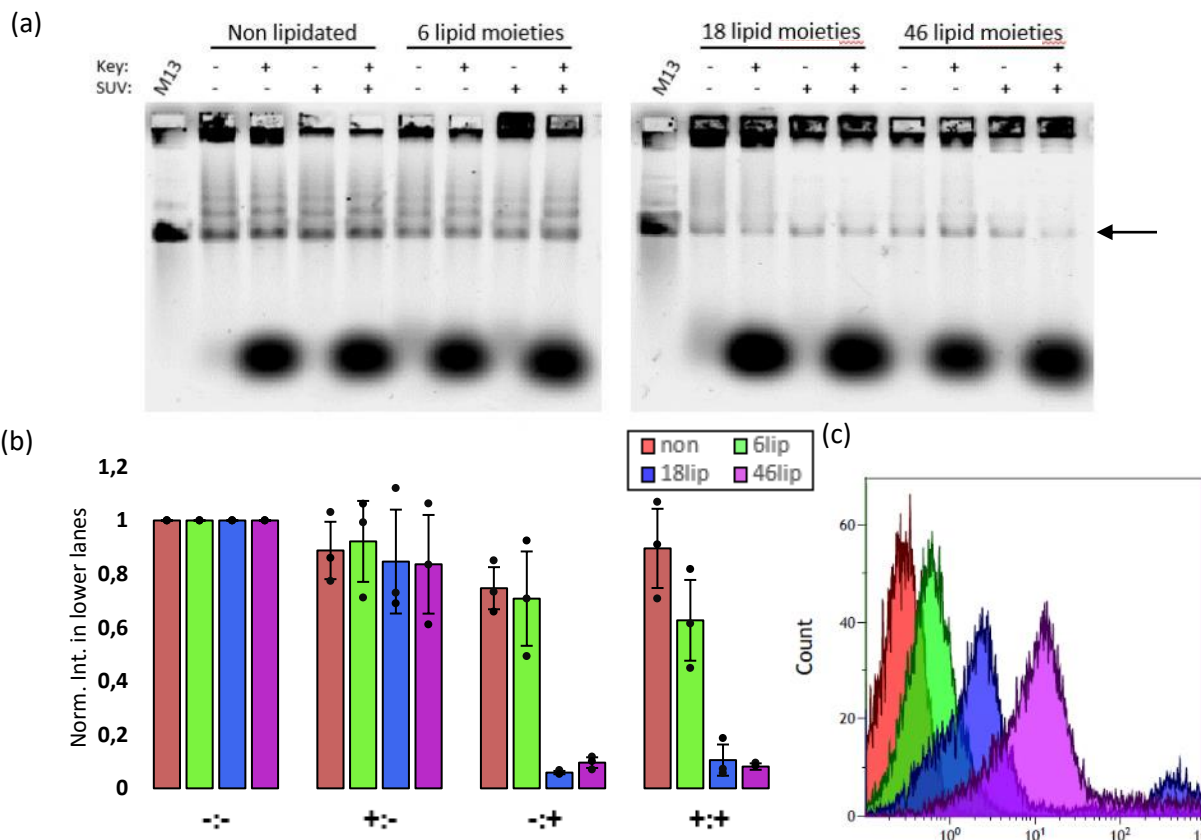
Supplementary Figure 3 – Hydrophobic modification of flap-channel compartments.

(a) Top view of all positioned lipid moieties in the flap-channel system. Some helices have 1 while others have 2 hydrophobic moieties. 17 are on the channel while 29 is on the flaps. 6 of the modifications on each flap are cholesterol (bought from IDT as 3'-cholesterol TEG) while the 28 remaining are modified with a palmitoyl anchor using click chemistry. See Supplementary Figure 4 and materials and methods. (b) View of modification of each flap-channel system. Positions of cholesterol modifications are colored orange, while palmitoyl is black.



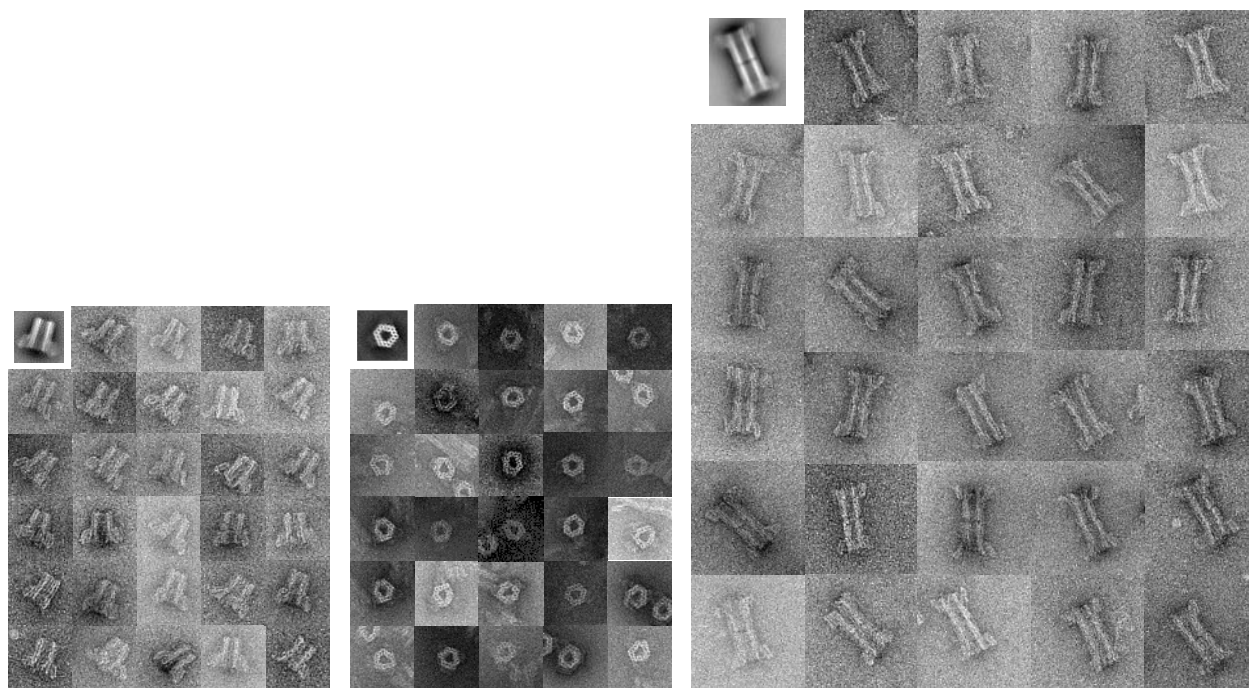
Supplementary Figure 4 – Functionalization of lipid anchors to DNA.

(a) Synthesis strategy of lipidated oligonucleotides. (b) The ddT(hexynyl)TP substrate to be attached with terminal transferase (TdT). (c) Palmitoyl lipid anchor attached by Cu-catalyzed conjugation to the alkyne of ddT(hexynyl)TP at the 3' end of the select staples. (d) Reaction flow of DNA strands, in a one-pot reaction – pulled staple strands. (e) RP-HPLC chromatogram of lipidated strands. Peak 1 are the unmodified plain oligonucleotides eluted after about 11 minutes. The broad peak 2 are the collected fraction of lipidated oligos used for functionalization. (f) 16% Denaturing gel of 28 strands which is modified with the palmitoyl moieties attached. Lane1: 10bp ladder. Lane2: 28 plain oligonucleotides. Lane3: Terminal transferase reacted oligonucleotides. Lane4: Cu-click reacted strands from 3. Lane5: RP-HPLC purified strands. Length of the 28 oligos before TdT reaction: 56-nts: 2 strands, 50-nts: 1, 49-nts: 1, 48-nts: 1, 47-nts: 1, 46-nts: 2, 42-nts: 2, 39-nts: 1, 38-nts: 3, 37-nts: 1, 35-nts: 2, 33-nts: 2, 30-nts: 1, 28-nts: 3, 27-nts: 1, 26-nts: 2, 25-nts: 1 and 22-nts: 1.



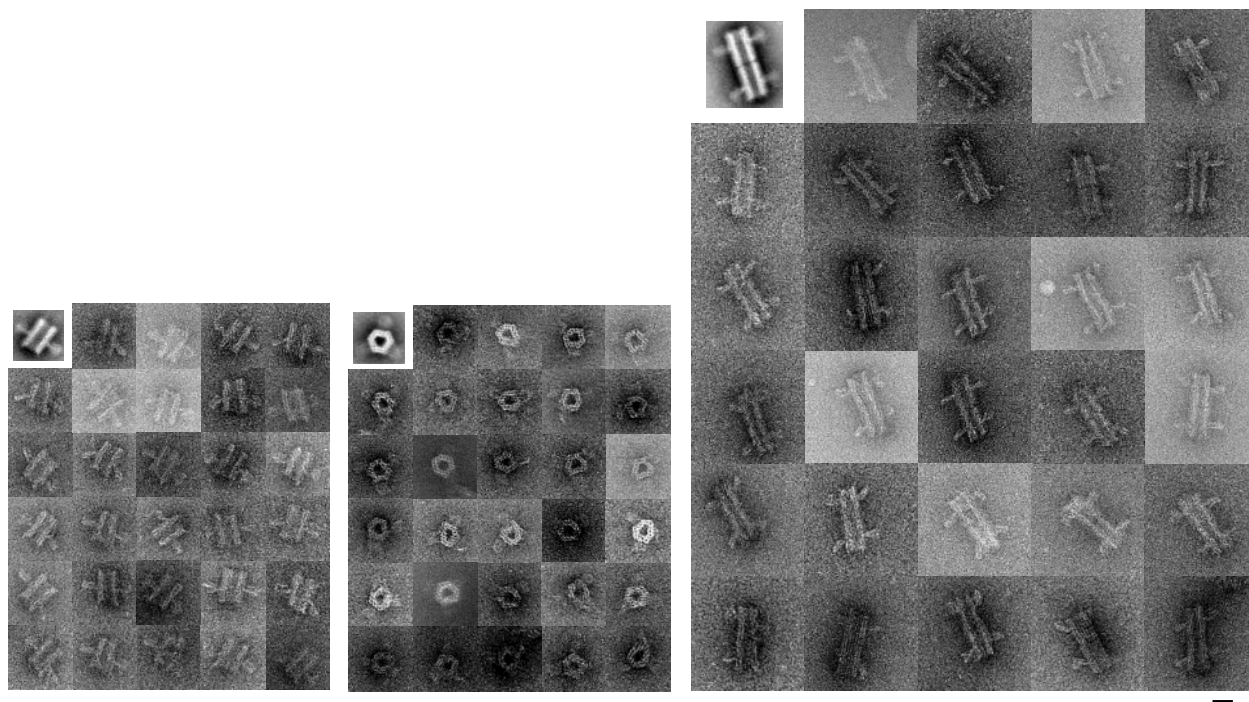
Supplementary Figure 5 – Studies of the effect of increasing number of hydrophobic moieties.

(a) 1% AGE of ATTO 488 labelled DNA nanopores with 0, 6, 18, or 46 lipid moieties attached. Legend denotes whether opening key has been added (+) or not (-); and whether the structures have been incubated with 200 nm extruded POPC SUVs for 1,5 h (+) or not. Arrow indicates monomer structure of interest. (b) ATTO 488 quantification of monomer band in AGE (arrow) which are normalized to each (-:-) control lane. Legend: **Key addition : SUV addition**. Independent replicates of 3 gels scanned with a typhoon scanner and quantified by ImageQuant software (GE healthcare) Error bars indicate standard deviation. (c) Flow cytometry of GUVs created by the inverted emulsion method⁶ (also used for CLSM), incubated with opened ATTO 488 labelled DNA nanopores containing, 0 (red), 6 (green), 18 (blue) and 46 (magenta) lipid moieties.



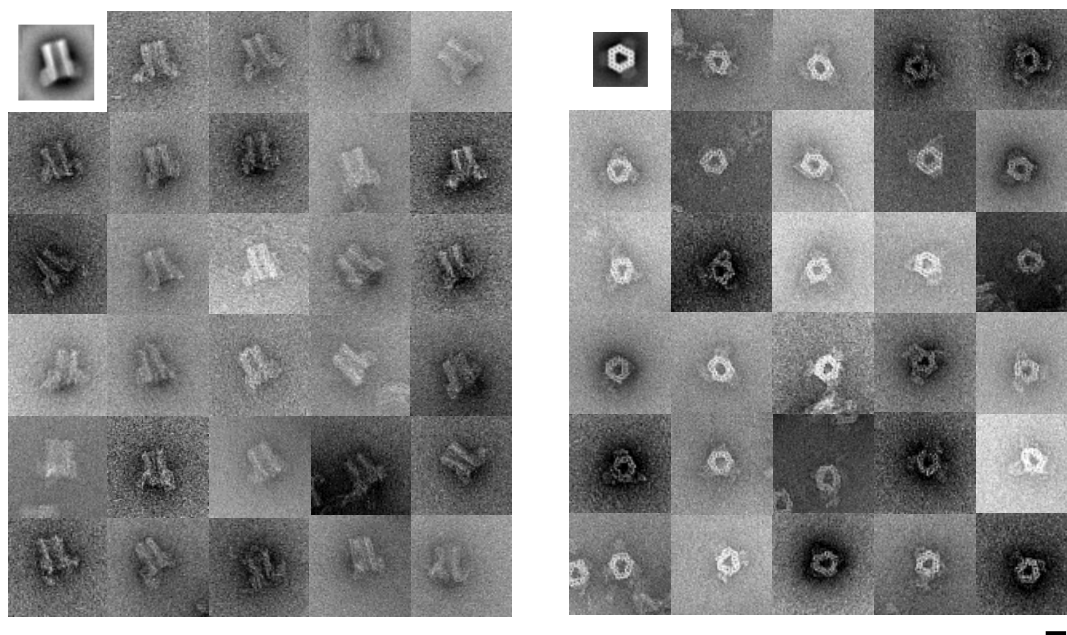
Supplementary Figure 6 – nsTEM gallery of closed DNA nanopore.

Gallery of DNA nanopore in the closed flap state. Monomers observed from side-view (a), and top-view (b) along with 2D class average in upper left corner. (c) Dimerized closed DNA nanopores observed from side-view along with 2D class average in upper left corner. Scale bar is 20 nm.



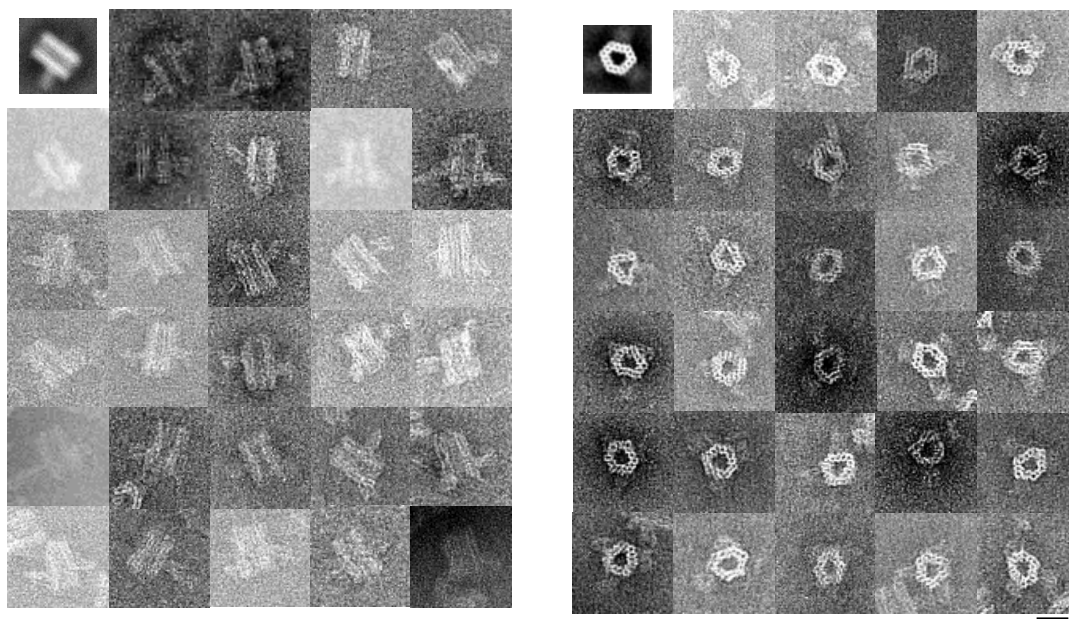
Supplementary Figure 7 – nsTEM gallery of opened DNA nanopore.

Gallery of DNA nanopore in the opened flap state. Monomers observed from side-view (a), and top-view (b) along with 2D class average in upper left corner. (c) Dimerized opened DNA nanopores observed from side-view along with 2D class average in upper left corner. Scale bar is 20 nm.



