SUPPORTING INFORMATION

Assessing the influence of small structural modifications in simple DNA-based nanostructures on their role as drug nanocarriers

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S1. Protocols, instrumentation and materials

DNS fabrication

1. Dissolve oligonucleotides (purchased from IDT, Macrogen or Biomers (Table S1)) in DNase-free water (IDT) to a concentration of 100 µM. Aliquot tubes and store them at -20 °C.
2. Before use, spin tubes containing oligonucleotides in a Mini-Centrifuge and check the concentration by UV-visible spectroscopy (UV-visible nanodrop spectrophotometer, DeNovix DS-11)
3. Assemble DNS, (a) SST, (b) Y-shaped:
   a. *Assembly of SST DNS*
      Mix appropriate oligonucleotides (Table S1) at equimolar concentrations into 1X PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4, pH ~7.4, ThermoFisher) for 6S or 20 mM $MgCl_2$ and 1X TE buffer for 10S and 14S, to a total volume of 100 µL in a 200 µL PCR tube (Axygen®). Place samples at the thermocycler (T100 Thermal Cycler,Bio-Rad) and run annealing protocol for SST (Figure S1a). Once assembled, keep samples at 4 °C for up to two weeks.
   b. *Assembly of Y-shaped DNS*
      Mix appropriate oligonucleotides (Table S1) at equimolar concentrations into 1XPBS buffer to a total volume of 100 µL in a 200 µL PCR tube. Place samples in the thermocycler and run annealing protocol for Y-shaped (Figure S1b). Once assembled, keep samples at 4 °C for up to two weeks.

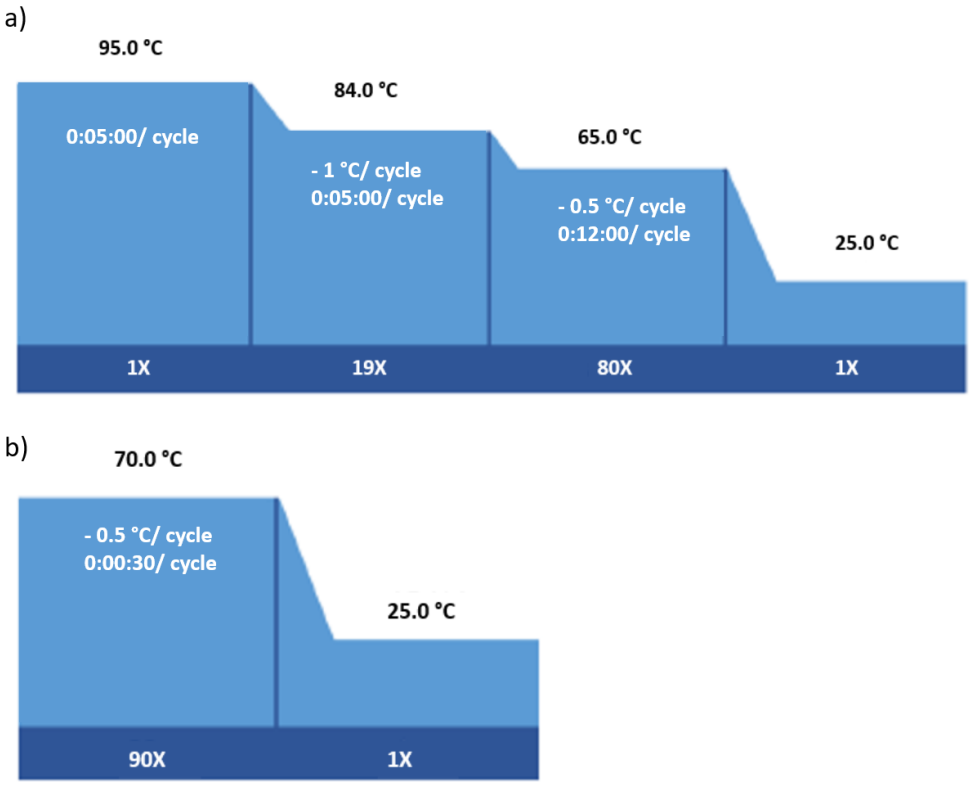


Figure S1. Thermal cycling protocol used for DNS. The initial temperature of each step is shown at the top of the diagrams. At the bottom of the diagram the number of cycles per step is indicated. The temperature drops from cycle to cycle and the time per cycle is shown below the starting temperatures. After finishing the thermal processes at 25 °C, the structures were kept at 4 °C. (a) Protocol for SST series. (b) Protocol for Y-shaped series.

4. For 10S and 14S DNS, dialysis is done to replace $MgCl_2$ and 1X TE buffer with 1X PBS buffer following this protocol:
    I. Place Slide-A-lyzer 2 kDa units (Slide-A-Lyzer™ MINI Dialysis Devices, 2K MWCO, ThermoFisher) in 2 ml tube and add milli-Q H_2O both in the tube and in the dialysis unit to hydrate the dialysis membrane. Wait 30 mins.
    II. Discard H_2O and add PBS both in the tube and dialysis unit to equilibrate membrane.
    III. Discard PBS and add fresh PBS in the tube. Add 100 µL of $MgCl_2$ 1XTE containing sample to dialysis unit.
    IV. Dialyze during 24h at a constant shaking speed of 200 rpm and change PBS buffer twice during that period.
    V. Check DNS concentration after dialysis by UV-visible low-volume spectrophotometry.

DNS characterisation

PAGE characterization

1. Prepare 10% (wt/vol) polyacrylamide gels in 1X TAE, 11 mM $MgCl_2$ (pH=8.3). Use as chemicals and buffers: Acrylamide/bisacrylamide, 30% (Sigma Aldrich); 10 X TAE buffer 400 mM Tris-acetate and 10 mM EDTA (pH=8.3) (ThermoFisher); 10% (wt/vol) Ammonium Persulfate (Sigma Aldrich); N,N,N,N-tetramethylethylenediamine (TEMED; ThermoFisher)
2. Dilute DNS to a concentration around 20- 60 ng/µL and add Loading Dye (Gel Loading Dye, Purple (6X); New England Biolabs).
3. Assemble gel crystals into electrode chamber and add 1X TAE, 11 mM $MgCl_2$ (pH=8.3) running buffer.
4. Load samples: 2 µL 20- 60 ng/µL with 1X loading dye. Load 100 bp DNA Ladder (New England Biolabs) as a reference.
5. Run gel electrophoresis at 100V during 100 mins in a Mini-PROTEAN Tetra Vertical Electrophoresis Cell (Bio-Rad).
6. Transfer the gel into Gel-Red–containing solution (GelRed® Nucleic Acid Gel Stain, Biotium) for nucleic acid staining. 20 mins at a constant 200 rpm shaking.
7. Image gel bands with a GelDoc device (GelDoc EZ Gel Imaging System (Bio-Rad)
8. Analyze and process images with ImageJ.

DLS measurements

1. Prepare DNS samples at a concentration of 2 µM in 1X PBS and load DLS micro cuvette (ZEN0040, Disposable plastic micro cuvette; Malvern) with 100 µL sample.
2. Measure size with Zeta Sizer Nano ZS (Malvern). Settings: 25 °C, 120 s temperature equilibration, 10 measurements of 5 runs for each sample.

Melting temperature study

1. Prepare DNS samples at a concentration of 32 µM bp (500 nM for 0T, 1T and 3T; 400 nM 6S, 200 nM 10S and 150 nM 14S) in PBS solution.
2. Load 100 µL of sample into Far UV Quartz Cuvette Ultra Micro Cells (Hellma™).

3. Measure absorbance in UV-vis spectrophotometer (Varian Cary 100 Bio). Set device to measure at a constant wavelength of 260 nm and a range of temperature from 20 ^{o}C to 95 ^{o}C with an increase of 1 ^{o}C /min. Data is recorded every 0.5 ^{o}C.
4. Analyze results using OriginPro:
    I. Plot absorbance *vs* temperature graph.
    II. Calculate the derivative of the melting curve.
    III. Fit derivative graph with a Gaussian function. Tm is determined by the maximum of the gaussian peak, and the disassembling thermal range is determined by sigma.

