SUPPORTING INFORMATION

Title: Glutaraldehyde crosslinking of oligolysines coating DNA origami greatly reduces susceptibility to nuclease degradation

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Supporting Experimental Procedures

Fabrication DNs. DNs were designed using caDNAno and assembled using previously published methods for folding of 3D DNA origami. Construction plans for each DN, scaffold and staple sequences are listed in **SI Tables 1-3** respectively. Synthetic DNA for staples was purchased in a 100 nmole scale from IDT. Scaffolds (p7308, p8064 and p8634) were produced in-house using previously published protocols, and purified from endotoxins before use.
For each shape, folding conditions used were 5 mM Tris, 1 mM EDTA (pH 8.0), 20 nM scaffold, 200 nM staple strands and 10 mM $MgCl_2$. Folding was performed in a thermocycler with the following program per shape: for DN1, denaturing at 80°C for 15 minutes; and then annealing at 60°C to 25°C at -1°C per 31 minutes; DN2, denaturing at 80°C for 15 minutes; and then annealing at 60°C to 25°C at -1°C per 31 minutes; for DN3, denaturing at 80°C for 15 minutes; and then annealing at 50°C to 40°C at -1°C per 108 minutes. All objects were purified using glycerol gradient purification and quality of DNs was analyzed via agarose gel eletrophoresis and transmission electron microscopy.

Fluorescent labeling of DNs. DNs were each labelled with Cy5 fluorophores. In each case, DNA oligonucleotides were modified with a 3' amine and covalently coupled to Cy5 fluorophores via NHS ester coupling (http://www.lumiprobe.com/p/cy5-nhs-ester). In the dark, 25 uL DNA oligonucleotide (0.5 mM in ddH_2O) was mixed with 12.5 uL of NHS-Cy5 (25 mM in DMSO) (25x excess) and 4.2 uL of $NaHCO_3$ (1M, buffer at pH 8.0, (sterile filtered) was combined for a total volume of 41.7 µL. The reaction was carried out in the dark for 2 h at 25°C. Zeba size-exclusion and desalting columns (7K MWCO; Thermo Scientific, Waltham, MA) were used to remove unreacted dye through centrifugation at 1000x *g* for 2 min. The columns were washed with 400 µL of ddH2O three times before use according to manufacturer's protocol.

Purifying DNs. DNs were purified using glycerol gradient purification as described by Lin et al.[1] Amicon ultracentrifugation filters (50 kDa) (Milipore Sigma) were washed with (5 mM Tris, 1 mM EDTA (pH 8.0), 0.01% tween, 10 mM $MgCl_2$) twice, before being used to concentrate DNs from folding (20 min, 4,000x *g*). DNs were then gently added to the top of 15-45% glycerol gradients in SW41 Ti compatible tubes. Samples were spun for 2.5 hours at 41,000 rpm at 4°C in a Beckman Coulter preparative ultracentrifuge. Gradients were fractionated and DN monomer containing fractions were collected and cleaned from glycerol using Amicon ultracentrifugation filters.

Oligolysine-PEG5K Coating of DNs. 30 uL of 90 nM DN was mixed 1:1 (v/v) with oligolysine-PEG5K (K10-PEG5K) (Alamanda polymers) such that nitrogen in amines:phosphates in DNA ratio was 1:1, according to the published method. Samples were incubated at room temperature for 1 hour.

Glutaraldehyde crosslinking of DNs. 60 uL of 45 nM DN was combined with 2% (v/v) glutaraldehyde (50% in H_2O) (Milipore Sigma), mixed gently and incubated at room temperature over two hours. Immediately prior to use, excess small molecule was removed using Zeba size-exclusion and desalting columns (7K MWCO; Thermo Scientific, Waltham, MA). Columns were prepared as described previously. DNs were then buffer exchanged into PBS using Amicon Ultra 0.5 mL centrifugation filters (Milipore Sigma) according to manufacturer's protocol.

DNase I degradation assays. DNs (10 nM final concentration) were incubated with 1.0 U/uL DNase I (NEB) with 1x DNase I buffer in sterile phosphate buffered saline (PBS) (Gibco). Samples were incubated in thermocycler at 37°C for specific timepoints and then analyzed using agarose gel electrophoresis. Immediately prior to gel analysis, 5x oligolysine-PEG5K was added to the samples, to ensure migration of the DN band out of the well. All time points were performed in at least triplicate.

TEM analysis. The structural integrity of each DN was verified using negative stain transmission electron microscopy (TEM). Prior to adding the samples, grids were cleaned using plasma discharge for 30 seconds. 3.0 uL of 1 nM DN solution (1 nM) was then deposited on a carbon coated Formvar grid (Electron Microscopy Sciences). After 3 minutes, the sample was wicked from the grid by gently touching filter paper to the grid edge. A drop of uranyl formate solution (2% w/v in H_2O) was then deposited onto the grid for 30 seconds, and the excess solution was wicked using filter paper. Studies were conducted using a JEOL JEM-1400 transmission electron microscope in brightfield mode at 80 kV.

Cell-based assays. Cell studies were performed using Human Embryonic Kidney (HEK293) cells maintained in high-glucose Dulbecco modified Eagle medium (Gibco, Gaithersburg) and 10% fetal bovine serum (FBS) (Lonza, Wakersville) with penicillin–streptomycin. For flow cytometry, HEK293 cells were seeded at a density of 250,000 cells/mL into tissue culture treated 48 well plates (BD Life Sciences) and allowed to grow for 24 hours in 200 uL of media. DNs (40 nM) were added to a final concentration of 0.2-2 nM and incubated with cells for the described amount of time. All samples were performed in triplicate.

Microscopy. 10,000 cells were seeded on tissue culture-treated 15-well ibidi slides (Cat. No. 81506) and allowed to adhere overnight. DNs were then added to a final concentration of 2 nM in 45 uL of media per well and incubated for 24 hours. After 24 hours, cells were washed with PBS with DAPI (300 nM) and incubated at room temperature for 30 min. Excess DAPI was then removed by washing with PBS and imaged on a Zeiss inverted microscope. Excitation for 640 nm for Cy5, and DAPI was 350 nm.

Flow Cytometry. Cells incubated with DNs were washed twice with PBS (-,-) (Gibco) and digested with trypsin (0.025%) (Invitrogen) for 2 minutes at 37°C. Cells were resuspended in 250 uL of PBS and stained using Propidium Iodide (ThermoFisher Scientific). Samples were then loaded onto a flow cytometer (BD LSR Fortessa) and data was analyzed using FlowJo (Flow Cytometry Analysis Software).

