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DNA with zwitterionic and negatively charged phosphate modifications: formation of DNA triplexes, duplexes and cell permeability studies

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Abstract

Two phosphate modifications were introduced into the DNA backbone using Staudinger reaction between the 3',5'-dinucleoside β-cyanoethyl phosphite triester formed during DNA synthesis and the sulfonyl azides, 4-(azidosulfonyl)-*N*,*N*,*N*-trimethylbutan-1-aminium iodide (N+ azide) or *p*-toluenesulfonyl (tosyl or Ts) azide, to provide either a zwitterionic phosphoramidate with N+ modification or a negatively charged phosphoramidate for Ts- modification in the DNA sequence. Incorporation of these N+ and Ts- modifications led to the formation of thermally stable parallel DNA triplexes, regardless of the number of modifications incorporated into the oligodeoxynucleotides (ONs). For both N+ and Ts- modified ONs, the antiparallel duplexes formed with complementary RNA were more stable than those formed with complementary DNA (except for ONs where the modification is in the middle of the sequence). Incorporation of N+ modifications led to the formation of duplexes whose thermal stability was less dependent on ionic strength than native DNA duplexes. Thermodynamic analysis of melting curves revealed that it is a reduction in unfavourable entropy, despite the decrease in favourable enthalpy, which is responsible for the stabilisation of duplexes with N+ modification.

N+ ONs also demonstrated greater resistance to nuclease digestion by snake venom phosphodiesterase I than the corresponding Ts-ONs. Cell permeability studies showed that Ts- ONs diffuse into the nucleus of mouse fibroblast NIH3T3 cells without the need

for transfection reagents. In contrast, N+ ONs were concentrated in vesicles within the cytoplasm.

These results indicate that both N+ and Ts- modified ONs are promising for various *in vivo* applications.

Keywords: Cell permeability, Charge neutral modification, DNA, modified phosphates, Staudinger reaction.

Introduction

The ability to detect changes in or modify the genome of living organisms is important for the diagnosis, prevention and treatment of many diseases.[1] The site-specific targeting and manipulation of genomic DNA or RNA using chemically modified short oligodeoxynucleotides (ONs) is considered to be a viable therapeutic strategy.[2-5] Anti-gene strategies use ONs to specifically bind native DNA and induce genomic changes that interfere with gene expression. Unlike the strategies that use modular enzymes such as zinc-finger nucleases[6] or transcription activator-like effector nucleases (TALENs)[7] to recognise and cut DNA sequences, or CRISPR-CAS9[8-10] and CAS9-constructs[11-14] that rely on large proteins to open the target duplex, triplex-forming oligonucleotides (TFOs)[15] can be designed to bind sequence-specifically to double-stranded DNA (dsDNA).[16] A polypyrimidine TFO binds to dsDNA through Hoogsteen base-pairing[17] to form a parallel triple-helix structure where the cytosine bases in the TFO are protonated at the N3 atom (Fig. 1B).