DNS stability in nuclease containing solutions

The structural stability of DNS against degradation by nucleases was studied with DNase I and with cell culture media supplemented with fetal bovine serum (FBS). Due to the different stabilities exhibited by DNS, times and nuclease concentrations varies between SST and Y-shaped series.

Stability in FBS containing solutions

1. Mix DNS at a concentration of 40 ng/µL with DMEM (high glucose, pyruvate; Gibco™) containing FBS (non-USA origin, sterile-filtered, suitable for cell culture; Sigma Aldrich) and incubate at 37 °C during different time periods:
    a. SST DNS: Mix DNS with DMEM containing 10% FBS and incubate them during 0, 2, 6, 18 and 24 hours.
    b. Y-shaped DNS: Mix DNS with DMEM containing 50% FBS and incubate them during 0, 2, 4, 8 and 24 hours.
2. Load and run samples in PAGE following steps 1-8 of *PAGE characterization* section.
3. Determine the degree of stability using ImageJ software:
    I. Localize bands which correspond to the full undegraded structure.
    II. Quantify the intensity of the bands corresponding to the full undegraded structure.
    III. Apply the following equation to determine the percentage of undegraded structure:

$$\frac{Intensity\ of\ full\ structure\ band\ at\ time\ x}{Intensity\ of\ full\ structure\ band\ at\ 0h} * 100$$

Stability in DNaseI containing solutions

1. Mix DNS and Dnase I (New England Biolabs) in Dnase I reaction buffer (New England Biolabs). Concentration of DNS constant to 40 ng/µL. Concentration of Dnase I varies from different samples:
    a. SST DNS: 20, 10 5, 2.5, 1 and 0.5 units of enzyme by ml (U/ml)
    b. Y-shaped DNS: 50, 20, 10, 5, 2.5 and 1 U/ml.
2. Incubate samples 1h at 37 °C.
3. Load and run samples on PAGE following steps 1-8 of *PAGE characterization* section.
4. Determine the degree of stability using ImageJ software like step 3 of *stability in FBS containing solutions* section.

Cell studies

Cell Culture

HeLa (Cervical cancer) and MIA PaCa-2 (Pancreatic cancer) cells were maintained for following cellular studies.

1. Seed cells at a density around 1-5 x 10^5 cells/mL on a cell flask with DMEM (high glucose, pyruvate; Gibco™) supplemented with 10% FBS (non-USA origin, sterile-filtered, suitable for cell culture; Sigma Aldrich) and 1% penicillin/streptomycin (Gibco™).
2. Incubate cells at 37 °C with 5% CO_2 in a humidified incubator.
3. Before reaching stationary phase, proceed with a cell passing:
    I. Discard cell medium.
    II. Wash cells with pre warmed PBS (Dulbecco's PBS for cell culture, Sigma Aldrich).
    III. Dissociate cells from flask surface using trypsin solution from porcine pancreas (Sigma Aldrich) during 5 mins at 37 °C.
    IV. Centrifuge cells at 1200 rpm 5 mins in a 15 mL tube and discard trypsin containing medium.
    V. Take a new cell culture flask and place in it 1-5 * 10^5 resuspended cells/mL in fresh cell medium.

Repeat a maximum number of 30 cycles of cell passing before discard cells due to possible accumulation of spontaneous mutations.

Flow cytometry

1. Seed cells into a 96-well culture plate (TPP®) at a density of 5x10^5 cells/mL and seed 100 µL/well of medium containing cells into a 96-well culture plate instead of a flask.
2. Incubate plate for 24 h at 37 °C with 5% CO_2. Then discard medium and wash twice with PBS.
3. Add 100 µL of fluorescently labelled DNS in complete DMEM medium to each well at a final concentration of 3 µM for the SST DNS and 7.5 µM for the Y-shaped DNS. Add respectively 3 µM and 7.5 µM of S (S1, table S1) as a reference control.
4. Incubate samples for 3h at 37 °C with 5% CO_2. Then remove DNS and wash three times with PBS.
5. Harvest incubated cells with 50 µL trypsin for 5 mins and collect them in 200 µL of non-phenol red DMEM (DMEM, high glucose, HEPES, no phenol red; Gibco™) supplemented with 10% FBS and 1% penicillin/streptomycin. Transfer cells to a 2 mL tube.
6. Determine geometric mean fluorescence intensity of the cells in each tube using a CytoFLEX flow cytometry (Beckman Coulter).
7. Analyze using CytExpert and Kaluza softwares (versions 2.4 and 2.1 respectively; Beckman Coulter).

Confocal microscopy

1. Seed MIA PaCa-2 cells on glass coverslips into 24-well culture plate (TPP®) at a density of 10^5 cells/mL. Seed 500 µL of medium containing cells on glass coverslips (Thor Labs) placed at the bottom of each well into 24-well culture plate.
2. Incubate plate for 24 h at 37 °C with 5% CO_2. Then discard medium and wash glasses in wells twice with PBS.

3. Add 500 µL of fluorescently labelled DNS in complete DMEM medium to corresponding wells at a final concentration of 3 µM.
4. Incubate samples 3h at 37 °C with 5% CO_2. Then remove DNS containing medium and wash wells thrice with PBS.
5. Fix cell in cold 4% (wt/vol) paraformaldehyde solution (avantor™) for 15 min at room temperature.
6. Wash cells twice with PBS and permeabilize them with PBS 0.1% Saponin (w/v) 1% BSA (w/v).
7. Stain the actin with 200 µL of 100 nM Alexa Fluor™ 546 Phalloidin (ThermoFisher) for 1 h at room temperature.
8. After staining, wash samples with PBS-Saponin, followed by PBS and, finally, Milli-Q®
9. Mount coverslips using 20 µL Fluoromont-G™ (ThermoFisher) which contains 0.1 µg/mL DAPI to stain cell nucleus. Let solution act overnight. Seal slides using nail polish to avoid evaporation and keep samples at -20 °C till observation.
10. Obtain images by Confocal Spectral Zeiss LSM 880 Microscope (Leica TCS SP8) with a 63× oil immersion objective using three different lasers 405, 488 y 561 nm to visualise DAPI (cellular nucleus), Atto488 (labelled DNS) and AlexaFluor546 (actin) respectively.
11. Analyze images using Zeiss Zen 3.4 software.

Cell viability study

1. Prepare samples.
    a. High concentrated DNS-encapsulated DOX: Mix DOX 20 µM with 80 ng/µL of DNS in PBS
    b. High concentrated free DOX: DOX 20 µM in PBS
    c. Low concentrated DNS-encapsulated DOX: DOX 2 µM with 8 ng/µL of DNS in PBS
    d. Low concentrated free DOX: DOX 2 µM in PBS
2. Seed cells into a 96-well culture plate at a density of 10^5 cells/mL
3. Incubate plate for 24 h at 37 °C with 5% CO2. Then discard medium and wash twice with PBS.
4. Add 50 µL/well of DMEM containing 20% of PBS and 2% penicillin/streptomycin and mix with 50 µL/well of samples to obtain 100 µL/well of DMEM 0.5X 10%FBS and 1% penicillin/streptomycin with free/encapsulated DOX 1 or 10 µM.
5. Incubate samples for 3h at 37 °C with 5% CO2. Then remove DNS, wash once with PBS and add fresh supplemented DMEM.
6. Incubate samples 24h at 37 °C with 5% CO2, then remove medium and wash once with PBS.
7. Add 100 µL/well of media without phenol red and with 5 mg/mL of Thiazolyl blue formazan (MTT; Sigma Aldrich) solution.
8. Incubate samples 2h at 37 °C with 5% CO2 for MTT reaction. Then remove solution and add 100 µL/well of DMSO.
9. Shake plates for 5 min at constant 200 rpm speed.
10. Measure absorbance at 540 nm with a microplate reader (Multiskan GO, ThermoScientific).
11. Analyze data and measure viability:
    I. Remove baseline absorbance of DMSO from measurements.
    II. Divide absorbance of every sample by absorbance of wells incubated with PBS (positive control) instead of DNS/DOX.
    III. Multiply by 100 to obtain percentage.

