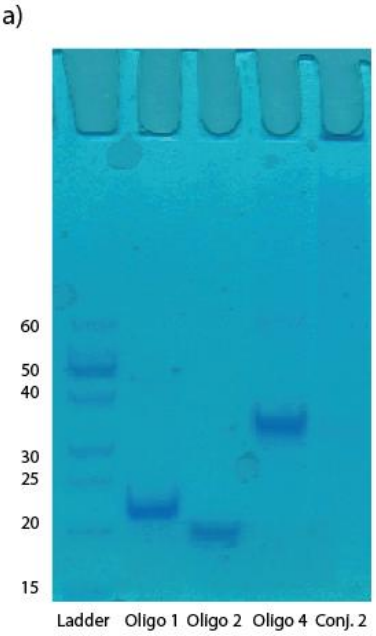
Electronic Supporting Information

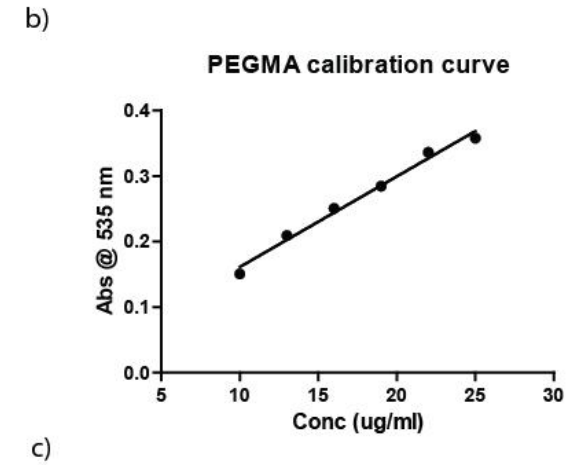
Multi-modal switching in responsive DNA block co-polymer conjugates

Gökçen Yaşayan,[a,b] Johannes P. Magnusson,[a] Giovanna Sicilia,[a] Sebastian G. Spain,[a] Stephanie Allen,[a] Martyn C. Davies, [a] and Cameron Alexander[a]*

[a] *School of Pharmacy, University of Nottingham, Nottingham, NG7 2RD, UK. Tel +44 115 846 7678; Fax +44 115 951 5102; E-mail cameron.alexander@nottingham.ac.uk*
[b] *Marmara Universitesi, Istanbul, Turkey*

Analysis of Conjugate 2:





c)

Sample	Oligonucleotide concentration (ug/mL) [i]	Absorbance at 535 nm [ii]	PEGMA Conc. (µg/mL) [iii]	Ratio of PEGMA to oligonucleotide [iv]	Molecular weight of PEGMA [v]
1	46.7	0.3285	219.66	4.708678756	28902
2	46.7	0.3397	227.28	4.872002392	29904
3	46.7	0.3431	229.59	4.921582781	30209

Figure S1 – a) PAGE analysis of Conjugate 2 (DC2), b) PEGMA calibration curve using a TriEGMA homopolymer (Mn 20000) as a calibrant (response linear between 10µg/mL to 25µg/mL). c) Calculation of PEGMA molecular weight (All samples were diluted 10 times before analysis). i) Oligonucleotide concentration was quantified using OD @ 260 nm, ii) Absorbance of the Conjugate 2 solutions at 535 nm after incubation with Barium/Iodine, iii) PEGMA concentration calculated from absorbance and calibration curve, iv) Calculated by comparing the concentration of polymer to concentration of oligonucleotide, v) Calculated by multiplying the ratio of polymer to oligonucleotide by the molecular weight of the double stranded oligonucleotide and dividing by the number of polymers.

Conjugate 1 PAGE analysis and gel displacement:

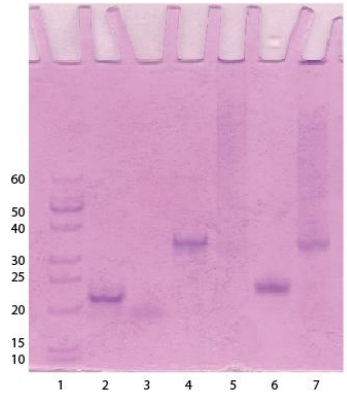


*Figure S2 – Oligonucleotide displacement – **Lane 1**: 10/60 Ladder, **Lane 2**: Oligo 1, **Lane 3**: Oligo 2, **Lane 4**: Initiator 1, **Lane 5**: Conjugate 1 (DC1), **Lane 6**: Oligo 3(complementary), **Lane 7**: Conjugate 1 (DC1) + Oligo 3 (1:1 ratio).*
Lane 5 shows that the initiator functionalised oligonucleotide successfully initiated the polymerisation of PEGMA, the band for the oligonucleotide initiator is no longer apparent and the molecular weight (MW) has increased and broadened. Lane 7 demonstrates that the fully complementary sequence (Lane 6) successfully displaced the partial complementary strand from the polymer structure, e.g. Oligo 3 band does not appear in lane 7. A new band formed at a higher MW, assigned to Oligo3:Oligo 1 + single polymer chain.