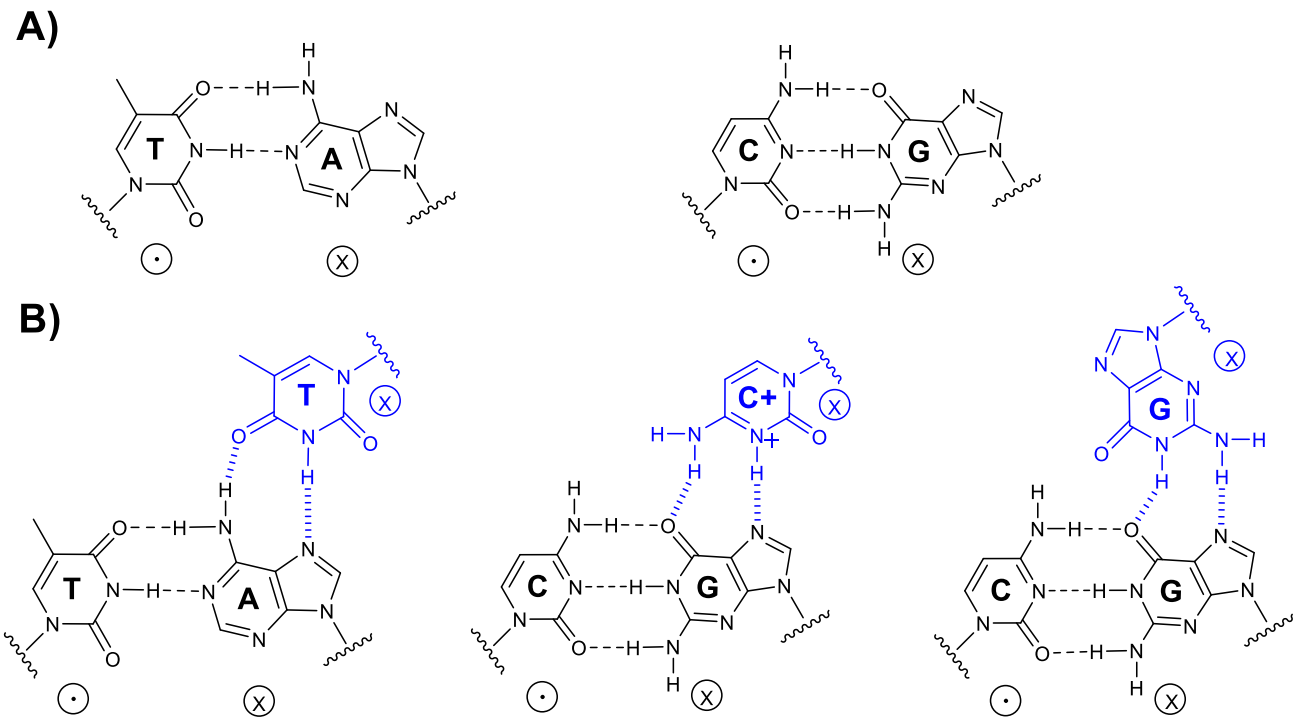


Figure 1. Illustration of H-bonding in a DNA duplex and a parallel triplex. A) Depiction of Watson-Crick base-paring (left: T-A and right: C-G); B) Parallel triple helices: Pyrimidine-rich third strand interactions are stabilised by Hoogsteen hydrogen bonds (the duplex is in black, TFO is in blue, Watson-Crick base-paring is shown with dashed bonds, and Hoogsteen base-paring is shown with hashed bonds) The relative orientation of phosphodiester backbones is indicated by the symbols "⊗" and "⊙".

Various strategies have been developed for targeting genomic sites, including triplex formation (Fig. 2, a)[18] and triplex invasion (Fig. 2, b),[19-21] which rely on designing TFO probes to form stable triplexes with dsDNA. In strand invasion (Fig. 2, c)[22] and double duplex invasion (Fig. 2, d) strategies,[23] chemically modified ONs invade into the duplex and form H-bonds with complementary DNA via Watson-Crick base-pairing.

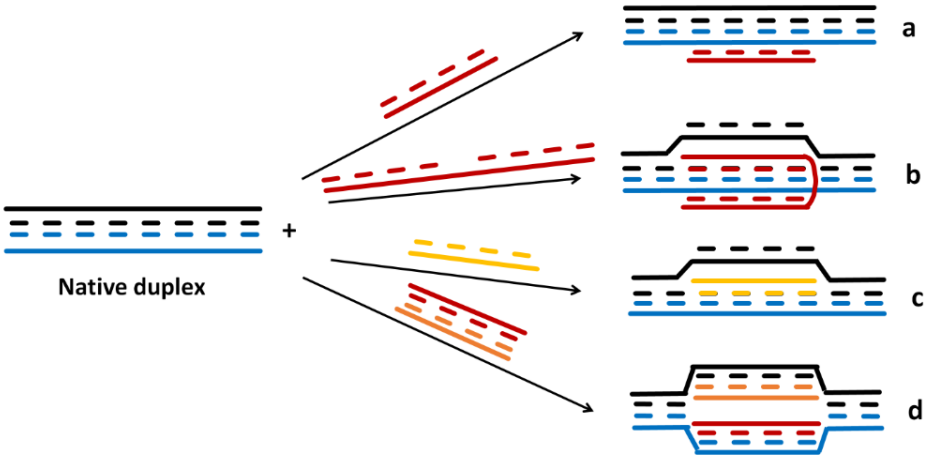


Figure 2. dsDNA targeting strategies by modified nucleic acids**.** a) Triplex formation by TFO; b) Triplex invasion: the two modified ON strands clamp onto the complementary DNA strand, leaving the non-complementary strand displaced as a loop; c) Strand invasion: ssDNA invades the dsDNA target forming Watson-Crick hydrogen bonds, leaving the non-complementary strand as a loop; d) Double duplex invasion: modified duplex invades into dsDNA, occupying both target strands via Watson-Crick base pairing.

In antisense strategies, antisense ONs (AOs) interact with RNA molecules to interfere with protein expression.[24] The major challenge in designing chemically modified ONs as anti-gene/anti-sense agents is to ensure efficient cellular uptake and nuclease resistance while still maintaining or ideally, increasing the binding affinity and specificity of the ONs towards their DNA or RNA target.