DOX encapsulation and release studies

DOX encapsulation by fluorescence measurements

1. Prepare starting doxorubicin (Sigma Aldrich) containing solutions:
    a. DOX solution: 10 µM of DOX in 1X PBS buffer.
    b. DOX with DNS solution: 10 µM of DOX and 100 µM of bps of DNS in 1X PBS buffer.
2. Place 100 µL of DOX solution into a quartz fluorescence microcuvette (Hellma®).
3. Measure fluorescence of DOX solution in a fluorimeter (LS-55 Perkin Elmer). Set excitation wavelength at 487 nm (maximum absorbance of DOX), and the emission spectrum in a range from 520 to 700 nm.
4. Without removing cuvette from fluorimeter, make serial additions of DOX with DNS solution, wait 5 mins after each addition for sample equilibration and measure fluorescence. DOX concentration will remain at 10 µM and DNS concentration will constantly increase to 65 µM of bps.
5. Analyze Data and determine the lowest residual fluorescence in which all DOX has been trapped using OriginPro2016 software:
    I. Plot variation of maximum DOX fluorescence at 595 nm as function of the pb/DOX ratio.
    II. Run a polynomial fit to the graph.
    III. Determine equation of the fitted graph and determine the lowest concentration of DNS needed to reach residual DOX fluorescence, in which all DOX has been trapped.

Isothermal calorimetry study

1. Load 0T or 6S into the sample cell (400 µL) of Auto-iTC200 calorimeter (MicroCal, GE Healthcare) at a concentration of 105 µM of bps in TNaK buffer (600 mM NaCl, 20 mM Tris-HCl and 5 mM KCl).
2. Load DOX into a 150 µL syringe at a concentration of 260 µM.
3. Proceed with isothermal calorimetry study in which 19 injections (2 µL per injection) of the DOX solution are done every 150 s into the sample cell at 25 °C.
4. Analyze data using Origin Microcal LCC ITC software.

DOX release study

1. Prepare a mix of DOX 10 µM and DNS at 40 ng/µL concentration of, in which all DOX is trapped into DNS. Prepare DOX at 10 µM without DNS as a control of 100 % released DOX.
2. Mix DOX samples with nuclease containing solutions (DMEM 10 % FBS or DNase I)
3. Measure DOX fluorescence as described in step 3 of *DOX encapsulation* section after different incubation times:
    a. DMEM 10 % FBS. Incubation times: 0, 4, 8, 24hat 37 °C.
    b. DNase I. 1h 37 °C incubation. DNase I concentrations for SST samples: 20, 10 5, 2.5, 1 and 0.5 U/mL and for Y-shaped samples: 50, 20, 10, 5, 2.5 and 1 U/mL.
4. Analyze the variation of maximum DOX fluorescence at 595 nm and determine % DOX release for every sample:
    I. Subtract residual DOX fluorescence to every sample (sample x – sample DOX with DNS at 0h of incubation in DMEM 10% or with 0 U/mL of DNase I)
    II. Divide fluorescence of the sample at specific incubation time or DNase I concentration by fluorescence of 10 µM solution (without DNS) at the same incubation time or DNase I concentration.
    III. Multiply per 100 to obtain released DOX percentage.

S2. Oligonucleotide sequences

6S was assembled by mixing: S1, S2, S3, S4, S5_6S and S6_6S.

10S was assembled by mixing: S1, S2, S3, S4, S5, S6, S7, S8, S9_10S and S10_10S.

14S was assembled by mixing: S1, S2, S3, S4, S5, S6, S7, S8, S9_14S, S10_14S, S11__14S, S12_14S, S13_14S and S14_14S.

0T was assembled by mixing: T1, T2 and T3.

1T was assembled by mixing: T1_T, T2_T and T3_T

3T was assembled by mixing: T1_TTT, T2_TTT and T3_TTT

S1 was the labelled ssDNA strand used in FACs as reference.

Table S1. Sequences of the oligonucleotide used to prepare DNS. Note that atto488 labelled strands are indicated. *S1 was used in FACS as a control of internalization (S).

Name	Sequence
S1 (S in FACS)*	Atto488/AAAACGCTAAGCCACCTTTAGATCCAAA
S2	AAAGTGCAGAGGCACGAATTCCCTCAAA
S3	GAGGGAATTCGCCCGTCTCGACCGCACGACCTGGCTTAGCGT
S4	GGATCTAAAGGACTTCTATCAAAGACGAGGCTGCCTCTGCAC
S5	GGTCGTGCGGACTGTCGAACACCAACGATGCCTGATAGAAGT
S6	GCCTCGTCTTTGGATCCGAAAGCCATAATATATCGAGACGGG
S7	TATATTATGGCGCGATCATACACAATTTGAGTGTTCGACAGT
S8	GGCATCGTTGGGCACCGAGCCAAAACACTTCTTTCGGATCCA
S5_6S	GGTCGTGCGGAAAAAATGATAGAAGT
S6_6S	GCCTCGTCTTAAAAAATCGAGACGGG
S9_10S	GAAGTGTTTTAAAAAAGTATGATCGC
S10_10S	CTCAAATTGTAAAAAAGGCTCGGTGC
S9_14S	CTCAAATTGTAGTGATCCAGCAATATCTCATGGGCTCGGTGC
S10_14S	GAAGTGTTTTCCGATGCAGCCATCGAAGGTGCGTATGATCGC
S11_14S	GCACCTTCGATCTTTTTTGGCCCTAAGTTGGGCTGGATCACT
S12_14S	CATGAGATATTACCGCAAATACCACTACGTGGGCTGCATCGG
S13_14S	CACGTAGTGGAAAAAAGCCAAAAAAG
S14_14S	CCAACTTAGGAAAAAATATTTGCGGT
T1_0T	GAGGCGCCCAGGCTAGCTACAATTCCAGACGACTCCGCCGAG
T2_0T	Atto488/AACATAGTAGTTAGTCTAGACTGTAGCTAGCCTGGGCGCCTC
T3_0T	CTCGGCGGAGTCGTCTGGAATGTCTAGACTAACTACTATGTT
T1_1T	GAGGCGCCCAGGCTAGCTACATATTCCAGACGACTCCGCCGAG
T2_1T	Atto488/AACATAGTAGTTAGTCTAGACTTGTAGCTAGCCTGGGCGCCTC
T3_1T	CTCGGCGGAGTCGTCTGGAATTGTCTAGACTAACTACTATGTT
T1_3T	GAGGCGCCCAGGCTAGCTACATTTATTCCAGACGACTCCGCCGAG
T2_3T	Atto488/AACATAGTAGTTAGTCTAGACTTTTGTAGCTAGCCTGGGCGCCTC
T3_3T	CTCGGCGGAGTCGTCTGGAATTTTGTCTAGACTAACTACTATGTT

S3. Hydrodynamic diameter values of DNS and DLS data

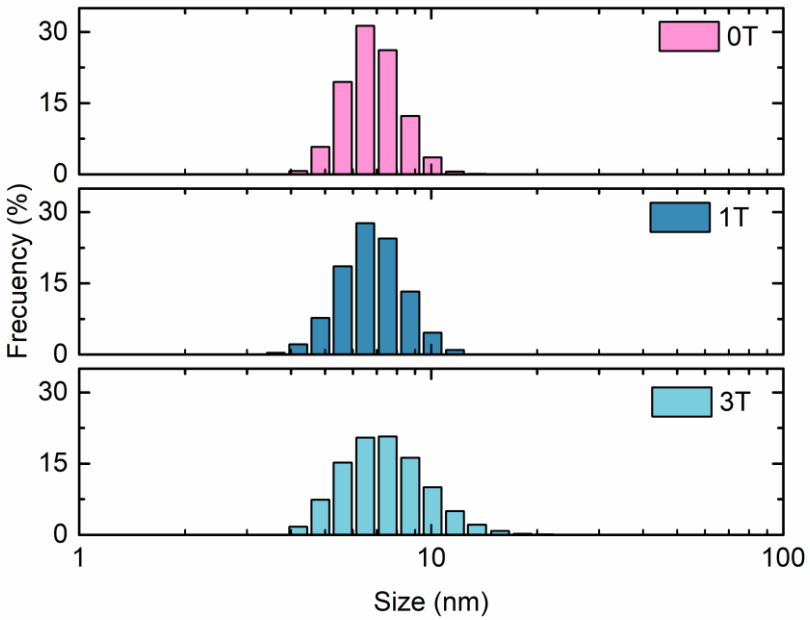


Figure S2. Histograms of the hydrodynamic diameter (Dh) values (in number) obtained by DLS for the Y-shaped DNS series (n=10).