Table S1 –DNA Conjugates characteristics:

	Double stranded oligonucleotide with ATRP initiators (g/mol)	Molecular weight of polymer (g/mol)	PDI	Total molecular weight of polymer conjugate (g/mol)	% ODN	% Polymer
DC1	12940	8000	1.19	28940	44.7%	55.3%
DC2	13000	29700	*	72400	18.0%	82.0%

** PDI (Polydisperity index) could not be evaluated for Conjugate 2 (DC2) as attempts to cleave the oligonucleotide cleanly from the polymer were not successful.*

AFM, DLS, and zeta potential studies of DNA Conjugates

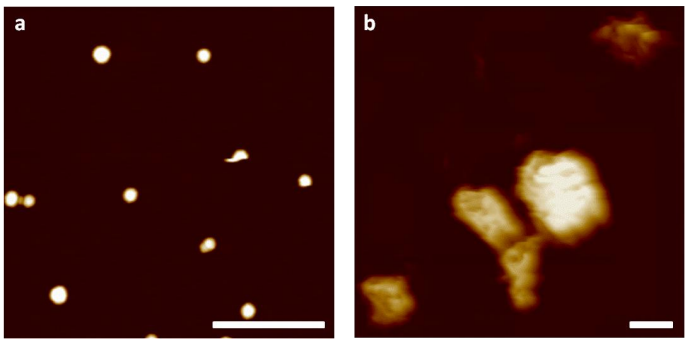


Figure S3 – AFM topography images of in situ (a) and ex situ (b) aggregation studies of Conjugate 1 at 40 °C. (Scales bars: 1 µm for both images. Vertical scales: 80nm for a, 2µm for b.)

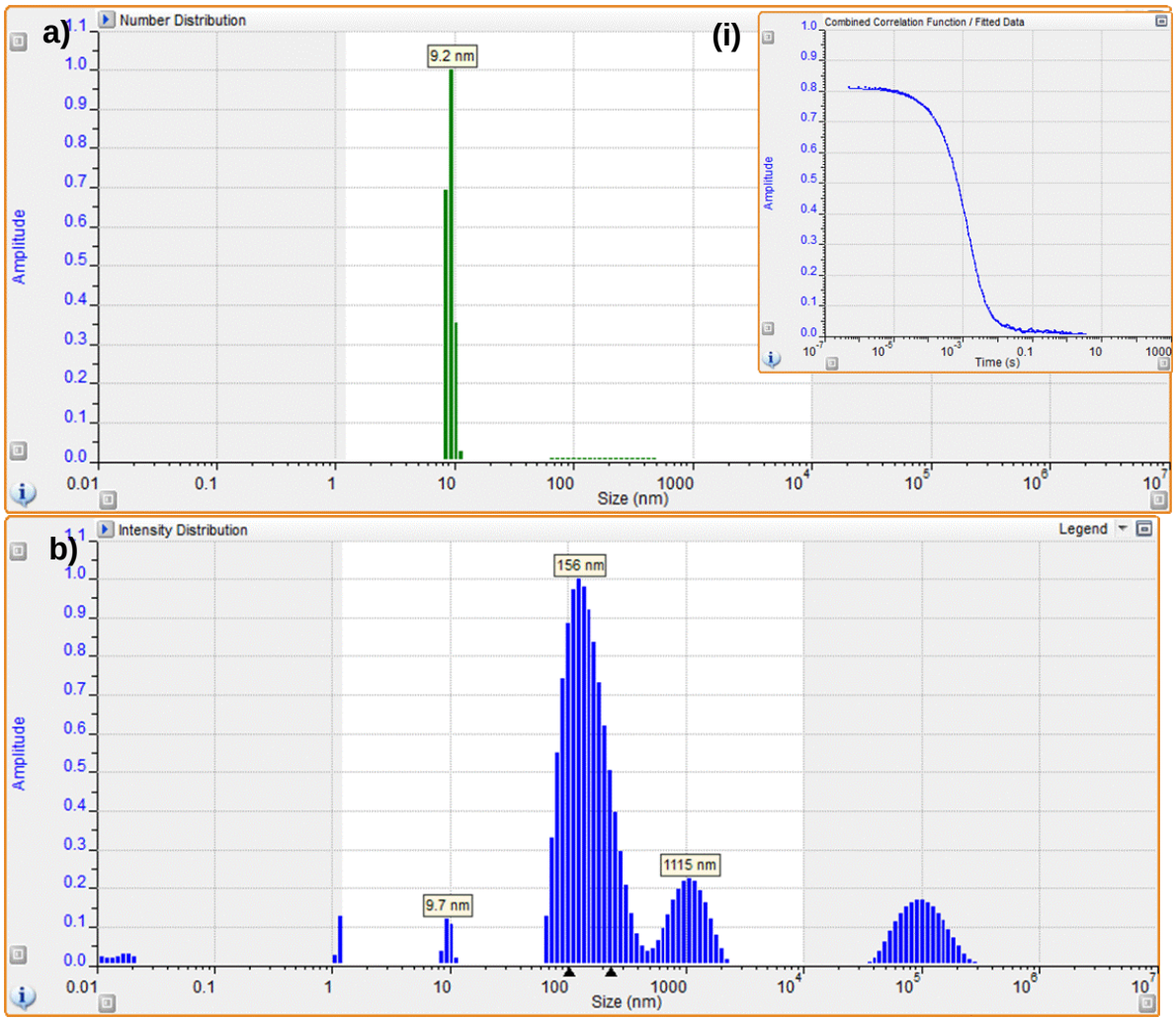


Figure S4 – Representative Dynamic Light Scattering (DLS) studies of Conjugate 1 in DPBS buffer. Instrument readouts show number (a) and intensity (b) distributions of Conjugate 1 at 30 °C. Inset (i) shows the corresponding correlation function from which the number and intensity distributions were obtained.

