Supporting Information for

Electrostatically PEGylated DNA Enables Salt-Free Hybridization in Water

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1. General

All chemicals and reagents were purchased from commercial suppliers and were used without further purification, unless mentioned otherwise. ^{1}H NMR spectra were recorded on a Varian Mercury NMR spectrometer, 400 MHz. The absorbance spectra and melting curves were measured on a V-630 UV-Vis spectrophotometer (JASCO Benelux B. V.) equipped with water-cooled Peltier thermostatted cell holder (JASCO ETCS-761). Inductively coupled plasma optical emission spectrometry (ICP-OES) was performed on a Perkin Elmer Optima 7000 DV and inductively coupled plasma mass spectrometry (ICP-MS) measurements were done on a Varian 820-MS ICP mass spectrometer. Scanning electron microscope (SEM) images were acquired using a JEOL 6320F field emission microscope working at 2 kV with a beam current of $1 \cdot 10^{-10}$ A. The circular dichroism (CD) measurements were performed on a JASCO J-810 spectropolarimeter. Quartz cuvette with 1 cm light-path was used for both absorbance and CD measurements. Small-angle X-ray scattering (SAXS) measurements were performed using MINA X-ray instrument at the University of Groningen. X-rays of 8 keV (wavelength λ = 1.5413 Å) are produced by a rotating anode operating at 45 kV and 60 mA. SAXS patterns have been acquired using Vantec 500 and 2000 Bruker detectors placed at two different sample-to-detectors distances (S-to-D = 24 and 300 cm, respectively). The peak positions from a standard silver behenate sample were used to calibrate the angular range 2θ. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-ToF) spectra were recorded on a Voyager-DE™ PRO Biospectrometry™ Workstation (Applied Biosystems).

2. Materials

Sodium chloride was obtained from Merck KGaA. 4-(hexyloxy)aniline (99 %) and sulfuric acid (99.9 %) were purchased from Sigma Aldrich. Methanol absolute AR (anhydrous, 99.8 %) was obtained from Biosolve Chimie SARL, France. Diethyl ether AR was purchased from Macron Fine Chemicals™ brand. mPEG-Amine 350 Da, 750 Da and 2000 Da were obtained from Creative PEGWorks, Sigma Aldrich and Iris Biotech GmbH, respectively. Ultrapure water (specific resistance > 18.2 MΩ cm) obtained by a Milli-Q water purification system (Sartorius) was used for all the experiments, unless noted otherwise.

3. Synthesis and characterization of DNA sequences

The four sequences ss22 (5'-CCTCGCTCTGCTAATCCTGTTA-3'), css22 (5'-TAACAGGATTAGCAGAGCGAGG-3'), ss14 (5'-CCTCGCTCTGCTAA-3') and css14 (5'-TTAGCAGAGCGAGG-3') were synthesized by employing an ÄKTA OligoPilot 100 automated DNA synthesizer (GE Healthcare) at 45 μmol scale on an universal solid support (Primer Support 5G UnyLinker 350, GE Healthcare) according to standard protocols. Deprotection and cleavage from the solid support were carried out in 25 % ammonia solution (Boom B. V., Netherlands) at 65 ºC for 16 hours. Purification of the oligonucleotides was performed on an ÄKTA Explorer (GE Healthcare) using a HiTrap Q HP 5 mL anion-exchange column (GE Healthcare) under a gradient of buffer A (10 mM NaOH; pH 12.0) and buffer B (10 mM NaOH, 1 M NaCl; pH 12.0). The oligonucleotides were desalted after purification on a HiTrap Desalting 5 mL column (GE Healthcare) under an isocratic flow of 20 % ethanol. Finally, the oligonucleotides were filtered using Whatman 0.45 μm syringe filter containing a cellulose acetate membrane. The identity and purity of the oligonucleotides were confirmed by MALDI-ToF mass spectrometry measurements (Figure S1).

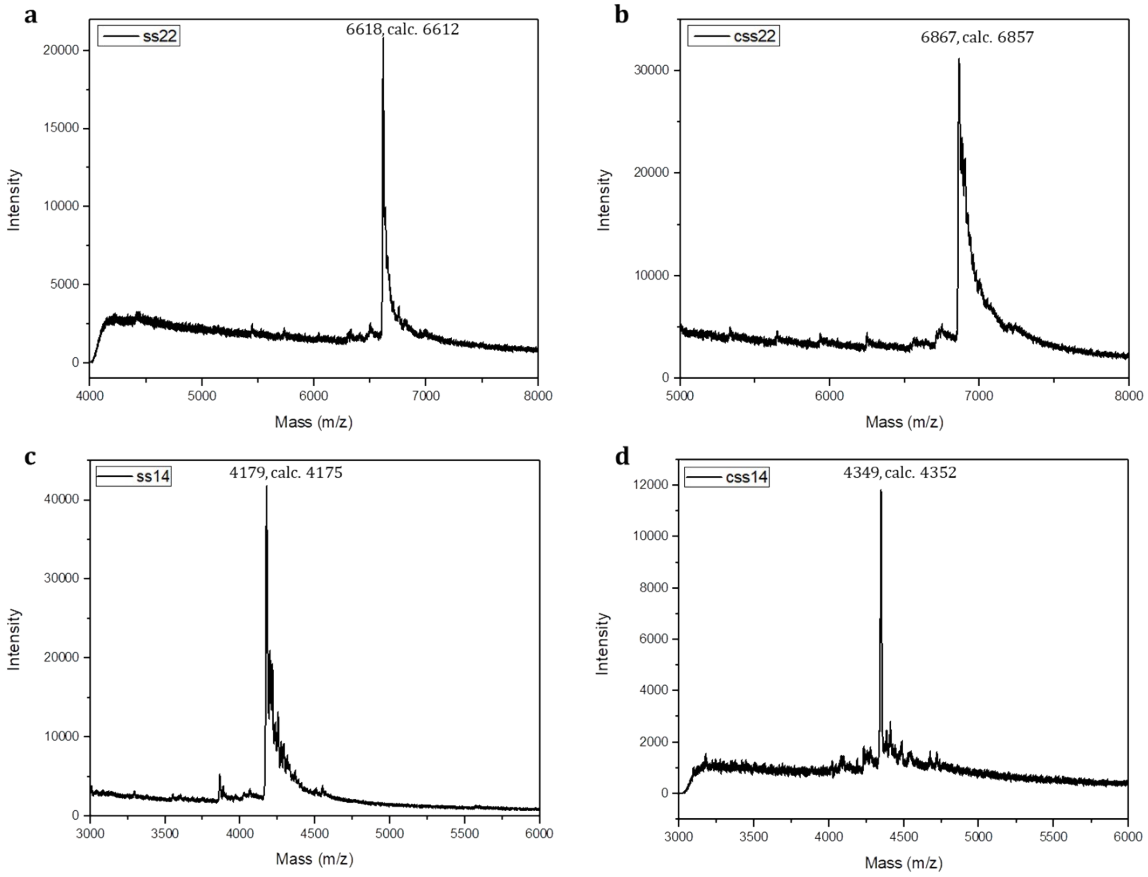
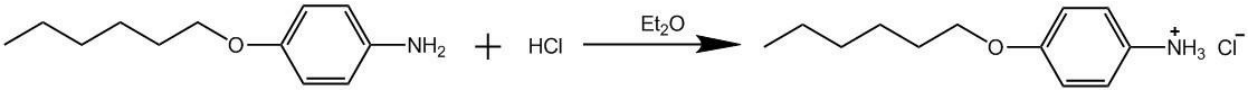


Figure S1. MALDI-ToF mass spectra of a) ss22, b) css22, c) ss14 and d) css14.

4. Synthesis and characterization of 4-(hexyloxy)anilinium chloride



Scheme S1. The synthesis of 4-(hexyloxy)anilinium chloride.

4-(hexyloxy)anilinium chloride (ANI.HCl) was synthesised by following a previously reported method.[1] In brief, 4-(hexyloxy)aniline (25 g, 130 mmol) was placed in a 1000 mL round bottom flask equipped with a magnetic stir bar. 500 mL diethyl ether was added. 4-(hexyloxy)aniline was allowed to dissolve completely under vigorous stirring. Freshly prepared hydrochloride gas, obtained by mixing sodium chloride with sulfuric acid, was passed into the solution of 4-(hexyloxy)aniline. A turbid solution was observed after few minutes of stirring and a precipitate

was formed. The solution was flushed with hydrochloride for further 30 minutes and then the reaction was ceased. Subsequent to the collection of the precipitate by filtration, it was rinsed with diethyl ether (2 × 300 mL). The purple colored solid was dried overnight under vacuum (27.4 g, 91.7 % yield). ^{1}H NMR spectrum was obtained to confirm the identity and purity of the aforementioned compound (Figure S2).

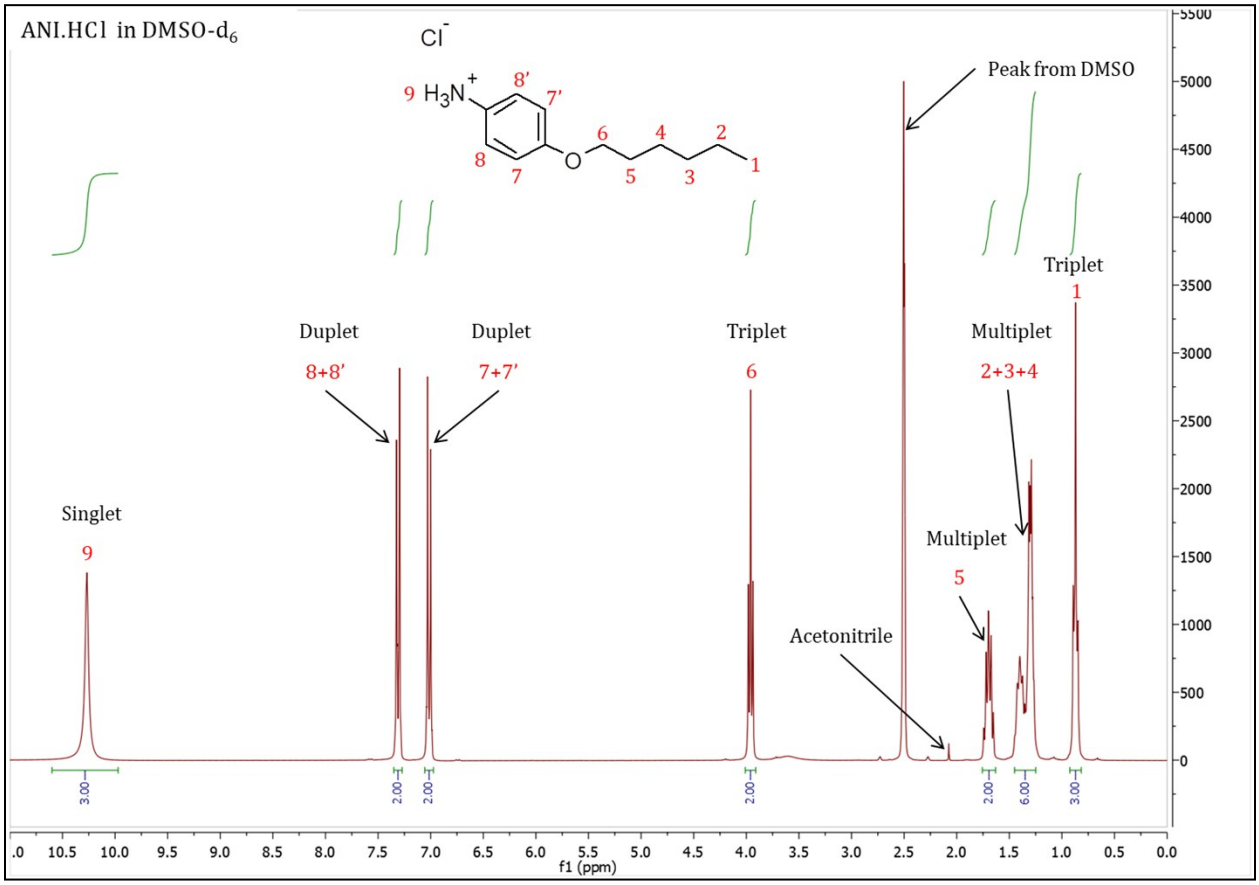


Figure S2. ^{1}H NMR spectrum of 4-(hexyloxy)anilinium chloride in DMSO-d$_6$.

5. ^{1}H NMR spectrum of methoxyPEG-Amine, molecular weight 350 Da (PEG350)

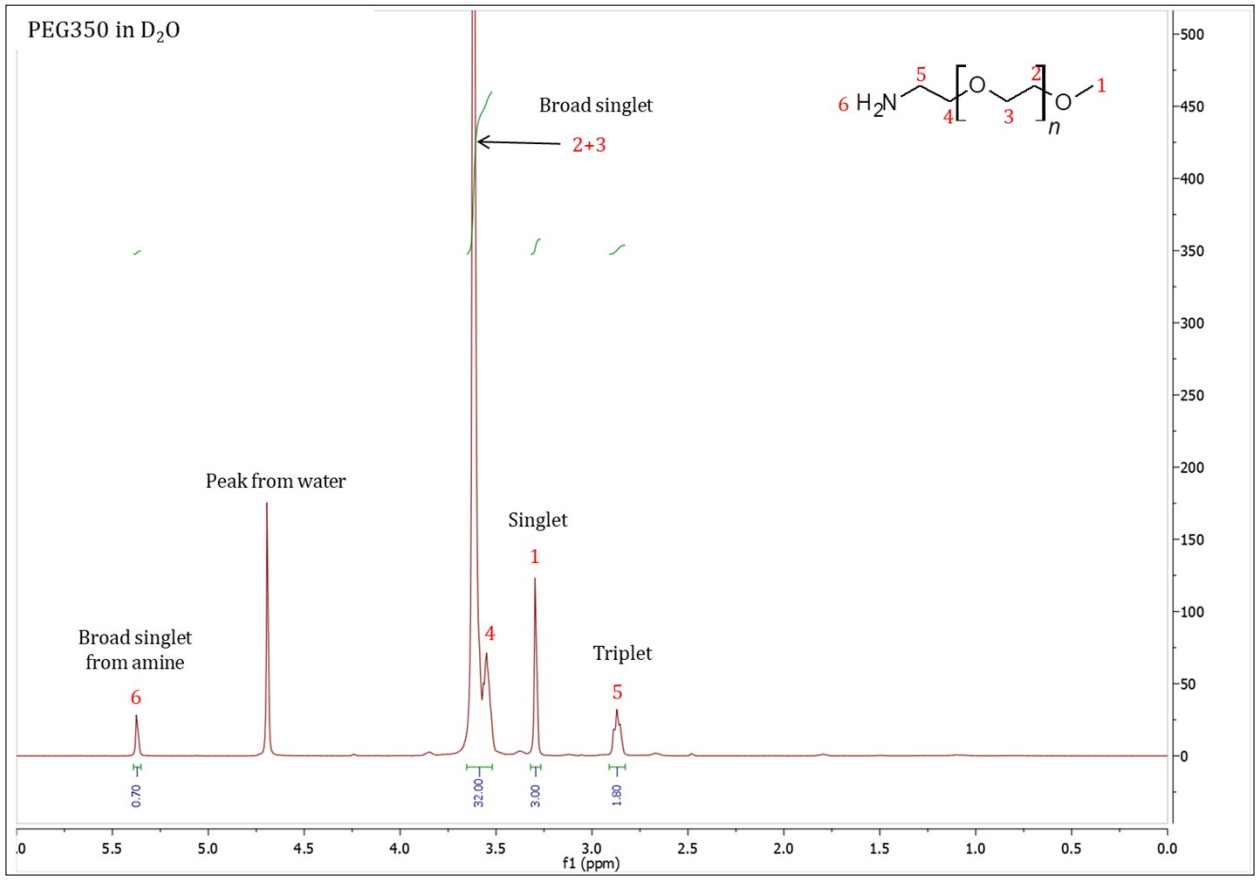


Figure S3. ^{1}H NMR spectrum of PEG350 in D_2O.