DNS	*PBS*		*PBS/DMEM (50%)*	
	Number	***Intensity***	***Number***	***Intensity***
6S	7.1±1.6	10.1±1.0	8.3±0.5	11.3±0.6
10S	10.9±0.4	16.5±0.8	11.0±0.7	15.4±1.1
14S	14.2±1.5	19.5±2.0	13.1±0.1	24.1±1.0
0T	6.8±1.1	8.0±0.6	7.4±0.9	9.4±0.9
1T	6.5±1.1	8.2±0.5	6.5±1.1	9.6±1.3
3T	7.2±1.9	10.8±2.0	6.6±0.7	9.9±1.1

Table S2. Hydrodynamic values of the different DNS obtained at a concentration of 2 µM, measured either in PBS or in PBS/DMEM (50%). Average and standard deviation of 10 measurements.

S4. DNS thermal stability

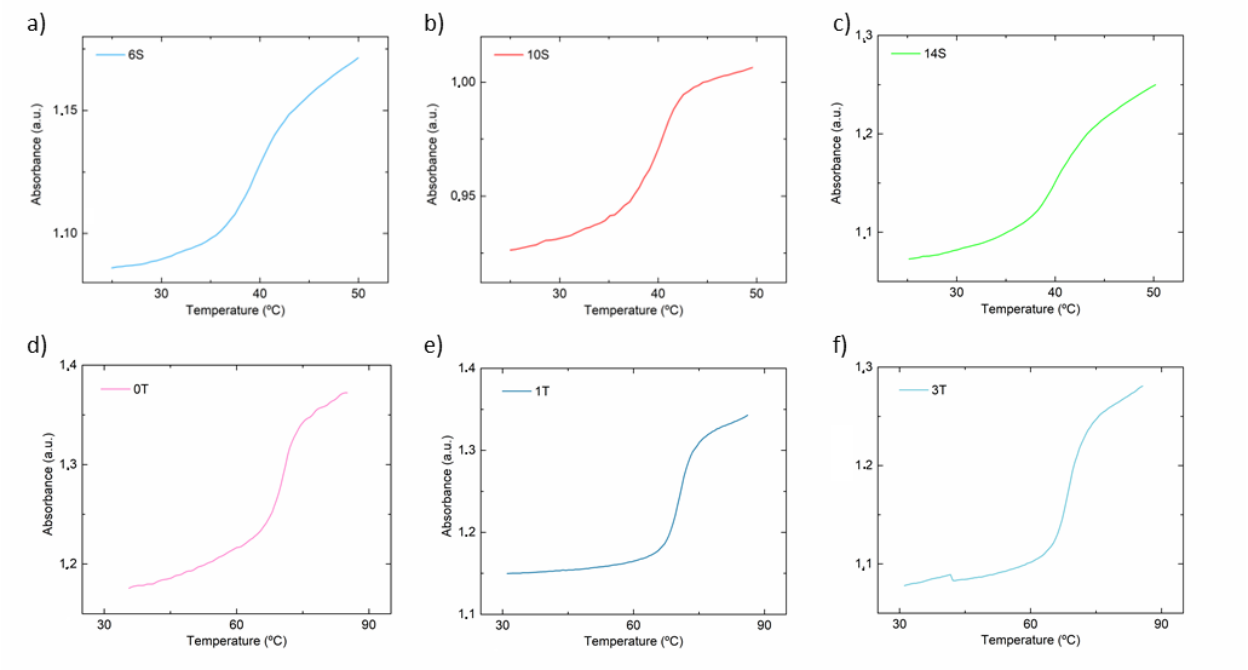
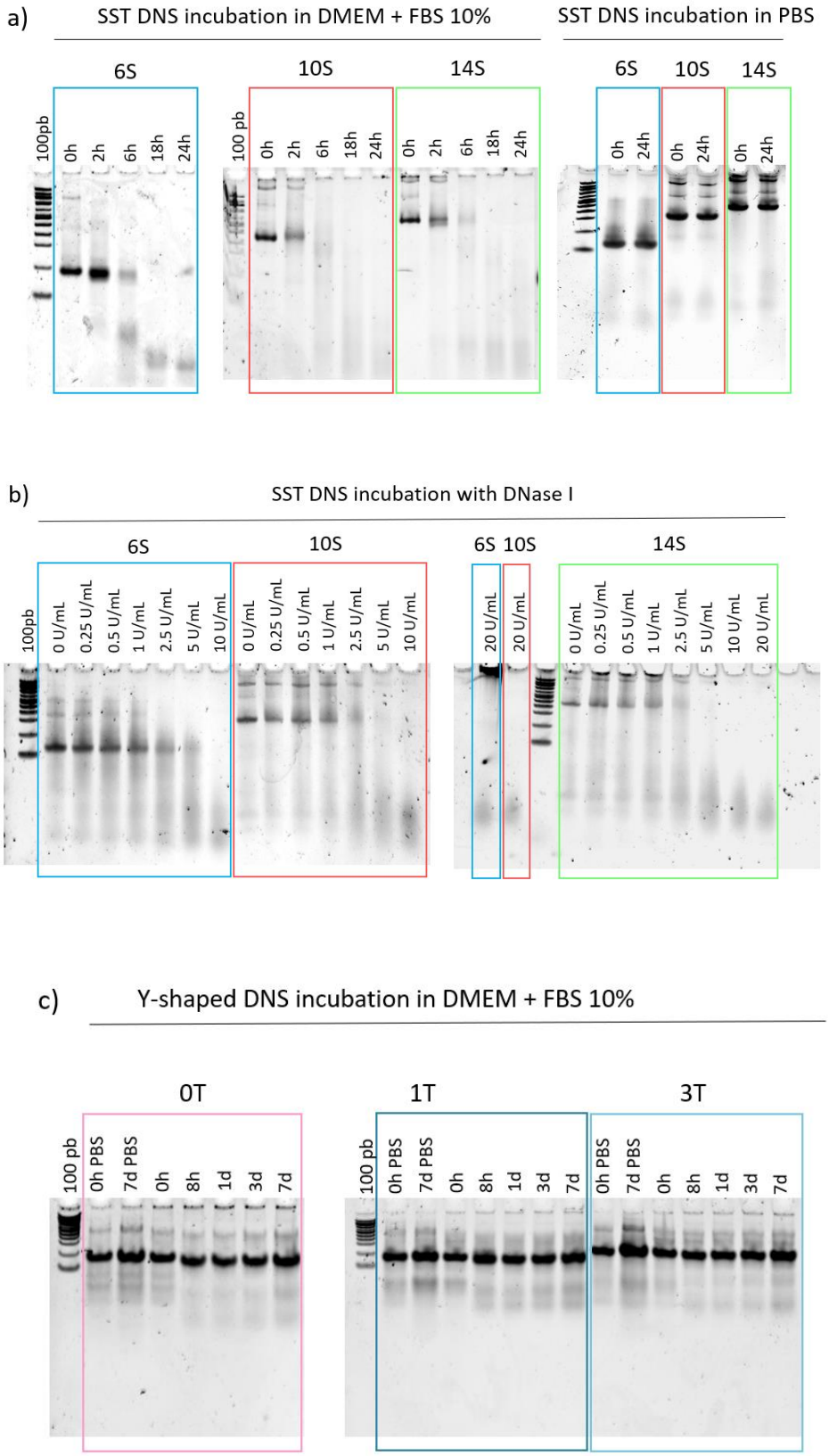


Figure S3. Thermal stability spectra for 32 µM bp DNS: a) 6S, b) 10S, c) 14S, d) 0T, e) 1T and f) 3T. Absorbance at 260nm in relation to the temperature.