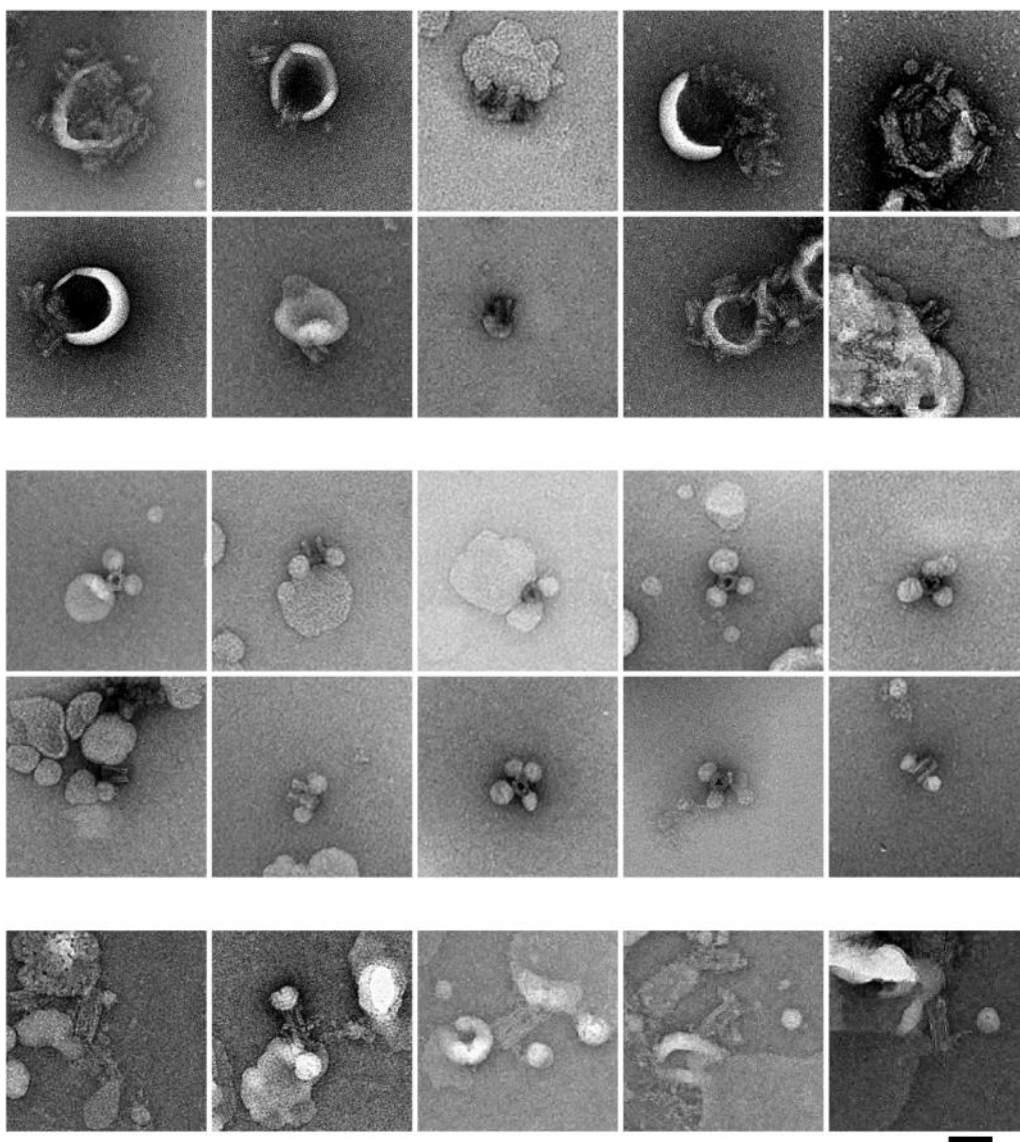
Supplementary Figure 8 – nsTEM gallery closed lipidated DNA nanopore.

Gallery of lipidated DNA nanopore in the closed flap state. Monomers observed from side-view (a), and top-view (b) along with 2D class average in upper left corner. Scale bar is 20 nm.



Supplementary Figure 9 – nsTEM gallery of opened lipidated DNA nanopore.

Gallery of lipidated DNA nanopore in the opened flap state. Monomers observed from side-view (a), and top-view (b) along with 2D class average in upper left corner. Scale bar is 20 nm.



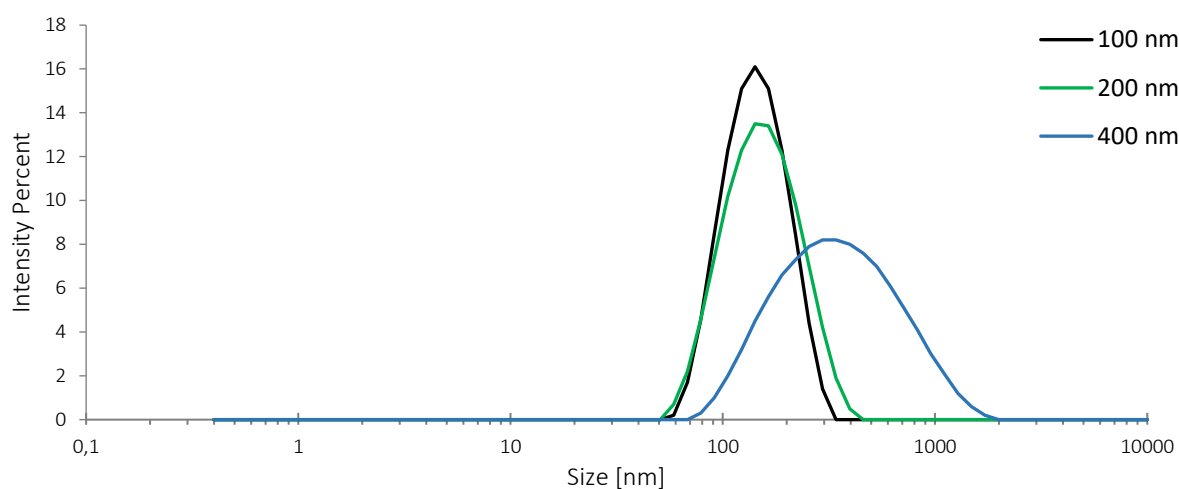
Supplementary Figure 10 – nsTEM gallery of lipidated DNA nanopores incubated with SUVs.

Upper panel: Inserted DNA nanopores into SUVs.

Mid panel: DNA nanopore flaps interacting with SUVs without insertion.

Lower panel: Dimerized DNA nanopores inserted into liposomes.

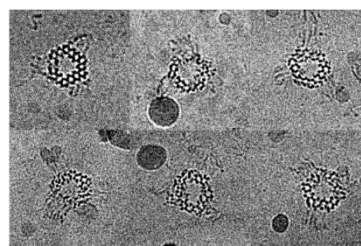
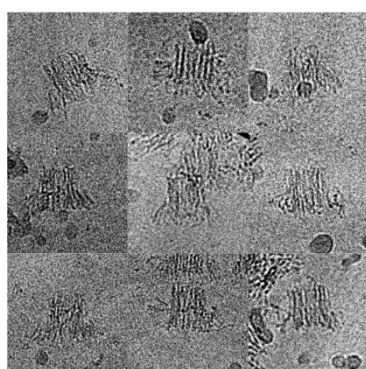
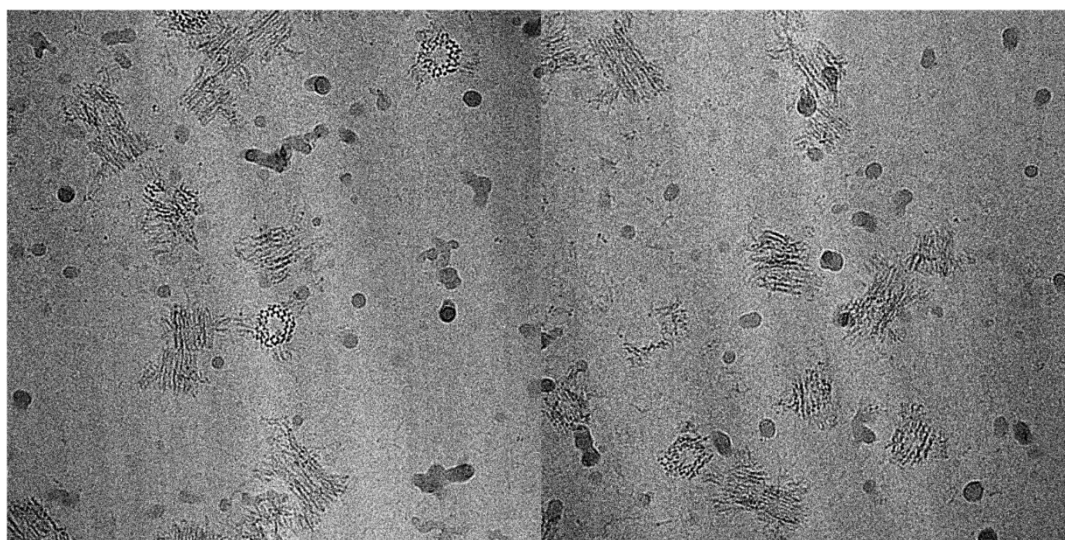
In general, smaller SUVs seems to be able to passivate insertion by interacting favorably with hydrophobic flap-channel compartments. Meanwhile, bigger SUVs are penetrated to a much larger extend. Scale bar is 50 nm.



PC membrane size	100 nm ♦	200 nm ♦	400 nm ♦
Avg. Peak size	148.8 nm	163.0 nm	417.2 nm
PDI	0.094	0.134	0.269

Supplementary Figure 11 – DLS measurements of SUVs created by extrusion using 100 nm, 200 nm and 400 nm filters.

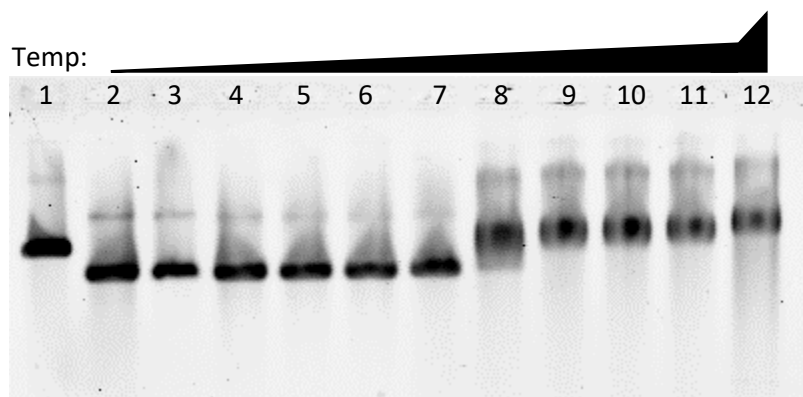
Extrusion was done with up to 1 ml of 2 mg/ml POPC emulsion sample in the assembled Avanti aperture with PC membrane filters containing 100-400 nm sized pores depending on desired diameter of the liposomes. Liposome samples were then passed back and forth a total of 13 times and size was checked by DLS analysis. For the Analysis the sample was diluted to 0.2 mg/ml.



Average Length	n
31.6 nm $\pm$ 1.4 nm	45

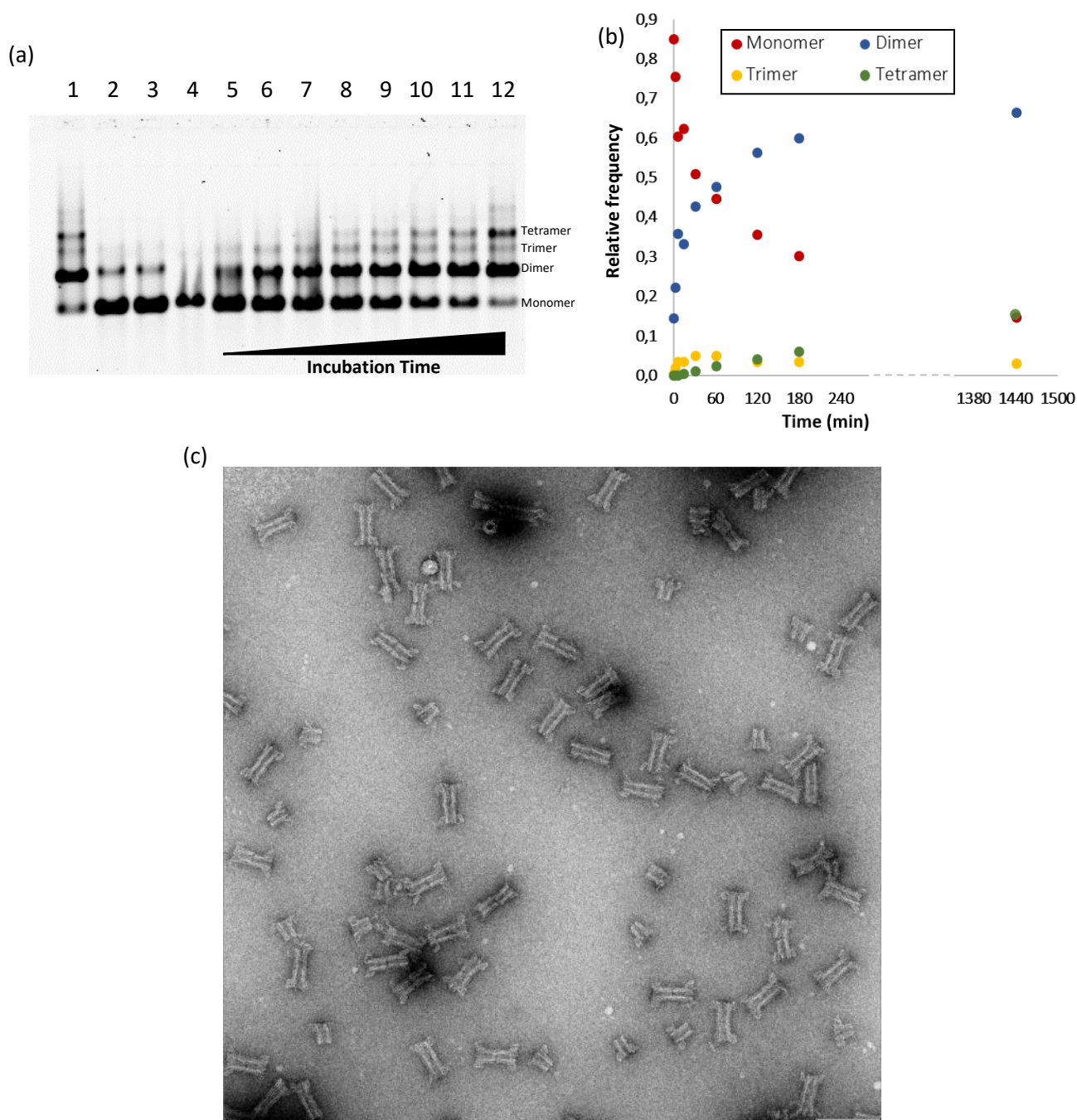
Supplementary Figure 12 – CryoEM images of the DNA nanopore.

Two Cryo EM images of the DNA nanopore on prepared quantifoil grids. Below are cutouts of DNA nanopore monomers from side- and top-view. While the 2D-class average side-view showed a length of 35 nm, quantification of 45 individual DNA nanopore monomers yielded an average length of 31.61 nm $\pm$ 1.38 nm was measured, which is very close to the expected 32 nm. Scale bar is 50 nm.



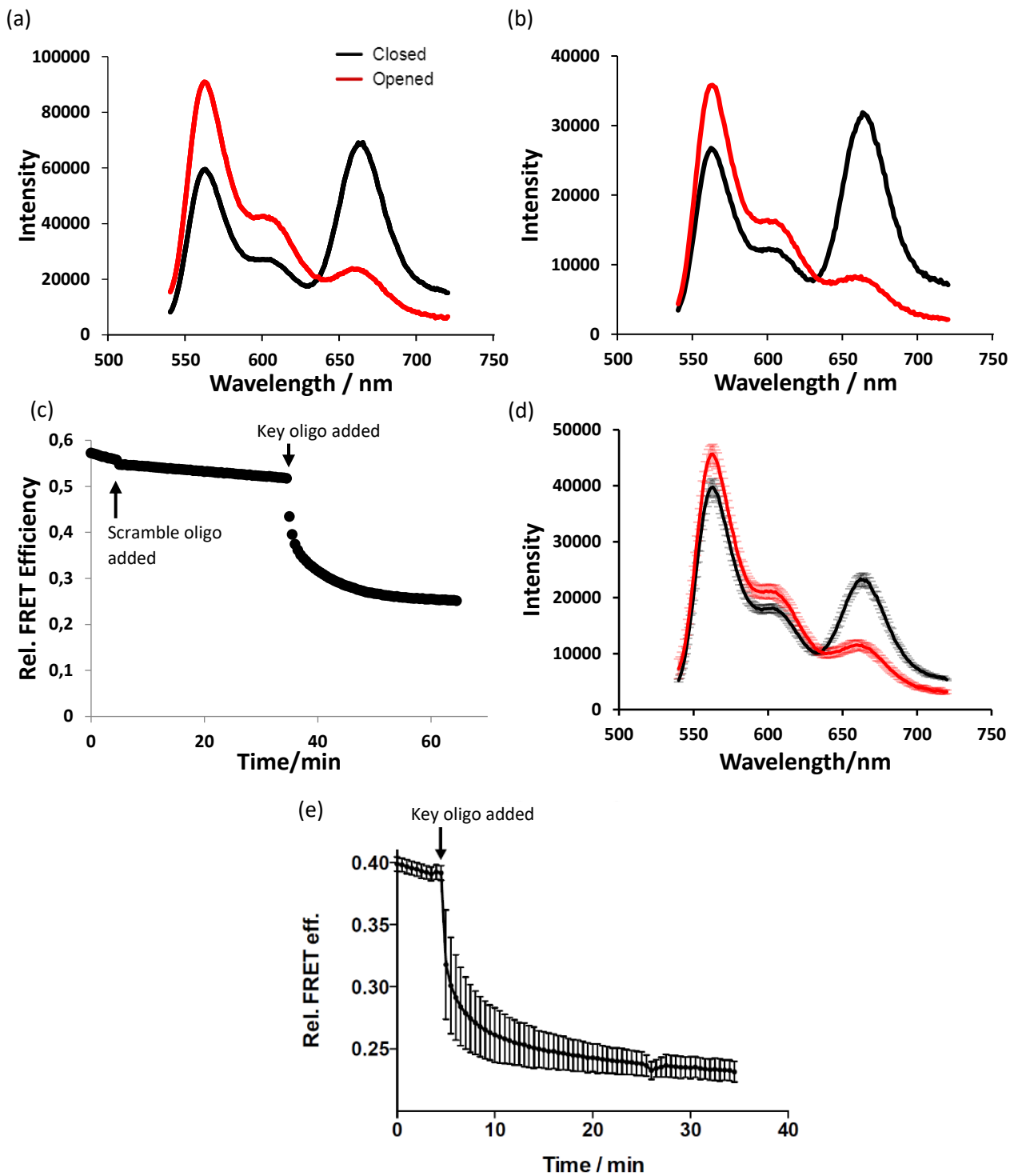
Supplementary Figure 13 – Structural stability of DNA nanopore at increasing temperature.