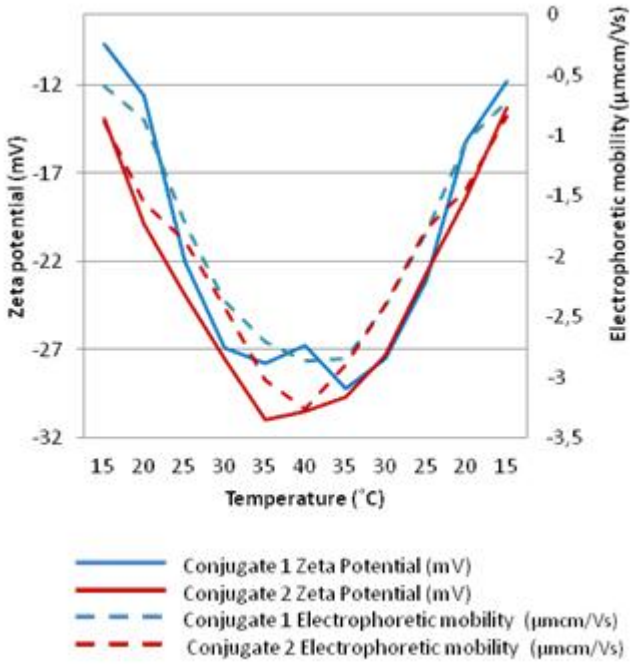


Figure S5 – Zeta potential measurements of Conjugate 1 and Conjugate 2 from 15 - 40 °C and 40 - 15 °C.

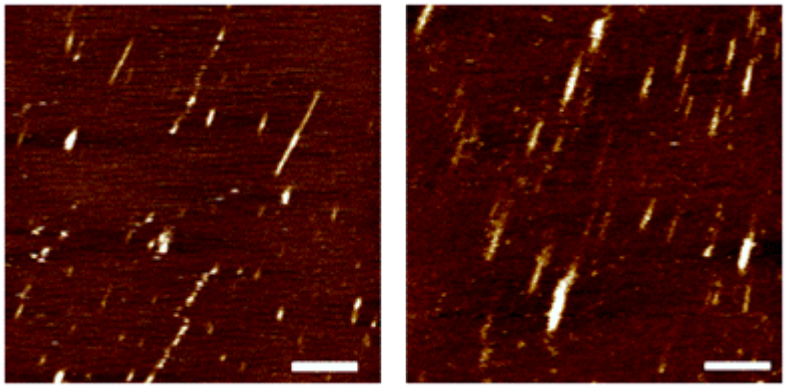


Figure S6 – AFM topography images of Conjugate 2. Samples were heated above polymer phase transition temperature ex situ then inserted in to the AFM sample chamber. Conjugates were poorly resolved in these micrographs due to low attachment of the conjugates to the mica surface (Scale bars: 100 nm for all images, vertical scales: 3nm)

Table S2 –AFM particle analysis for in situ and ex situ aggregation studies. The table shows the number of features counted in the AFM image

	Conjugate 1 /*in situ* studies	Conjugate 1 /*ex situ* studies	Conjugate 2 /*in situ* studies
Below LCST	100	112	100
25 ˚C	109	98	150
30 ˚C	391	129	55
35 ˚C	222	143	119
40 ˚C	17	38	115

Table S3 – AFM measurements of particle heights during in situ and ex situ aggregation studies

AFM Height Measurements (nm)

	Conjugate 1 /*in situ* studies	Conjugate 1 /*ex situ* studies	Conjugate 2 /*in situ* studies
30 °C	1.7 ± 0.6	1.7 ± 0.7	1.6 ± 0.6
35 °C	2.0 ± 1.0	1.6 ± 0.8	1.9 ± 1.3
40 °C	63.8 ± 14.8	2.4 ± 0.7	122 ± 253

Ex situ disaggregation studies

To investigate whether aggregation of the conjugates was reversible, an *ex situ* study was performed with AFM. Particles were heated to 40 ˚C for 15 minutes, and then cooled down below the polymer phase-transition temperature (hereafter approximated to the Lower Critical Solution Temperature or LCST) by using an ice-bath. Afterwards the polymer suspensions were injected onto the mica surface. The sizes of the features by grain analysis were around 7 nm for Conjugate 1 and 11 nm for Conjugate 2, confirming that the aggregation of the conjugates was reversible. DLS and zeta potential measurements were performed under the same conditions, confirming that co-polymer self-association was reversible.

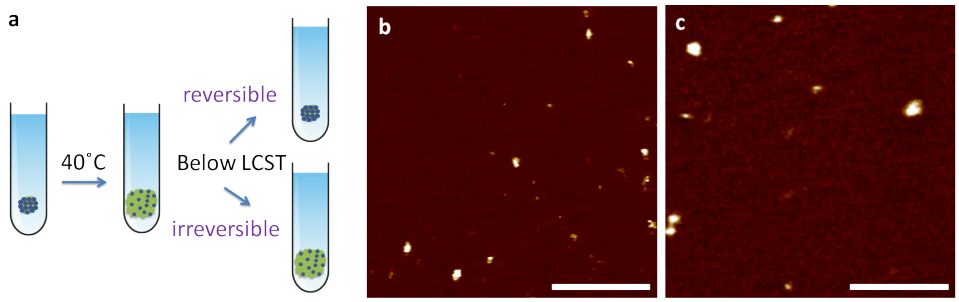


Figure S7 – Cartoon representation of ex situ disaggregation studies (a). AFM topography images of ex situ disaggregation studies of DNA-polymer conjugates below the phase-transition temperature; b, Conjugate 1 and c, Conjugate 2. (Scale bars: 100 nm, Vertical scales: 3nm)

Table S4 –Analysis of features observed in ex situ disaggregation studies, by AFM, DLS (mass and number distribution) and zeta potential measurements of Conjugate 1 and Conjugate 2 below the phase-transition temperature.