DNS	*PBS*		*PBS/DMEM (50%)*	
	Tm	Sigma	Tm	Sigma
6S	40	3	42	2
10S	40	2	41	6
14S	41	3	43	6
0T	70	2	69	2
1T	71	2	71	3
3T	69	3	72	2

Table S3. Tm and sigma values obtained for each DNS at a constant concentration of 32 µM bps. Values were determined either in PBS or in a solution containing PBS and DMEM at 50% to mimic cell culture conditions.

S5. PAGE images of DNS stability in cell culture media and with DNase I



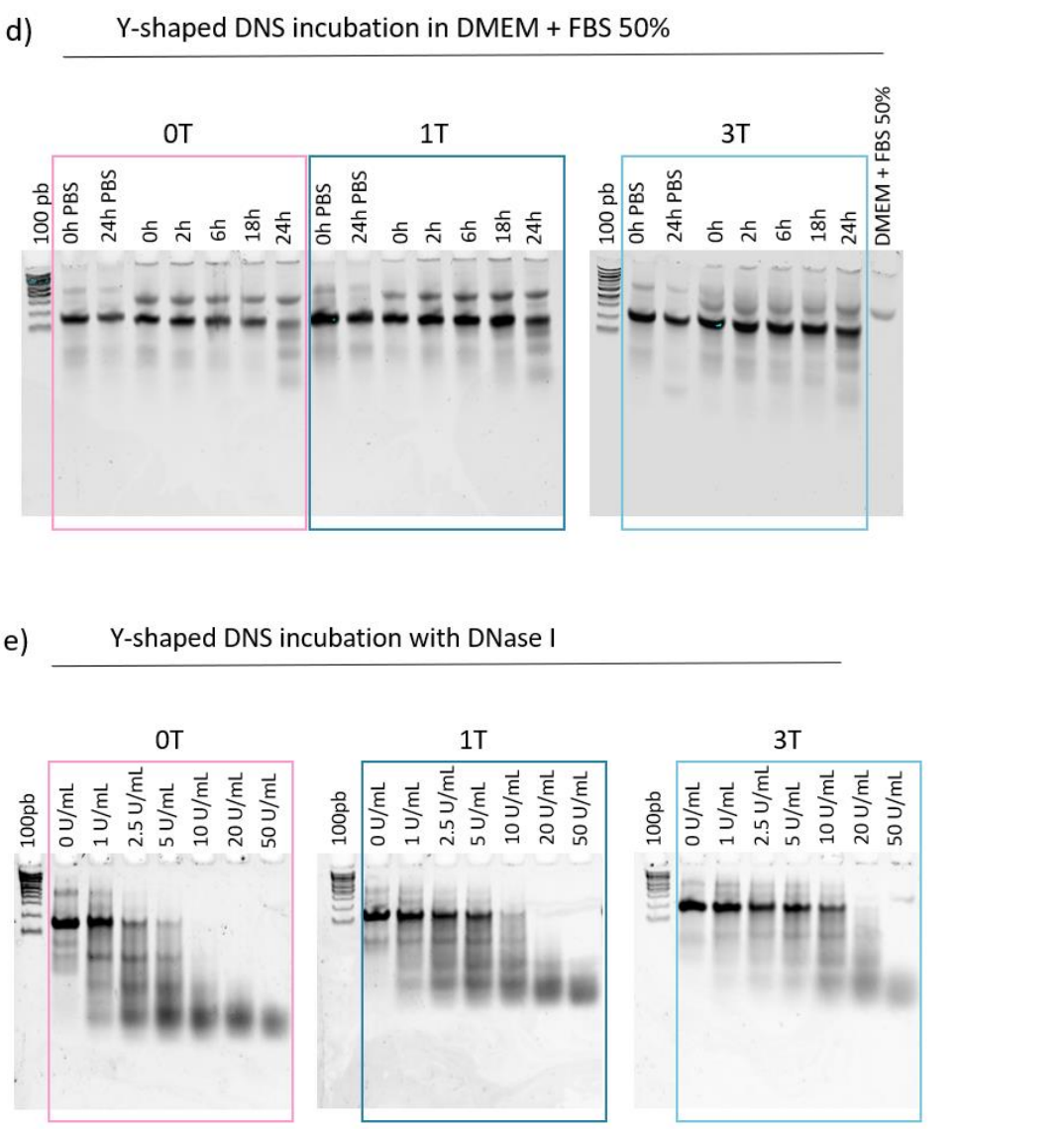
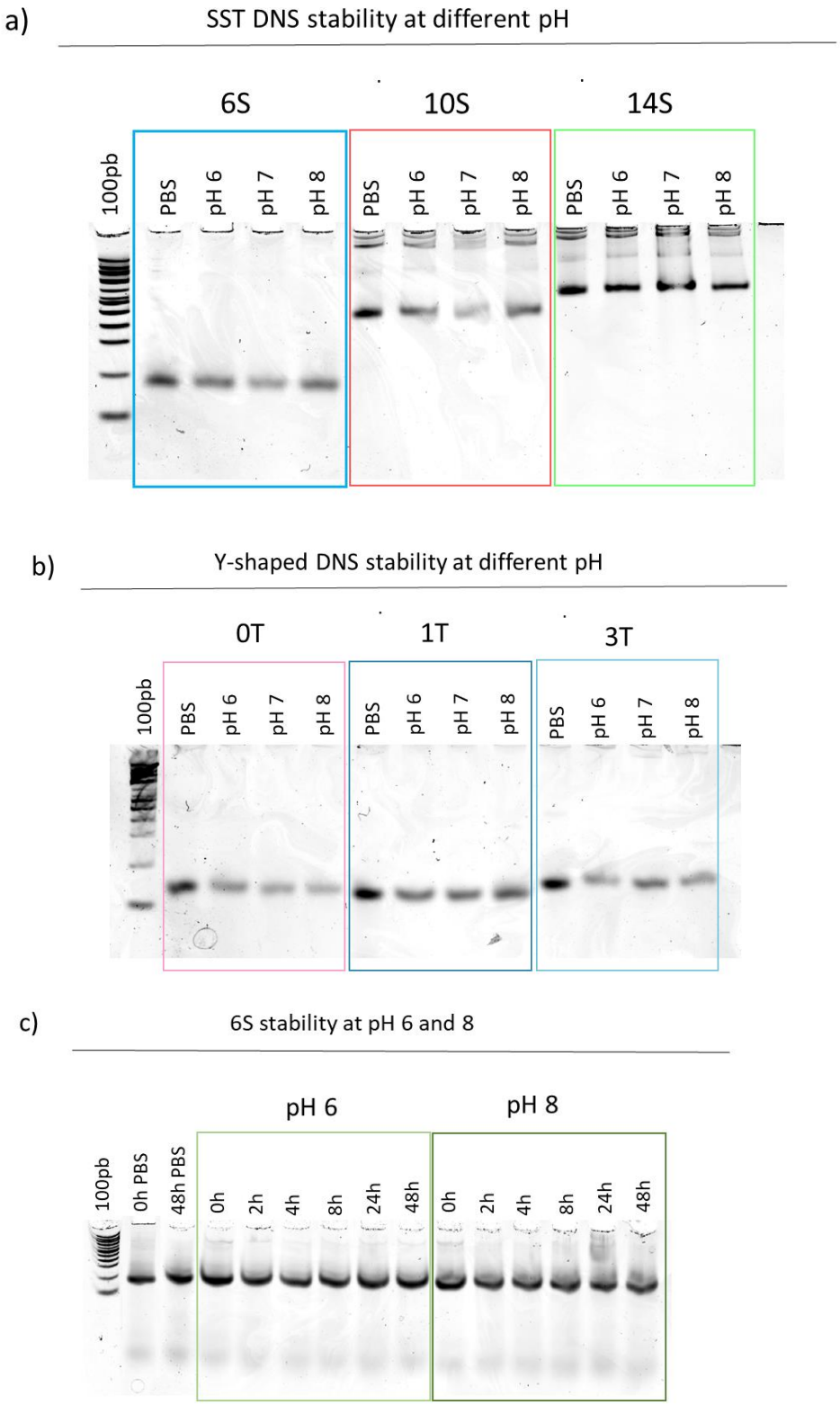
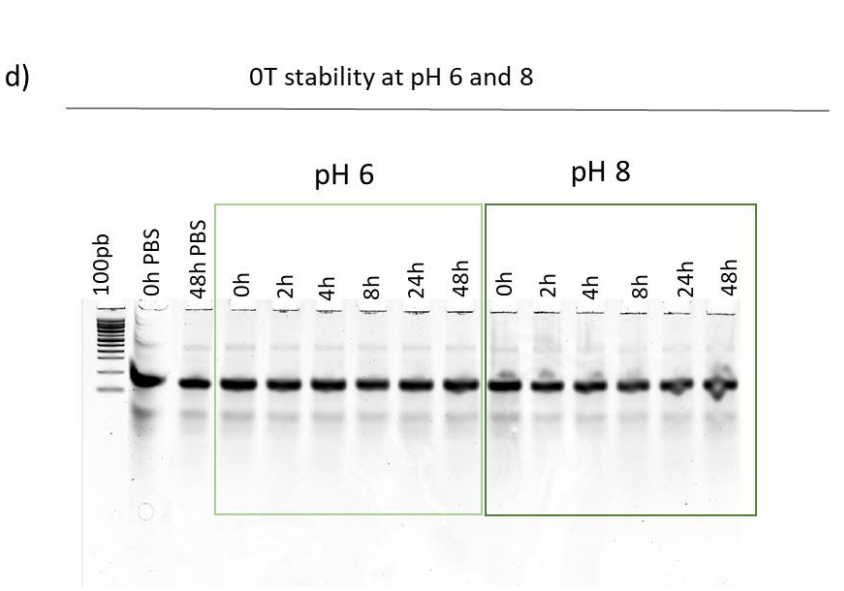