Figure S1 | caDNAno illustration of DN1 (C-shape). Arrows indicate 3'-ends of DNA; squares indicate 5'-ends of DNA. Scaffold is shown in blue; staple strands are shown in purple, grey and red

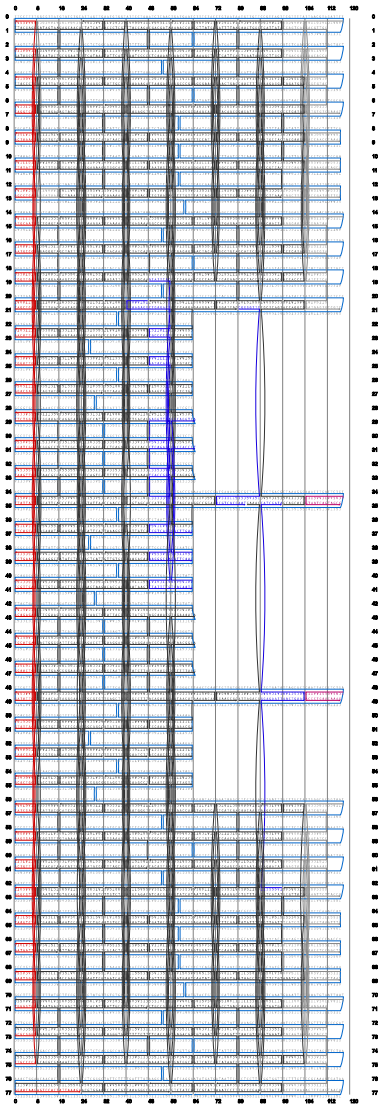


Figure S2 | caDNAno illustration of DN2 (90 nm barrel). Arrows indicate 3'-ends of DNA; squares indicate 5'-ends of DNA. Scaffold and mini-scaffold strands are shown in blue; staple strands are shown in light and dark green, light and dark orange.

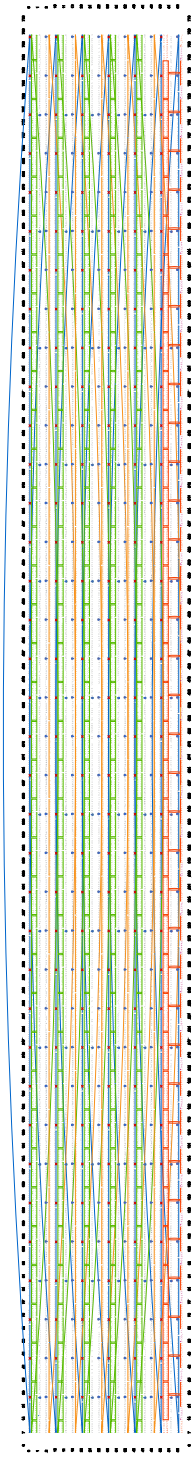


Figure S3 | caDNAno illustration of DN2 (60 nm barrel). Arrows indicate 3'-ends of DNA; squares indicate 5'-ends of DNA. Scaffold and mini-scaffold strands are shown in blue; staple strands are shown in green and orange.

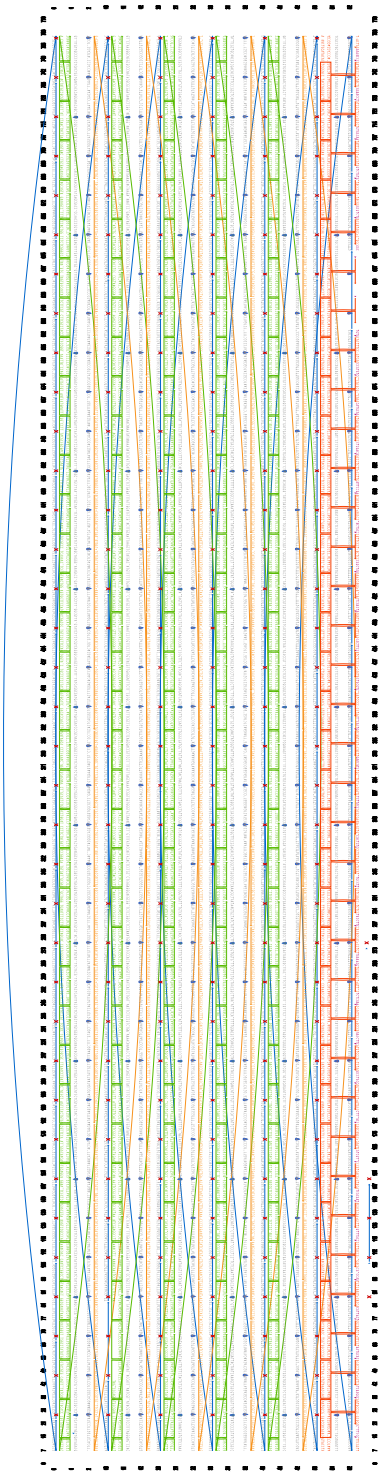


Figure S4 | AGE of DN1-3 after purification. DNA nanostructures were annealed according to the described protocol and purified using density gradient ultracentrifugation with 15-45% glycerol. Purified samples were loaded onto a Sybrsafe stained 2% agarose gel and run in 0.5x TBE buffer with 10 mM $MgCl_2$ at 70 V for 2 hour.