	Conjugate 1				Conjugate 2			
Below LCST*	D_{AFM} (nm)[a]	D_h (nm)[b]	D_h (nm)[c]	Zeta potential (mV)	D_{AFM} (nm)[a]	D_h (nm)[b]	D_h (nm)[c]	Zeta potential (mV)
	7 ± 1	2 ± 1	2 ± 1	-14 ± 2	11 ± 4	11 ± 4	10 ± 4	-13 ± 2

[a] Diameter of particles by AFM *ex situ* disaggregation studies, [b] diameter of particles by DLS mass distribution, [c] diameter of particles by DLS number distribution, *DLS and zeta potential studies were performed at 15 ˚C. The AFM results are the average diameters of 81 particles for Conjugate 1 and 95 particles for Conjugate 2 measured from image data.

Polymer-DNA conjugate response to competitive hybridisation

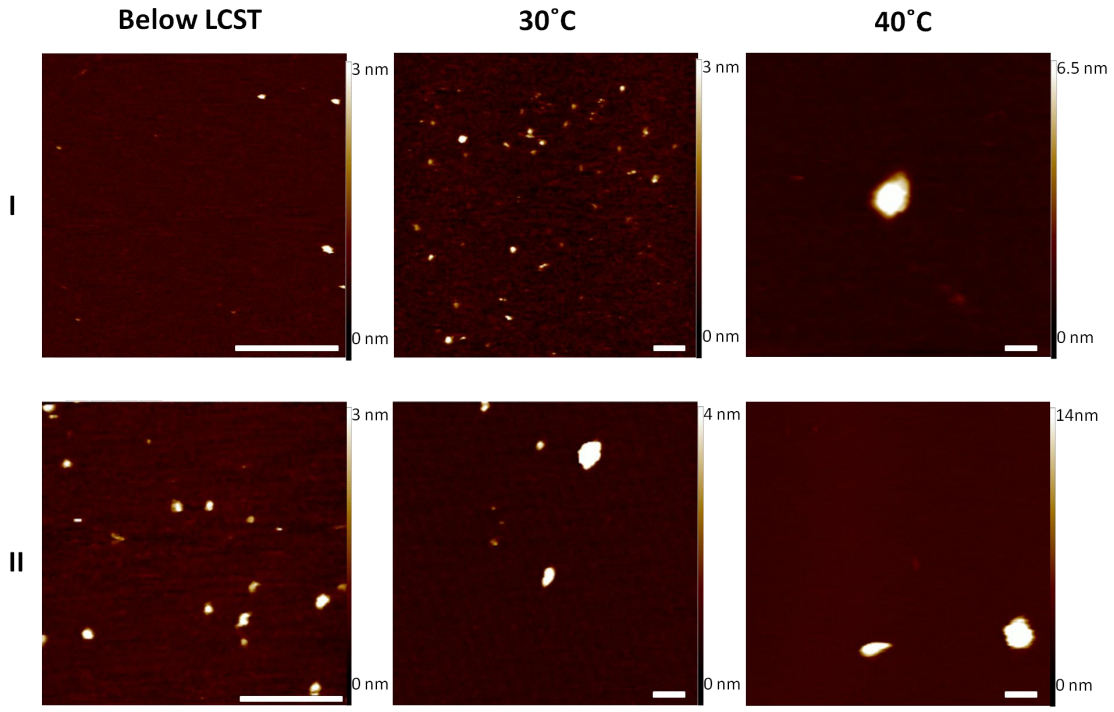


*Figure S8 – AFM topography images of DNA-polymer conjugates after adding complementary DNA stands below the polymer phase transition temperature, at 30 °C and 40 °C. **I**; Conjugate 1; **II**; Conjugate 2. (Scale bars: 100 nm for all images.)*

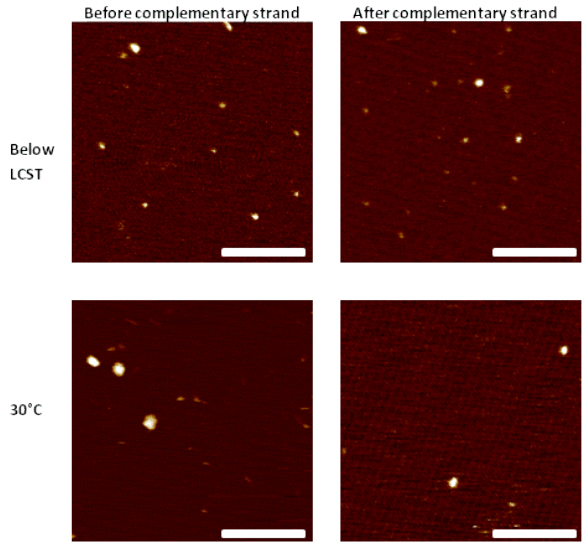
Table S5 – AFM particle analysis of Conjugate 1 and Conjugate 2 below the below the polymer phase transition temperature, and at 30 °C and 40 °C after the addition of complementary DNA strand. The table shows the number of features counted in the AFM image.

	Conjugate 1	Conjugate 2
Below LCST	105	107
30 ˚C	214	106
40 ˚C	40	53

Table S6 – Particle height measured during in situ aggregation studies at 30 °C and 40 °C after the addition of complementary DNA strand.