Figure S4. Example of PAGE images of DNS degradation studies conducted at 37 °C. a) SST DNS incubated in DMEM+ 10% FBS during different times: 0h, 2h, 6h, 18h and 24h. 0h PBS and 24h PBS: control samples incubated in PBS with no DMEM+ 10% FBS. b) SST DNS incubated with different concentrations of DNase I: 0 U/mL, 0.25 U/mL, 0.5 U/mL, 1 U/mL, 2.5 U/mL, 5 U/mL, 10 U/mL, 20 U/mL. c) Y-shaped DNS incubated in DMEM+ 10% FBS during different times: 0h, 8h, 1d, 3d and 7d. 0h PBS and 24h PBS: control samples incubated in PBS with no DMEM+ 10% FBS. d) Y-shaped DNS incubated in DMEM+ 50% FBS during different times: 0h, 2h, 6h, 18h and 24h. 0h PBS and 24h PBS: control samples incubated in PBS with no DMEM+ 50% FBS. e) Y-shaped DNS incubated with different concentrations of DNase I: 0 U/mL, 1 U/mL, 2.5 U/mL, 5 U/mL, 10 U/mL, 20 U/mL, 50 U/mL. A 100 bps ladder was run in each gel. A sample of DMEM + FBS 50% was run as evidence of the band that appears when incubated the samples with DMEM + FBS 50%.

S6. DNS stability at pH 6 and pH 8





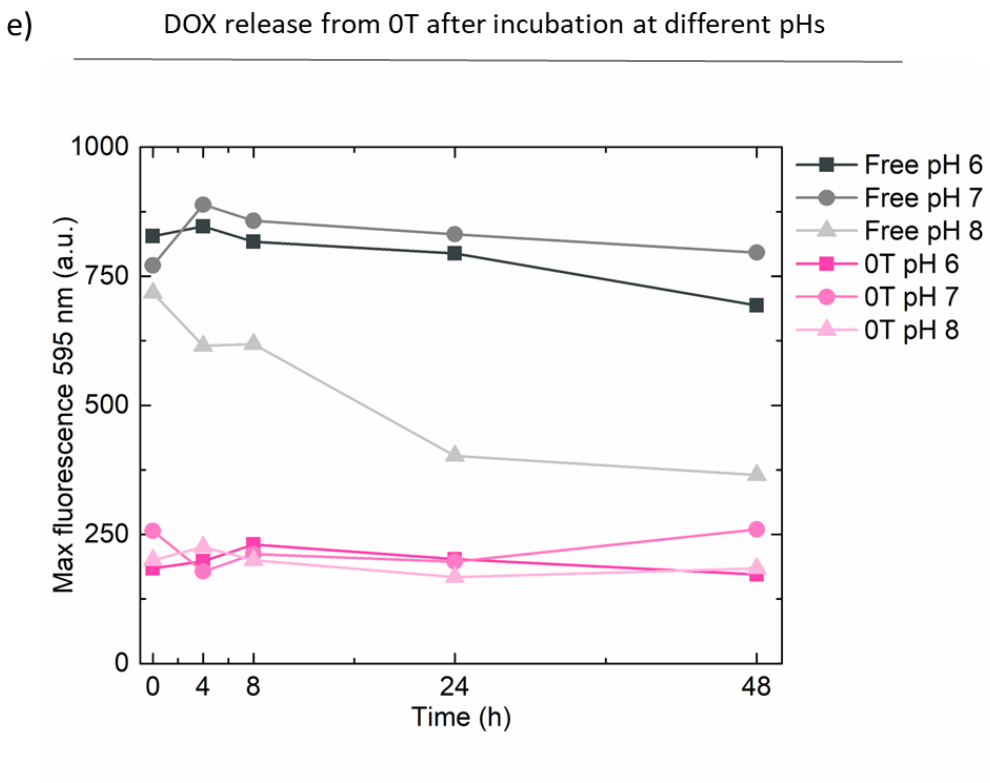


Figure S5. Effect of pH on DNS stability, degradation and DOX release. a) SST DNS assembled at different pHs (6, 7, and 8) in sodium phosphate buffer, and in PBS as control. b) Y-shaped DNS assembled at different pHs (6, 7, and 8) in sodium phosphate buffer, and in PBS as control. c) 6S stability after incubation in sodium phosphate buffer at pH 6 or 8 during distinct times: 0h, 4h, 8h, 24h and 48h. Incubation in PBS during 0 and 48 h as control. d) 0T stability after incubation in sodium phosphate buffer at pH 6 or 8 during distinct times: 0h, 4h, 8h, 24h and 48h. Incubation in PBS during 0 and 48 h as control. e) Free or 0T encapsulated DOX's max fluorescence variation after samples incubation in sodium phosphate at different pH during 0, 4, 8, 24 and 48 h.