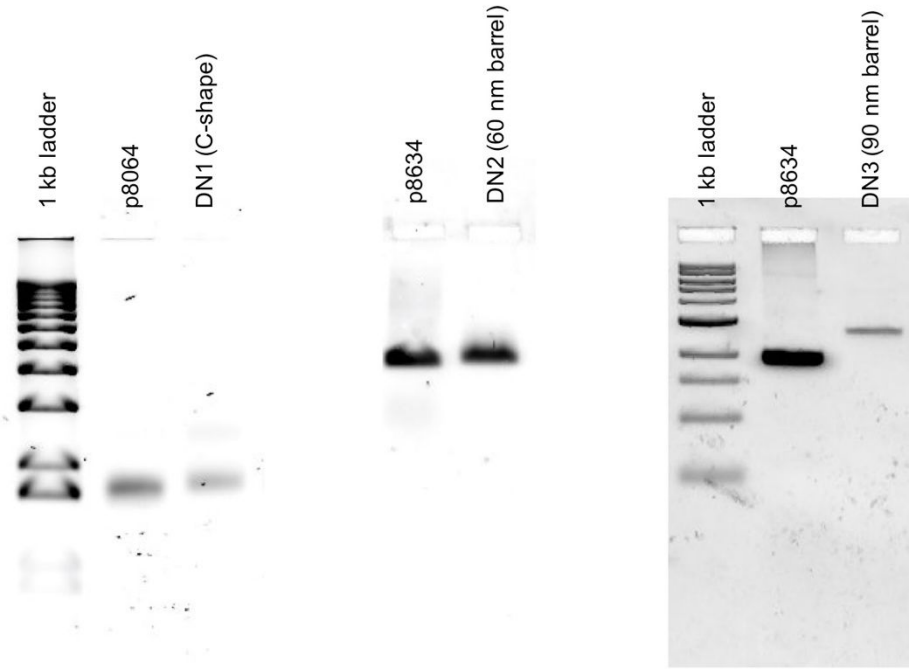


Figure S5 | (a) Schematic showing chemical structure of crosslinkers tested in this study: (top to bottom) 8-methoxypsoralen, cisplatin, formaldehyde and glutaraldehyde. (b) K10P-DN1 was incubated with chemical crosslinkers and then run on a denaturing polyacrylamide gel. This shows disappearance of DN1 staples on addition of different crosslinkers indicating successful crosslinking of staples. We observe concentration dependent disappearance of staples. Samples were loaded onto a 10% denaturing PAGE and run in 0.5x TBE buffer at 150 V for 30 min, followed by SYBR Gold staining.

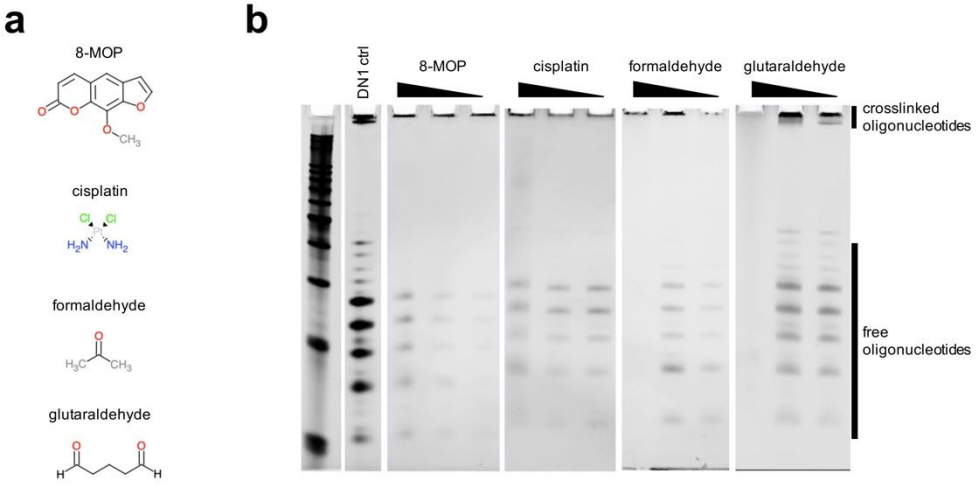


Figure S6 | Agarose gel electrophoresis showing: (a) DN1, K10P-DN1 and XK10P-DN1, (b) DN3, K10P-DN3 and XK10P-DN3. This shows bands to show no mobility (trapped in the well, K10P-DN1) or upward mobility (XK10P-DN1, K10PDN3 and XK10P-DN3) indicating neutral or positively charged entities when coated with oligolysine-PEG5K. Uncoated DNs travel downwards towards the cathode indicating negatively charged particles.

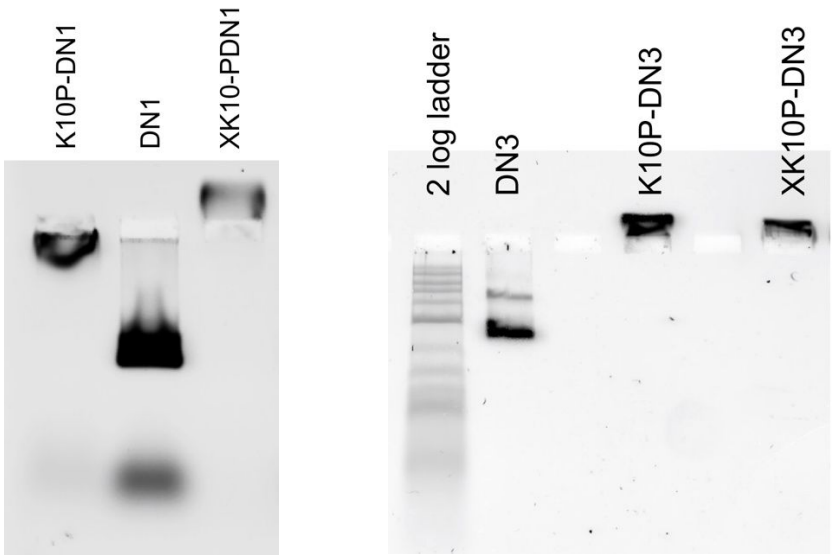


Figure S7 | Agarose gel electrophoresis showing complete digestion K10P-DN2 and K10P-DN2 coated with K10PEG5K and then crosslinked with formaldehyde after 3 and 24 hours of incubation with DNaseI (1 U/uL). In contrast, we observe survival of K10P-DN2 coated with glutaldehyde after 3 and 24 hours suggesting the glutaraldehyde-mediated crosslinking results in resistance to DNAseI digestion of DNs.