AFM Height Measurements (nm)

	Conjugate 1	Conjugate 2
30 °C	*1.5 ± 0.7*	*2.6 ± 1.0*
40 °C	*18.2 ± 9.4*	*25.0 ± 8.9*



	Before complementary strand	After complementary strand
Below LCST	84	135
30 ˚C	249	101

Figure S9 – Left: AFM topography images of Conjugate 1 before and after adding complementary DNA strands in ex situ studies below the polymer phase transition temperature and at 30 °C (Scale bars: 100 nm for all images, vertical scales: 1.5 nm for all images except before complementary strand at 30 °C; which is 5nm).Right: The number of features counted in AFM particle analysis below the polymer phase transition temperature, and at 30 °C before and after the addition of complementary Oligo 3 strand.

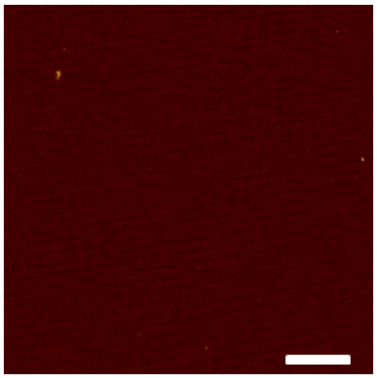


Figure S10 – AFM topography images of reducing agent (control study) (Scale bars: 100 nm for all images, vertical scales: 3nm)

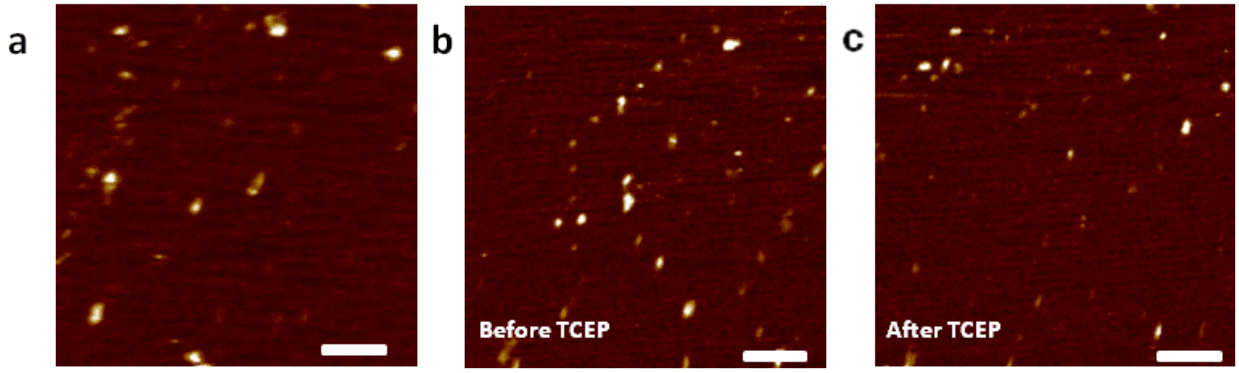


Figure S11 – AFM topography images of Conjugate 2 a) after addition of reducing agent in situ, and b) before and c) after reducing agent ex situ (control study). The sizes of the features were found to be 20 ± 7 nm in all conditions. (Scale bars: 100 nm for all images, vertical scales: 3 nm). (The AFM results are the average diameters of 92 particles during in situ studies (a), and 111 particles before the addition of reducing agent and 94 particles after the addition of reducing agent during ex situ studies (b).

Oligonucleotide - Doxorubicin hydrochloride binding studies

A solution of doxorubicin HCl (1.5 µM) was prepared in Dulbecco PBS buffer. The fluorescence of the solution was measured with a fluorescence spectrophotometer (5 mm slits) with excitation of doxorubicin at 480 nm and recording of the emission spectrum in the range 520–620 nm. The doxorubicin hydrochloride solution was then used to dissolve Oligo 3 (15 µM oligonucleotide contentration). The emission spectra of doxorubicin hydrochloride was recorded in the presence of Oligo 3. Sequential dilutions were performed on the oligonucleotide solution using the 1.5 µM doxorubicin solution to vary the ratio of oligonucleotide to drug and the emission spectra for doxorubicin was recorded each time. The binding affinity of doxorubicin to the oligonucleotide was determined by plotting the maximum fluorescence at 560 nm as a function of oligonucleotide concentration.