S7. Histograms of flow cytometry study of cells incubated with DNS

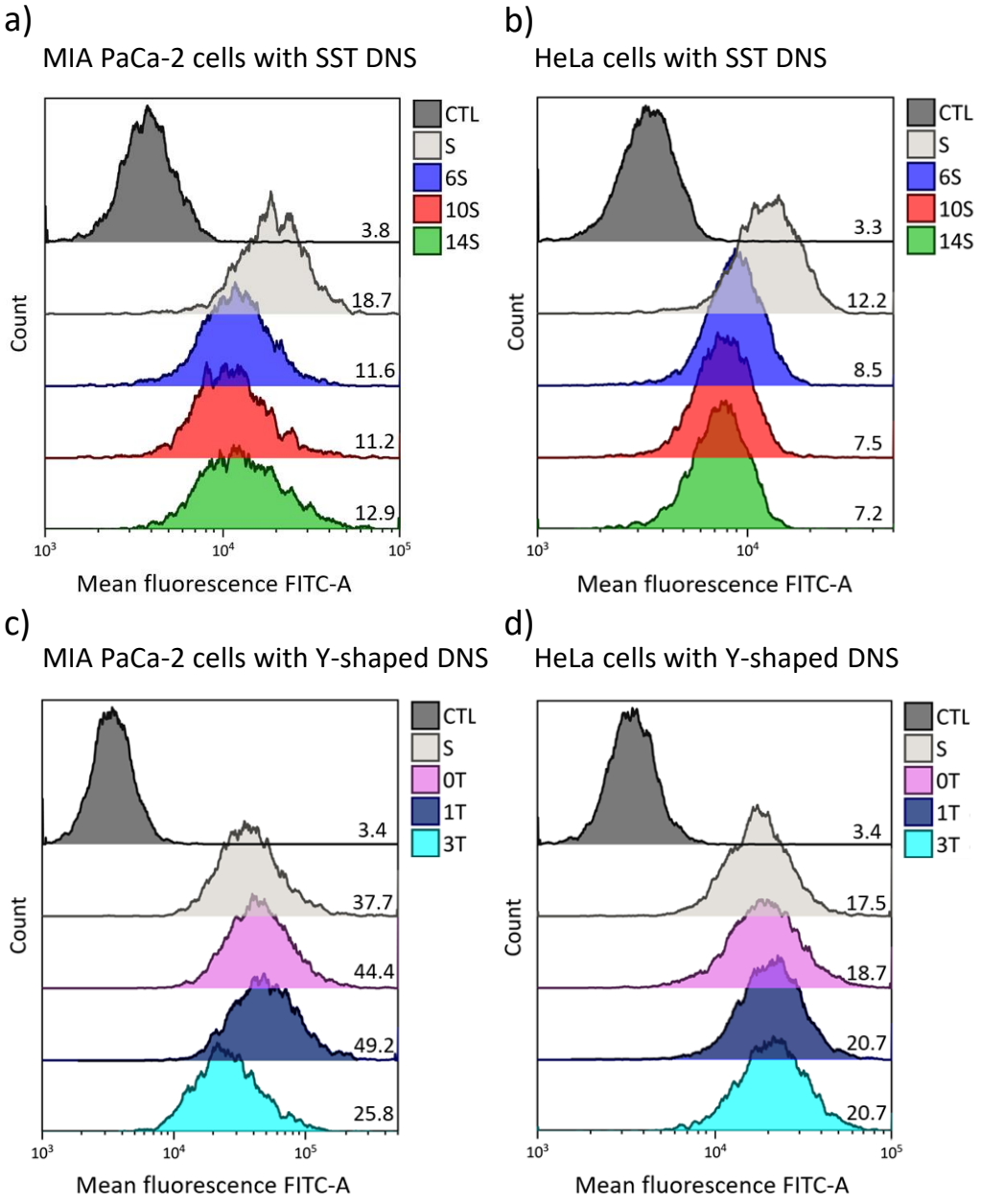


Figure S6. Flow cytometry histograms of Mia PaCa-2 or HeLa cells after incubation with SST or Y-shaped DNS labelled with Atto488. Geometric mean fluorescence intensity of samples (a.u.) divided by factor 10^3 is indicated in each measurement.

S8. Internalization study by flow cytometry and DNase I addition

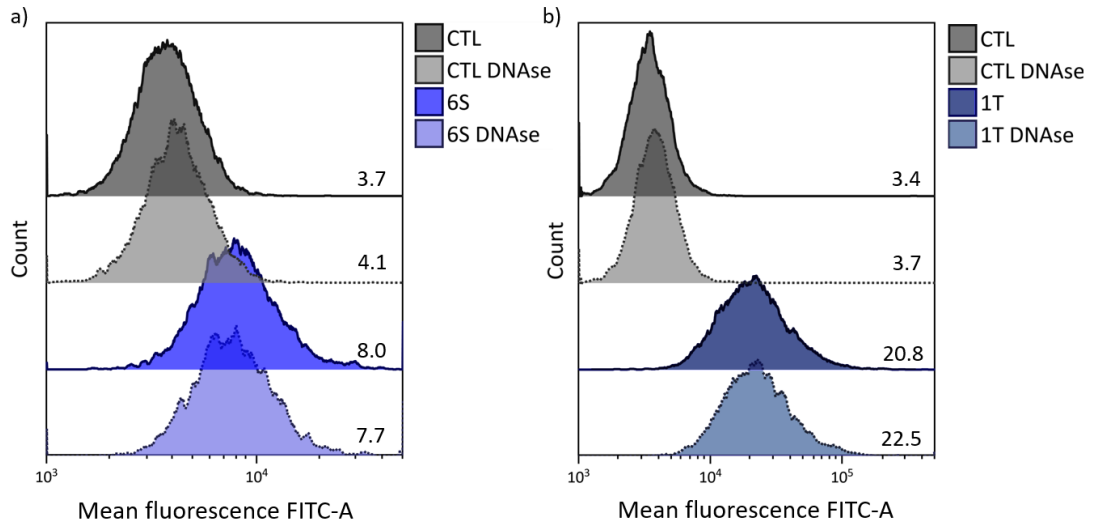


Figure S7. Flow cytometry histograms of internalisation study using DNase I. MIA PaCa-2 cells were incubated with 6S or 1T for 3 h. Samples were subsequently washed with PBS and subjected to nuclease digestion with 50 U/mL DNase I during 1h. Not internalised DNS are supposed to be degraded by DNase I. Finally, samples were harvested and washed three times with PBS. Geometric mean fluorescence intensity (a.u.) of samples divided by a factor of 10^3 is indicated in each measurement.

S9. Confocal images of control sample

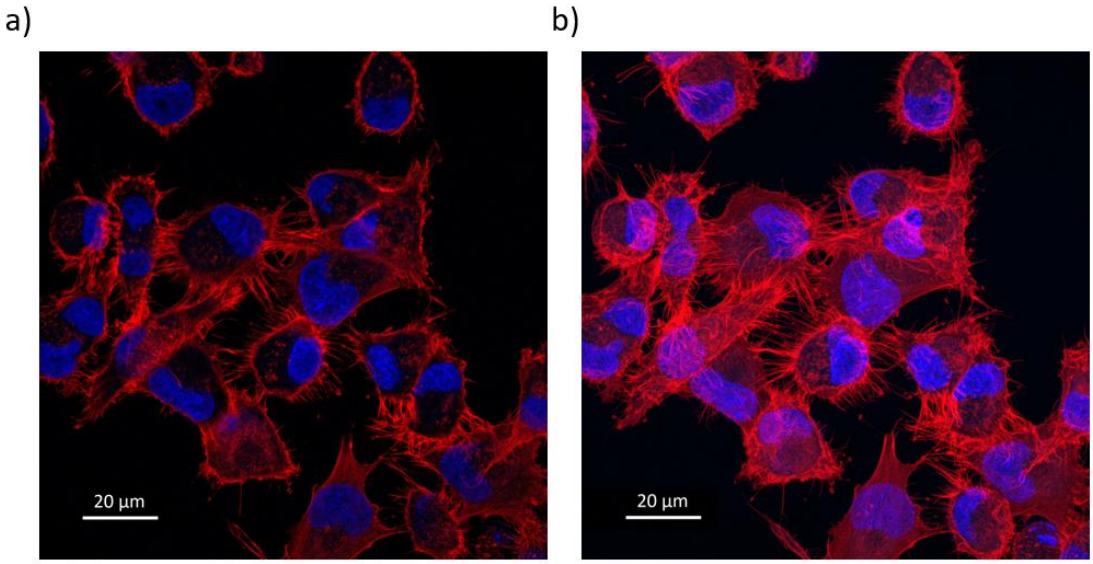


Figure S8. Confocal laser scanning microscopy images of MIA-PaCa-2 cells control, i.e. not incubated with any DNS. a) Image of a single slice. b) Maximum intensity projection of all z images which correlate with the plane of the cell nucleus.