1% Agarose gel electrophoresis of DNA nanopores incubated at the increasing temperature for 30 minutes before running the gel. (1) M13, (2-12) DNA nanopore at RT (2), 40 °C (3), 50 °C (4), 52 °C (5), 54 °C (6), 56 °C (7), 58 °C (8), 60 °C (9), 62 °C (10), 64 °C (11), 90 °C (12).



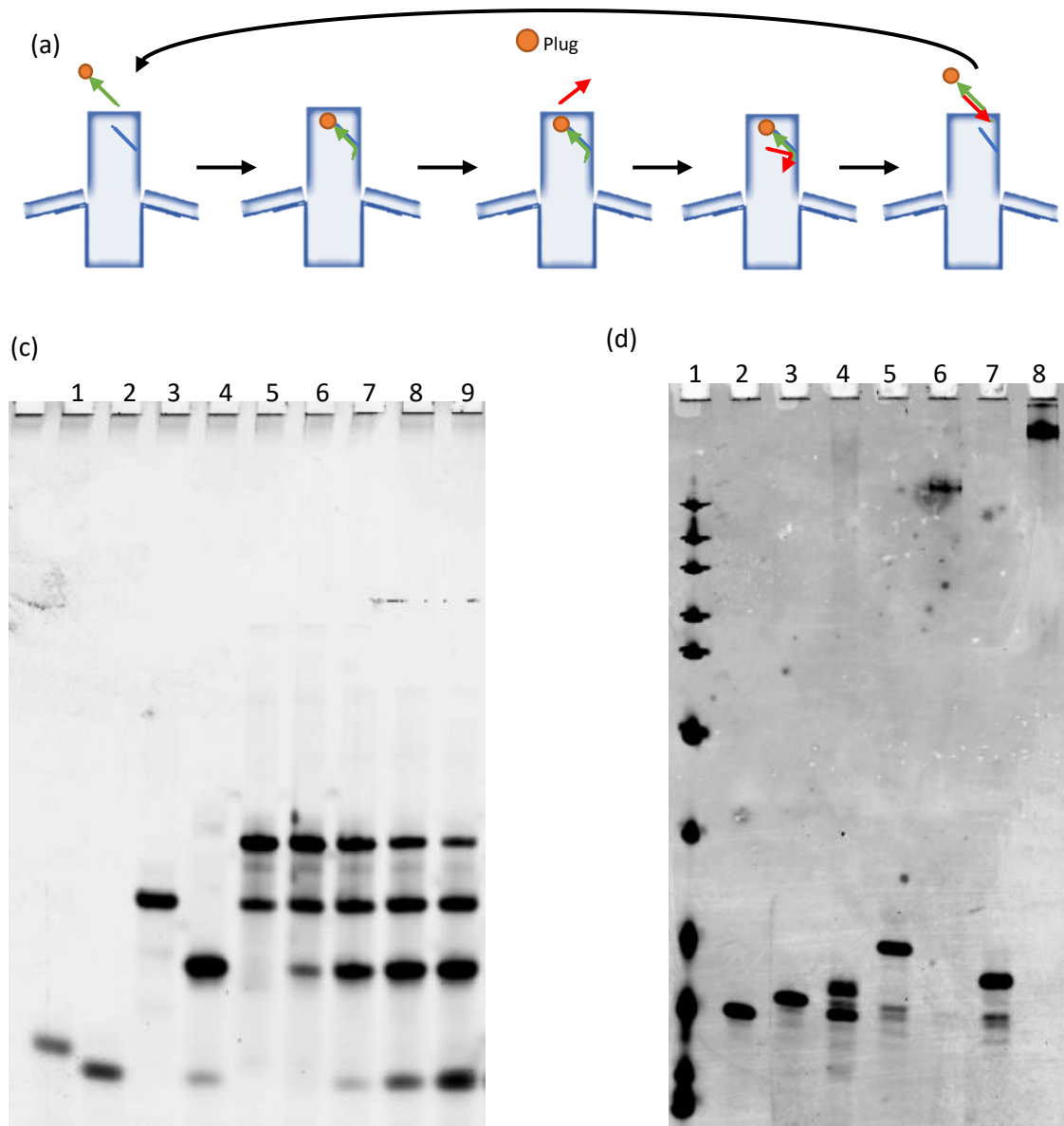
Supplementary Figure 14 – DNA nanopore dimerization kinetics.

(a) 1% AGE of A and B versions of the DNA nanopore. Lane 2 and 3 is A and B monomers alone respectively, incubated for 30 min. Lane 4: M13, Lane 5-12 and lane 1 are A and B incubated for 0min, 5min, 15min, 30min, 60min, 120min, 180min, 24h at 40C and 6days at RT. (b) Quantification of monomers, dimers, trimers and tetramer bands from the Agarose gel. A plateau is reached at around 70%. From the gel unspecific aggregation is apparent but sticky-end dimerization has a significant effect. (c) Raw nsTEM image of nanopore incubated with matching sticky ends scale bar is 100 nm.



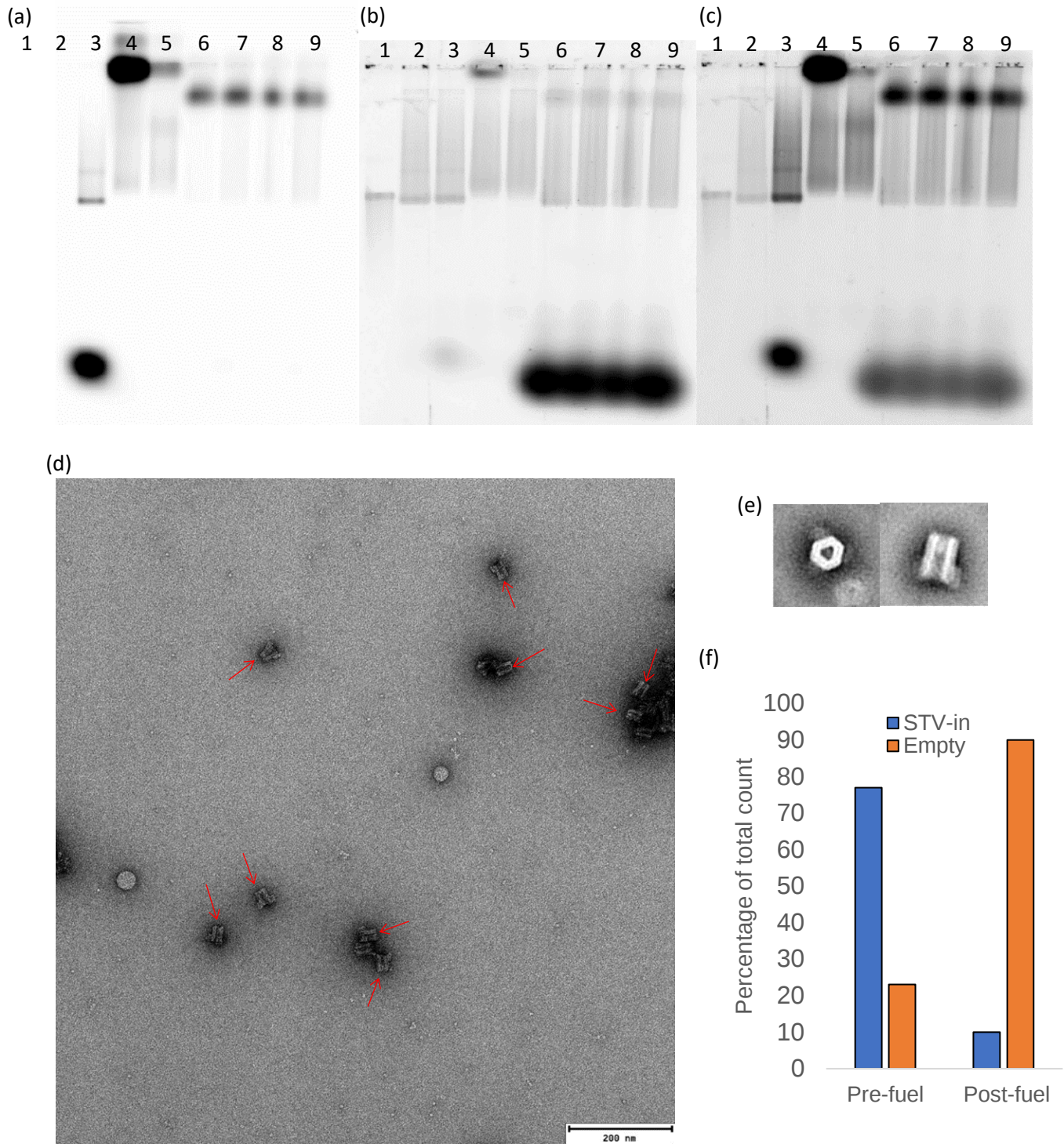
Supplementary Figure 15 – FRET studies of DNA nanopore flaps.

(a) FRET of DNA nanopore with fluorophore in flap-channel system annealed in purified in TAEM₅N₅. (b) FRET of DNA nanopore with fluorophore in flap-channel system annealed in purified in KCl. (c) Specificity and kinetics of lock. (d) FRET of lipidated DNA nanopore similar setup to (a). (e) Kinetics of flap opening for lipidated DNA nanopore. (d) and (e) are averages of 3 separate measurements with the standard deviations as error bars. Staple strands used in the FRET measurements are listed in Supplementary Table 3.



Supplementary Figure 16 – Plugging mechanism of DNA nanopores.

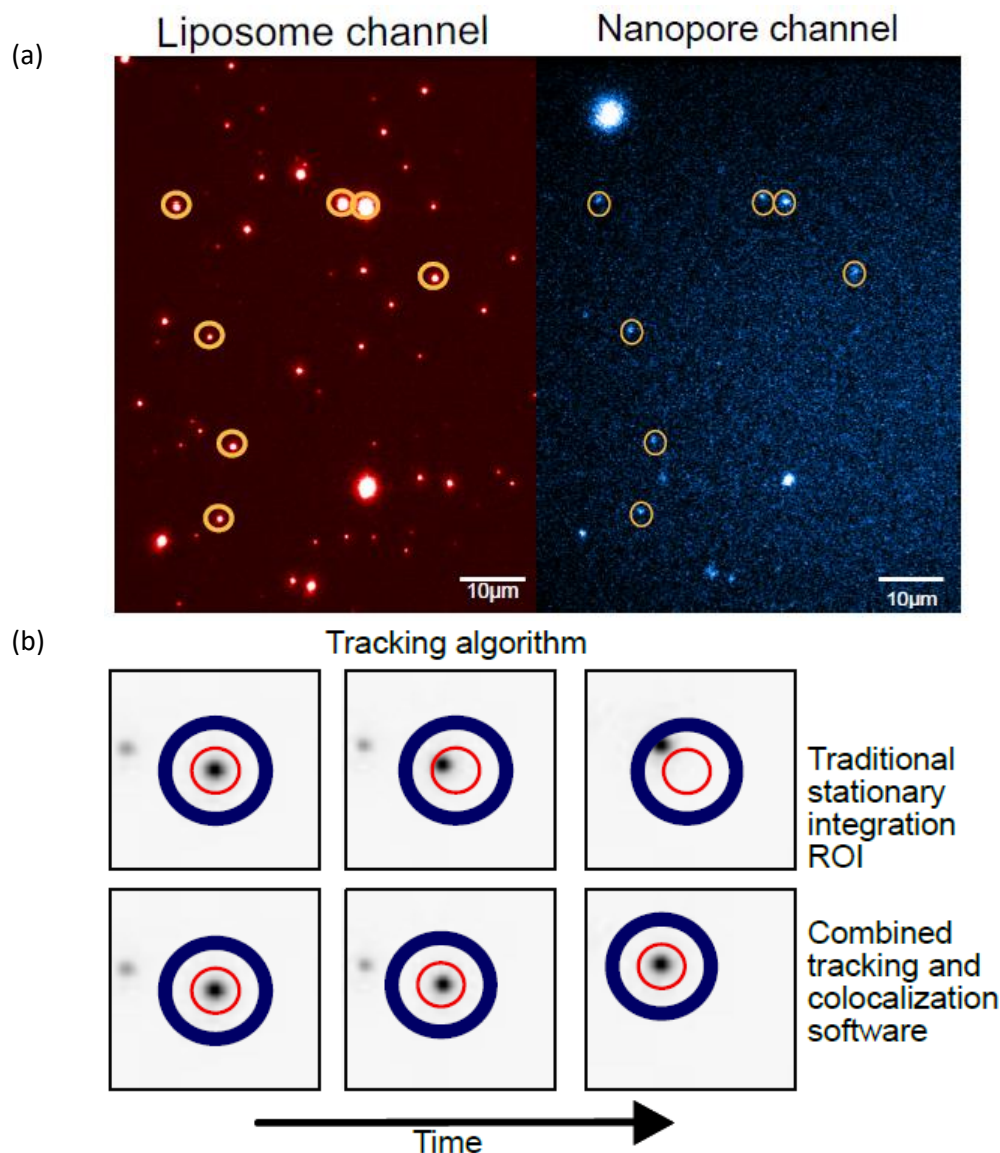
(a) Mechanism of plug capture and unzipping of DNA nanopore. (b) 10 % native PAGE of the plug and unzip mechanism. Lane 1: Plug oligo (green). Lane 2: unplugging oligo (red). Lane 3: Inner channel capture oligo (blue). Lane 4: Plug and unplugging oligos. Lane 5: Plug and inner capture oligos. Lane 6-9: Plug, unplug and capture oligos incubated with increasing unplug oligo excess of 0.1x, 0.5x, 1x or 2x, respectively. Mixtures of oligos in lanes 4 and 5 have been incubated 20 minutes. Mixtures of oligos in lanes 6-9 have been premixed 20 minutes with plug and inner capture strands, followed by 20 min of incubation with unplugging oligos in the given excess. (c) Analysis of modified plug strands using 12% denaturing PAGE. Lane 1: generuler. Lane 2: Plug oligo oligo. Lane 3: 3' NH₂ plug oligo. Lane 4: NHS-DBCO modified plug oligo. Lane 5: Az-biotin modified oligo. Lane 6: As lane 5 but incubated with streptavidin. Lane 7: Az-lipid modified oligo. Lane 8: Az-PEG (20kDa) modified oligo.



Supplementary Figure 17 – Plugging and unplugging of DNA nanopores.