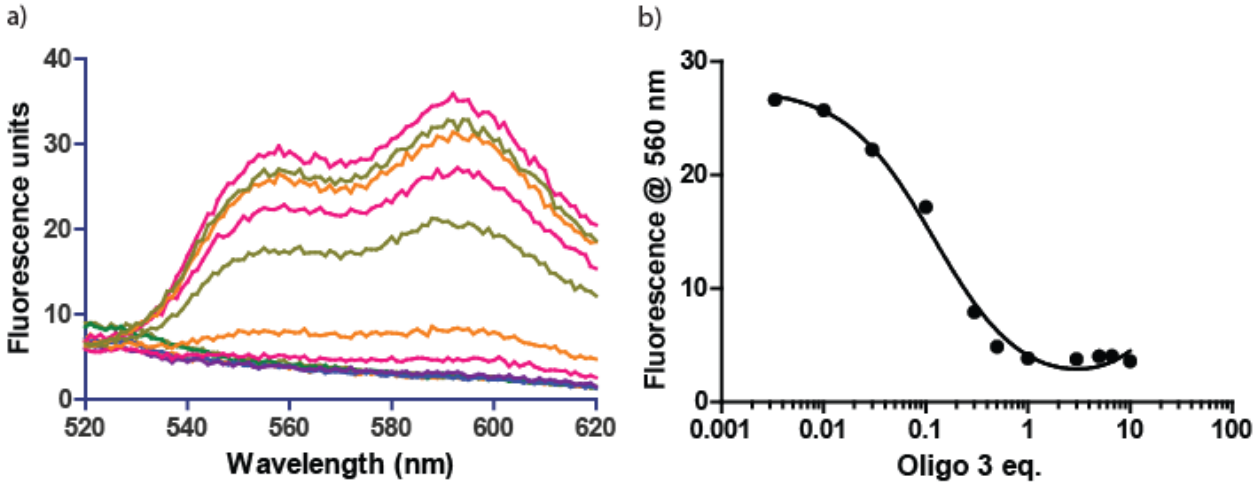


Figure S12 – a) Fluorescence spectra of a doxorubicin solution (1.5µM) as function of oligo 3 concentration (from top to bottom , 0, 0.01,0.03, 0.1, 0.3, 0.5, 1, 3, 5, 7, and 10 equivalents of the oligo). b) Doxorubicin fluorescence at 560 nm as a function of oligo 3 equivalents. (K_d =0.182 µM)

Effects of doxorubicin on a human alveolar adenocarcinoma cell line (A549).
Suspensions of A549 cells (100 µL, 50K cells/mL) were seeded on a 96 well plate using Eagle minimum media containing 10% FCS and 2 mM L-glutamine. Cells were allowed to attach for 24 hours before the media was removed and replaced with 100 µL of media which contained the appropriate doxorubicin HCl concentration. Cells were seeded in the wells for 72 hours, before the media was aspirated. Metabolic activity of each well was determined using a MTT assay, 6 wells were used to test each drug concentration. Doxorubicin HCl IC50 values for the A549 cell line was determined by incubating the cell line over a range of doxorubicin HCl concentration.

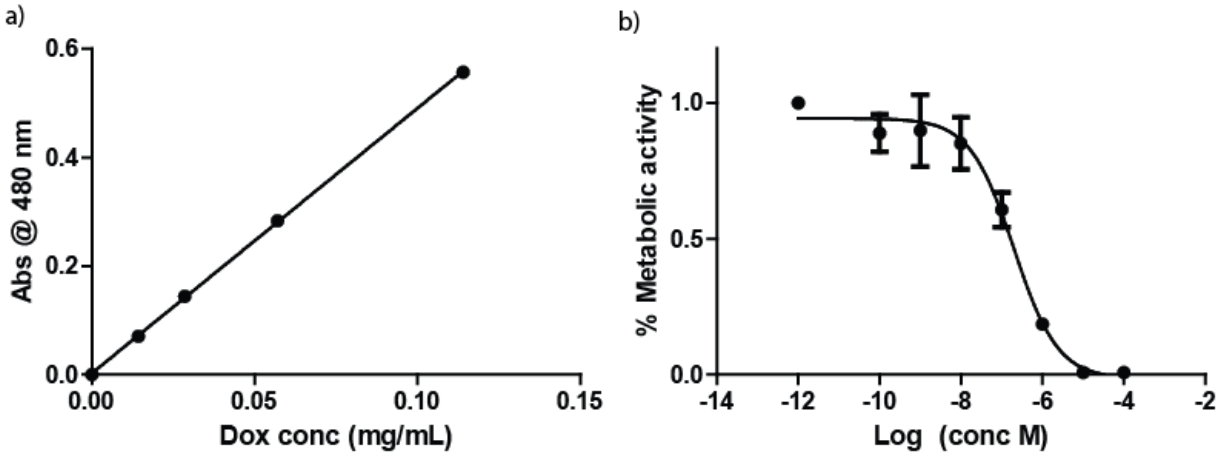
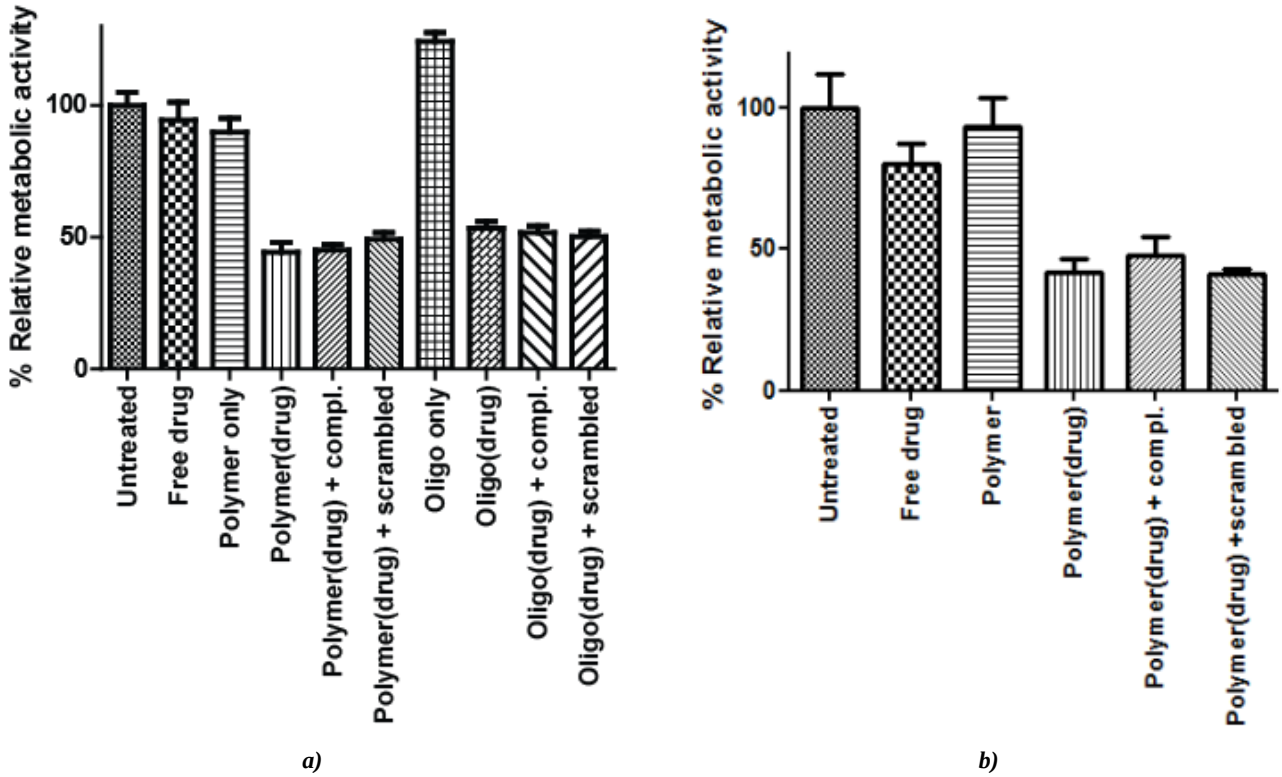