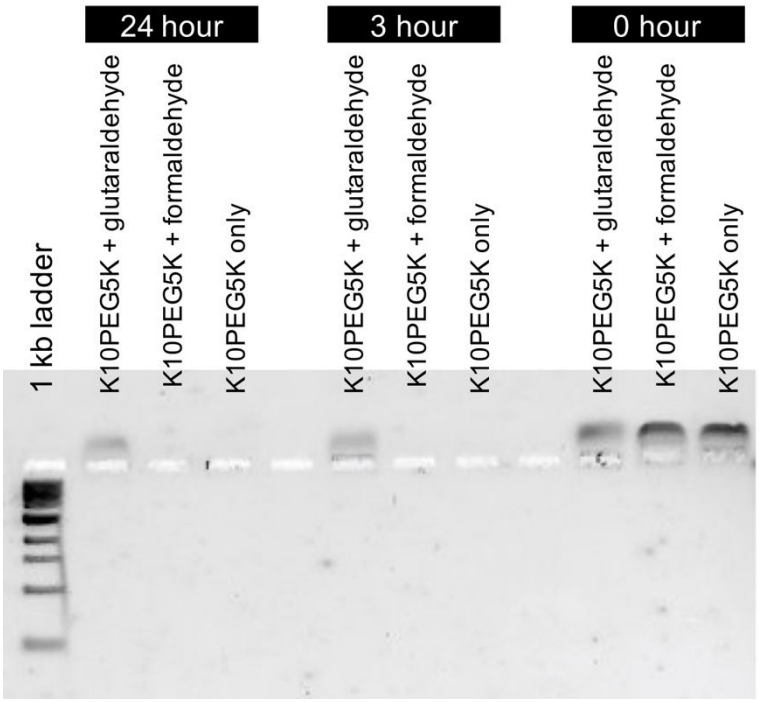


Figure S8 | Schematic showing chemical reaction of polymeric glutaraldehyde with lysine side chain to form secondary amine links between oligolysine molecules.

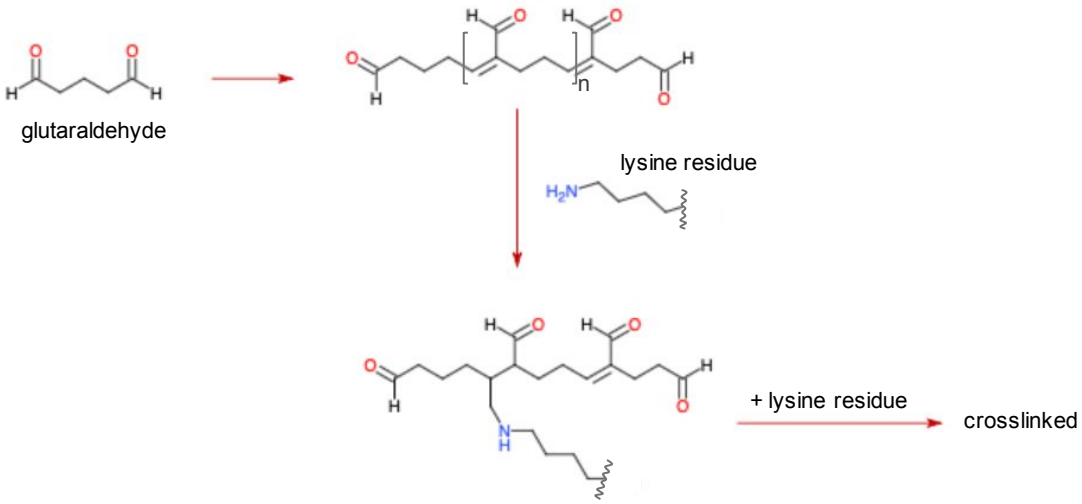


Figure S9 | Negative stain TEM of DNs 1-3 once coated with oligolysine-PEG5K and then crosslinked with glutaraldehyde. Here, no structural deformations Structures were stained using 2% uranyl formate for 30 seconds. Scale bar is 100 nm in all images.

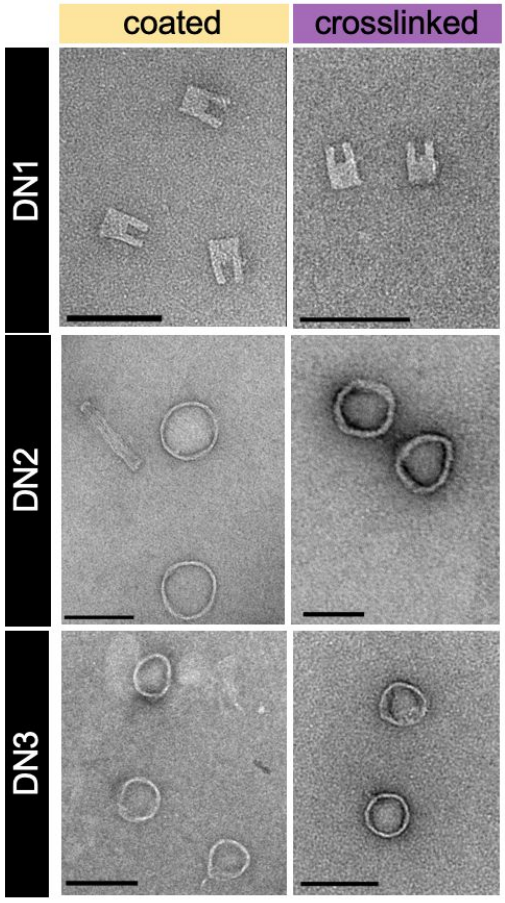


Figure S10 | AGE showing oligolysine-PEG5K coated structures (DN1-3) after incubation with different concentratios of DNase I (0.001-1.0 U/uL DNase I) for 1.5 hour and 12 hours at 37°C. Samples were loaded onto a Sybrsafe stained 2% agarose gel and run in 0.5x TBE buffer with 10 mM $MgCl_2$ at 70 V for 2 hours. Here, we observed varying degradation of DNs in a structurally dependent manner.

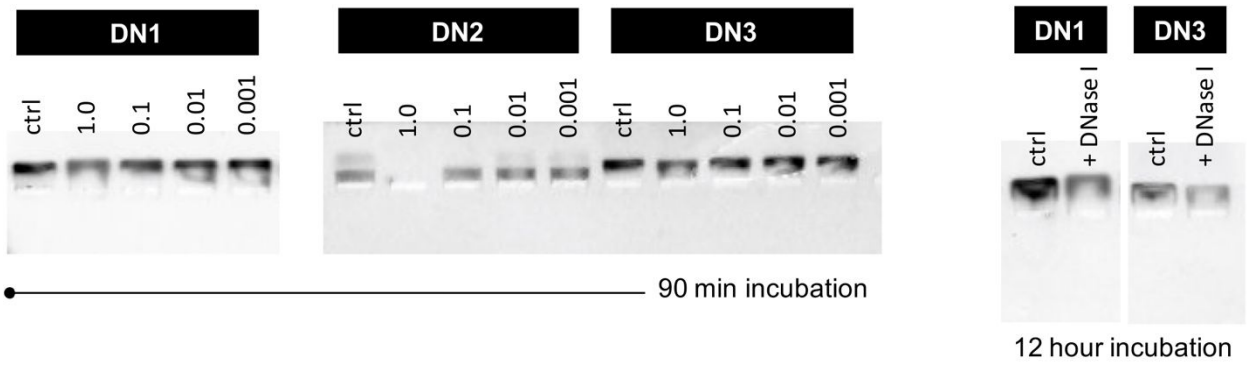


Figure S11 | AGE showing DN3 before and after glutaraldehyde crosslinking (no oligolysine-PEG5K).