6. Standard procedure for the synthesis of a DNA-PEG complex

DNA-PEG complex was obtained by the substitution of DNA-ANI complex with methoxyPEG containing a terminal primary amine group (mPEG-Amine). The DNA-ANI complex was prepared according to a method described by Chen, *et al.*[1] ANI.HCl (three equivalents to the charge present in the DNA i.e., 3 × 21 × 1.5 µmol for ss22 and css22 or 3 × 13 × 1.5 µmol for ss14 and css14) dissolved in 3 mL of ultrapure water was added to an aqueous solution of DNA. Then the solution was mixed thoroughly for 1 hour on a shaker at 800 rpm and centrifuged at 4500 rpm for 10 minutes. A precipitate of DNA-ANI complex was obtained. The supernatant was discarded and the precipitate was washed three times as follows: addition of 3 mL ultrapure water, mixing thoroughly followed by centrifugation for 10 minutes at 4500 rpm, to ensure the removal of unbound ANI molecules and salts from the complex by discarding the supernatant. Then the DNA-ANI complex was freeze-dried overnight and resuspended in 5 mL methanol. It

was mixed thoroughly for 5 minutes. Excess amount ($3 \times 21 \times 1.5 \times 10^{-6}$ mol × 350 g/mol for ss22-PEG350 and css22-PEG350, $3 \times 21 \times 1.5 \times 10^{-6}$ mol × 750 g/mol for ss22-PEG750 and css22-PEG750, $3 \times 21 \times 1.5 \times 10^{-6}$ mol × 2000 g/mol for ss22-PEG2000 and css22-PEG2000, $3 \times 13 \times 1.5 \times 10^{-6}$ mol × 350 g/mol for ss14-PEG350 and css14-PEG350) of mPEG-Amine, dissolved in 1 mL methanol, was added to the dispersed solution of DNA-ANI. The mixture was allowed to shake for two hours on a shaker at 800 rpm, to ensure the complete exchange of ANI molecules by cationic amino PEGs. The resulting DNA-PEG complex solution was diluted by adding 6 mL of ultrapure water. The entire DNA-PEG complex solution was transferred to a 3 kDa molecular weight cut-off (MWCO) tube (GE Healthcare) and concentrated by centrifugation at 4500 rpm. The MWCO tube was activated with 1:1 water-methanol mixture, prior to use. The concentrated DNA-PEG complex solution was subjected to five more purification steps employing a 1:1 water-methanol mixture in centrifugal filtration tubes to remove all the unbound PEG and free ANI molecules. The DNA-PEG complex solution on the membrane was then concentrated in a speed-vac to remove methanol and then freeze-dried overnight to obtain the final DNA-PEG complex.

7. ^{1}H NMR spectra of DNA, DNA-ANI and DNA-PEG complexes

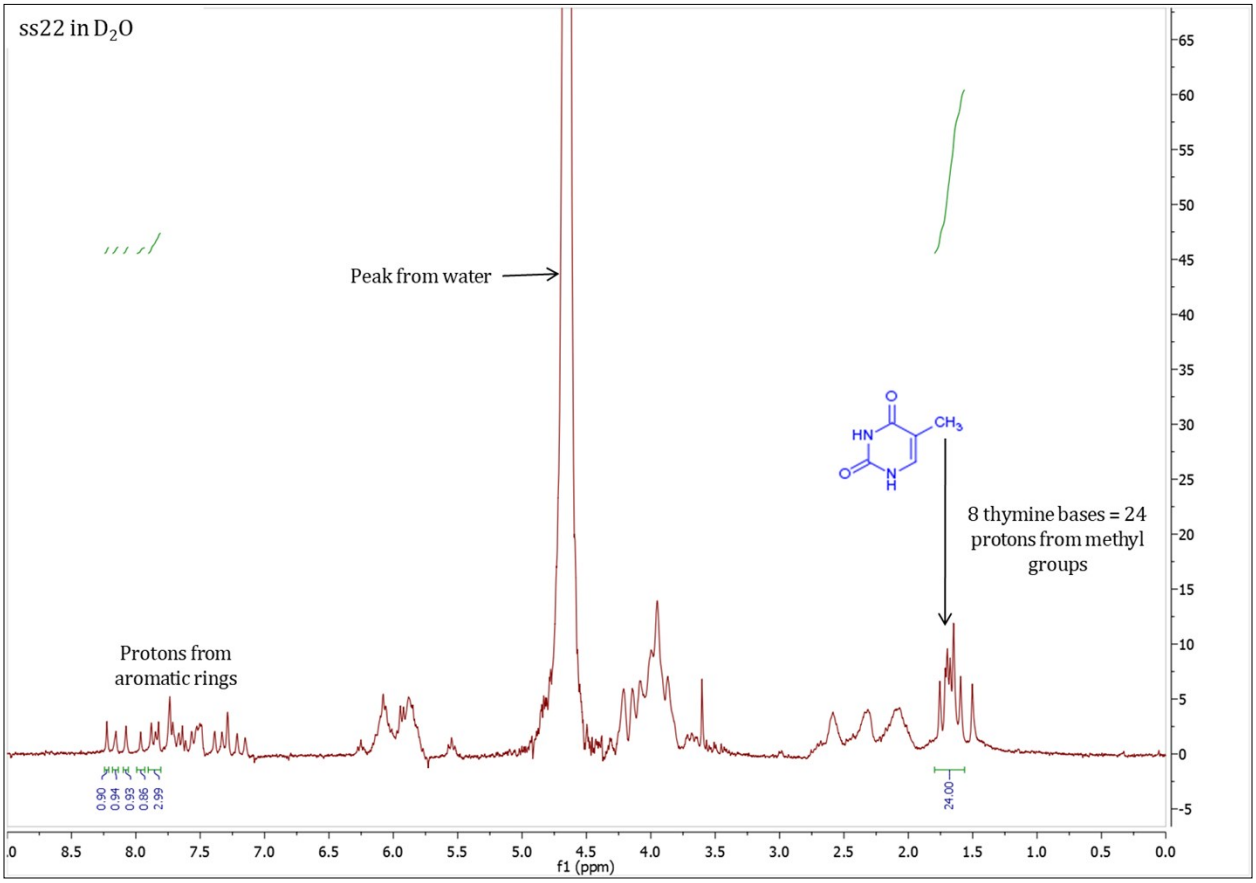


Figure S4. ^{1}H NMR spectrum of ss22 in D_2O.

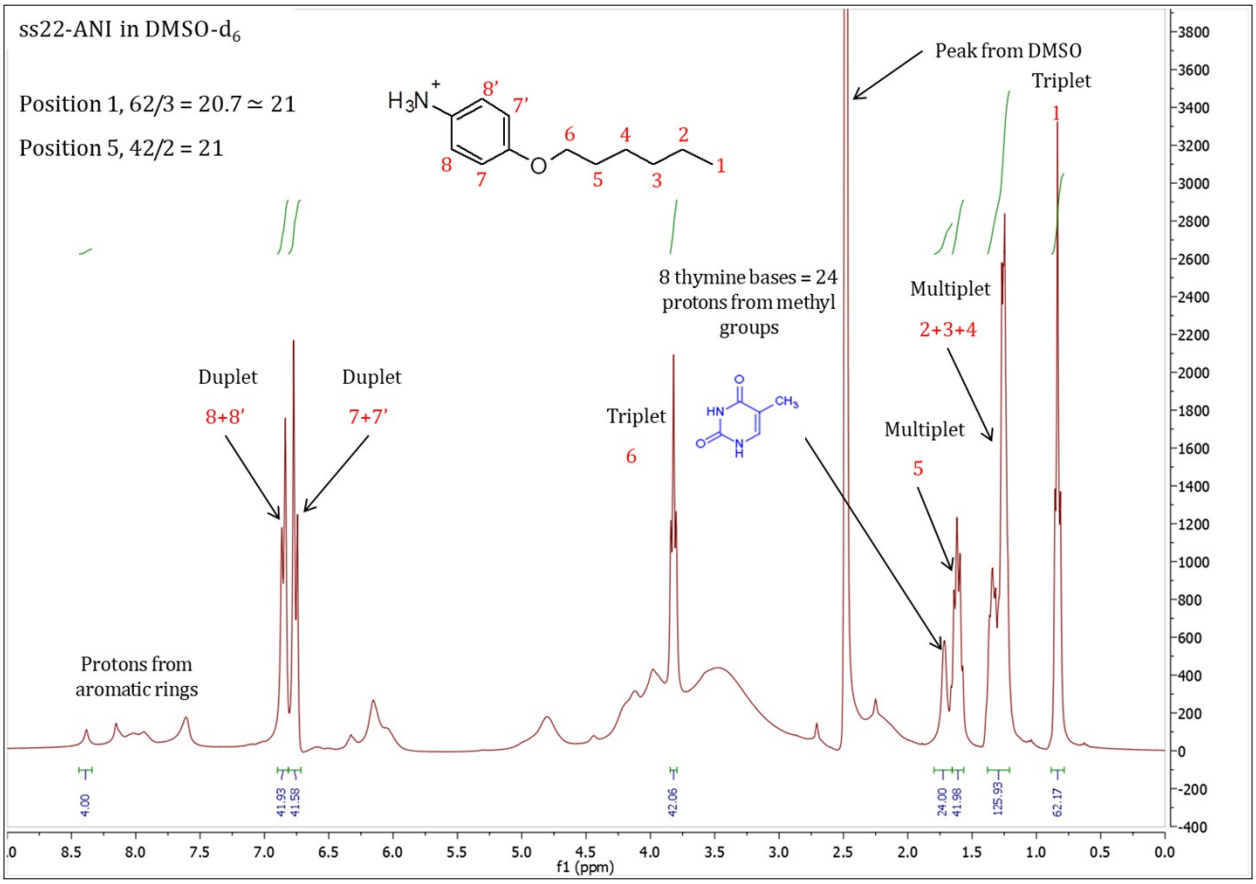


Figure S5. ^{1}H NMR spectrum of ss22-ANI in DMSO-d_6.

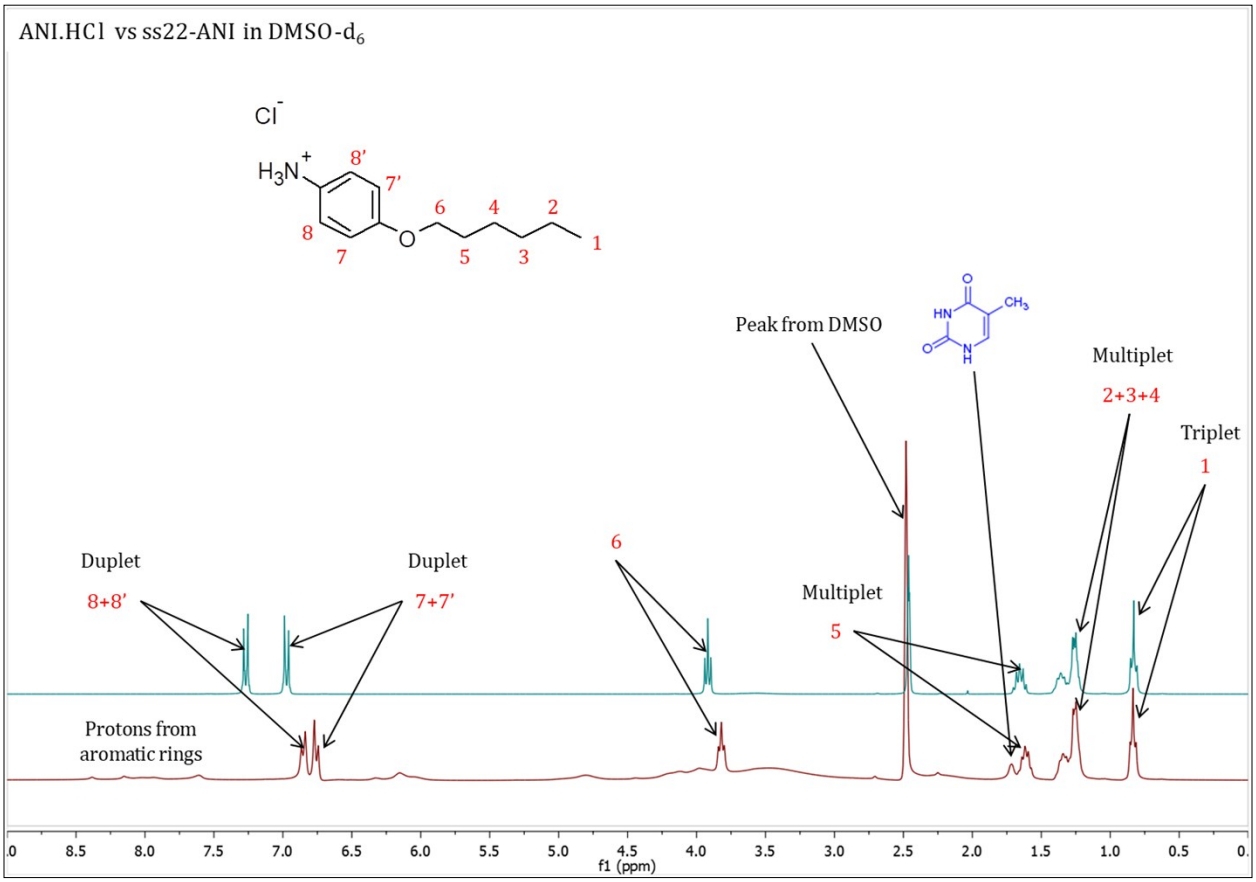


Figure S6. ^{1}H NMR spectra of ANI.HCl (green) and ss22-ANI complex (red) in DMSO-d_6.