Figure S13 – a) Doxorubicin HCl calibration curve. Absorbance at 480 nm as a function of doxorubicin HCl concentration; b) Doxorubicin HCl sensitivity – Metabolic activity of A549 cell line as a function of doxorubicin HCl concentration. IC50 : 0.2 µM.



a) ***b)***

Figure S14– Effects of free doxorubicin, doxorubicin bound to oligonucleotides and Conjugate 1 (i) and doxorubicin bound to Conjugate 2 (b) on A549 cells.

Table S7 – Drug loading of Oligonucleotide and Oligonucleotide polymer conjugates

Sample	Amount used(µg)	Drug loaded(µg)	Drug loading (µg)	Loading efficiency %	Drug Loading (%w/w)	Dox-ODN ratio
Hybrid 1	689	204	87.3	42.79%	12.67%	0.1267
DC1	300	85	8.03	9.45%	2.68%	0.0611
DC2	3500	400	10.21	2.55%	0.29%	0.0172

Conjugate uptake studies via cell imaging

Suspensions of A549 cells (1 mL, 50K cells/mL) were seeded on a 24 well plate using Eagle's minimum media containing 10% FCS and 2 mM L-glutamine. Cells were allowed to attach for 24 hours before the media was removed and replaced with 1 mL of media which contained the appropriate test substance. Cells were incubated for 40 h before the media was aspirated and the cells fixed using a 4% paraformaldehyde (PFA) solution in PBS for 15 minutes. The PFA solution was removed from the cells with repeated washes of PBS. Mounting media was placed on top of the wells and the wells were subsequently covered with cover slips. Excess mounting media was wiped off the edges and the wells were sealed by adding clear nail polish on the edges of the cover slips. Cells were imaged on a Leica DM IRB microscope.