DN3 after crosslinking was incubated with DNase I (0.1 U/uL) for 1 hour at 37°C. Samples were loaded onto a Sybrsafe stained 2% agarose gel and run in 0.5x TBE buffer with 10 mM $MgCl_2$ at 70 V for 2 hours. Here, we observed full degradation of DN3 crosslinked with glutaraldehyde after 1 hour indicating that oligolysine-PEG5K is necessary for protection from nuclease degradation.

Lanes are denoted 1-9 (left-right)

Lane 2: $DN3_{bare}$
Lane 3: DN3 crosslinked with glutaraldehyde **(without oligolysine-PEG5K)**
Lanes 4-6: DN3 crosslinked with glutaraldehyde (**without oligolysine-PEG5K**) incubated with 0.1 U/uL of DNase I for 1 hour at 37°C

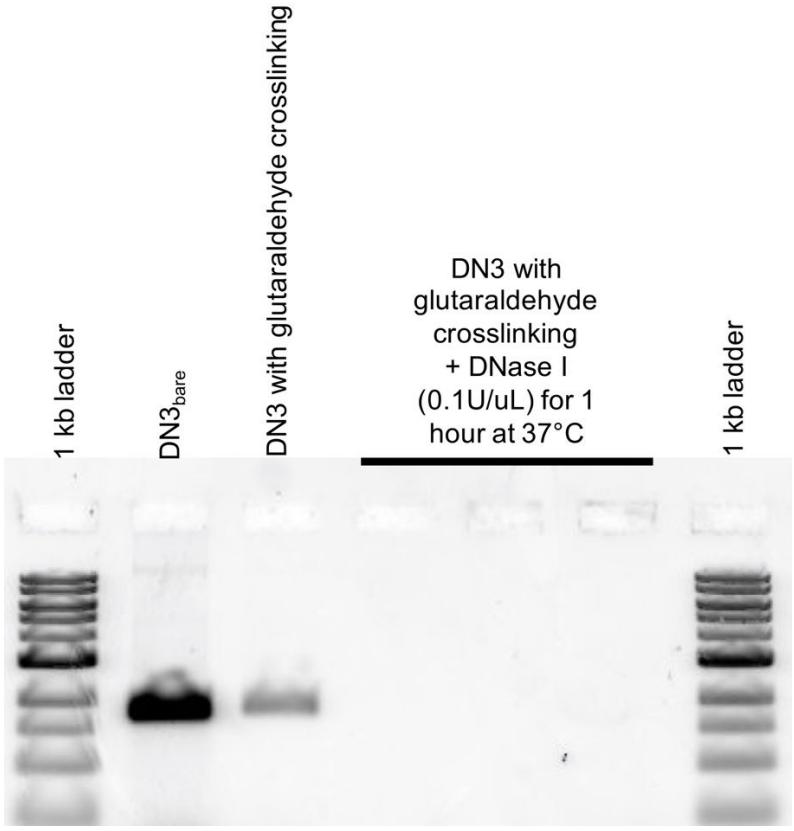
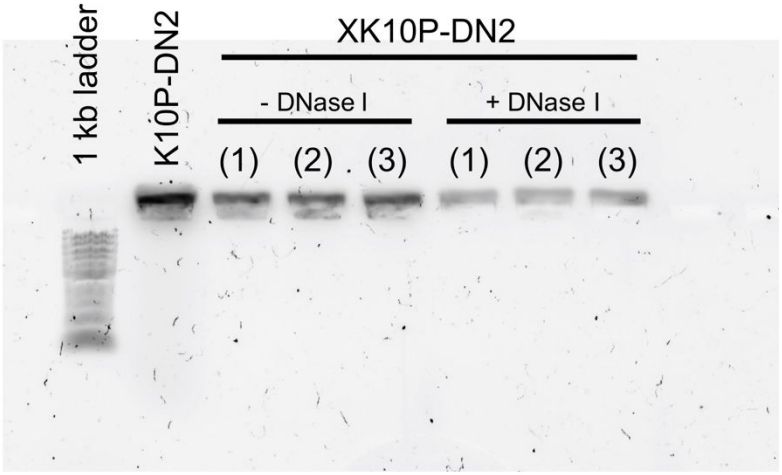


Figure S12 | AGE showing DN2 crosslinked with aged vs. freshly-opened glutaraldehyde solution show comparable resistance to nuclease degradation and staple strand dissociation

The effect of crosslinking using differently aged glutaraldehyde formulations on stability against nuclease degradation and low salt denaturation was studied.
We investigated use of glutaraldehyde that had been opened 18 months prior and was stored in a hood; freshly opened glutaraldehyde, and freshly opened glutaraldehyde that had previously been stored at -20°C.
Coating of DN2 with oligolysine-PEG5K and then subsequent crosslinking reaction was performed as described in the Methods section. Each version of XK10P-DN2 was then subjected to 12 hours of incubation with 1 U/uL of DNase I at 37°C. 5x oligolysine-PEG5K was then added to each sample prior to running on a 1 % agarose gel at 65V over 2 hours at room temperature.

These results show comparable nuclease resistance and thus staple strand dissociation afforded through crosslinking using each of the glutaraldehyde conditions tested.



(1) X – glutaraldehyde opened 18 months prior

(2) X – freshly opened glutaraldehyde

(3) X – freshly opened glutaraldehyde stored in -20℃

Figure S13 | AGE showing that DNase I incubated for 14 days with crosslinked DNs is still able to digest uncoated DN.