The DNA-ANI complex formation was followed by ^{1}H NMR spectroscopy. To confirm the complex formation, NMR spectra of pure ANI.HCl (Figure S2) and DNA-ANI complex (ss22-ANI, Figure S5) in DMSO-d_6 were compared (Figure S6). The number of protons corresponding to the terminal methyl group of ANI.HCl was found to be 62 in ss22-ANI complex whereas the same group in pure ANI.HCl exhibited only 3 protons. The ratio obtained by dividing the number of protons related to the specified methyl group in ss22-ANI complex by the ones in pure ANI.HCl allows determining the grafting stoichiometry of the complex. The number of ANI molecules grafted onto the backbone of ss22 was found to be $\simeq$ 21, this is equivalent to the total number of negative charges of ss22. This validates the specific charge-to-charge interaction leading to the grafting of cationic ANI molecules onto the anionic backbone of DNA.

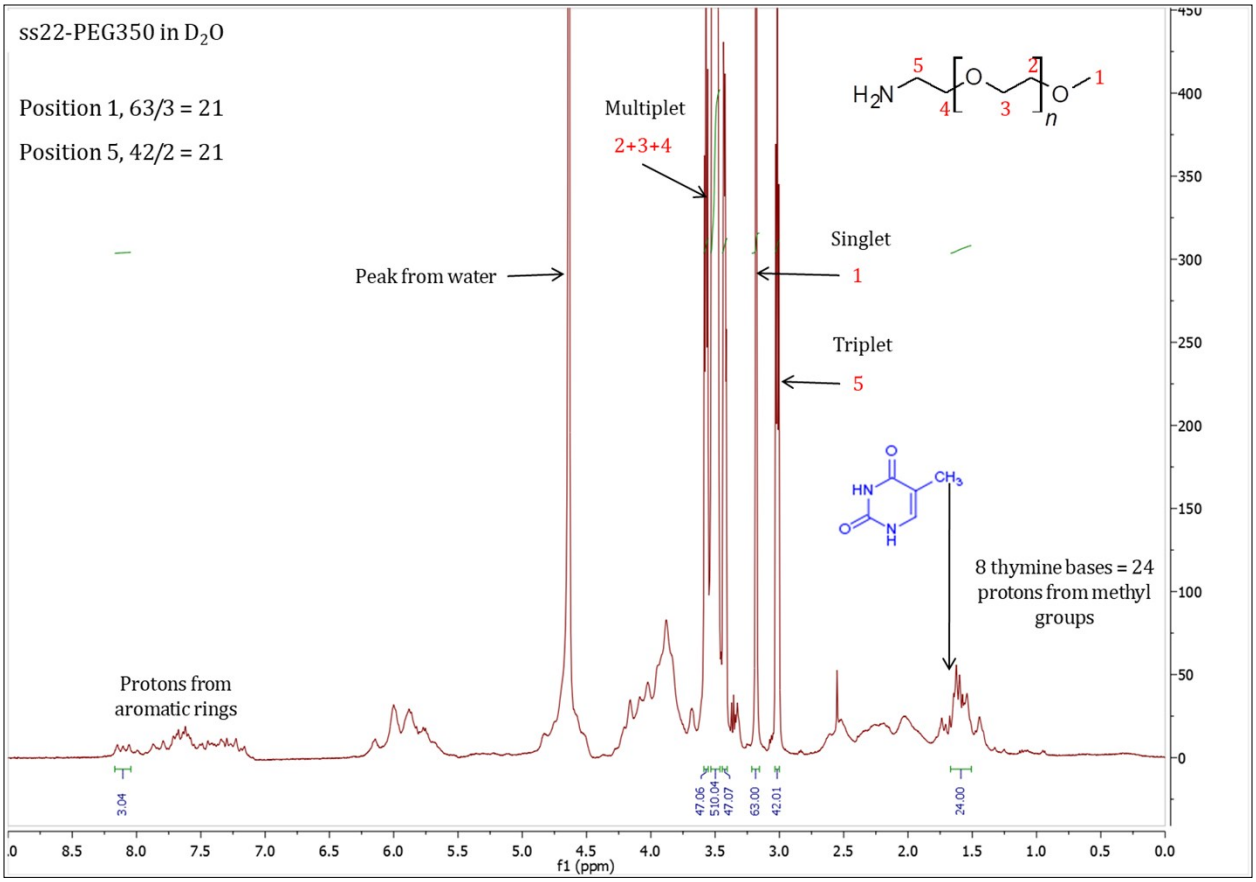


Figure S7. ^{1}H NMR spectrum of ss22-PEG350 in D_2O.

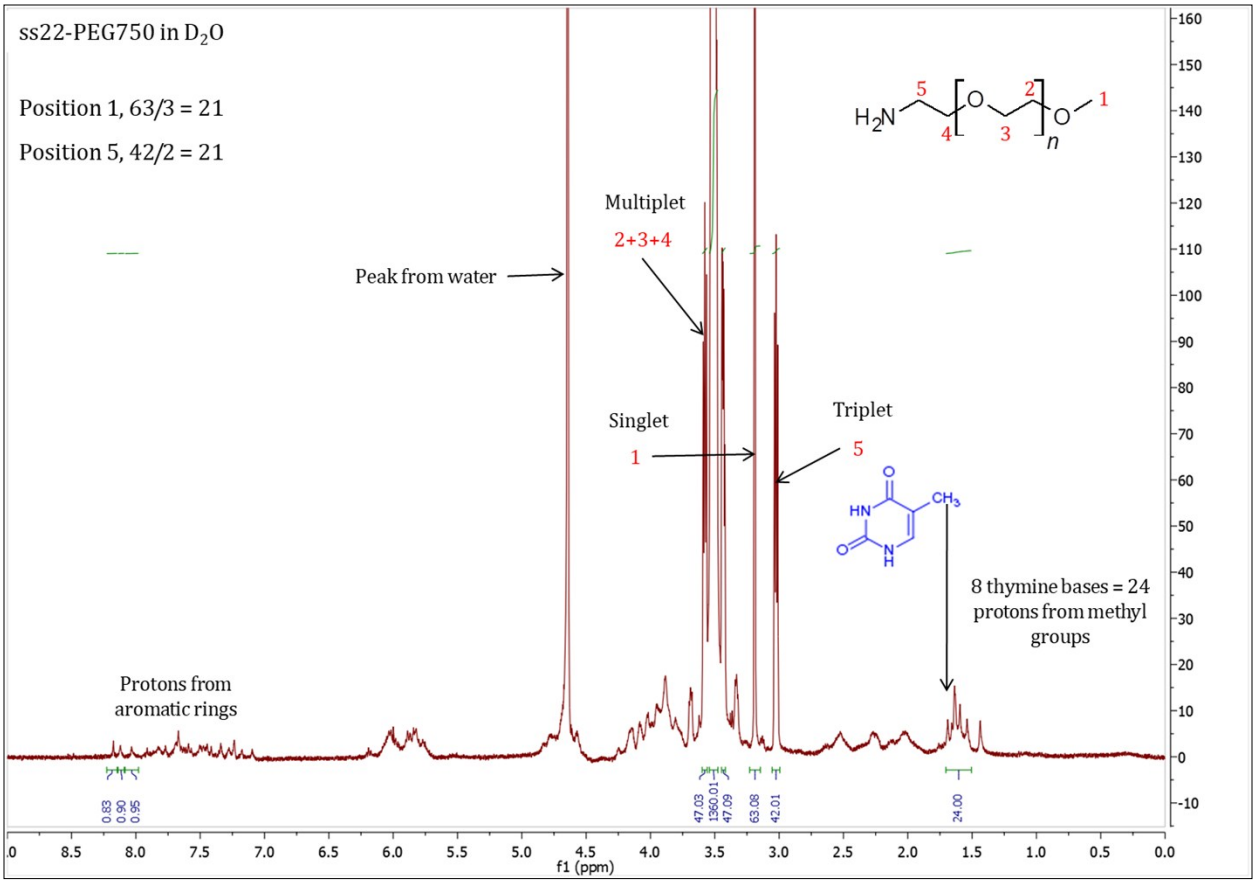


Figure S8. ^{1}H NMR spectrum of ss22-PEG750 in D_2O.

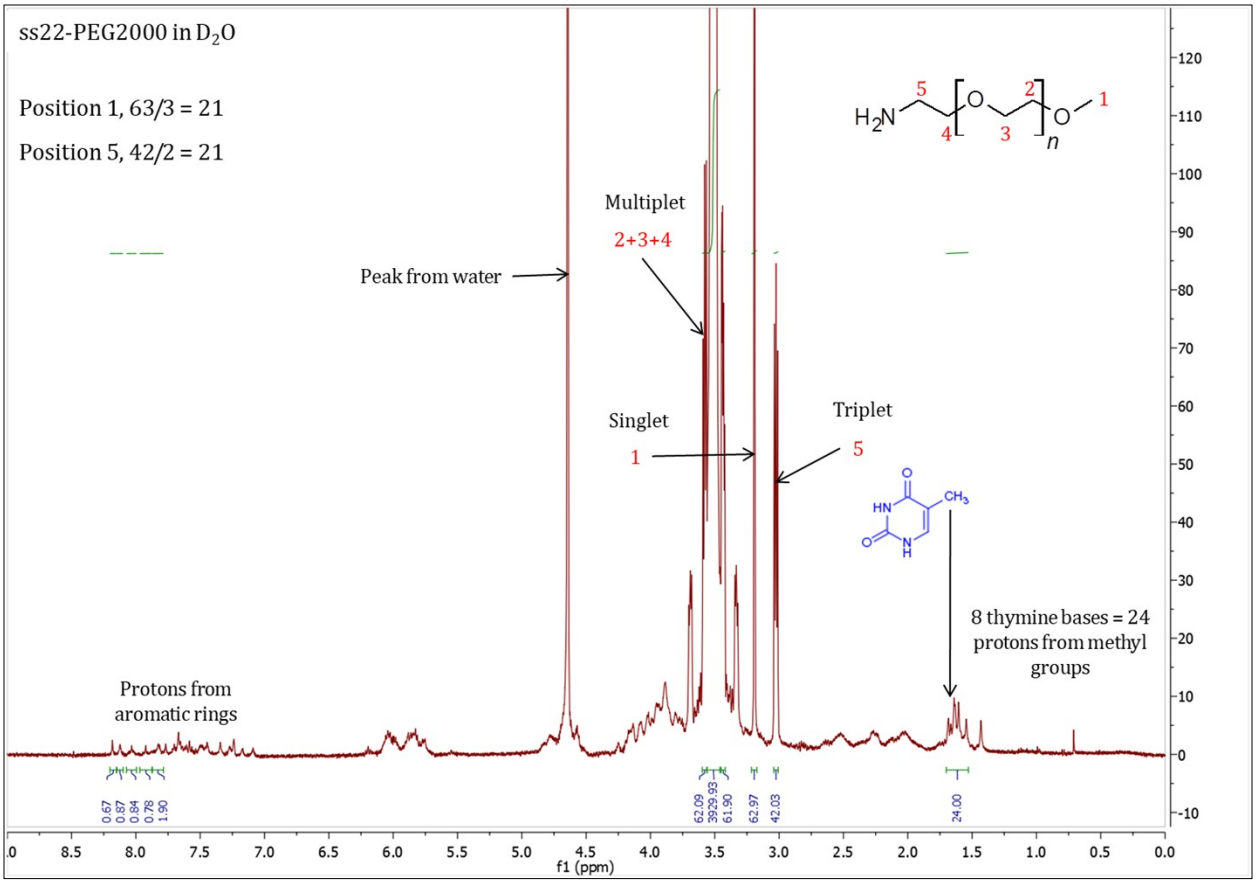


Figure S9. ^{1}H NMR spectrum of ss22-PEG2000 in D_2O.

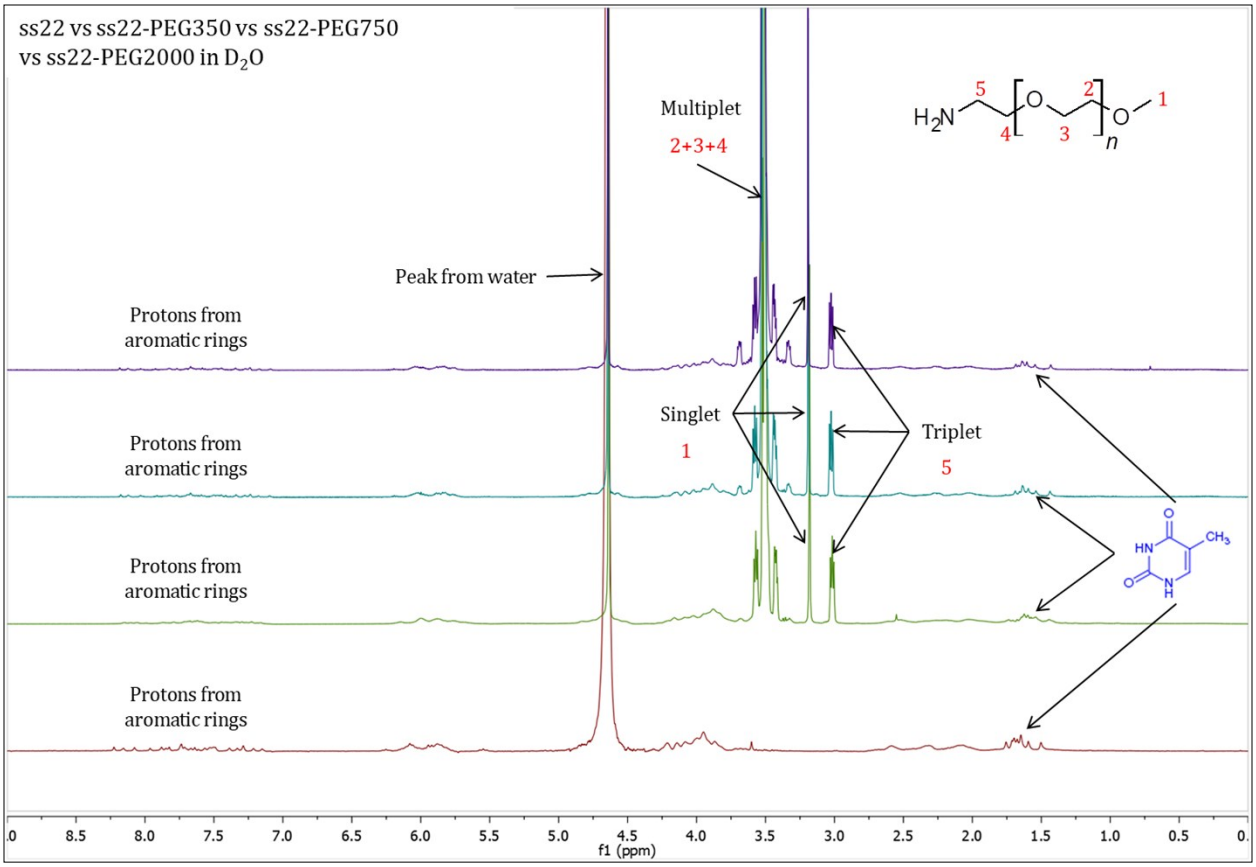


Figure S10. ^{1}H NMR spectra of ss22 (red), ss22-PEG350 (light green), ss22-PEG750 (dark green) and ss22-PEG2000 (violet) in D_2O.