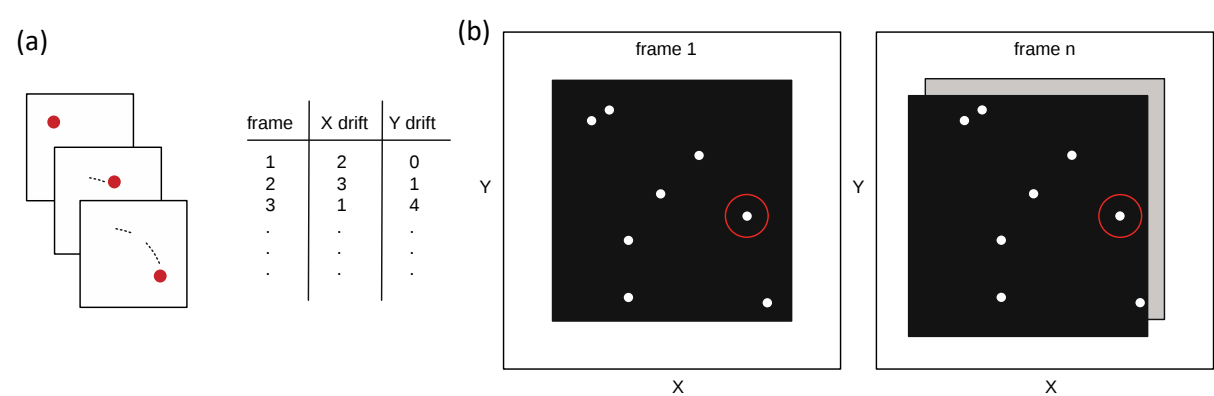
(a-c) 1% AGE of plugged and unplugged DNA nanopores with 3'-Cy5-Plug scanned for Cy5 before Sybr Gold staining (a) or after Sybr Gold staining for Sybr Gold (b) and 488 nm to Cy5 (c). Lane 1: M13. Lane 2: DNA nanopore without plug. Lane 3: DNA nanopore with 3'-Cy5-DNA plug strands. Lane 4: DNA nanopore with 3'-Cy5-DNA-PEG plug strand. Lane 5: DNA nanopore with 3'-Cy5-DNA-PEG plug strand (+). Lane 6-9: DNA nanopore with 3'-Cy5-DNA-PEG plug strand (+) incubated with the “unplugging” strand for 30, 15, 5, or 1 minute(s), respectively. Samples 5-9 have been purified after plug addition, denoted by (+). The plug is nicely integrated and provides a Cy5 fluorescence of nanopore (3 vs. 4). By using PEG plug strand the migration becomes slower, while maintaining

Cy5 tagged structures by plug incorporation (4 and 5 vs. 3). Incubation with the “unplugging” strand quickly detaches the nanopore plug and revert the nanopore migration and releases Cy5-tagging. (d) Raw nsTEM image of DNA nanopores with streptavidin immobilized in inner pore for visualization. Arrows indicate immobilized streptavidin in the nanopore channel. Scale bar is 200 nm. (e) Single particle class average of streptavidin-plugged DNA nanopores from top- and side-view. (f) Quantification of efficiency of plugging and unplugging from several TEM images. Biotin oligo - green in (a) incubated for 30min before addition of streptavidin for 1 hour. Incubation with “unplugging” strand - red in (a) - was done for another hour before imaging.



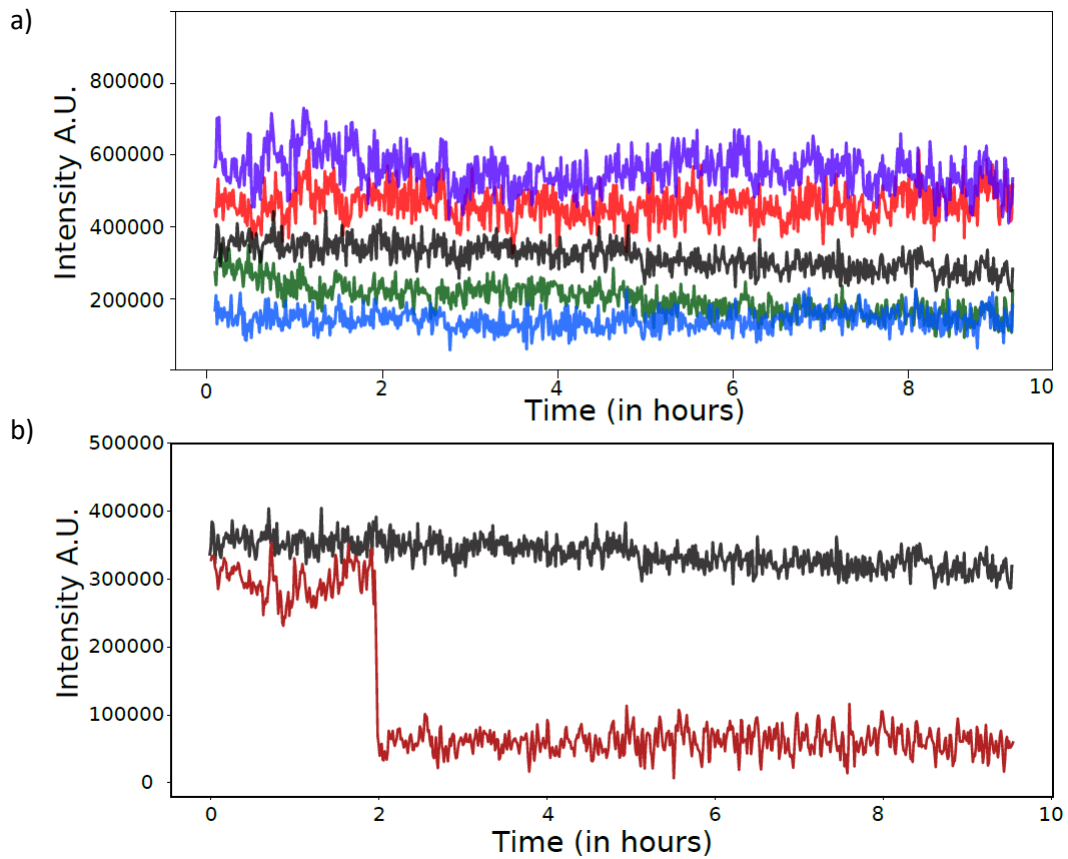
Supplementary Figure 18 – Overview of TIRF setup.

(a) Real image of the liposome channel (left) and the nanopore channel (right) used to correlate the docking of nanopores to specific liposomes, here highlighted in circles. Using simultaneous imaging, it is possible to, through this correlation, directly observe docking and leaking kinetics for individual liposomes. (b) Schematics of tracking algorithm used to stabilize time dependent movements. Due to long experimental timeframes (+9 hours), inevitable drifting in the x-y directions was observed. To correct for this, without biasing data, we deployed single particle tracking (SPT), that allowed us to follow the movement of liposomes throughout the experiment and hence correct for any drift present. Essentially this allows the integration area to move with the liposome, instead of attaining a stationary position.



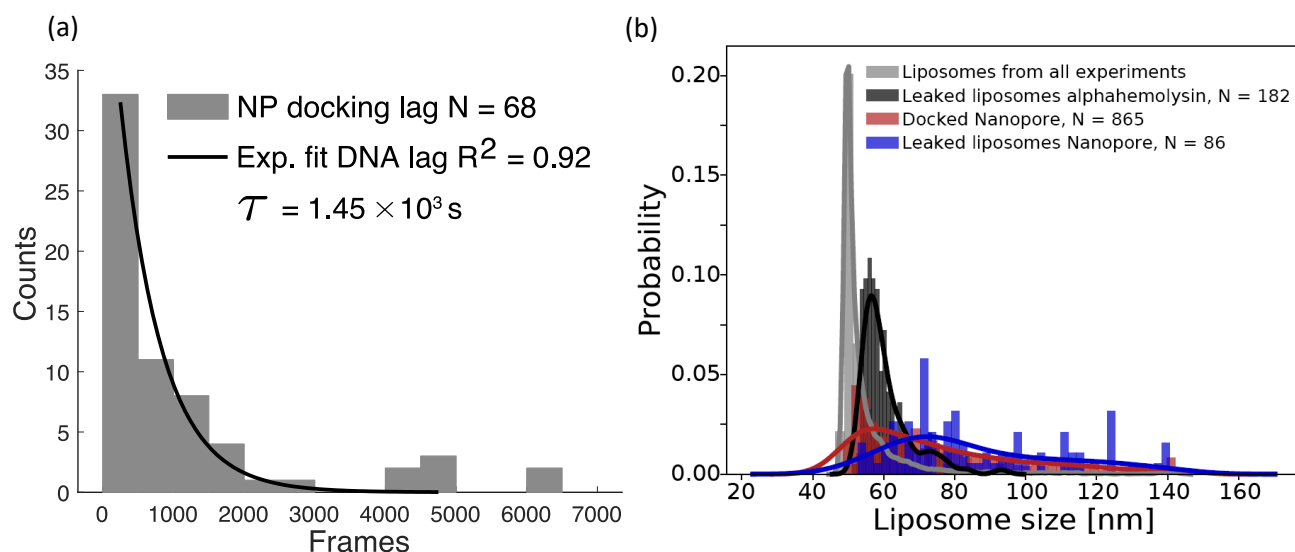
Supplementary Figure 19 – Single particle tracking and drift correction.

(a) Schematic representation (left) of the single particle tracking methodology used. By averaging the movement of all irreversibly bound liposomes simultaneously, a table of x and y displacement for each frame can be generated (right). (b) Using the generated table of drift, each frame in a given experiment can be replaced accordingly, thus removing any stage drift. To do so, the first frame (512 by 512 pixels) is placed in the middle of a significantly larger image (562 by 562 pixels) (left). Each consecutive frame from the experiment is then moved within the larger image (right), thus maintaining the absolute position of liposomes, here indicate by red circle. Iterating through each experiment, a new video is obtained where liposomes no longer move. A video to visualize this process can be found as Supplementary Video 1. By localizing each liposome in the first frame, the intensities can now reliably be extracted.



Supplementary Figure 20 – Control showing no significant photobleaching of ATTO 655 in the experimental time frame

The figure shows intensity traces for ATTO 655 loaded SUV's recorded over a time period of 9.6h with a temporal resolution of 4.6s. (a) The photobleaching under these imaging conditions is negligible. (b) Comparing the stable non-leaking SUV's (black) to the DNA nanopore induced leaking SUVs (red) confirms the negligible effect of photobleaching in our results.



Supplementary Figure 21 – Study of nanopore docking, insertion and size dependence.

(a) Histogram of the docking lag, which shows a colocalization time of $\tau_1 = 1450$ seconds. (b) Liposome size distributions for i) all liposomes ii) liposomes with dye efflux due to α -hemolysin iii) liposomes where a DNA nanopore is found to dock and iv) liposomes with dye efflux due to a DNA nanopore penetration. Liposome sizes based on calculations from encapsulation efficiency (Supplementary Fig. 30). The distributions for docked and leak liposomes due to DNA nanopores are shifted towards bigger liposome sizes, indicating a preference for smaller curvatures. The majority (80%) of the SUVs exhibited no detectable nanopore docking or ATTO transport, which combined with a single docking event on the blue channel strongly support the presence of a single nanopore docking on each individual SUV.

All liposomes tracked for DNA nanopore: 4412 liposomes

Total registered insertions: 86

All liposomes tracked for alpha hemolysin: 400 liposomes

Total registered insertions: 182

Supplementary Table 1 – Statistics on size distributions for both Alpha-Hemolysin and DNA Nanopore experiments.

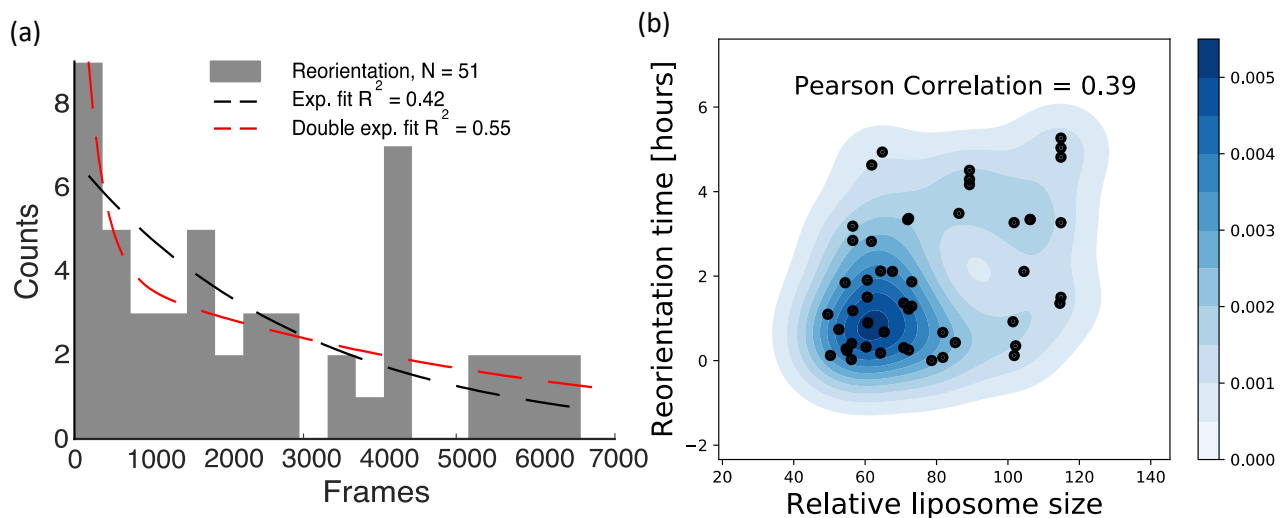
Liposomes sizes where DNA nanopore successfully form pores

Liposome selection	Diameter size [nm]*	Membrane tension [mN·m ⁻¹]*
Median	78.37 ± 35.91	48.73 ± 22.60
Max	139.30 ± 0.78	27.40 ± 1.87
Min	52.18 ± 0.28	73.25 ± 4.99

Liposomes size information where alpha hemolysin successfully forms pores.

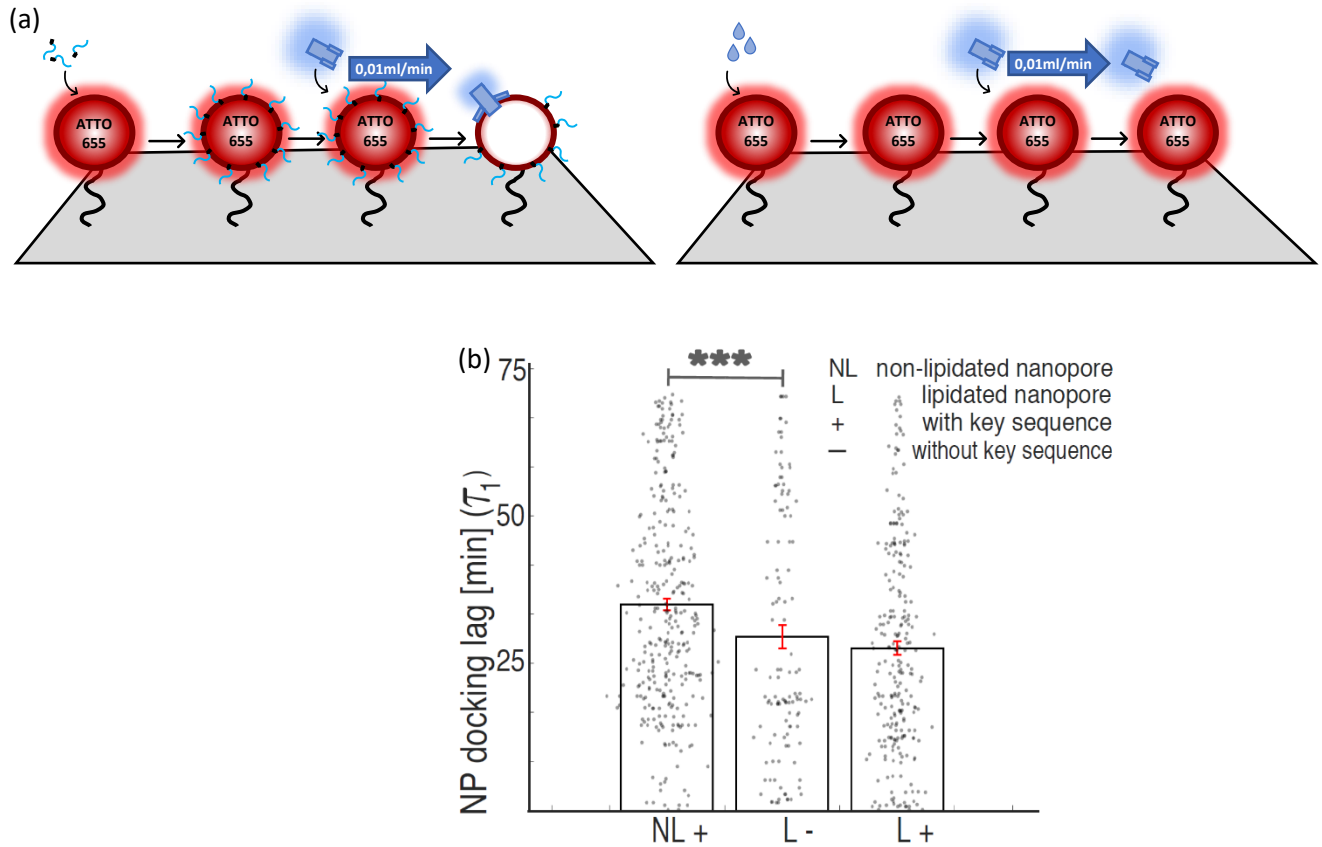
Liposome selection	Diameter size [nm]*	Membrane tension [mN·m ⁻¹]*
Median	57.67 ± 10.26	66.25 ± 12.64
Max	92.89 ± 0.52	41.10 ± 2.80
Min	50.71 ± 0.27	75.38 ± 5.14