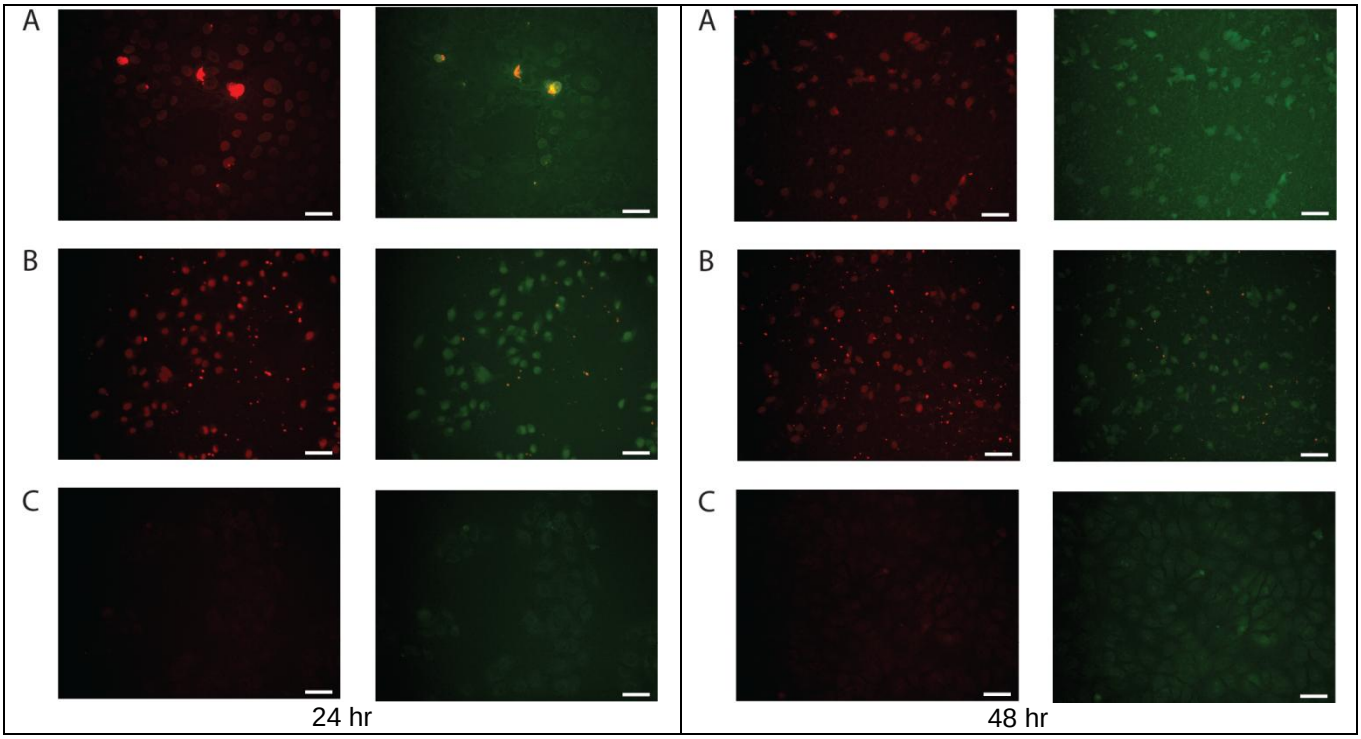


Figure S15: Fluorescence microscopy of A549 cells following incubation over 24 hr (left-hand panels) and 48 hours (right-hand panels) with (A) free doxorubicin (0.11 µM), (B) Conjugate 2 loaded with doxorubicin (0.11 µM), (C) Conjugate 2 without drug. For each panel, the left-hand block of images shows red fluorescence due to doxorubicin (residual colour is cell autofluorescence) and the right-hand block is the green channel corresponding to labelled polymer and doxorubicin.

Oligonucleotide stability/drug release

Oligo3 and ODN-polymer-doxorubicin complexes were incubated in triplicate at 37 °C in 1X PBS using a Slide A Lyzer MWCO 3.5KDa (0.5 mL). Equivalent concentrations of doxorubicin HCl was used for all sets. At set time points 200 µL samples were taken and the volume replaced with 200 µL DPBS buffer. Doxorubicin.HCl release was quantified by measuring fluorescence of solutions (Excitation 479 nm, Emission 590 nm).

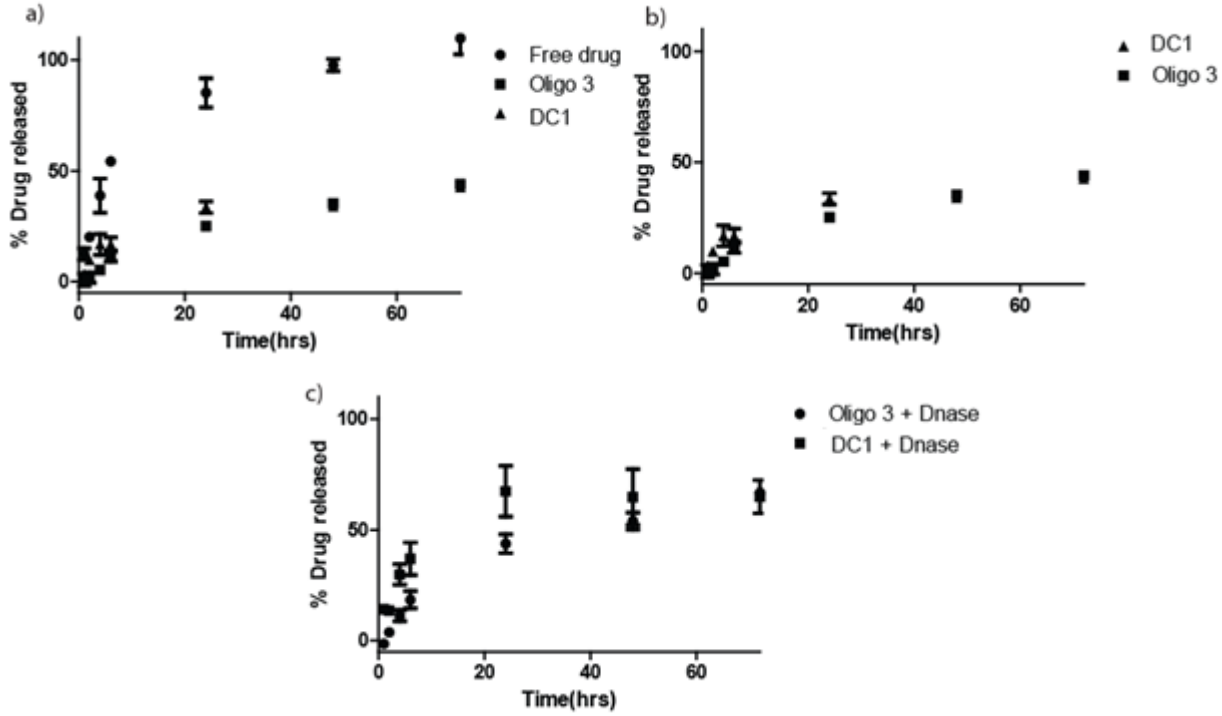


Figure S16– Drug release of Doxorubicin HCl. a) Drug release in 1XPBS at pH 7.4. Comparison of free drug, Oligo 3 and Conjugate 1. b) Drug release in 1XPBS at pH 7.4. Comparison between Oligo 3 and Conjugate 1 c) Effect of DNase on the drug release profiles of Oligo 3 and Conjugate 1.