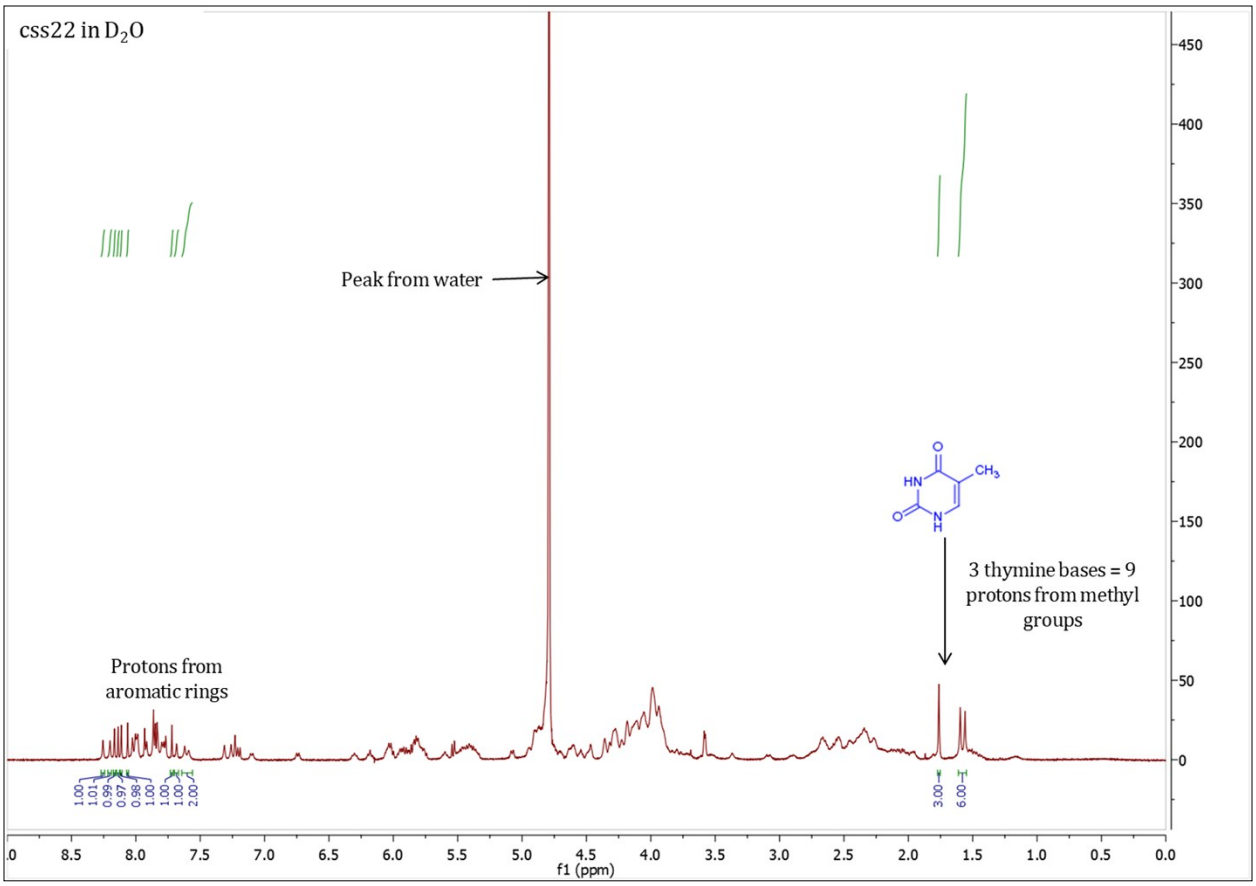


Figure S11. ^{1}H NMR spectrum of css22 in D_2O.

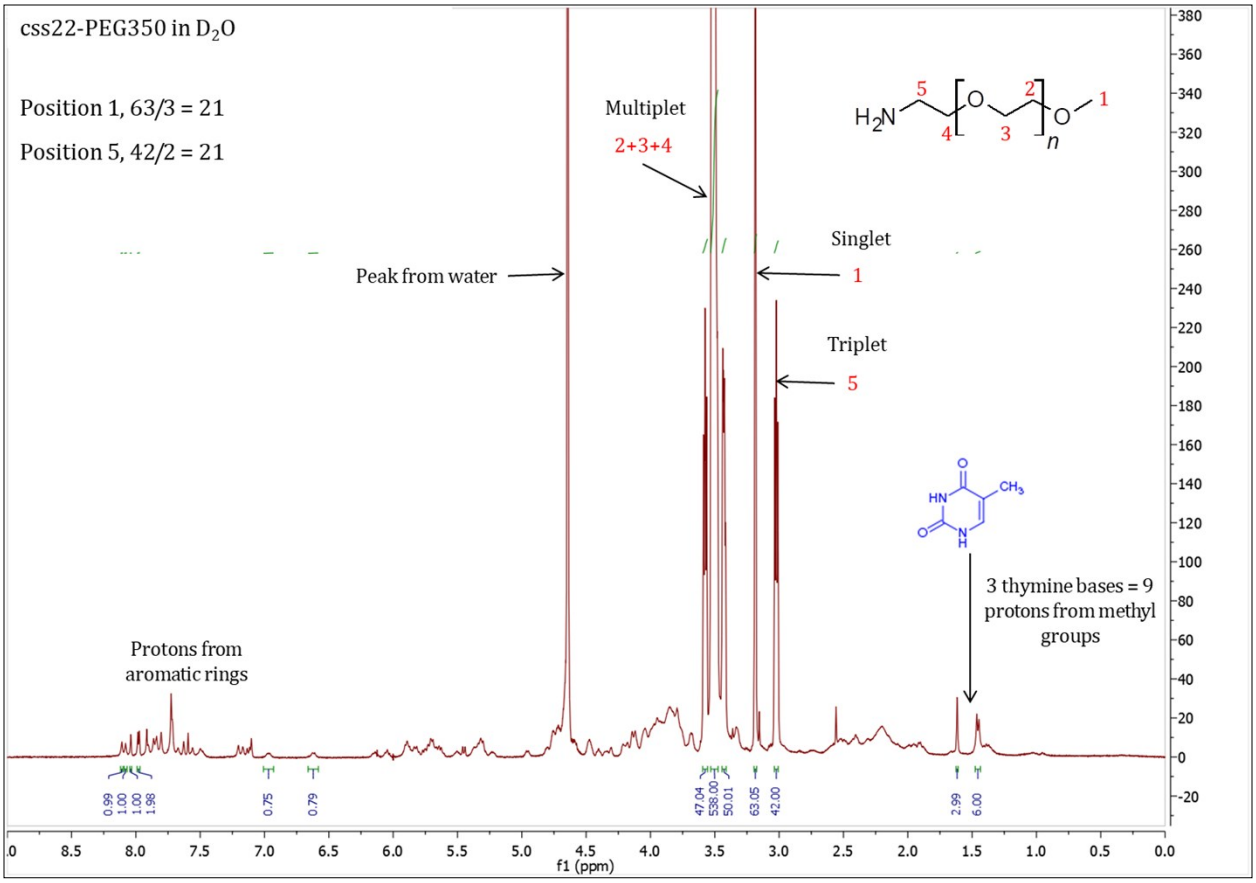


Figure S12. ^{1}H NMR spectrum of css22-PEG350 in D_2O.

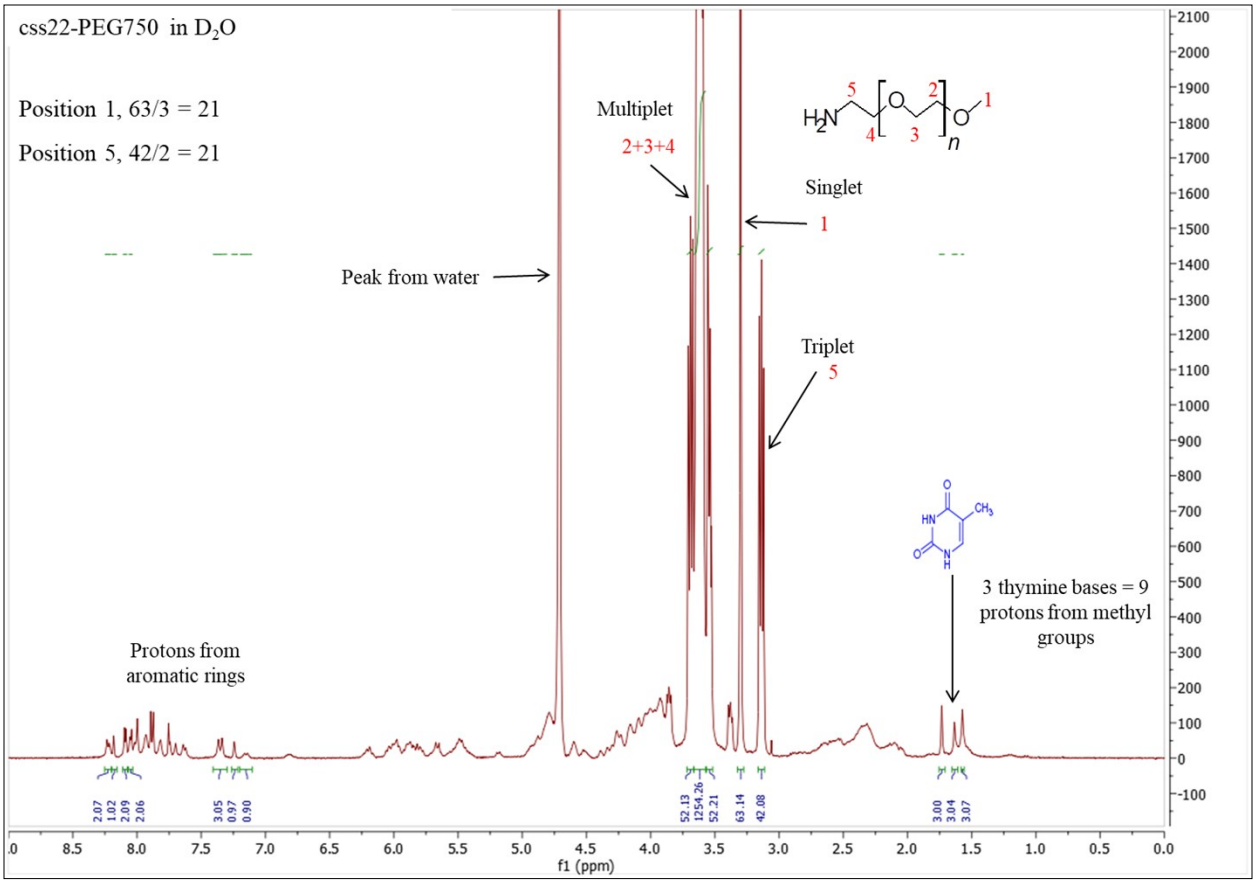


Figure S13. ^{1}H NMR spectrum of css22-PEG750 in D_2O.

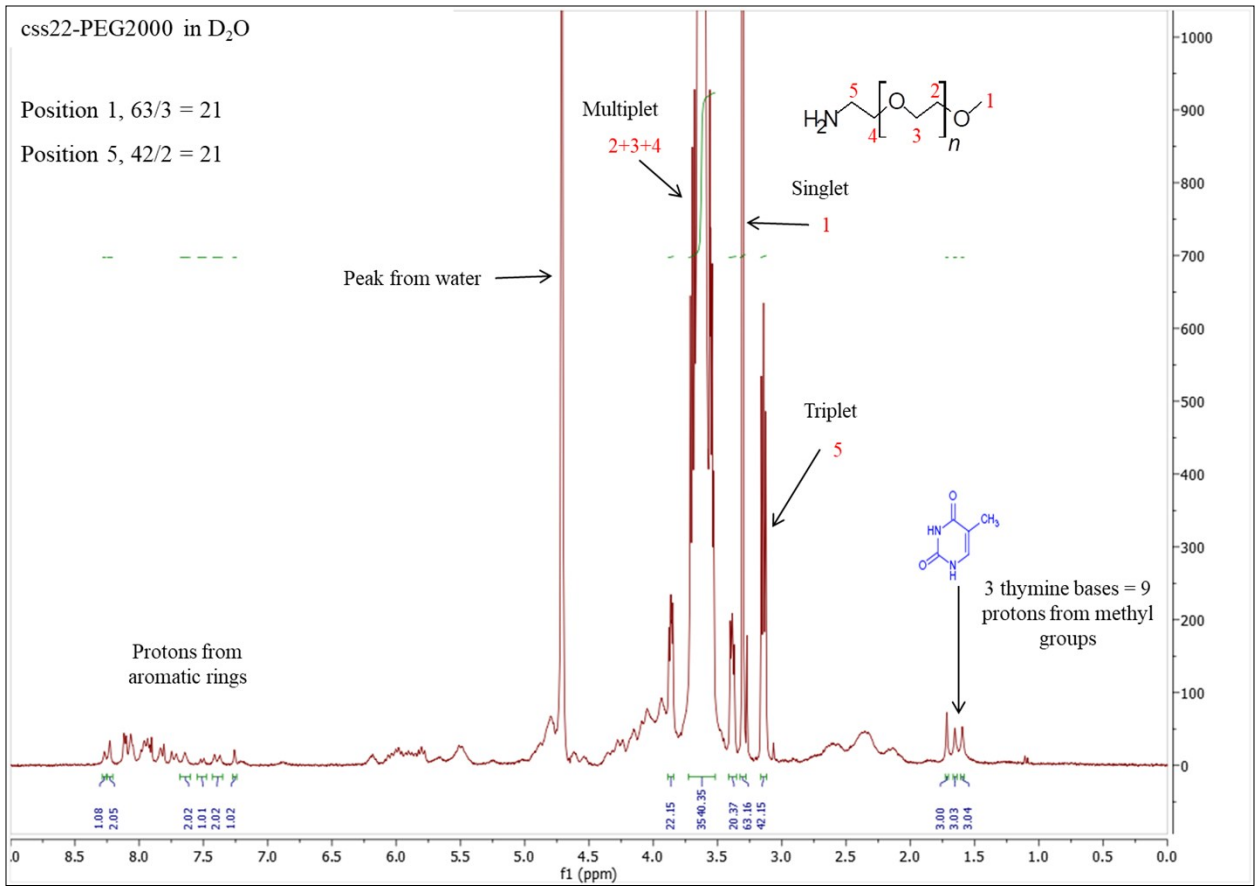


Figure S14. ^{1}H NMR spectrum of css22-PEG2000 in D_2O.