* Provided errors is given as standard deviation



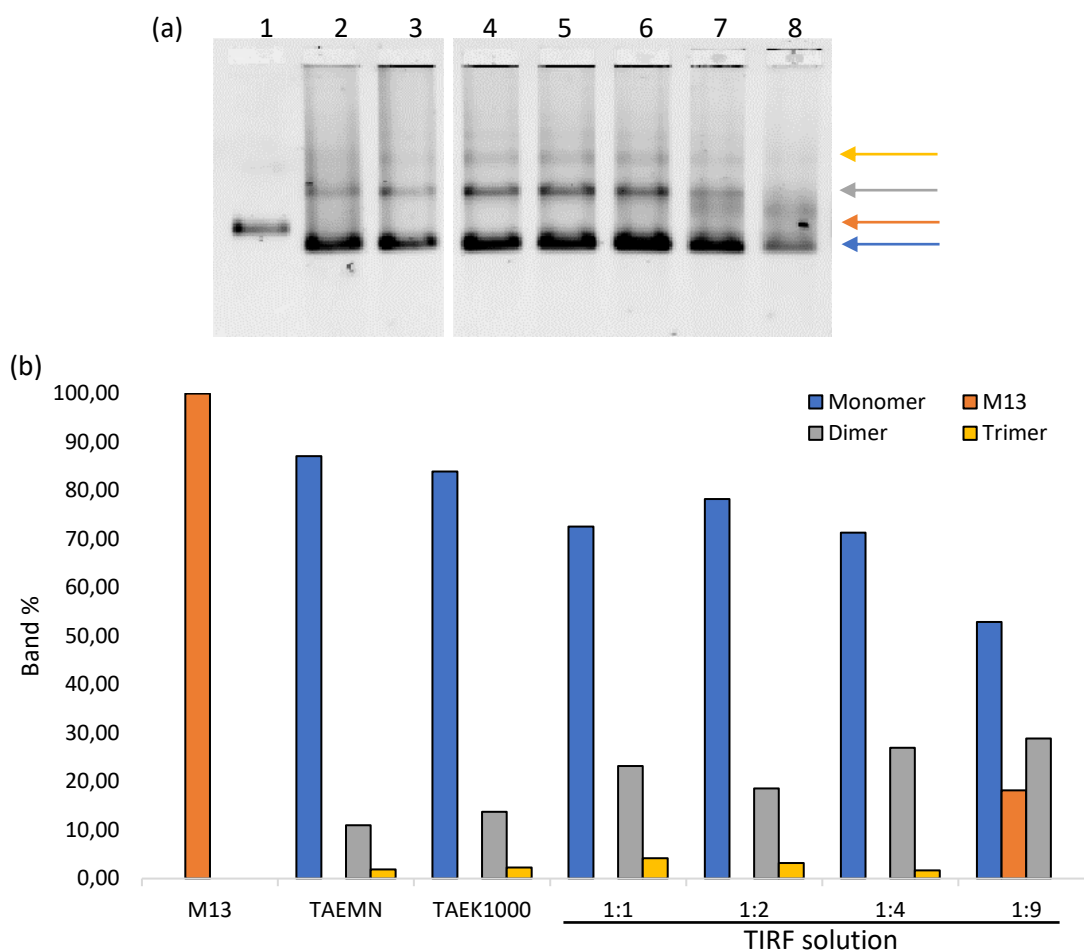
Supplementary Figure 22 – Reorientation and pore formation kinetics upon binding to SUVs.

(a) Histogram of the observed reorientation lag times for the DNA nanopores inserted, τ_2 showing insertion not to be a single step process (b) Distribution of reorientation and its dependence to liposome size. Reorientation time is positively correlated to the size of the liposome with a Pearson correlation coefficient at 0.39 and a p-value of 0.004. This supports that when the DNA nanopore is docked, it reorients faster on liposomes with smaller sizes and higher curvature.



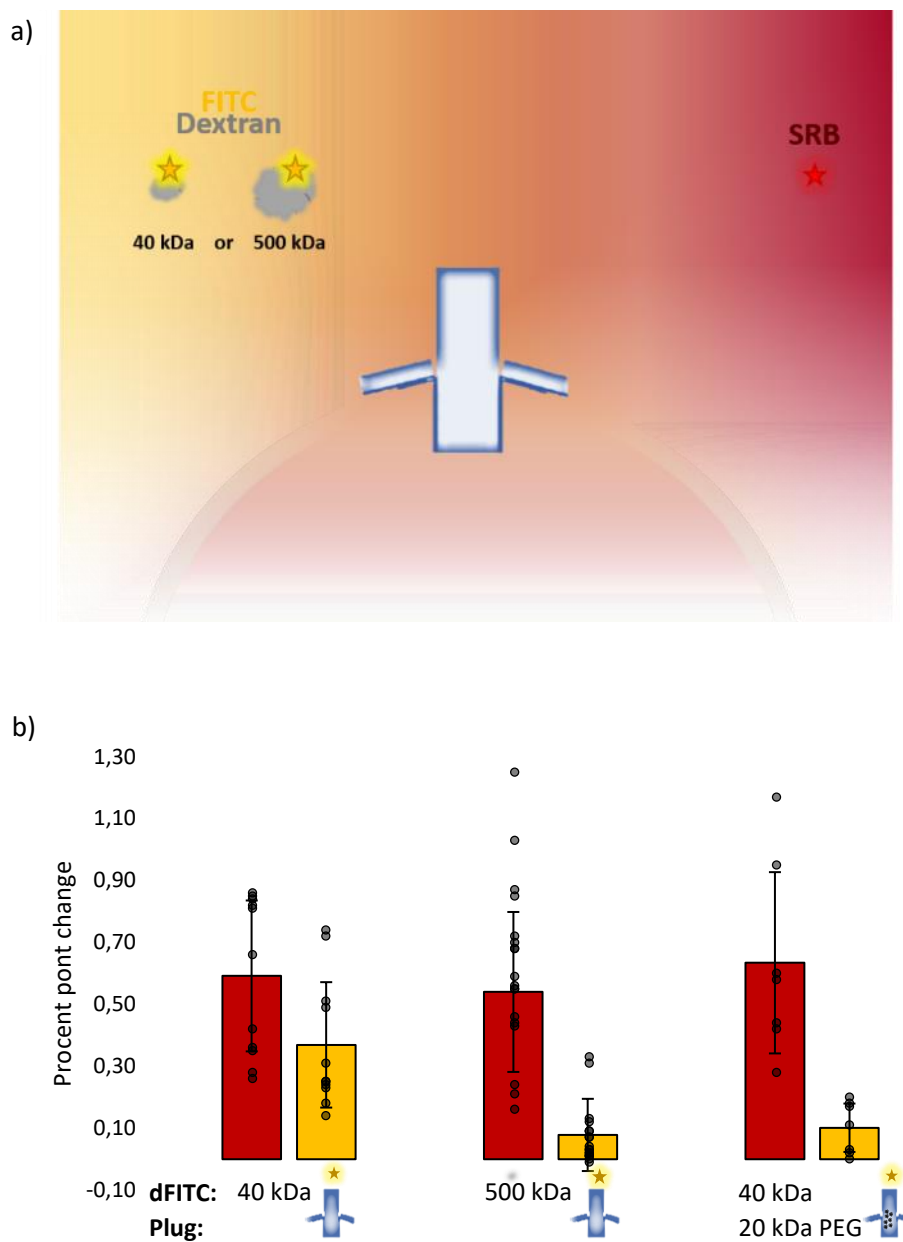
Supplementary Figure 23 – TIRF key experiments.

(a) Schematic setup of TIRF studies of SUV target insertion directed by lipidated DNA key oligos. Left, setup with keys flowed in (+) or right, control setup (-). Lipid anchored key strands was flowed into the immobilized SUVs and equilibrated for 30 min before 3x full chamber rinsing. Following, a continues flow of DNA nanopores with (L) or without (NL) lipidation was setup. Flow of nanopores in TIRF solution with a continuous rate of 0.01 ml/min for 50 min. (b) Nanopore colocalization lag time in setup. Asterisks (***) indicate a p-value of $<10^{-5}$ based on a two-sample t-test, showing prior lipidation of structures lower docking time significantly, while error bars show the standard error of the mean. No significant difference is found between key-decorated (+) and plain (-) SUVs with lipidated structures (p-value = 0.212).



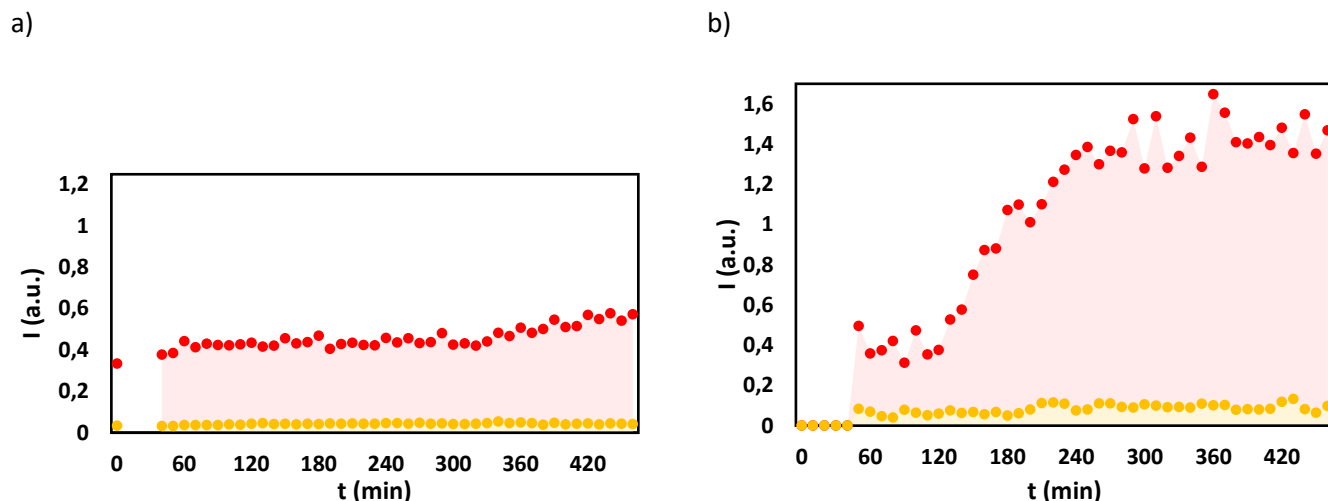
Supplementary Figure 24 – Stability of DNA nanopore in TIRF solution.

(a) 1 % AGE of DNA nanopores incubated in TIRF solution for 26 hours. In TIRF setup, DNA nanopores (in TAEMN) is incubated in TIRF buffer (400 mM Sucrose, 200 mM KCl) in a 1:4 mixture for up to 9 hours. Lane 1: M13. Lane 2: DNA nanopore (NP) in 1x TAEMN (1:1). Lane 3: NP in TAEK₁₀₀₀ (1:1). Lane 4-8: NP in TIRF buffer of 1:1, 1:2, 1:4, and 1:9 dilution. (b) Gel quantification of the indicated bands. A good stability in TIRF setup conditions (1:4) during the 26 hours is observed. Even at 1:9 dilution for 26 hours DNA nanopores is still detectable.



Supplementary Figure 25 – Confocal setup.

(a) Schematic of the CLSM setup of dyes included with either small (left) or large (right) dFITC together with SRB dye. (b) Summary of insertions from frame -1 to 0 (filling) based on percent point change from the insertions depicted in Figure 4. Error bars show the standard deviation.



Supplementary Figure 26 – Non-filled and leaky CLSM traces of SRB and 40kDa dextran-FITC inside GUVs.

Representative traces of non-filled (a) and leaky (b) GUVs observed in CLSM influx assay with SRB and FITC-dextran(40kDa). (a) Non-filled traces show no internalization of SRB or FITC-dextran while the GUV bilayer remains intact. (b) Leaky GUV traces show a slow SRB dye increase behavior over extended periods of time, while FITC-dextran does not enter. Such traces were not included in the analysis as only single frame large SRB influx observations was used.

Supplementary Table 2 – CLSM normalized intensities of internal color (dFITC-40k)

SRB traces

Frame	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	Avg	s.d.
-4	0,15	0,21	0,18	0,58	0,56	0,54	0,21	0,21	0,36	0,16	0,76	0,36	0,20
-3	0,18	0,36	0,18	0,55	0,53	0,54	0,19	0,22	0,40	0,18	0,73	0,37	0,19
-2	0,19	0,33	0,18	0,52	0,53	0,49	0,21	0,26	0,46	0,17	0,76	0,37	0,18
-1	0,33	0,29	0,18	0,58	0,53	0,53	0,21	0,20	0,52	0,17	0,85	0,40	0,21
0	1,14	1,14	1,02	0,84	0,95	0,89	0,87	1,06	0,87	0,99	1,13	0,99	0,11
1	0,97	1,04	1,05	0,81	0,98	0,99	0,84	1,11	0,92	1,08	1,15	0,99	0,10
2	1,03	1,14	1,03	0,83	0,98	0,91	0,85	1,07	0,87	1,22	1,24	1,02	0,14
3	0,98	1,04	1,01	0,80	1,03	0,92	0,86	1,02	0,84	0,95	1,23	0,97	0,11
4	1,05	0,98	1,02	0,84	1,02	0,92	0,85	1,03	0,89	0,98	1,16	0,98	0,09
5	1,02	0,99	1,03	0,83	1,07	0,98	0,88	1,00	0,90	1,01	1,22	0,99	0,10
6	1,00	1,05	0,99	0,83	1,02	0,96	0,89	0,99	0,92	1,03	1,19	0,99	0,09

FITC traces

Frame	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	Avg	s.d.
-4	0,03	0,03	0,03	0,03	0,04	0,07	0,05	0,05	0,05	0,06	0,05	0,04	0,01
-3	0,04	0,04	0,03	0,03	0,04	0,06	0,05	0,04	0,04	0,07	0,05	0,05	0,01
-2	0,04	0,03	0,03	0,03	0,04	0,06	0,05	0,04	0,04	0,06	0,05	0,04	0,01
-1	0,04	0,03	0,03	0,03	0,04	0,06	0,05	0,04	0,04	0,06	0,06	0,04	0,01
0	0,22	0,26	0,52	0,17	0,55	0,31	0,30	0,78	0,28	0,37	0,78	0,41	0,21
1	0,30	0,26	0,64	0,19	0,68	0,29	0,41	0,92	0,33	0,49	0,92	0,49	0,25
2	0,33	0,27	0,70	0,23	0,76	0,33	0,44	0,92	0,35	0,57	0,96	0,53	0,25
3	0,36	0,27	0,74	0,26	0,79	0,33	0,49	0,92	0,38	0,50	0,98	0,55	0,25
4	0,39	0,28	0,77	0,29	0,82	0,31	0,52	0,91	0,40	0,52	0,99	0,56	0,25
5	0,40	0,28	0,79	0,32	0,84	0,32	0,55	0,91	0,41	0,53	1,01	0,58	0,25
6	0,43	0,27	0,79	0,32	0,86	0,33	0,56	0,91	0,43	0,55	0,98	0,58	0,24

The 11 traces observed of GUV dye 1-frame filling observed at normalized frame 0 used for Figure 4d. There is 10 minutes between each frame. The listed values are the internal intensity normalized to the outer intensity to observe the relative intensity increase.