We first incubated DNase I with crosslinked DN2 and DN3 over 14 days at 37°C (1 U/uL). This DNase I was then incubated with 100 femtomoles of bare DN2 over 1 hour at 37°C such that the final DNase I concentration is 0.5 U/uL. We observe rapid degradation of the bare DN2, indicating that glutaraldehyde does not poison DNase I activity. Samples were loaded onto a Sybrsafe stained 2% agarose gel and run in 0.5x TBE buffer with 10 mM $MgCl_2$ at 70 V for 2 hours.

Lanes are denoted 1-9 (left-right)

Lanes 1-3: 0.5 U/uL of DNase I is able to digest all 100 fmoles of $DN2_{bare}$ within 1 hour.
Lanes 5-6: 1 U/uL of DNase I is incubated with $DN3_{crosslinked}$ over 14 days at 37°C. This is then added to a solution of 100 fmoles of $DN2_{bare}$ such that the final DNase I concentration is 0.5 U/uL. We show that after this incubation with $DN3_{crosslinked}$, the DNase I is still able to full digest $DN2_{bare}$ within 1 hour indicating limited (if any) poisoning of DNase I activity. As expected faint residue band of $DN3_{crosslinked}$ is visible.
Lanes 7-8: 1 U/uL of DNase I is incubated with $DN2_{crosslinked}$ over 14 days at 37°C. This is then added to a solution of 100 fmoles of $DN2_{bare}$ such that the final DNase I concentration is 0.5 U/uL. We show that after this incubation with $DN2_{crosslinked}$, the DNase I is still able to full digest $DN2_{bare}$ within 1 hour indicating limited (if any) poisoning of DNase I activity. As expected faint residue band of $DN2_{crosslinked}$ is visible.
Lane 9: Control band of 100 fmoles of $DN2_{bare}$ (without incubation with DNase I).

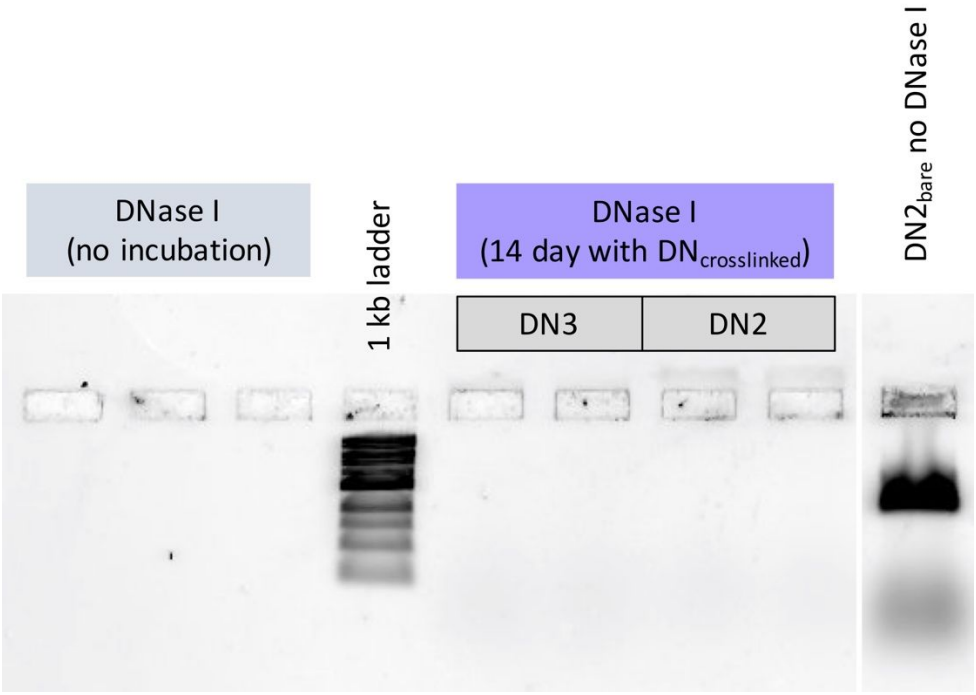
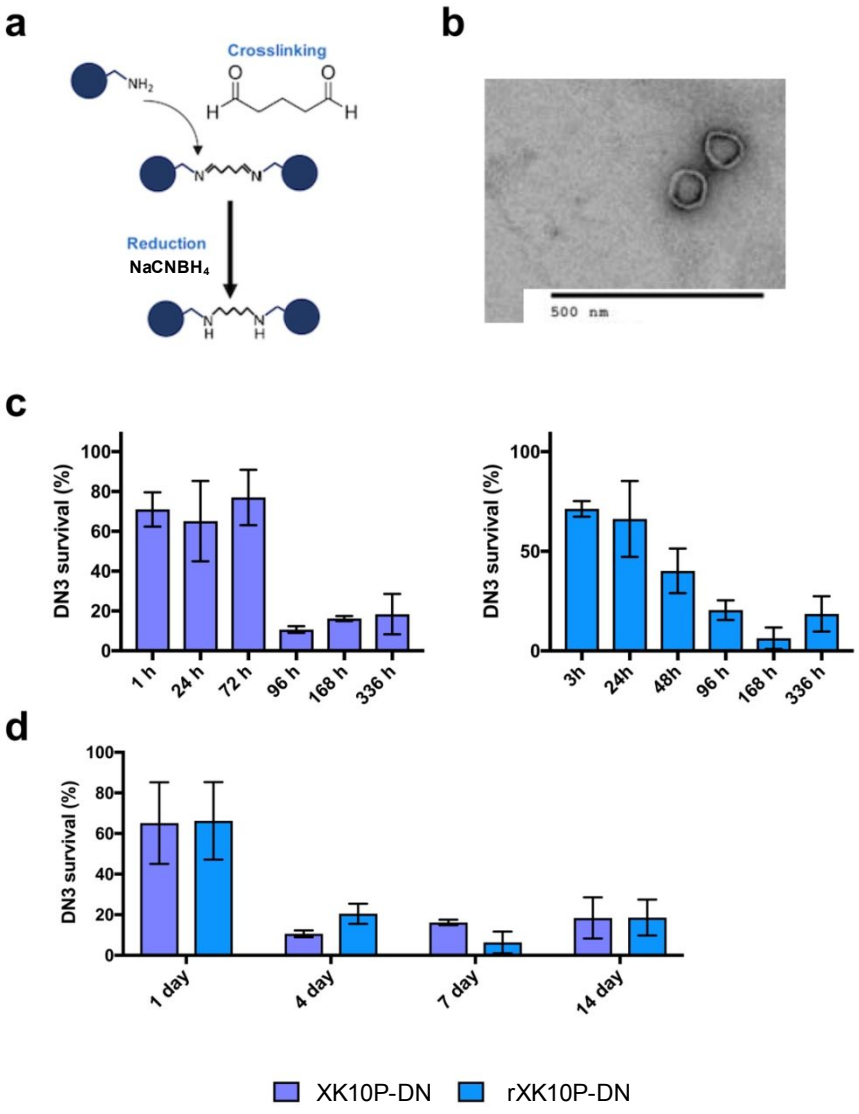


Figure S14 | Reduction using sodium cyanoborohydride did not improve nuclease resistance.