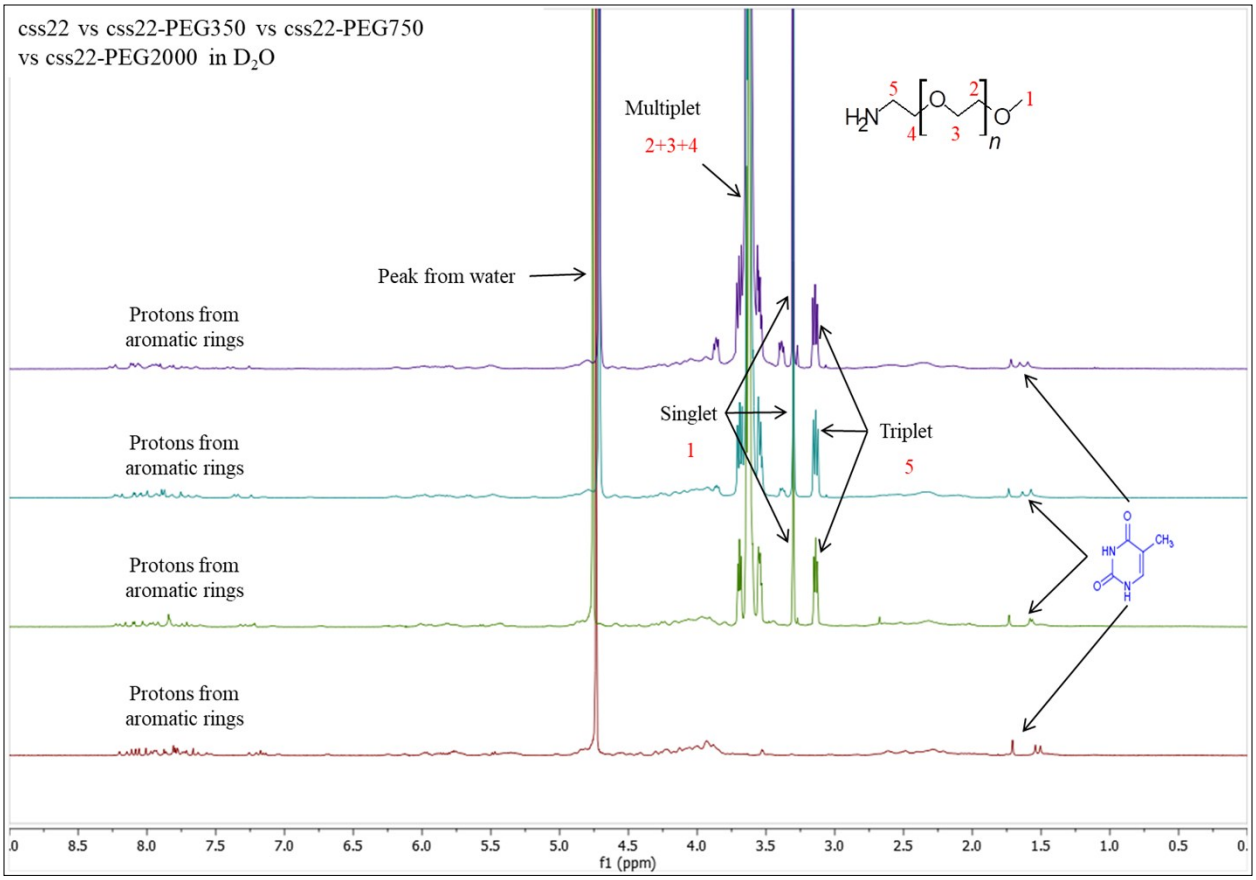


Figure S15. ^{1}H NMR spectra of css22 (red), css22-PEG350 (light green), css22-PEG750 (dark green) and css22-PEG2000 (violet) in D_2O.

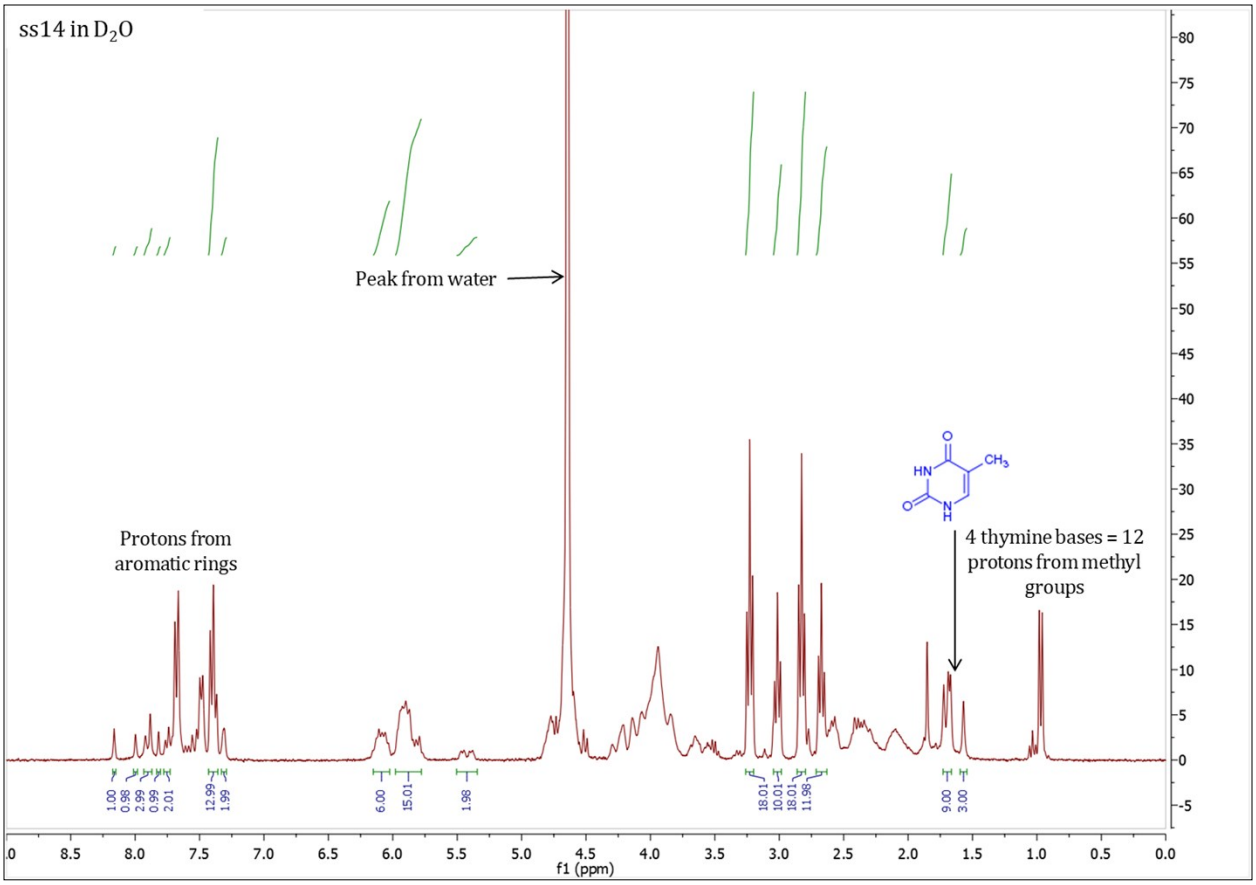


Figure S16. ^{1}H NMR spectrum of ss14 in D_2O.

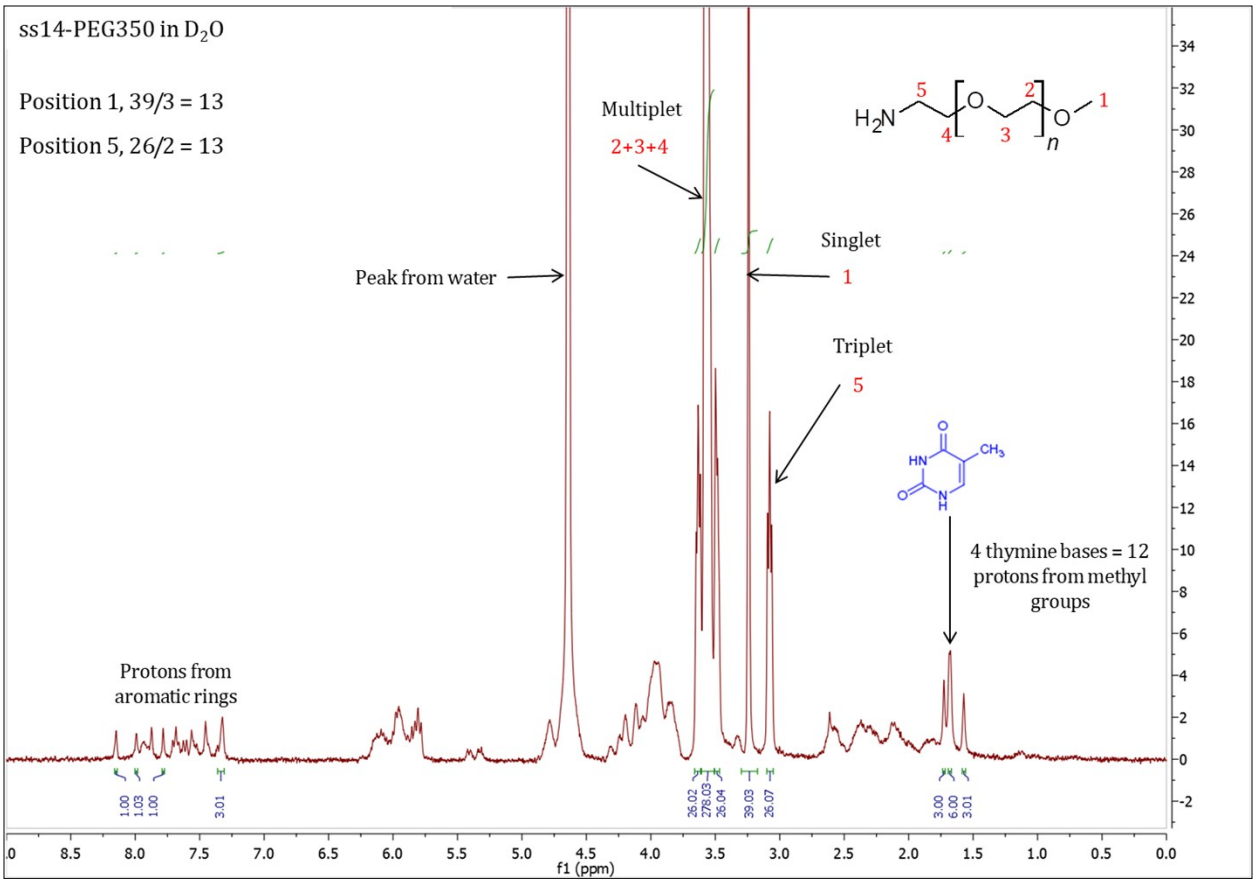


Figure S17. ^{1}H NMR spectrum of ss14-PEG350 in D_2O.

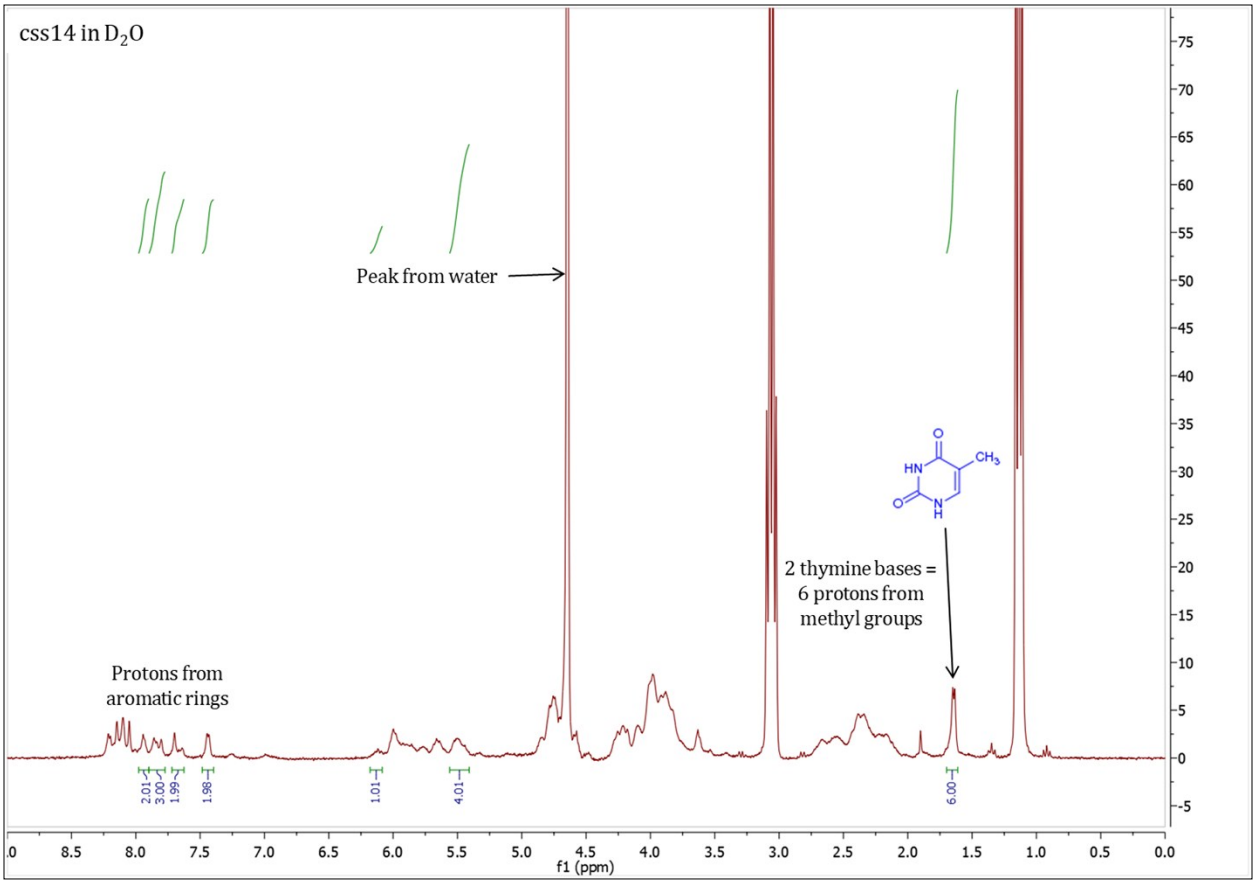


Figure S18. ^{1}H NMR spectrum of css14 in D_2O.