S10. DOX encapsulation by fluorescence

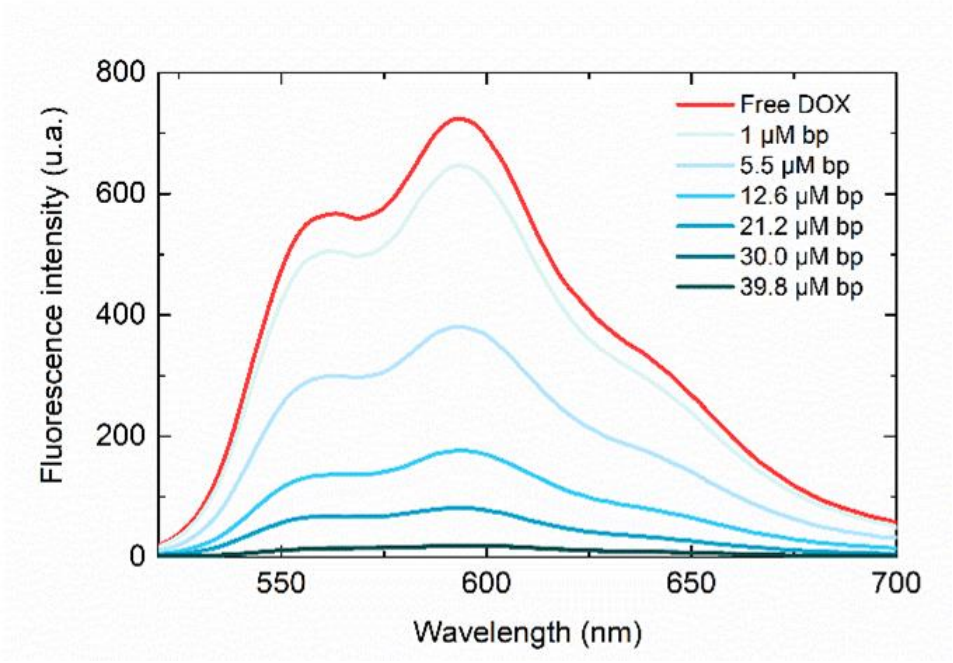


Figure S9. DOX loading and release study. Fluorescent spectra for a constant concentration of 10 µM DOX titrated with 0T.

DNS	bp/DOX
6S	4.0
10S	3.7
14S	4.5
0T	4.3
1T	4.2
3T	3.8

Table S4. bp/DOX obtained value after analysis of titration 10 µM DOX titrated with each DNS.

S11. ITC data and analysis

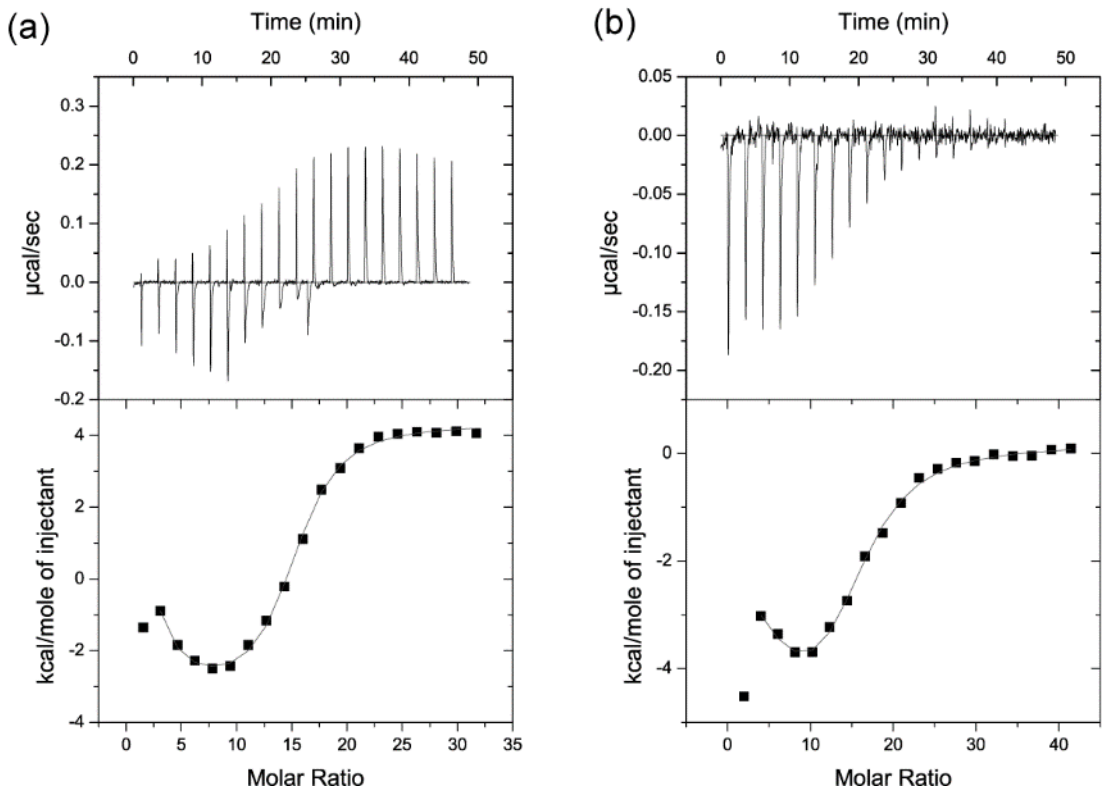


Figure S10. Calorimetric titration for the a) DOX:0T and (b) DOX:6S interactions. A "two-site" model was employed to fit the binding isotherm.[1]

The solutions containing the DNA nanostructures were titrated with increasing amounts of DOX. In analogy to other DOX-DNA titrations,[1] the obtained data (Figure S10) showed that, potentially, two different modes of binding could occur. Indeed, a two-site model has served to fit the resulting binding isotherms. The two different modes of binding depend on the DOX/bp concentration ratio. The titrations, similarly to previous findings,[1] display a minimum at a molar ration around 7-10. The two modes of binding may be attributed to the formation of an intercalative complex, at a lower molar ratio, and an external complex at higher molar ratio. According to the fitted data, when all the binding sites have been occupied, the number of bp per DOX molecule was 4.4 and 4.5 for 0T and 6S respectively.

S12. HeLa cytotoxicity

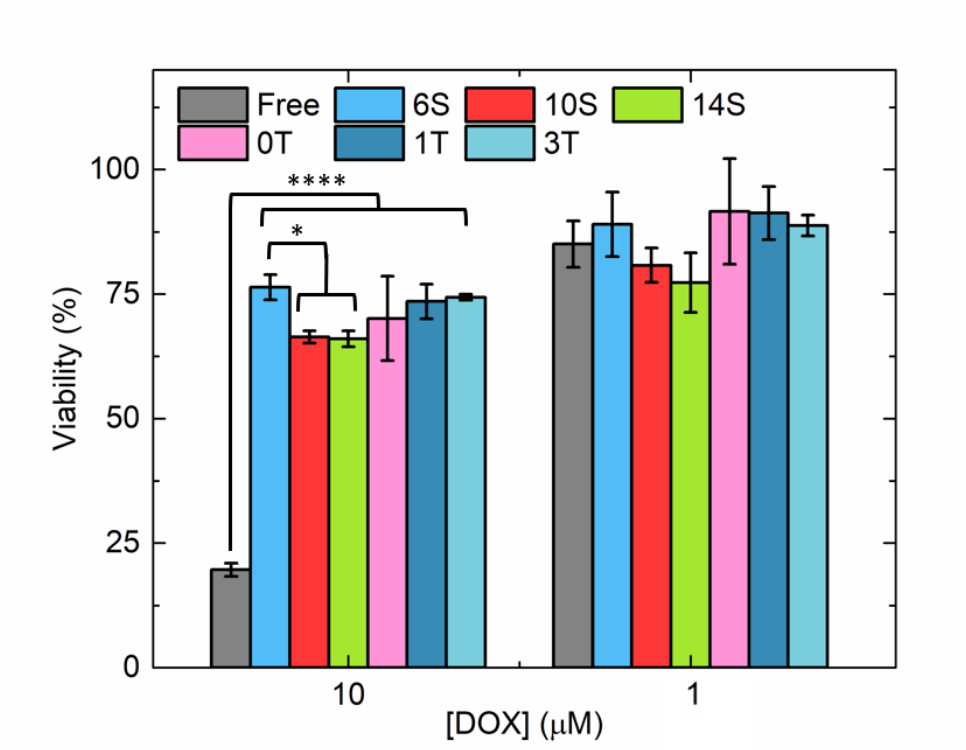


Figure S11. HeLa cells viability analysis by MTT assay for DOX encapsulated in DNS after 3h of incubation with cells, followed by a washing step to eliminate DOX not interacting with cells, and subsequent 24 hours of incubation. Data are the averaged values (n=3) with associated standard P-values calculated using one-way ANOVA, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 comparing every group with each other.

References

1. Pérez- Arnaiz *et al. J. Phys. Chem. B* **2014**, *118*, 1288−1295.