Supplementary Table 3 – CLSM normalized intensities of internal color (dFITC-500k)

SRB traces

Frame	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12	T13	T14	T15	T16	T17	T18	Avg	s.d.
-4	N/A	0,33	N/A	0,35	0,12	0,12	0,14	0,80	0,11	0,38	0,60	0,15	0,49	0,26	0,35	0,14	0,32	0,19	0,30	0,19
-3	N/A	0,38	N/A	0,32	0,12	0,17	0,19	0,88	0,12	0,38	0,63	0,16	0,53	0,27	0,36	0,14	0,29	0,20	0,32	0,20
-2	0,29	0,48	0,31	0,36	0,10	0,16	0,22	0,88	0,12	0,41	0,65	0,19	0,56	0,28	0,37	0,13	0,27	0,18	0,33	0,21
-1	0,50	0,53	0,50	0,38	0,11	0,17	0,28	0,79	0,12	0,37	0,67	0,17	0,58	0,27	0,34	0,15	0,31	0,20	0,36	0,20
0	0,71	1,09	0,74	1,10	0,66	0,72	1,15	0,95	1,15	0,96	1,13	1,42	1,01	0,95	1,02	1,00	1,01	0,64	0,97	0,20
1	0,80	0,95	0,78	1,15	0,73	0,67	1,21	0,90	1,16	0,99	1,09	1,32	0,96	1,03	1,12	1,04	0,99	0,59	0,97	0,19
2	0,81	0,97	0,90	1,14	0,77	0,68	1,15	0,84	1,26	0,97	1,14	1,11	0,99	1,05	1,08	1,03	0,99	0,58	0,97	0,17
3	0,81	1,00	0,90	1,12	0,70	0,66	1,13	0,86	1,20	1,02	1,12	1,12	0,94	1,04	0,97	1,09	1,03	0,58	0,96	0,17
4	0,86	0,98	0,92	1,05	0,69	0,66	1,15	0,91	1,13	0,98	N/A	1,12	0,91	0,99	1,04	1,03	1,04	0,57	0,94	0,16
5	0,86	1,03	0,87	0,99	0,63	0,70	1,14	0,85	1,18	1,00	N/A	1,11	0,92	1,08	1,05	1,02	1,01	0,59	0,94	0,17
6	0,85	0,99	0,94	1,03	0,64	0,65	1,12	0,84	1,17	1,06	N/A	1,19	0,91	1,01	1,07	1,06	1,04	0,60	0,95	0,18

FITC traces

Frame	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12	T13	T14	T15	T16	T17	T18	Avg	s.d.
-4	N/A	0,04	N/A	0,09	0,03	0,03	0,07	0,04	0,04	0,03	0,04	0,06	0,03	0,04	0,07	0,03	0,06	0,03	0,04	0,02
-3	N/A	0,04	N/A	0,07	0,03	0,03	0,09	0,04	0,04	0,03	0,04	0,06	0,02	0,04	0,06	0,03	0,06	0,04	0,05	0,02
-2	0,04	0,05	0,04	0,09	0,03	0,03	0,09	0,03	0,04	0,04	0,04	0,07	0,03	0,04	0,07	0,03	0,08	0,04	0,05	0,02
-1	0,03	0,05	0,04	0,08	0,03	0,02	0,10	0,04	0,05	0,04	0,05	0,06	0,02	0,04	0,07	0,03	0,06	0,03	0,05	0,02
0	0,04	0,12	0,04	0,15	0,05	0,04	0,09	0,05	0,38	0,07	0,36	0,18	0,11	0,13	0,10	0,16	0,10	0,04	0,12	0,10
1	0,05	0,13	0,05	0,15	0,04	0,04	0,09	0,03	0,43	0,09	0,38	0,16	0,12	0,15	0,15	0,17	0,14	0,05	0,13	0,11
2	0,04	0,14	0,05	0,14	0,05	0,04	0,11	0,03	0,47	0,10	0,36	0,16	0,12	0,15	0,16	0,18	0,15	0,04	0,14	0,11
3	0,04	0,16	0,04	0,15	0,04	0,03	0,10	0,04	0,50	0,10	0,37	0,16	0,12	0,16	0,16	0,18	0,16	0,04	0,14	0,12
4	0,05	0,17	0,05	0,15	0,04	0,03	0,11	0,05	0,51	0,11	N/A	0,19	0,13	0,17	0,17	0,21	0,16	0,04	0,14	0,11
5	0,05	0,18	0,04	0,14	0,04	0,03	0,12	0,04	0,54	0,11	N/A	0,18	0,13	0,19	0,18	0,20	0,18	0,04	0,14	0,12
6	0,05	0,18	0,05	0,13	0,04	0,03	0,12	0,05	0,54	0,12	N/A	0,18	0,12	0,20	0,19	0,21	0,20	0,05	0,14	0,12

The 18 traces observed of GUV dye 1-frame filling observed at normalized frame 0 used for Figure 4h. There is 10 minutes between each frame. The listed values are the internal intensity normalized to the outer intensity to observe the relative intensity increase. N/A values indicate frames where the values were not possible to determine as these frames was before or after CLSM acquisition.

Supplementary Table 4 – CLSM normalized intensities of internal color (dFITC-40k + plug)

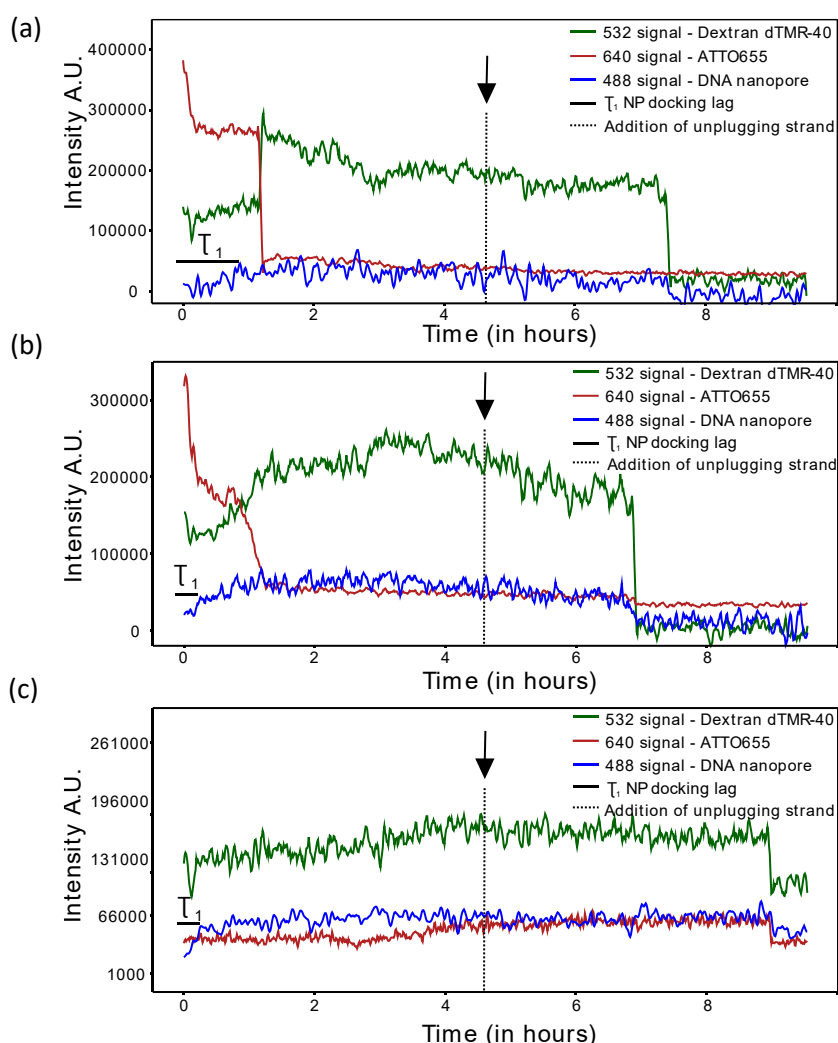
SRB traces

Frame	T1	T2	T3	T4	T5	T6	T7	Avg	s.d.
-4	0,35	0,17	N/A	N/A	0,82	0,44	0,34	0,42	0,22
-3	0,40	0,24	N/A	N/A	0,84	0,47	0,33	0,45	0,21
-2	0,39	0,18	N/A	N/A	0,80	0,46	0,46	0,46	0,20
-1	0,53	0,13	0,32	0,36	0,72	0,54	0,37	0,42	0,17
0	1,48	1,30	0,90	0,78	1,00	0,98	0,97	1,06	0,23
1	1,15	1,00	1,04	0,76	0,94	1,04	1,14	1,01	0,12
2	1,13	1,27	0,99	0,69	0,86	1,06	1,12	1,02	0,18
3	1,18	1,08	0,98	0,75	0,92	1,17	1,17	1,04	0,15
4	1,12	1,11	1,02	0,68	0,91	1,17	1,11	1,02	0,16
5	1,37	1,00	0,98	0,76	0,87	1,14	1,06	1,02	0,18
6	1,17	0,97	0,98	0,86	0,88	1,18	1,07	1,02	0,12

FITC traces

Frame	T1	T2	T3	T4	T5	T6	T7	Avg	s.d.
-4	0,07	0,06	N/A	N/A	0,04	0,06	0,16	0,08	0,04
-3	0,11	0,08	N/A	N/A	0,05	0,06	0,13	0,09	0,03
-2	0,09	0,07	N/A	N/A	0,06	0,04	0,11	0,07	0,02
-1	0,07	0,06	0,09	0,09	0,04	0,05	0,16	0,08	0,04
0	0,25	0,26	0,20	0,09	0,06	0,08	0,33	0,18	0,10
1	0,33	0,22	0,21	0,12	0,04	0,07	0,33	0,19	0,11
2	0,47	0,27	0,32	0,11	0,04	0,10	0,28	0,23	0,14
3	0,39	0,38	0,18	0,12	0,06	0,12	0,31	0,22	0,13
4	0,36	0,44	0,21	0,19	0,05	0,14	0,31	0,24	0,12
5	0,35	0,39	0,22	0,17	0,05	0,16	0,31	0,24	0,11
6	0,36	0,43	0,19	0,14	0,06	0,17	0,31	0,24	0,12

The 7 traces observed of GUV dye 1-frame filling observed at normalized frame 0 used for Figure 4I. There is 10 minutes between each frame. The listed values are the internal intensity normalized to the outer intensity to observe the relative intensity increase. N/A values indicate frames where the values were not possible to determine as these frames was before or after CLSM acquisition.



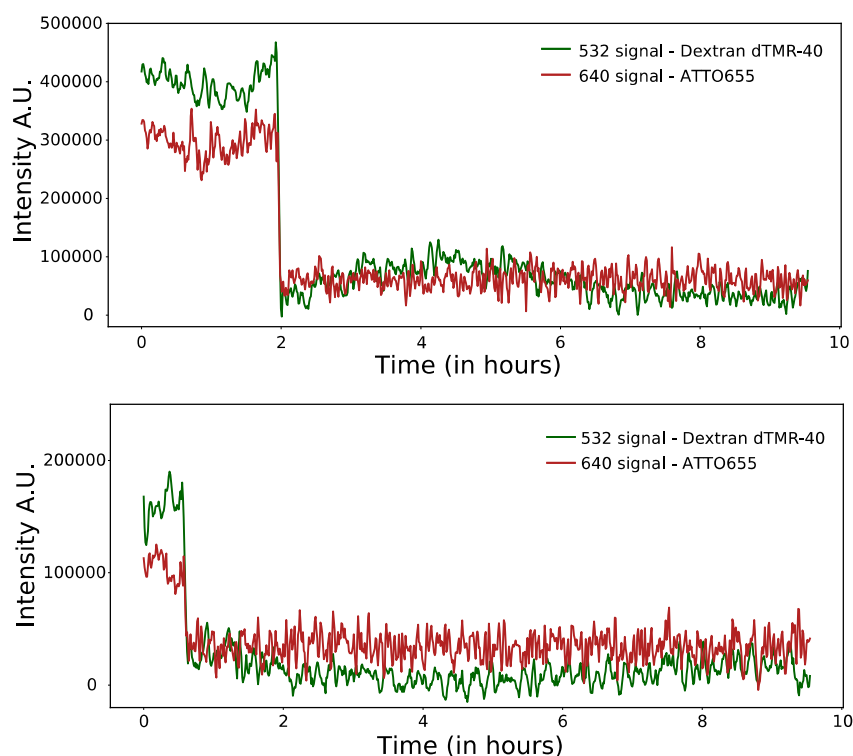
Supplementary Figure 27 – Real-time sensing of “unplugging” oligonucleotide.

Real-time sensing of unplugging of DNA nanopore insertion into ATTO 655 and 40 kDa dextran-tetramethyl rhodamine (dTMR-40k) encapsulated SUVs. (a) and (b) shows 2 representative sequentially leaking liposomes, where the small ATTO 655 dyes translocate through the inserted and plugged DNA nanopore. Note that due to spectral overlap of ATTO 488 with TMR, the signal of individual nanopore docking on TMR loaded SUVs resulted in small signal in the blue channel. 4.4 hours into the experiment the “unplugging” strand is added, opening the nanopore and allowing translocation of the larger dTMR-40k. (c) If docking/penetration of the DNA nanopore occurs after addition of the “unplugging” strand the two dye molecules are translocating simultaneously. This behavior is observed two times.

Supplementary Table 5 – Registered dye flow events in real-time sensing assay sorted by event type.

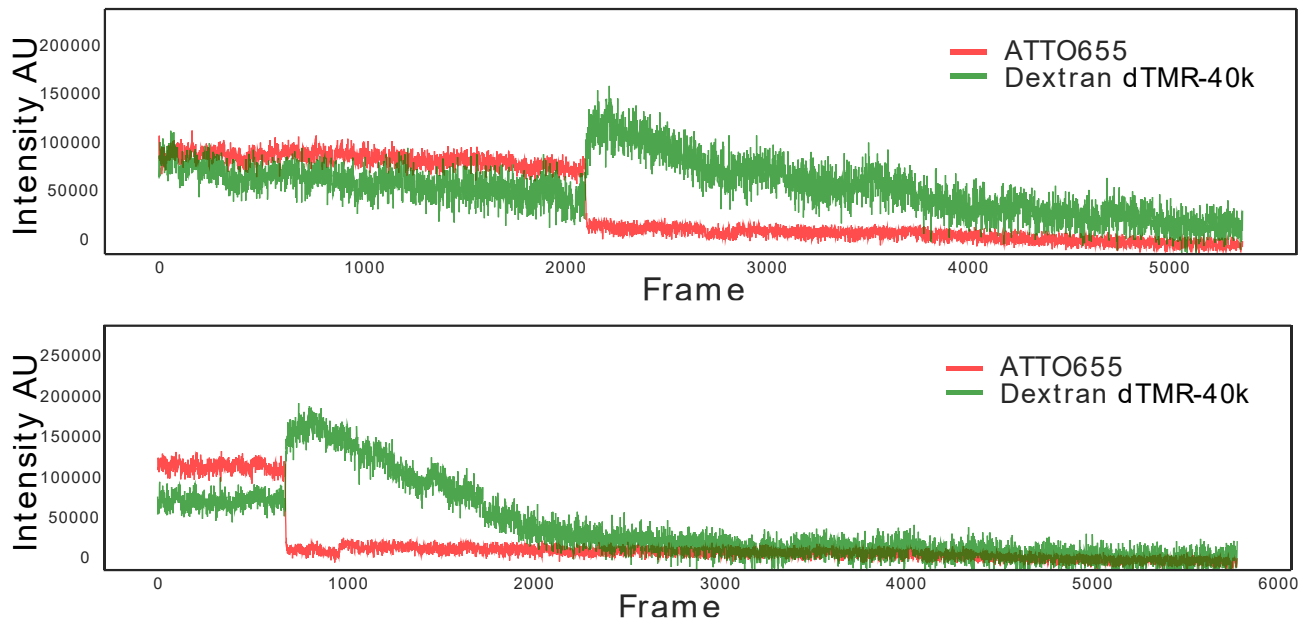
Event (pre/post addition of “unplugging strand”)	# Recorded events
ATTO 655 flow only (pre)	125
ATTO 655 flow only (post)	3
ATTO 655 flow (pre) and dTMR-40k flow (pre)	1
ATTO 655 flow (pre) and dTMR-40k (post)	5
ATTO 655 and dTMR-40 simultaneous flow (pre)	0
ATTO 655 and dTMR-40 simultaneous flow (post)	2

A total of 3174 SUV's have been investigated for the real-time sensing experiment. It is found that 128 SUVs shows translocation of ATTO655 which corresponds to an efficiency of 4%. 5 cases demonstrate a successful sensing by unzipping the plugs. In 2 cases the two dye molecules are found to translocate simultaneously post addition of “unplugging” strand. Presumably, the single outlier for which dTMR-40k release is observed already before the pore is unplugged corresponds to a pore, which has only partially been plugged in the first place.



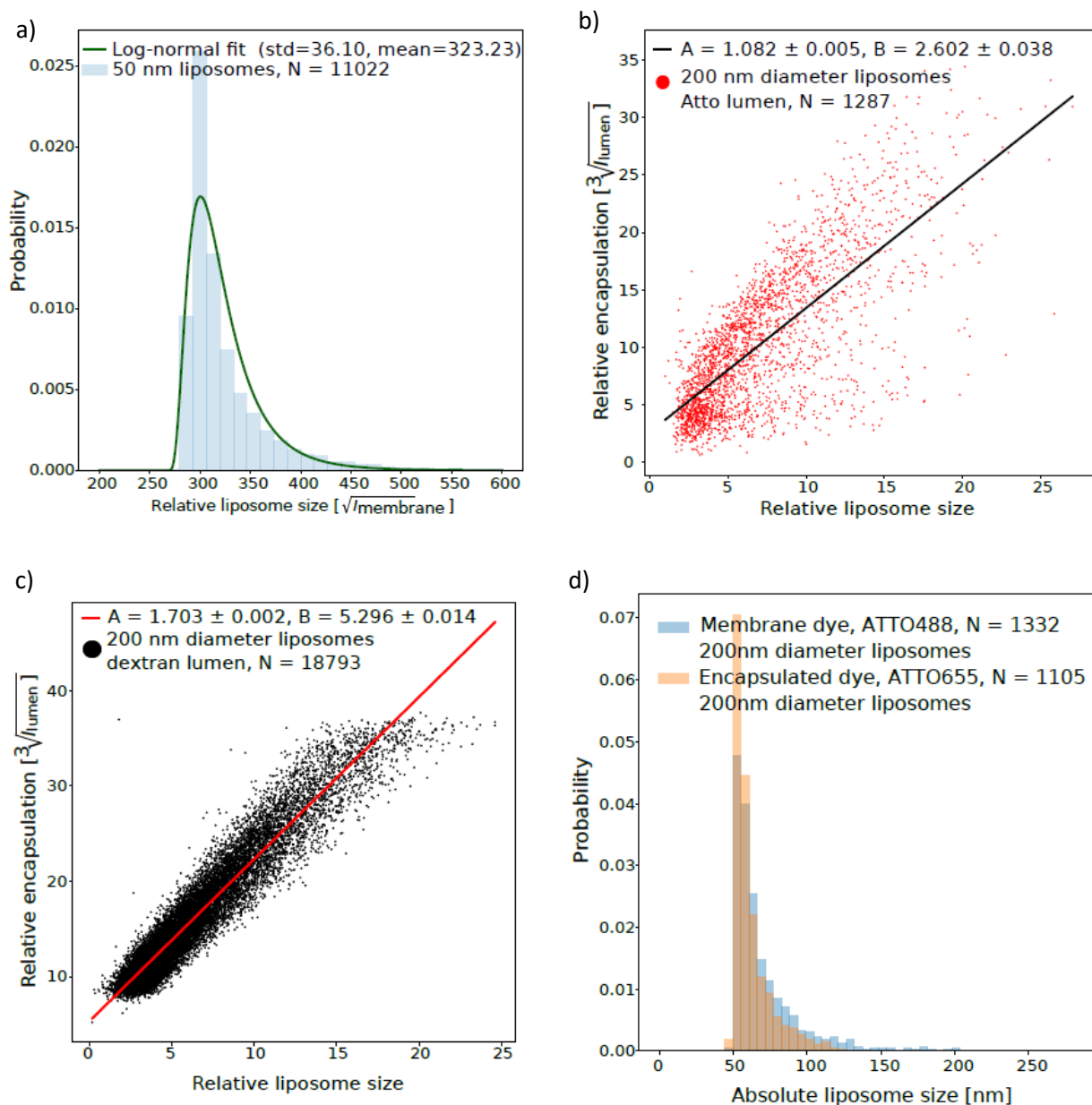
Supplementary Figure 28 – Control for real-time sensing with non-plugged DNA nanopores.