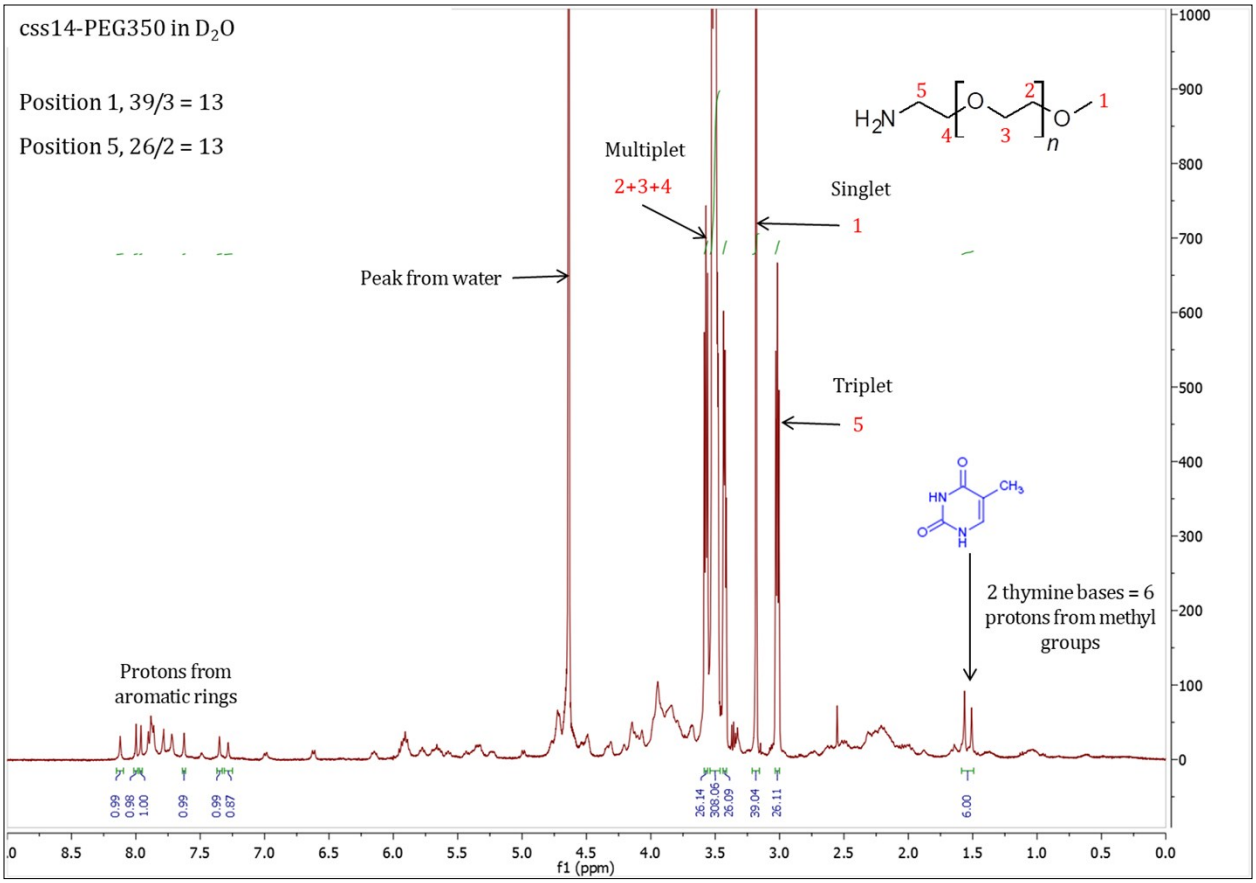


Figure S19. ^{1}H NMR spectrum of css14-PEG350 in D_2O.

8. Determination of DNA and DNA-PEG stock concentrations

The stock concentrations of ss22, css22, ss14 and css14 were determined by measuring the UV absorption at 260 nm. DNA stock solutions were prepared in ultrapure water. The stocks were diluted to certain dilution factors using ultrapure water. Ultrapure water was used for blank (background) measurement and then absorbance for each sequence was recorded, individually. The DNA sequences together with their respective values of absorbance were entered in "Oligo Calc: Oligonucleotides Properties Calculator" to determine the concentrations of the diluted samples. The concentrations of the diluted samples were multiplied with their respective dilution factors to obtain the concentrations of DNA stock solutions. The determination of stock concentrations of DNA-PEG complexes were based on the grafting stoichiometries of PEG molecules onto the DNA backbones, obtained from ^{1}H NMR spectra. To explain, the freeze-dried form of DNA-PEG complexes were weighed precisely (up to 6-digits after decimal). The molecular weights of the complexes were calculated as follows:

Molecular weight of ss22-PEG350 = 6612 + 21 × 350 = 13,962 g/mol
Molecular weight of ss22-PEG750 = 6612 + 21 × 750 = 22,362 g/mol
Molecular weight of ss22-PEG2000 = 6612 + 21 × 2000 = 48,612 g/mol
Molecular weight of css22-PEG350 = 6857 + 21 × 350 = 14,207 g/mol
Molecular weight of css22-PEG750 = 6857 + 21 × 750 = 22,607 g/mol
Molecular weight of css22-PEG2000 = 6857 + 21 × 2000 = 48,857 g/mol
Molecular weight of ss14-PEG350 = 4175 + 13 × 350 = 8,725 g/mol
Molecular weight of css14-PEG350 = 4352 + 13 × 350 = 8,902 g/mol

It is well-known that C = n/V, here
C = concentration (moles/L= molarity)
n = moles of solute (DNA-PEG complex)
V = volume of solution in L

The amount of the DNA-PEG complexes (in moles) was obtained by dividing the weighed mass (m) of the DNA-PEG complexes with their respective molecular weights (M), n = m/M. All the DNA-PEG stocks were prepared to be of 0.003 M by adding ultrapure water to the respective complexes.

9. Dialysis of DNA samples

Spectra/Por Float-A-Lyzer G2 dialysis devices containing Biotech Grade Cellulose Ester (CE) membrane (MWCO 3.5-5 kD) of 1 mL volume size were used for the dialysis of 100 μM each, of the desalted ss22 and css22. The DNA strands were dialysed against 2 L of ultrapure water (pH 4.5-5.0) for four days. The water baths were refilled with fresh batch of ultrapure water at a regular interval to ensure maximum removal of metal-cations from the DNA backbones. The concentrations of the dialysed stocks were determined by measuring the UV absorption at 260 nm.

10. Confirmation of the absence of aggregation of DNA-PEG complex in ultrapure water

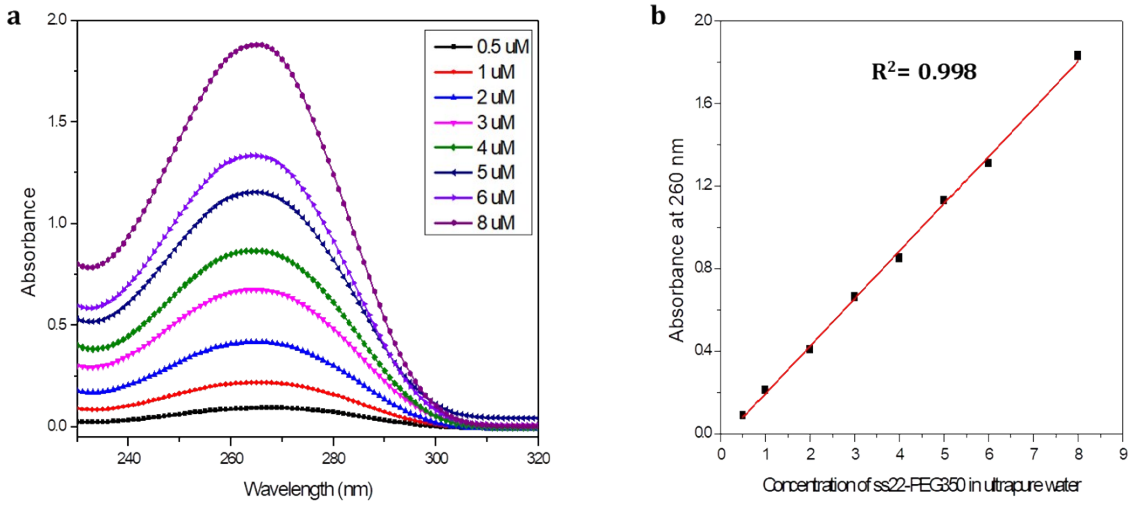
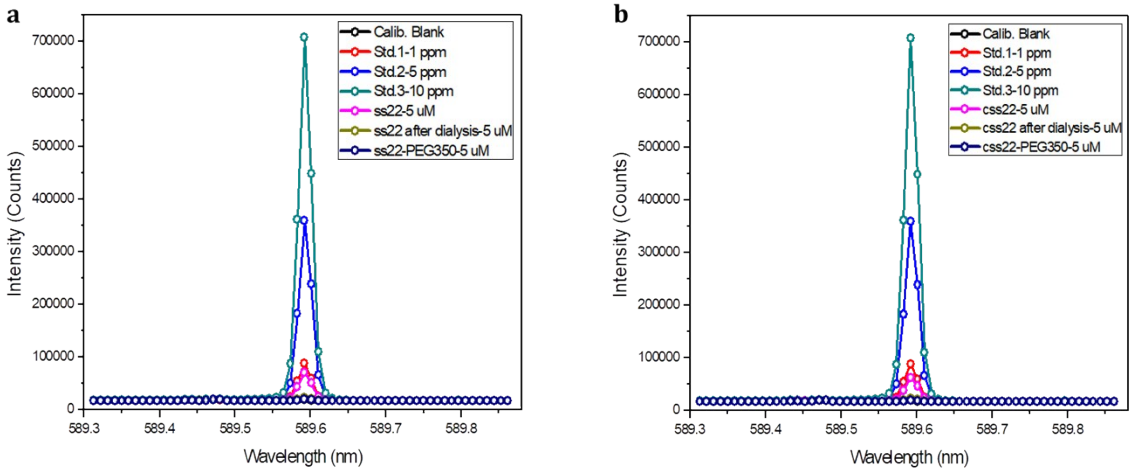


Figure S20. a) Absorbance spectra of ss22-PEG350 in ultrapure water. b) Linear correlation plot of absorbance at 260 nm vs concentration of ss22-PEG350 in ultrapure water.

11. Determination of Na^+, K^+ and Mg^{2+} in DNA and DNA-PEG complex solutions



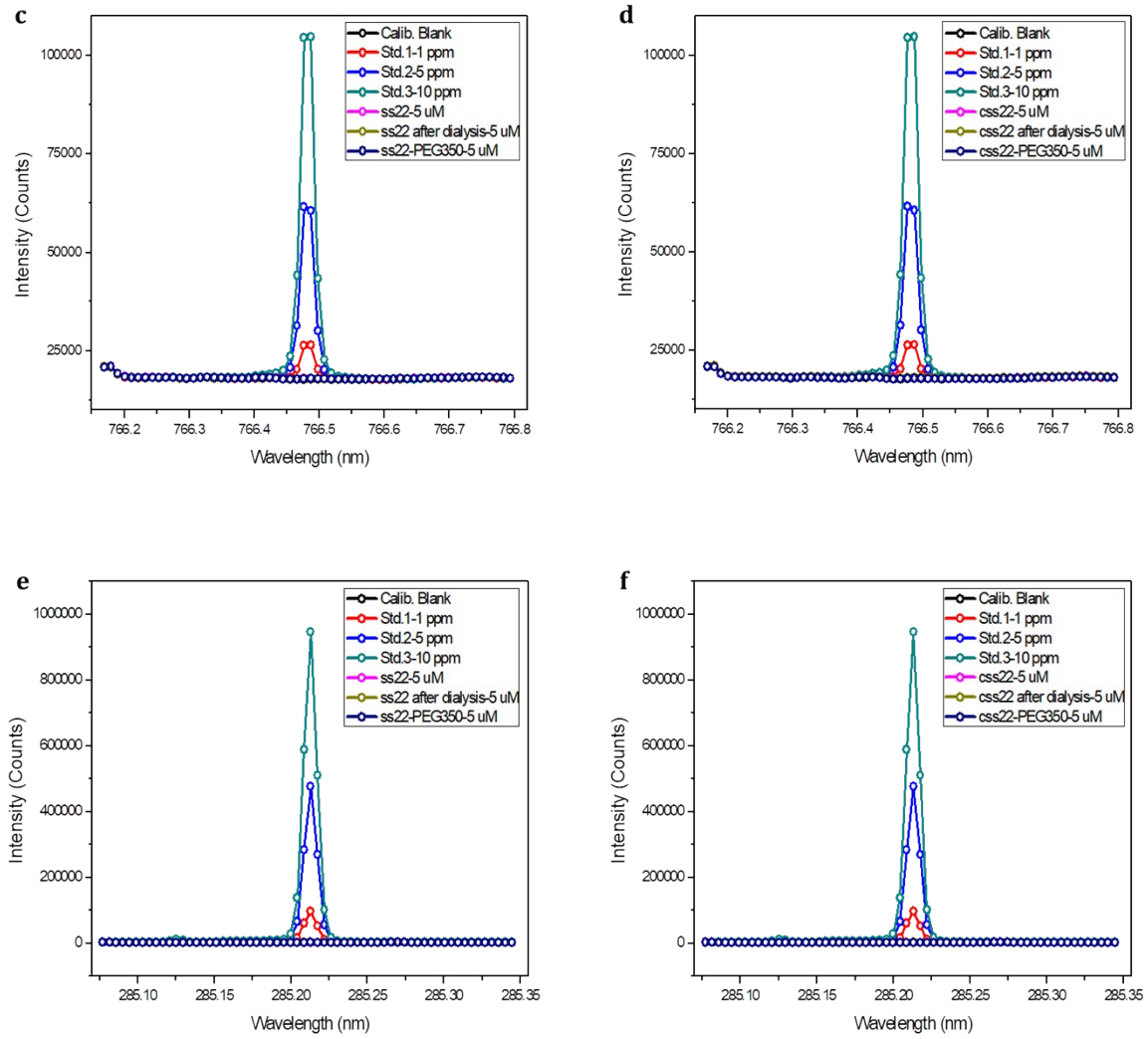


Figure S21. ICP-OES spectra of unmodified and PEG-modified ss22 and css22 at 5 µM, for sodium, Na (a-b), for potassium, K (c-d) and magnesium, Mg (e-f).

Table S1. ICP-OES analysis of non-PEGylated and PEGylated ss22 and css22.