In these crosslinking reactions, glutaraldehyde forms imines bonds with the primary amines. These imine bonds decrease the off-rate of the oligolysine-PEG5K coating, but can act as a Schiff base and are thus are reversible. We therefore hypothesized that reduction of these bonds (to irreversible amine bonds), would further decrease the oligolysine-PEG5K off-rate and thus improve nuclease resistance. However, in our tests we saw no meaningful improvement in nuclease resistance over crosslinking alone.
XKP10-DN3 that are subsequently reduced are referred to as rXRKP10-DN3.



(a) Schematic showing reduction of imine bonds by reducing agent sodium cyanoborohydride to form secondary amine bonds **(b)** transmission electron micrography of rXK10P-DN3 confirming no structural deformities caused by reduction process **(c)** AGE-based analysis to compare survival of XK10P-DN3 (left) and rXK10P-DN3 when incubated with DNase I (1.0 U/uL) over 14 days. *n*>3, error bars are ±s.d. **(d)** side-by-side comparison of XK10P-DN3 and rXK10P-DN3 at 1 day, 4 day, 7 day and 14 day. This shows limited difference between degradation rates.

Figure S15 | AGE showing that DN1can be loaded cargo after coating with oligolysine-PEG5K and crosslinking with glutaraldehyde.

Here, we are interested in testing the availability of handles on DN1 before and after coating with oligolysine-PEG5K and crosslinking with glutaraldehyde. We designed DN1 to have 20 handles – i.e. attachment sites for antihandles. Our antihandles each carry a Cy3 dye.
To load our nanostructures with antihandle-Cy3, we incubated 10x excess antihandle-Cy3 with respective DNs and incubate over 2 hours at 37°C. Samples were loaded onto a 2% agarose gel and run in 0.5x TBE buffer with 10 mM $MgCl_2$ at 70 V for 2 hours.

Lane 1: DN1 loaded with antihandle-Cy3. This shows a typical DN1 band in the Cy3 channel.
Lane 2: DN1 loaded with antihandle-Cy3 and then coated with oligolysine-PEG5K and crosslinked with glutaraldehyde. This shows a typical DN1 crosslinked band in the Cy3 channel.
Lane 3: DN1 **without** antihandle-Cy3. As expected, no band is visible in the Cy3 channel.
Lane 4: DN1 coated with oligolysine-PEG5K and crosslinked with glutaraldehyde, and then loaded with antihandle-Cy3. Here, we observe an intense band in the Cy3 channel, suggesting that antihandle-Cy3 is now loaded onto DN1.

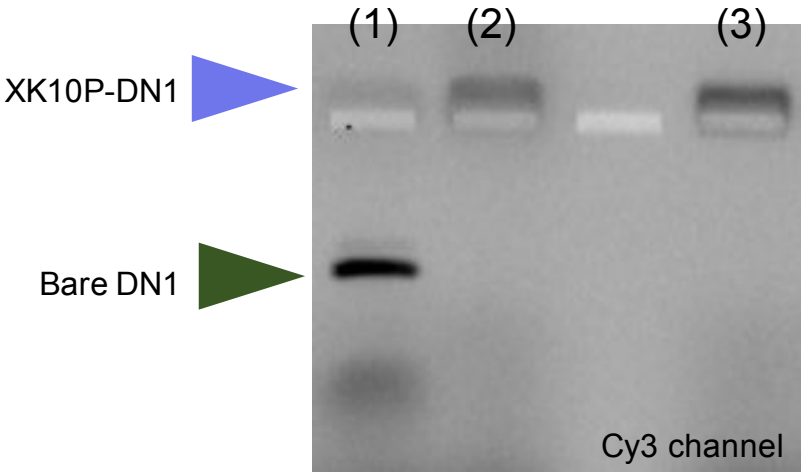


Figure S16 | Flow cytometry of HEK293 cells incubated DN3 bare, coated or crosslinked over 8 hours. Dead cells are stained with Propidium Iodide and can be excluded using flow cytometry. Our findings show our system to be non-toxic: we observe no significant difference in cell viability compared with addition of vehicle only. Error bars are s.d.; n=3.