Control sensing experiment with non-plugged DNA nanopores and ATTO 655 and 40 kDa dextran-tetramethyl rhodamine (dTMR-40k) encapsulated SUVs. The inner pore of the DNA nanopore without plug is as reported 9nm which allows both ATTO 655 and dTMR-40k to translocate.



Supplementary Figure 29 – Control for real-time efflux of ATTO 655 via the α -hemolysin pore.

Representative traces from control experiment for real-time specific efflux of the small ATTO 655 through the narrow α -hemolysin pore. 100 μ mol α -hemolysin is flowed into the surface with immobilized ATTO 655 and 40 kDa dextran-tetramethyl rhodamine (dTMR-40k) encapsulated SUVs. A total of 244 SUV's are tracked and investigated and 97 (40%) of these show translocations of ATTO 655 as shown in the figure. ATTO 655 translocation results in dTMR-40k signal increase due to FRET between the two chromophores when they are entrapped together in the SUVs (The TMR signal increase can also be seen after ATTO 655 efflux with the plugged DNA nanopore in Supplementary Fig. 27). The TMR bleaching seems faster after ATTO 655 translocation, probably because the absorbed energy in dTMR-40k can no longer be transferred which results in a higher intensity signal and more photobleaching.



Supplementary Figure 30 – Loading efficiency of various dye encapsulated SUVs.

Histograms of size distributions of liposomes labeled on membrane with lipidated ATTO 488 and data recorded with identical settings as throughout the paper as to correlate membrane intensity to size calibration was done following our well-established methodology⁷.

(a) Liposome extruded around 50 nm. The distribution follows a clear log-normal distribution. From here the mean integrated square root of the intensity from the membrane stained dye is found which is used to convert all membrane intensities to absolute sizes⁷. (b-c) Quantification of the amount of (b) ATTO 655 and (c) 40 kDa dextran-tetramethyl rhodamine (dTMR-40k) encapsulated for all liposome sizes. A linear dependence of the third root of the integrated lumen intensity with liposome sizes as shown earlier⁸. This conversion is found to be $S = A_{\text{corr}} \cdot \sqrt[3]{I_{\text{lumen}}} + B_{\text{corr}}$, $A_{\text{corr}} = 1.049 \pm 0.006$, $B_{\text{corr}} = 2.671 \pm 0.055$ for ATTO655 encapsulated (b) and $A_{\text{corr}} = 1.703 \pm 0.002$, $B_{\text{corr}} = 5.296 \pm 0.014$ for dTMR-40k encapsulated (c). The linear fit allows to extract liposome sizes based on the encapsulated dye. (d) Similar Size distribution of liposomes is obtained when calculated by their membrane dye⁷ or their encapsulated dye⁸.

Supplementary Note 3 – Numerical relation between observed flow rate and the pore size described by the Hagen-Poiseuille relation.

In order to gain further insights in translocation of molecules through a transmembrane pore, we have investigated the numerical relation between the pore diameter and the observed flow rate. As a simplification the diffusion can be approximated with a flow that follows the Hagen-Poiseuille relation.

For the 1.4nm wide alpha hemolysin pore we measured a flow rate of 0.014 ± 0.010 l/s. For the 9.6nm wide DNA nanopore we measured a flow rate of 0.098 ± 0.087 l/s. We report a 7.0 ± 8.1 -fold increase in flow rate (error bars correspond to one standard deviation). The pore diameter of DNA nanopore on the other hand is 6.8-fold larger than the Hemolysin one (9).

The relation between pore size and flow can be described by the pressure difference in a Newtonian and incompressible fluid under laminar flow through a pore with constant cross section. The flux is given as

$$J = \frac{\Delta p d_p^2 \varepsilon}{32 x_p \mu_p} \quad (2)$$

Where Δp is the transmembrane pressure, d_p is the diameter of the pores, ε is the surface porosity of the liposome membrane, x_p is the length of the pore, and μ_p is the viscosity of the permeating fluid.

Assuming Δp , ε and μ_p are unchanged from the two experiments we get the relation:

$$\frac{d_{NP}^2}{x_{NP}} = \text{const.} \cdot \frac{d_{\alpha}^2}{x_{\alpha}} \quad (3)$$

$$\frac{(9.6 \cdot 10^{-9})^2}{35 \cdot 10^{-9}} = \text{const.} \cdot \frac{(14 \cdot 10^{-10})^2}{100 \cdot 10^{-10}}$$

$$\rightarrow \text{const} = \mathbf{13.43}$$

The theoretical 13.4-fold increase for the ATTO 655 flow though the DNA nanopore are thereby in relatively good agreement within the observed experimentally flow increase. This is under the approximation of the diffusion with a flow and despite a) the different pore charges which is not taken into consideration in the theoretical calculation, but might affect the efflux rates b) the pore diameter in Hemolysin is not constant along the channel tunnel (9) c) the fact the membrane curvature and appended tension varies the narrow point for SUVs of different membrane curvature (10)

Supplementary Note 4 – Freely diffusion model of the translocated dye molecules.

The diffusion of a dye molecule outside of the vesicle entails a) finding the pore following free diffusion and b) translocating across the pore following free diffusion and any potential mechanical hindrance.

The Stokes-Einstein equation describes diffusive processes of spherical particles in liquid that undergo Brownian motion, but it does not adequately describe the highly restricted motion within the pore nor the repulsive interactions of similarly charged species. So, we note it can only be used as an approximation.

The unrestricted diffusion of a particle is given by

$$D = \frac{k \cdot T}{6 \cdot \pi \cdot R_H \cdot \eta} \quad (4)$$

Where R_H is the hydrodynamic radius of the translocated molecule (here an ATTO 655 carboxy and a 40 kDa Dextran-tetramethyl rhodamine).

The root mean square displacement of the diffusive dye molecules within a time t in the 3 dimensions of the vesicle is given as:

$$\langle r^2 \rangle = 6Dt \quad (5)$$

The flow rate constant k and the half-life time τ are inverse correlated. This gives that the diffusion coefficient and the rate constant are proportional:

$$k \propto \frac{1}{\tau} \propto \frac{D}{r^2} \quad (6)$$

where r is the radius of the vesicle.

The half-life time for an ATTO 655 dye encapsulated within a vesicle with a radius of 35nm would thereby theoretically be expected to be $\tau \cong 3.3 \cdot 10^{-6}$ sec. We would therefore expect that within microseconds, half of the dye molecules have searched the vesicle found the pore. The unhindered diffusion out of the pore is expected to occur in the same time scale. Our direct experimental observation of rates however shows efflux rates in the order of milliseconds to seconds (dTMR-40k translocating through the DNA nanopore of: 0.0057 ± 0.0036 l/s, while ATTO 655 flow rate was found to be 0.098 ± 0.087 l/s).

An explanation for this observed reduced translocation could be that the translocated molecules interact electrostatically with the pore or interact with the inner membrane of the vesicle, both of which could result in hindrance and therefore a delayed translocation.

Because we have used the same pore and membrane composition, we can assume that the effect of Pore and membrane characteristic are similar for the Atto-655 and dTMR-40k the and the relation between the observed and theoretical flow rates and pore size can be analyzed.

Comparison of flow rates yield a 17.09 ± 18.50 -fold faster flow rate for translocation of the small ATTO 655 molecule (error bars correspond to one standard deviation). We note that the temporal resolution of the confocal experiments limits the accurate extraction of flow rates.

Using the Stokes-Einstein Kinetics we would expect:

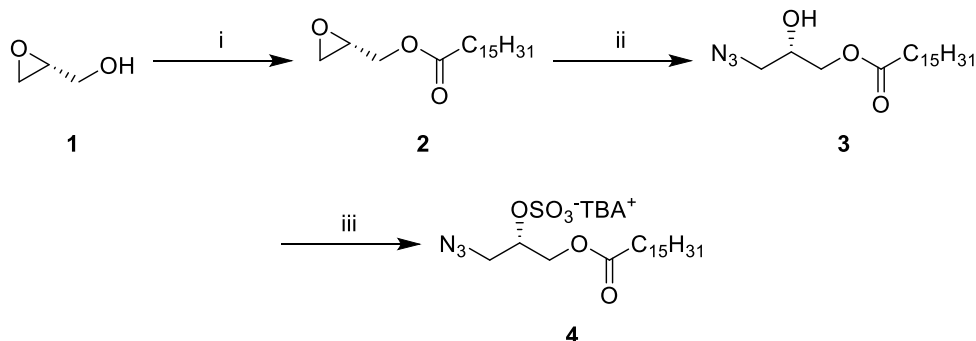
$$D_{\text{ATTO}} = \text{const} \cdot D_{\text{dextran}} \quad (7)$$

$$\frac{k \cdot T}{6 \cdot \pi \cdot R_{\text{H ATTO}} \cdot \eta} = \text{const} \cdot \frac{k \cdot T}{6 \cdot \pi \cdot R_{\text{H Dextran}} \cdot \eta}$$

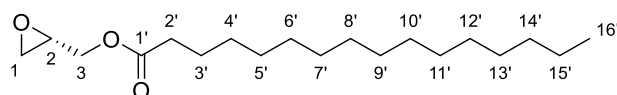
$$\frac{1}{R_{\text{H ATTO}}} = \text{const} \cdot \frac{1}{R_{\text{H Dextran}}}$$

const = **7.59** times higher flow rate for the ATTO 655, using a hydrodynamic radius of 5.86 Å for ATTO 655 and 44.5 Å for the 40 kDa Dextran-tetramethyl rhodamine. While Stoke-Einstein does not include the highly restricted motion within the pore nor the repulsive interactions of similarly charged species. The calculated rates relation is in relatively good agreement with the recorded values.

Supplementary Note 5 – Synthesis of Azido-palmitoyl building block ((S)-1-azido-3-(palmitoyloxy)propan-2-yl sulfate).

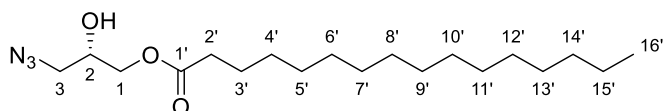


i) palmitoyl chloride, DMAP, DCM, r.t., 19h, 95%, ii) NaN_3 , NH_4Cl , DMF, mw, 100°C , 15min, 88%, iii) a) pyridine- SO_3 complex, toluene, mw, 100°C , 20min, b) $\text{Bu}_4\text{N}^+\text{OH}^- \cdot 30\text{H}_2\text{O}$, H_2O , r.t., 1h, 87%.



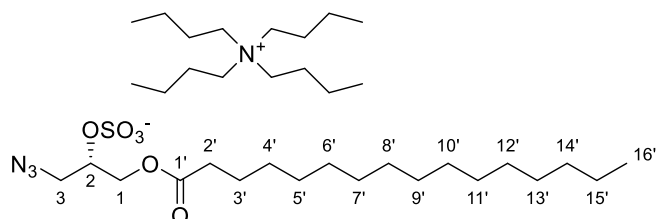
To a stirred solution of **1** (R)-(+)-glycidol (0.40 mL, 6.02 mmol, 1.0 eq) in dry dichloromethane (5 mL) under nitrogen atmosphere at 0°C 4-dimethylaminopyridine (882 mg, 7.22 mmol, 1.2 eq) was added and stirred for 30 min. Palmitoyl chloride (2.2 mL, 7.25 mmol, 1.2 eq) was added dropwise and the cooling bath was removed. Stirring was continued for 19h at room temperature. The reaction mixture was filtered through a short pad of silica (dichloromethane) and the solvent was removed under reduced pressure. The crude product was purified by column chromatography (SiO_2 , petroleum ether/ethyl acetate 6:1) yielding 1.79 g (5.73 mmol, 95 %) of **2** as a white solid.

R_f: 0.55 (petroleum ether/ethyl acetate 3:1); **¹H NMR** (400 MHz, CDCl_3): δ [ppm] = 0.86 (t, 3H, J = 6.8 Hz, 16'-H₃), 1.16-1.36 (m, 24H, 4'-H₂-15'-H₂), 1.62 (tt, 2H, J = 7.3, 7.6 Hz, 3'-H₂), 2.33 (t, 2H, J = 7.6 Hz, 2'-H₂), 2.63 (dd, 1H; J = 4.7, 2.6 Hz, 1-H_a), 2.82 (dd, 1H, J = 4.7, 4.7 Hz, 1-H_b), 3.15-3.21 (m, 1H, 2-H), 3.90 (dd, 1H, J = 12.3, 6.3 Hz, 3-H_a), 4.39 (dd, 1H, J = 12.3, 3.1 Hz, 3-H_b); **¹³C NMR** (101 MHz, CDCl_3): δ [ppm] = 14.21 (C-16'), 22.80, 29.23, 29.36, 29.47, 29.56, 29.70, 29.76, 29.77, 29.78 (C-4'-C15'), 24.99 (C-3'), 34.18 (C-2'), 44.74 (C-1), 49.48 (C-2), 64.84 (C-3), 173.62 (C-1'); **MS** (ESI): 335.26 $[\text{M}+\text{Na}]^+$, 647.52 $[2\text{M}+\text{Na}]^+$; **HRMS** (ESI): calcd. for $\text{C}_{19}\text{H}_{36}\text{NaO}_3$ 335.2557 $[\text{M}+\text{Na}]^+$, found 335.2566.



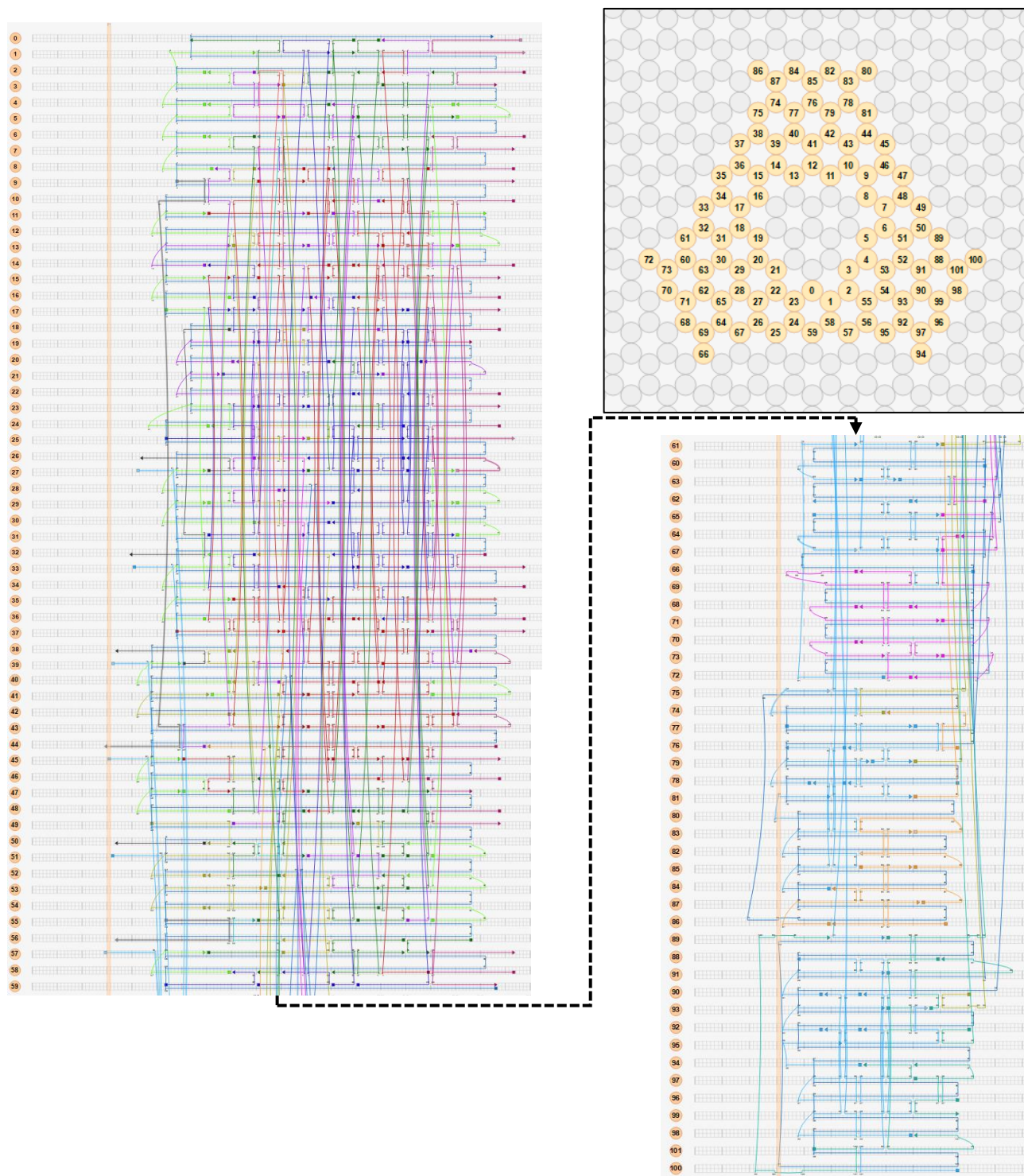
A suspension of **2** (200 mg, 0.64 mmol, 1.0 eq), sodium azide (125 mg, 1.92 mmol, 3.0 eq) and ammonium chloride (103 mg, 1.93 mmol, 3.0 eq) in dry DMF (3 mL) was stirred under microwave irradiation at 100°C for 15 min. Water (10 mL) was added and the aqueous layer was extracted with ethyl acetate (3x 10 mL). The combined organic layers were washed with water (1x 10 mL) and dried over magnesium sulphate. The solvent was removed under reduced pressure and the residue was purified by column chromatography (SiO_2 , petroleum ether/ethyl acetate 4:1) yielding 200 mg (0.56 mmol, 88 %) of **3** as white solid.