Sl. No.	Sample	Sodium (mg/L)	Potassium (mg/L)	Magnesium (mg/L)
1.	5 µM ss22	4.5	0.2	0.05
2.	5 µM ss22 after dialysis	0.5	0.2	0.06
3.	5 µM ss22-PEG350	0.2	0.2	0.05
4.	5 µM css22	4	0.2	< 0.05

5.	5 µM css22 after dialysis	0.4	0.2	0.06
6.	5 µM css22-PEG350	0.1	0.2	< 0.05
7.	Ultrapure water	< 0.1	< 0.1	< 0.05

Yttrium (Y) standard 1000 mg/L (PerkinElmer, N9303810) was used for internal standard and ICP multi-element standard solution IV 1000 mg/L (No. 1.11355.0100, Merck KGaA, Germany) was used to prepare the standards 1 to 3. The standards and samples were prepared in 2 % nitric acid solution. The samples were diluted by 6-fold with 2 % nitric acid solution. Double-distilled water was used for the preparation of 2 % nitric acid solution. The calibration blank is the 0 mg/L standard and the most sensitive wavelength was used for the analysis.

Table S2. ICP-MS analysis of non-PEGylated and PEGylated ss22 and css22.

Sl. No.	Sample	Sodium (mg/L)	Potassium (mg/L)	Magnesium (mg/L)
1.	5 µM ss22	4	< 0.1	< 0.05
2.	5 µM ss22 after dialysis	0.7	< 0.1	< 0.05
3.	5 µM ss22-PEG350	0.1	< 0.1	< 0.05
4.	5 µM css22	4	< 0.1	< 0.05
5.	5 µM css22 after dialysis	0.5	< 0.1	< 0.05
6.	5 µM css22-PEG350	< 0.1	< 0.1	< 0.05
7.	Ultrapure water	< 0.1	< 0.1	< 0.05

Yttrium ICP standard 1000 mg/L (Merck 1.70368.0100) was used for internal standard in ICP-MS measurements. Sodium 10,000 ppm (VWR, Prod: 457132T), potassium 10,000 ppm (VWR, Prod: 457122R) and magnesium 10,000 ppm (VWR, Prod: 457082D) plasma emission standards were used for the preparation of different standard solutions. Each sample was measured against six different standards of sodium, potassium and magnesium (0.2 mg/L, 0.4 mg/L, 0.8 mg/L, 1.2 mg/L, 1.6 mg/L and 2 mg/L). 1 % nitric acid solution was used for the dilution of Yttrium standard and ultrapure water was used for the preparation of other standards.

12. Procedure for the preparation of hybridized structures

The dsDNA structures were formed from their corresponding ssDNA molecules. The reaction mixtures containing complementary DNA strands in ultrapure water or buffer and salts (for samples containing salts) were heated to 90 ℃ for 10 minutes and cooled to 25 ℃ with a rate of 2 ℃/min using a Mastercycler (Eppendorf). Subsequently, the samples were kept at 4 ℃.

13. Circular dichroism spectra of DNA and DNA-PEG complexes

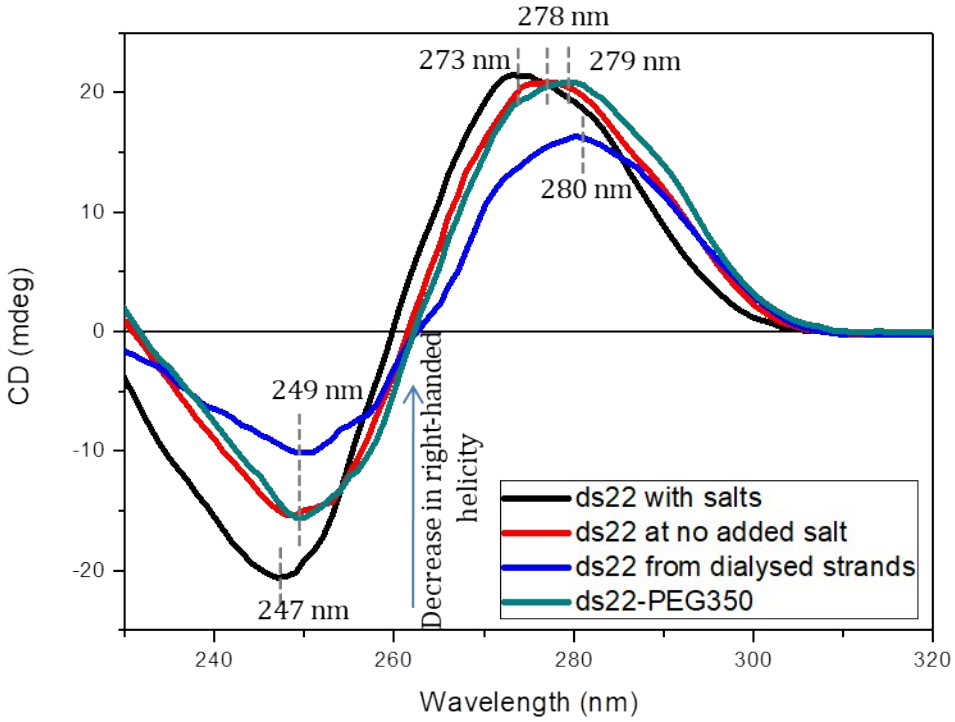


Figure S22. CD spectra of 5 μM ds22 at different salt conditions and ds22-PEG350 complex without salt in ultrapure water.

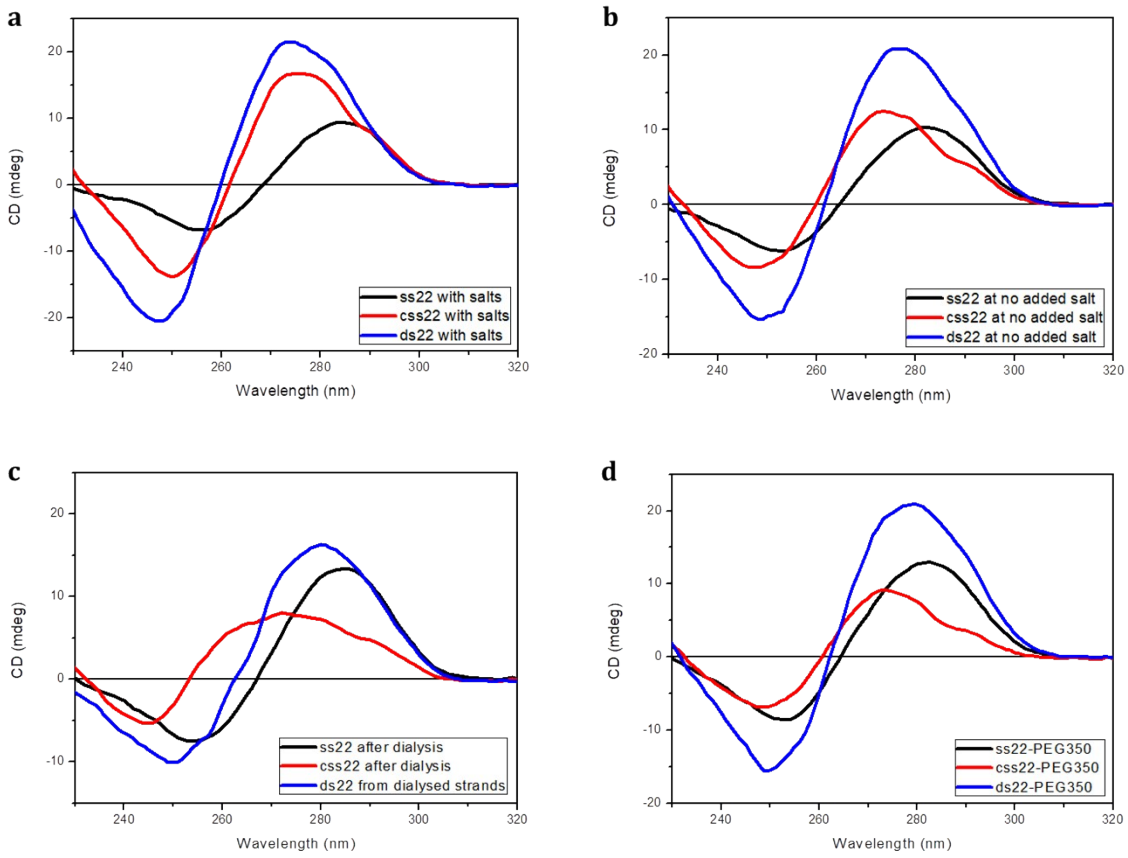


Figure S23. CD spectra of unmodified (a-c) and PEG-modified (d) ssDNA and dsDNA oligonucleotides at 5 μM.

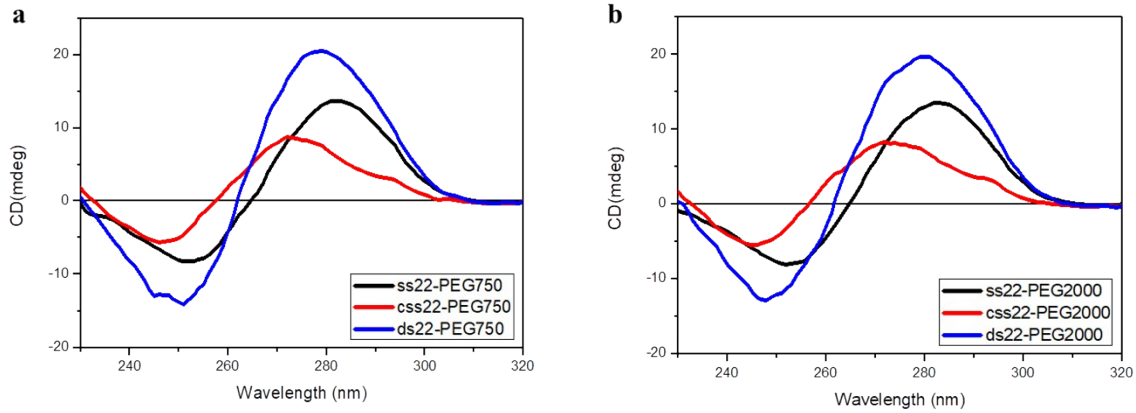


Figure S24. CD spectra of 5 μM ssDNA and dsDNA enveloped with a) PEG750 and b) PEG2000.

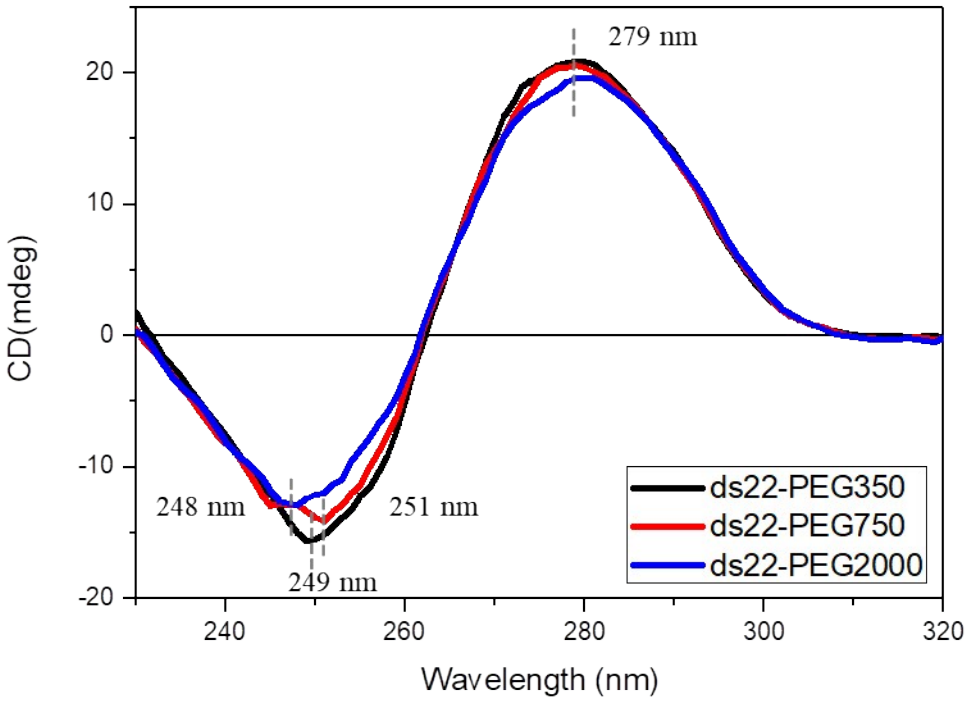


Figure S25. CD spectra of different PEGylated DNA double helices at 5 μM.

The final reaction mixture used for CD measurements was composed of 5 μM of the oligonucleotide in ultrapure water or 20 mM Tris-HCl (pH 7.5), 100 mM NaCl and 1.2 mM $MgCl_2$.

14. Small-angle X-ray scattering profiles of DNA and DNA-PEG complexes

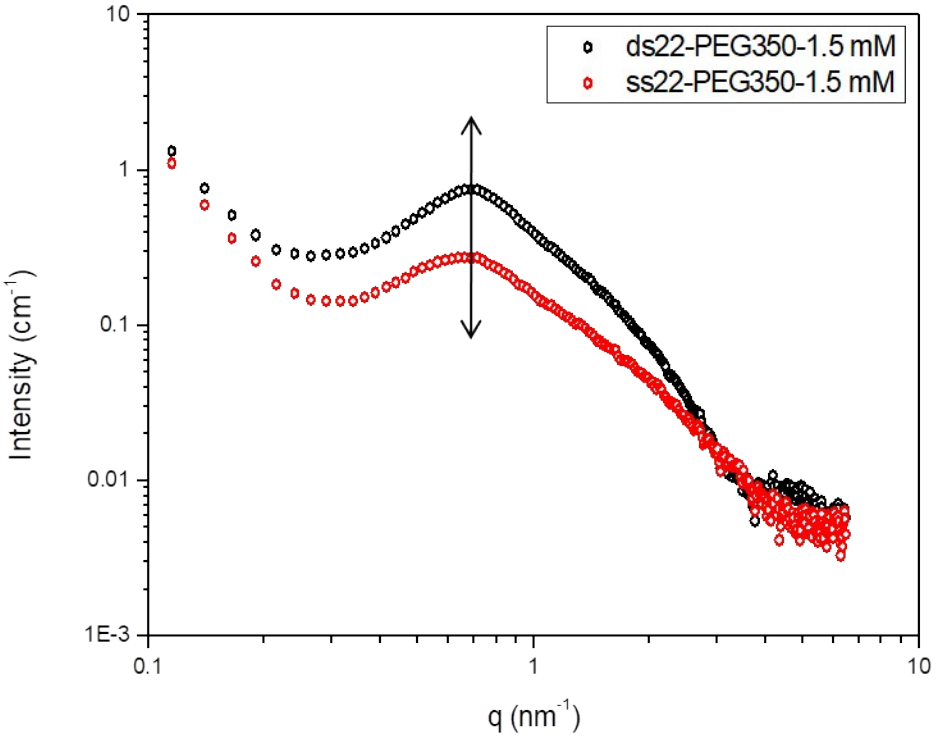


Figure S26. SAXS profiles of ss22-PEG350 and ds22-PEG350 without salt in ultrapure water.