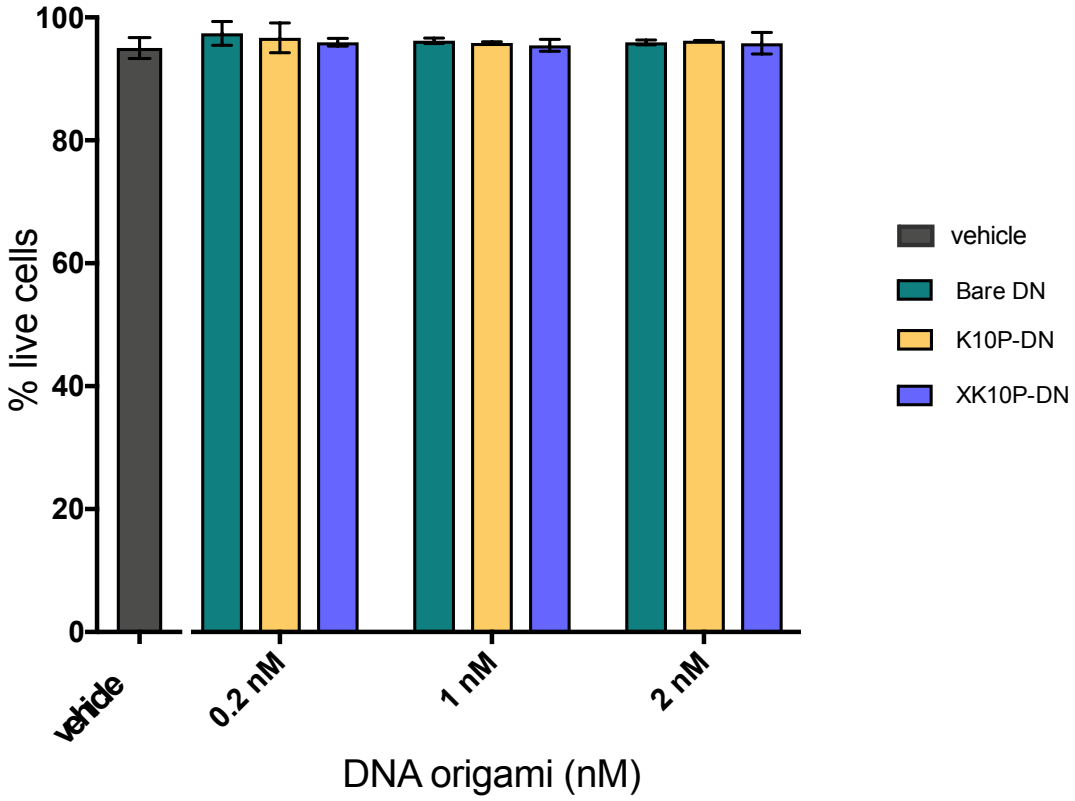
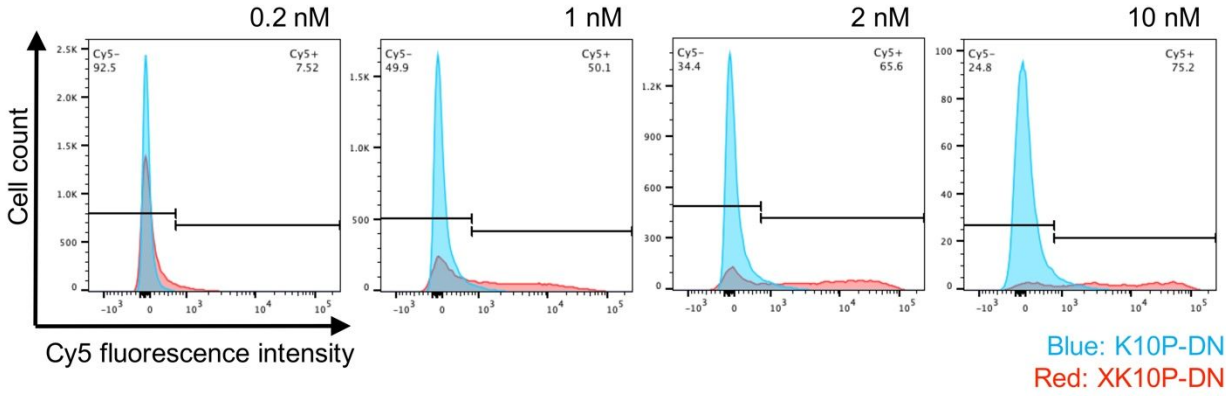
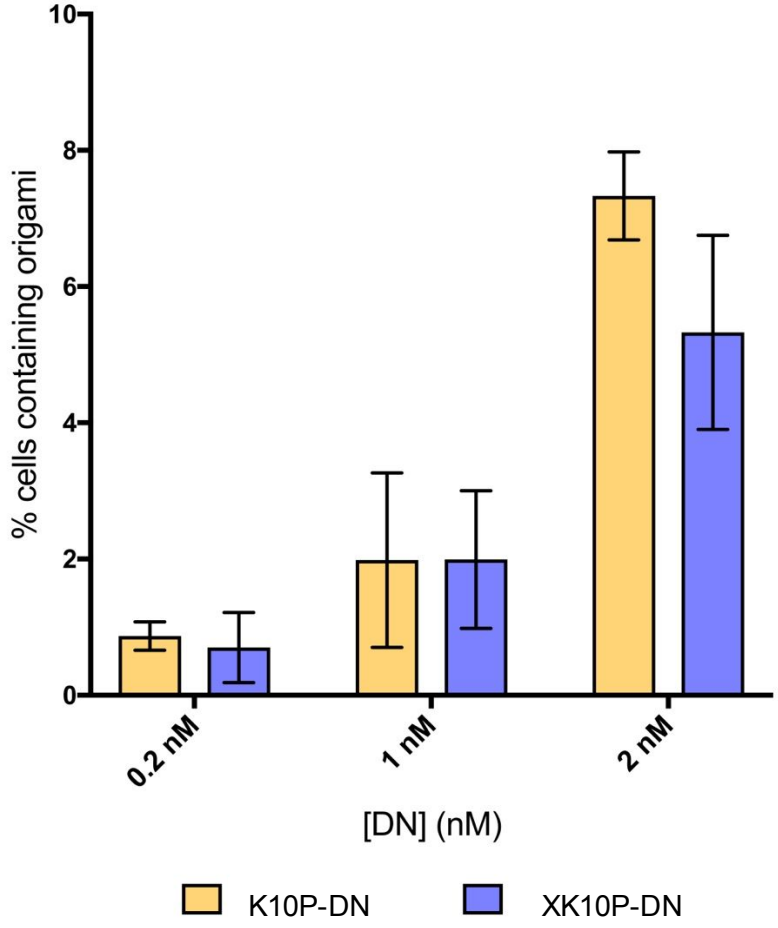


Figure S17 | Flow cytometry histograms of HEK293T cells incubated DN3 coated or crosslinked over 24 hours. DN3 is labelled with 18 Cy5 dyes which allow for monitoring of DN presence within the cells. Here, we observe a dose-dependent rightward shift of XK10P-DN area and an increased mean fluorescence with increasing concentration.



XK10P-DN	0.2 nM	1 nM	2 nM	10 nM
Cy5+ cells	7.52%	50.1%	65.6%	75.2%

Figure S18 | Flow cytometry of HEK293T cells incubated DN3 coated or crosslinked over 8 hours. DN3 is labelled with 18 Cy5 dyes which allow for monitoring of DN presence within the cells. At 8 hours, we do not observe a statistically significant difference between uptake of $DN3_{coated}$ and $DN3_{crosslinked}$. Error bars are s.d. and n=3



DNA Scaffold for DN1 | p8064 sequence

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DNA Scaffold for DN2 | p8634 sequence

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DNA Scaffold for DN3 | p7308 sequence

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DNA Oligonucleotides for DN1

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DNA Oligonucleotides for DN2

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DNA Oligonucleotides for DN3

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