R_f: 0.36 (petroleum ether/ethyl acetate 3:1); **¹H NMR** (400 MHz, CDCl₃): δ [ppm] = 0.87 (t, 3H, *J* = 6.8 Hz, 16'-H₃), 1.18-1.36 (m, 24H, 4'-H₂-15'-H₂), 1.56-1.67 (m, 2H, 3'-H₂), 2.34 (t, 2H, *J* = 7.6 Hz, 2'-H₂), 2.61 (bs, 1H, OH), 3.37 (dd, 1H, *J* = 12.7, 6.4 Hz, 3-H_a), 3.41 (dd, 1H, *J* = 12.7, 4.7 Hz, 3-H_b), 3.96-4.03 (m, 1H, 2-H), 4.12 (dd, 1H, *J* = 11.9, 6.1 Hz, 1-H_a), 4.16 (dd, 1H, *J* = 11.9, 4.7 Hz, 1-H_b); **¹³C NMR** (101 MHz, CDCl₃): δ [ppm] = 14.23 (C-16'), 22.81, 29.24, 29.36, 29.48, 29.57, 29.71, 29.76, 29.77, 29.79 (C-4'-C-15'), 25.01 (C-3'), 34.22 (C-2'), 53.62 (C-3), 65.62 (C-1), 69.26 (C-2), 174.13 (C-1'); **MS** (ESI): 378.27 [M+Na]⁺, 733.55 [2M+Na]⁺; **HRMS** (ESI): calcd. for C₁₉H₃₇N₃NaO₃ 378.2727 [M+Na]⁺, found 378.2715.



A mixture of **3** (200 mg, 0.56 mmol, 1.0 eq) and pyridine-sulfur trioxide complex (87 mg, 0.55 mmol, 1.0 eq) in dry toluene (5 mL) was stirred at 100 °C for 20 min under microwave irradiation. The mixture was treated with water (1 mL), stirred for 5 min at room temperature and subsequently tetrabutylammonium hydroxide 30-hydrate (448 mg, 0.56 mmol, 1.0 eq) was added. Stirring was continued for 1 h at room temperature, water (2 mL) was added and the aqueous layer was extracted with ethyl acetate (3x 5 mL). The combined organic layers were dried over sodium sulfate and the solvent was removed under reduced pressure yielding 332 mg (0.49 mmol, 87 %) of **4** as clear oil.

¹H NMR (400 MHz, CDCl₃): δ [ppm] = 0.84 (t, 3H, *J* = 6.8 Hz, 16'-H₃), 0.96 (t, 3H, *J* = 7.3 Hz, TBA-CH₃), 1.16-1.31 (m, 24H, 4'-H₂-15'-H₂), 1.40 (qt, 8H, *J* = 7.3, 7.3 Hz, TBA-CH₂CH₃), 1.50-1.66 (m, 10H, 3'-H₂, TBA-NCH₂CH₂), 2.22-2.33 (m, 2H, 2'-H₂), 3.16-3.27 (m, 8H, TBA-NCH₂), 3.46 (dd, 1H, *J* = 12.8, 4.6 Hz, 3-H_a), 3.66 (dd, 1H, *J* = 12.8, 5.3 Hz, 3-H_b), 4.23 (dd, 1H, *J* = 11.3, 6.3 Hz, 1-H_a), 4.28 (dd, 1H, *J* = 11.3, 4.6 Hz, 1-H_b), 4.62 (m, 1H, 2-H); **¹³C NMR** (101 MHz, CDCl₃): δ [ppm] = 13.62 (TBA-CH₃), 14.09 (C-16'), 19.66 (TBA-CH₂CH₃), 22.66 (C-3'), 23.89 (TBA-NCH₂CH₂), 29.14, 29.27, 29.33, 29.45, 29.59, 29.62, 29.66, 31.89 (C-4'-C-15'), 34.12 (C-2'), 51.19 (C-3), 58.61 (TBA-NCH₂), 62.91 (C-1), 72.78 (C-2), 173.35 (C-1'); **MS** (ESI): 242.28 [TBA]⁺, 434.22 [M-TBA]⁺; **HRMS** (ESI): calcd. for C₁₉H₃₆N₃O₆S 434.2330 [M-TBA]⁺, found 434.2222.



Supplementary Figure 31 – CaDNano design of DNA nanopore.

CaDNano blueprint of designed structure and with included top-view. The image is separated at the channel to flap transition.

Supplementary Note 6 – DNA origami staples.

A total of 219 staples are used for annealing the DNA nanopore (one of the “Top” sets, all “cyl”, “inner” and “flap” strands).

To create the lipidated structure, strands in the green module are used with a 3’ cholesterol or lipid functionalized anchor as described elsewhere.

For internal immobilization inner strands are elongated with handle strands inner -> inner (2) (blue module)

For TIRF of CLSM the 10 dye handle strands are added in excess to substitute top strands, purified and before addition of the fluorescent strand and a final purification.

Strands used for FRET are indicated below their original sequence with inserted IDT sequence.

Sequence-stretches of staples used for specific purposes other than structural use is highlighted in bold.

Supplementary Table 6 – DNA sequences.

Module	Notes	DNA Sequence
Top_A	a	TATGCAAAATTCGACAACCTCGTATTAAATCT ACACTCA
Top_A	a	GCACATAC ATTTTAGTTAATCAGTATAAAGCC
Top_A	a	CTAAAGGACGCTGTCCTTGAAAACAG GGATCATA
Top_A	a	CTTGTCAT TAGCGATAGCTTAGATTAAGACGCTGATTTTCCAAACATC
Top_A	a	CTGGTAATAGCGGATAAGTGC ACTGCATA
Top_A	a	GATCAAAC ACTAATAGTAGTTTTTAGAACCC TTAGTAGAC
Top_A	a	ACAGGGAAGTTTTCAATTTTATCCTGAATATTTTGACCCAGCTAG AACATTT
Top_A	a	ACCTTGTC GTACAACTACAAATTCGAATA AGAGGAACA
Top_A	a	GCTAGTAC ATGTTTTAAAGGCATCAATTCT
Top_A	a	GAGGCTAA CCCTCAATCAATATCTAACCTCACTGATAG
Top_A	a	CAGTAAGC ACAAGCAAGCCGTGGCGAGAAAGGAAGGGAA
Top_A	a	CTCAGGT ATAATGCAGAACGCACTCATCGAGAG CTAGTAT
Top_A	a	TCAAAAAGATTAAAGAGGAATGCTGTAGCTCAG ATTAAACG
Top_A	a	TCTCCATC TGAGCAAAAGTTAAAGGCCGCTTTTAATTTT
Top_A	a	TCAGAAT CGCTCGAGAGGGTTGATATATTTTCAAATATA AGTCATA
Top_A	a	CGGAATAC CTTTGCCGGAAGCGGATTGCATTTTTTTTGCAAAAGA
Top_A	a	TTGATAC CGTGGACTCCAACGTCATCCACTATTAAAGAAC GATAACGA
Top_A	a	GATCTTG AGTGTTTTTATAAAACAGGAAAAAC
Top_A	a	AGCAAC CGGCTGCGGTAACCACTACGCGAGAATCCTGAGAA AGGATACC
Top_A	a	CACTTAG ATTAATGCGCGAAATATCAAAT TTACGATA
Top_B	b	TATGCAAAATTCGACAACCTCGTATTAAATCT ATGATCC
Top_B	b	TCTAAGT GATTTTAGTTAATCAGTATAAAGCC
Top_B	b	CTAAAGGACGCTGTCCTTGAAAACAT AGTGTTGA
Top_B	b	TTAGCCTC TAGCGATAGCTTAGATTAAGACGCTGATTTTCCAAACATC
Top_B	b	CTGGTAATAGCGGATAAGTGC GTCTACTA
Top_B	b	CATTCTGA ACTAATAGTAGTTTTTAGAACCC TTATGCAGT
Top_B	b	ACAGGGAAGTTTTCAATTTTATCCTGAATATTTTGACCCAGCTAT CGTTATC
Top_B	b	GTACTAGC TACAACTACAAATTCGAATA ACGTTAATC
Top_B	b	GACAAGG TACATGTTTTAAAGGCATCAATTCT
Top_B	b	ATGACA AGCCCTCAATCAATATCTAACCTCACTGATAG
Top_B	b	GGTATCAA ACAAGCAAGCCGTGGCGAGAAAGGAAGGGAA
Top_B	b	TCAAGAT CTAATGCAGAACGCACTCATCGAG AGGTATCCT
Top_B	b	TCAAAAAGATTAAAGAGGAATGCTGTAGCTCAT TGTCCTC
Top_B	b	GTATTC CGTGAGCAAAAGTTAAAGGCCGCTTTTAATTTT
Top_B	b	GTTTGAT CCGTCGAGAGGGTTGATATATTTTCAAATAT TATCGTAA
Top_B	b	GATGGAG ACTTTGCCGGAAGCGGATTGCATTTTTTTTGCAAAAGA
Top_B	b	GCTTACT GGTGGACTCCAACGTCATCCACTATTAAAGAAC AAATGTTT
Top_B	b	TACCTG AGGTGTTTTTATAAAACAGGAAAAAC
Top_B	b	CGGTTG CTGCTGCGGTAACCACTACGCCAGAATCCTGAGAA ATACTAGC
Top_B	b	GTATGT GCTTAATGCGCGAAATATCAAAT ATGACTT
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cyl21	LockToe	CTAATAGATGAATTATCATCTTTCAAATGCAGGT CAGCTGCTTTTGGGATTCCGTTG
cyl12	LockToe	CCAACGCGCGGGGAGAGGCTGTTTGATGGTGGTTCCGA CAGCTGCTTTTGGGATTCCGTTG
cyl31	LockToe	CGTTAAATAAGATTTTCATTAAACCTATTA CAGCTGCTTTTGGGATTCCGTTG
cyl22	LockToe	CCGATAGGGTGAATTTCTTAAAC CAGCTGCTTTTGGGATTCCGTTG
cyl32	LockToe	AATAAGACAGCCATATTATTTAT CAGCTGCTTTTGGGATTCCGTTG
cyl_lip	Pal	AAATCGGTTGTACCAAAAACATTACAGTCA
cyl_lip	Chol	ATGGTCATAGCTTTTTATGATATTCAACCGTTCTCGTAATC
cyl_lip	Pal	TATCGGCTTAACCGCGCCAGGGTGGTGTCTCCAGTCGGG
cyl_lip	Chol	CCGACCGAAAAAGCCCAACATGTAATTTGCCTCCATTGGCC
cyl_lip	Pal	ACAATTCACACACATACTCGTAAACCGGGT
cyl_lip	Pal	TTTCAACTAATGTGTAGGTAAAGATTCAAAGGGT
cyl_lip	Pal	TCAGGACTGTGAATTACCTTATGCGATTTTAATTCTGTTTATCAGCTTG
cyl_lip	Pal	ACGGATTGATGAATACGAGGCATAGTAA
cyl_lip	Chol	GTCAATAATATTTCATTGAATCCCTTTTGATACATAACGCCAAAA
cyl_lip	Chol	GGAGGTTTCGCGCCCAATAGCAAGCAATCTCCCGAGTTACA
cyl_lip	Pal	TCAATCATTTAATATTTTAAACCTGTCTGCTATCCGCTC
cyl_lip	Pal	CACCAACCTAAATAATACTCGCGGAATC
cyl_lip	Pal	AATATCTGTCAATAATCCACCTTTTGATAAGAGAGCG
cyl_lip	Chol	CTTTGCTGTCGCCGACAATGGATTTAG
cyl_lip	Pal	GAATACCCAAAAATTTTTCGTATAAACAGTTAATGCC
cyl_lip	Chol	TTAATGAATCGGTTTTGTTCTGTGTGAAATTGTAGTGCA
cyl_lip	Pal	GCCCGAGATAGGGTGCTGTTTGCCCA
Top_dye_Ha	Dye_handle	TAACAGGATTAGCAGAGCGAGG CTGGTAATAGCGGATAAGTGC
Top_dye_Ha	Dye_handle	TAACAGGATTAGCAGAGCGAGG TCAAAAAGATTAGAGGAATGCTGTAGTCA
Top_dye_Ha	Dye_handle	TAACAGGATTAGCAGAGCGAGG ACAAGCAAGCCGTGGCGAGAAAGGAAGGGAA
Top_dye_Ha	Dye_handle	TAACAGGATTAGCAGAGCGAGG ATTTTAGTTAATCAGTATAAAGCC
Top_dye_Ha	Dye_handle	TAACAGGATTAGCAGAGCGAGG CCCTCAATCAATATCTAACCTCACTGATAG
Top_dye_Ha	Dye_handle	TAACAGGATTAGCAGAGCGAGG GTGTTTTATAAAACAGGAAAAAC
Top_dye_Ha	Dye_handle	TAACAGGATTAGCAGAGCGAGG ACTAATAGTAGTTTTAGAACCTT
Top_dye_Ha	Dye_handle	TAACAGGATTAGCAGAGCGAGG GTGGACTCCAACGTCATCCACTATTAAGAAGC
Top_dye_Ha	Dye_handle	TAACAGGATTAGCAGAGCGAGG TTAATGCGCGAAAAATATCAAA
Top_dye_Ha	Dye_handle	TAACAGGATTAGCAGAGCGAGG TACCGCCTGGCCTGAGAACAAGAG
Inner(2)	Immob_handle	CCTAAAGGGAGCCCCGATTTTGGCCCAAGGAGGCC CATGCGAGGCTACACT
Inner(2)	Immob_handle	TTATCAAAATCATAGGTGTAAATGCTGAT CATGCGAGGCTACACT
Inner(2)	Immob_handle	TAAATTTAATGGTTTGAAATACAAATCTGAAT CATGCGAGGCTACACT
Inner(2)	Immob_handle	AATACTTAGAAGAACAGTCCAGAGAGATAGAACCC CATGCGAGGCTACACT
Inner(2)	Immob_handle	CAGTTGAACAACAACCATCGCTTTAACATTGCTTT CATGCGAGGCTACACT
Inner(2)	Immob_handle	TTCTGACATTGGCAGATTACCTCAAAC CATGCGAGGCTACACT
Inner(2)	Immob_handle	ATGACCTGTAAATAAGCAAAACACGCTGTATCTAA CATGCGAGGCTACACT
Inner(2)	Immob_handle	AGGCATTTTCGAGTTTATAAACACCGGAATCATAA CATGCGAGGCTACACT
Inner(2)	Immob_handle	TTGAGAGTCGATGAACGGTAAGAGCCGGAATGGAT CATGCGAGGCTACACT
Inner(2)	Immob_handle	GAATACCAGATTTTCAGGTTTATATAAT CATGCGAGGCTACACT
Bio_plug capture oligo		AGCTCACTCAGGACGTTCAATTACCTTTTATCAAAATTTTGCGGGTTAGAACCTACCA
3'bio		CGACGACATAAACAACATGTTACGCTTTTAAACGCTCAACAGTAAGCTCACTCAGGACGT
Bio unplug oligo		TACCTGTGCGAGCTCCTGAGTGAGCT
		AGCTCACTCTGGACGTCTACAGGTA
Dye oligo		/DYE/CCTCGCTCTGCTAATCTGTGA
Key oligo		CAACGGAATCCCAAAAGCAGCTG
Scramble key oligo		TAGCTTATCAGACTGATGTTGA
PEG plug oligo		/5AmMO/AGTGTAGCCTCGCATGAGCCC
Unplugging oligo		GGGCTCATGCGAGGCTACACT

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