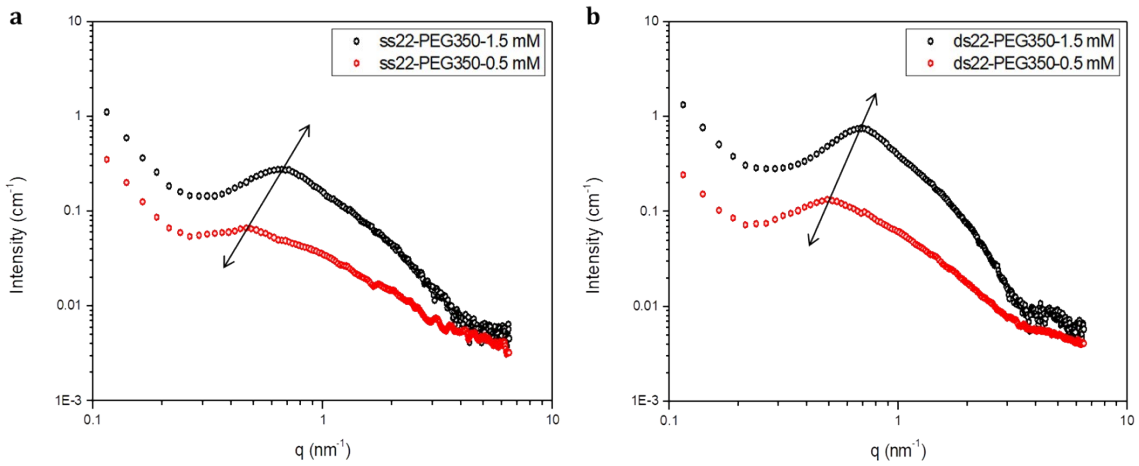


Figure S27. SAXS profiles of PEGylated oligonucleotides without salt in ultrapure water.

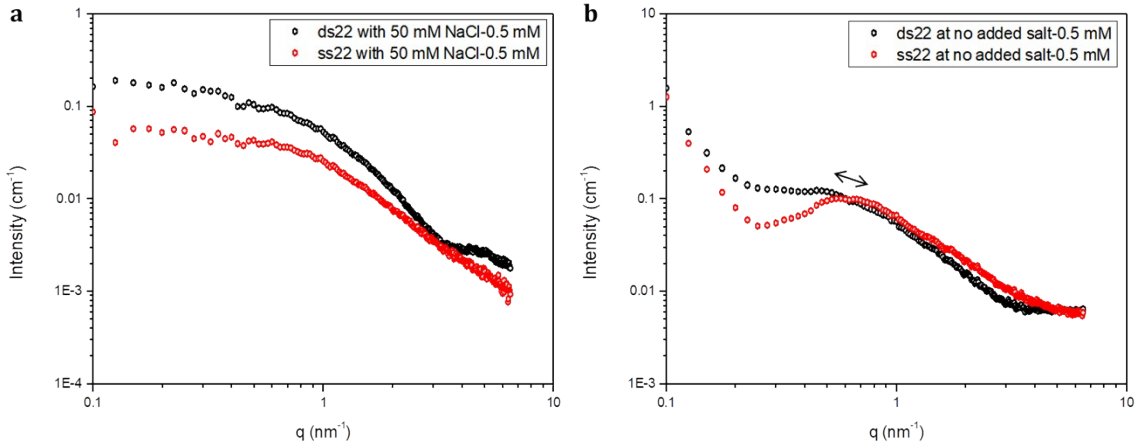


Figure S28. SAXS profiles of ss22 and ds22 at a) 50 mM NaCl and b) "no added salt" condition.

The final reaction mixture used for SAXS measurements was composed of 0.5 or 1.5 mM of the oligonucleotide in ultrapure water or 10 mM Tris-HCl (pH 7.5), 1 M (high concentrations) or 50 mM (low concentrations) NaCl and 20 mM $MgCl_2$. 30 µl of solutions were placed inside 1.5 mm Mark-tubes made of borosilicate glass (Hilgenberg GmbH). The capillary end was flame-sealed to avoid solvent evaporation over time. Before performing radial integration, images were normalized by their exposure time, sample transmission and background subtraction was performed. The scattering intensity from the capillary plus the buffer with the proper salt quantity was used as a background for each sample. The corrected images were analyzed using Fit2D and a Matlab code and the curves are finally reported as scattering intensity I(q) vs the modulus of the scattering vector $q = (4\pi/\lambda)sin\theta$. The two curves acquired at different S-to-D were finally merged using Matlab. Calibration to the absolute scale in cm^{-1} was achieved by the secondary standard method, using water as intensity standard.[2]

15. DNA thermal stability

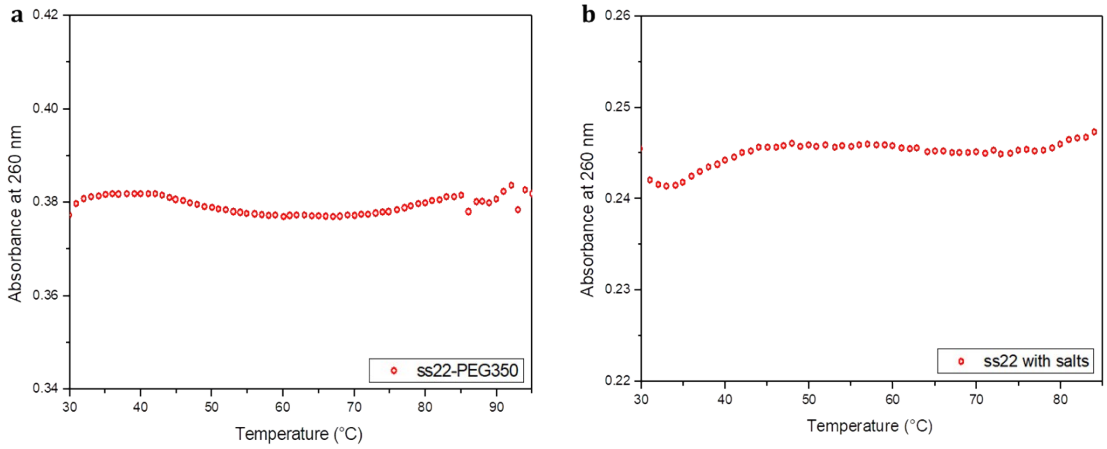


Figure S29. Melting profiles of a) ss22-PEG350 without salt and b) ss22 with salts in aqueous medium.

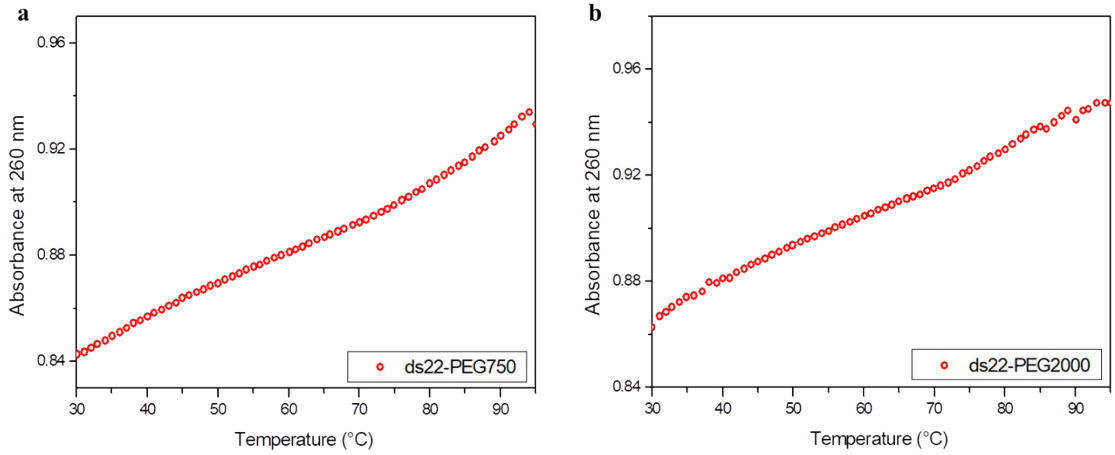


Figure S30. Melting curves of a) ds22-PEG750 and b) ds22-PEG2000 without salt in ultrapure water.

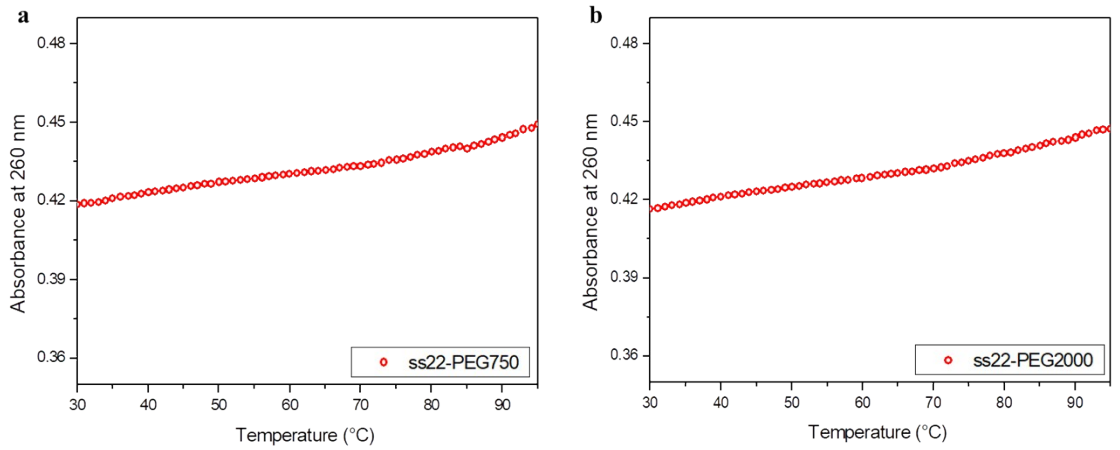


Figure S31. Melting profiles of a) ss22-PEG750 and b) ss22-PEG2000 without salt in ultrapure water.

The denaturation curves for both the PEGylated and non-PEGylated DNA were obtained at 260 nm at a heating rate of 1 °C/min and 1 °C interval. The final reaction mixture used for T_m measurements was composed of 2 μM of the oligonucleotide in ultrapure water or 20 mM Tris-HCl (pH 7.5), 100 mM NaCl and 10 mM $MgCl_2$.

16. Effect of salt on DNA-PEG complexes

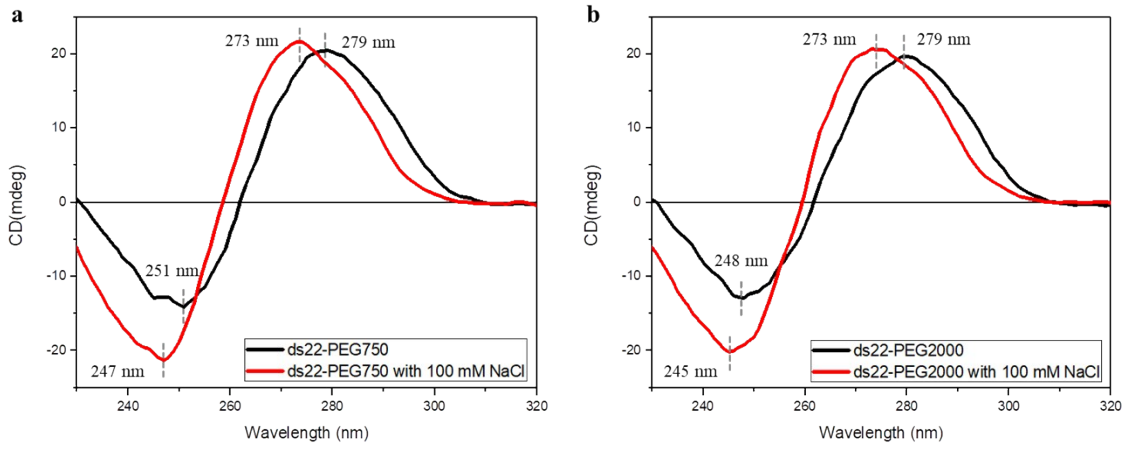


Figure S32. CD spectra of 5 μM a) ds22-PEG750 and b) ds22-PEG2000 without salt and with 100 mM NaCl in ultrapure water.

The final reaction mixture used to record the above CD spectra was composed of 5 μM of the oligonucleotide in ultrapure water or 100 mM NaCl.

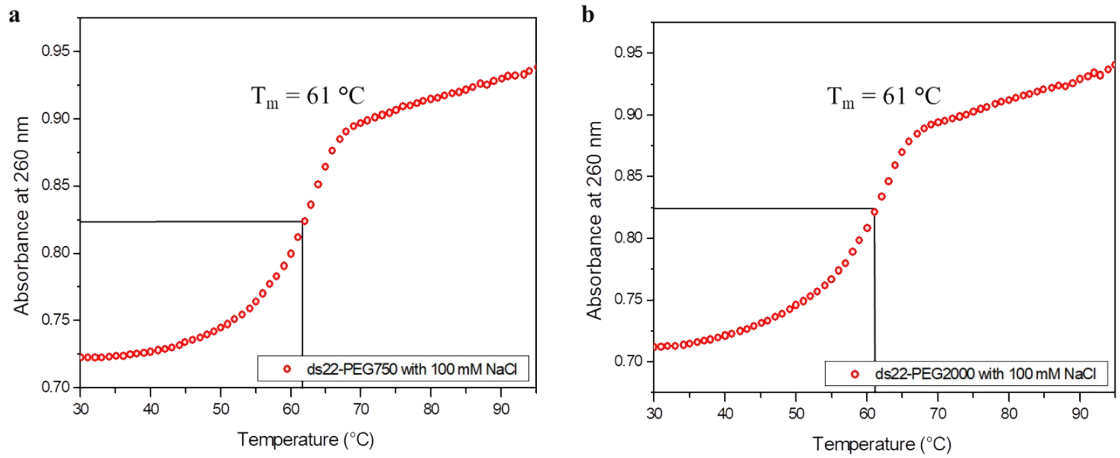


Figure S33. Thermal denaturation profiles of a) ds22-PEG750 and b) ds22-PEG2000 with 100 mM NaCl in ultrapure water.

The final reaction mixture used to obtain the above melting curves was composed of 2 μM of the oligonucleotide and 100 mM NaCl added in ultrapure water. The denaturation profiles were obtained at 260 nm at a heating rate of 1 °C/min and 1 °C interval.

17. References

1. W. Chen, J. Y. Gerasimov, P. Zhao, K. Liu and A. Herrmann, *J. Am. Chem. Soc.*, 2015, **137**, 12884-12889.

2. D. Orthaber, A. Bergmann and O. Glatter, *J. Appl. Cryst.*, 2000, **33**, 218-225.