Many synthetic analogues of natural ONs have been evaluated for antigene/antisense applications, however, none have met all the requirements. For example, both peptide nucleic acids (PNA, Fig. 3),[25] and modified PNAs have excellent chemical stability, are resistant to enzymatic degradation and have a high binding affinity towards complementary DNA and RNA, but they have a tendency to aggregate, require high salt conditions and have low solubility in water.[1, 23, 26] Locked nucleic acids[27] (LNA, also known as bridged nucleic acid (BNA),[28] Fig. 3) have enhanced thermal stability in DNA triplexes and duplexes, high binding affinity to RNA and are nuclease resistant.[26, 28-30]

These properties have led to LNA (BNA) being used in various therapeutic ONs that have reached clinical trials.[31] However, the multi-step synthesis of LNA and increased hepatotoxicity of some modified AOs ensure that further optimisation is required.[32] Chemical modification with a phosphorothioate linkage (PS, Fig. 3),[33-34] resulted in ONs resistant to nuclease degradation but with side effects due to nonspecific interactions with cellular components.[35]

In morpholino oligonucleotides (PMO, Fig. 3)[36-38] the phosphodiester bond is replaced by a charge neutral phosphorodiamidate linkage and the ribose replaced by a morpholino moiety. PMOs exhibit higher affinity for their target nucleic acid sequences, greater resistance to enzymatic degradation than conventional nucleic acids and extremely low toxicity. In contrast to DNA-based modified backbones, RNA-PMO hybrids are not substrates for RNase H, and thus the mRNA is not degraded. These properties were used to develop exon skipping PMOs that found their application in clinics with FDA-approved eteplirsen and golodirsen. However, despite of their nonionic nature, PMOs still have insufficient delivery into cells especially in the heart.[39]

Several intercalator-type modifications have been introduced in TFOs leading to formation of stable parallel triplexes at neutral pH.[40-51]

Modifications of the DNA/RNA phosphate backbone, especially charge neutral modifications, have gained attention in recent years because such modifications not only improve the nuclease resistance of ONs, but also enhance their affinity towards complementary DNA/RNA/dsDNA as well as their cell permeability. The lack of a negatively charged backbone also improved the binding of PNA to DNA or RNA

strands. Compared to negatively charged PNA, positively charged PNA bind more strongly to DNA and RNA at low salt concentrations (0 – 100 mM Na^+) whereas at medium to high salt concentrations (250 – 1000 mM Na^+) the trend is reversed.[52] Incorporation of a positively charged monomer into PNA, *N*-(2-aminoethyl)-D-lysine (chiral PNA monomer, Fig. 3), destabilised the PNA/PNA duplex whereas the corresponding PNA/DNA duplex was stabilised. This modification triggered the double-duplex invasion of chemically modified PNA duplexes into dsDNA with high specificity.[53]

As a charge-neutral phosphate mimic, methylphosphonate linkage (PMe, Fig. 3) has been introduced into the DNA backbone to improve the resistance to enzymatic digestion as well as DNA duplex and triplex binding affinity.[54] However, poor aqueous solubility,[55] reduced binding affinity with complementary RNA[56] and a destabilising effect on the thermal stability of G-quadruplexes[57] hinders its application. In contrast, phosphate methylated linkage (POMe, Fig. 3) marginally destabilised complementary DNA but improved sequence specificity.[58] Introduction of an internucleotide tetramethyl phosphoryl guanidine group (Tmg, Fig. 3) led to only marginal changes in the binding affinity of the ON towards complementary DNA and RNA sequences.[59] A similar phosphoryl guanidine group (PG, Fig. 3) was later introduced into G-rich DNA (dTGGGGT, TG_4T) [60-62] and the replacement of all native phosphates led to the formation of a G-quadruplex characterised by a fast association rate that was independent of ionic strength.[63] In comparison, substitution of all phosphates in TG_4T with mesyl phosphoramidate modification (μ-modification, Fig. 3) led to a less

thermally stable G-quadruplex that had a slow association rate. The μ-modification has been introduced into AOs, as an alternative to PS-ONs, and shows significantly higher nuclease resistance and a much lower cellular toxicity.[64] Introduction of a single sulfonamide RNA (SaRNA, Fig. 3) dimer into the DNA backbone led to decreased thermal stability of the duplex formed with either complementary DNA or RNA, with the ON-RNA duplex being less stable than the ON-DNA duplex.[65] Branched, charge-neutralising sleeves (BCNS, Fig. 3) incorporated on the ON backbone[66] formed self-neutralising ONs with good aqueous solubility, enhanced resistance to nucleases, low cytotoxicity and increased thermal stability in the context of 2'-OMe-RNA duplexes.

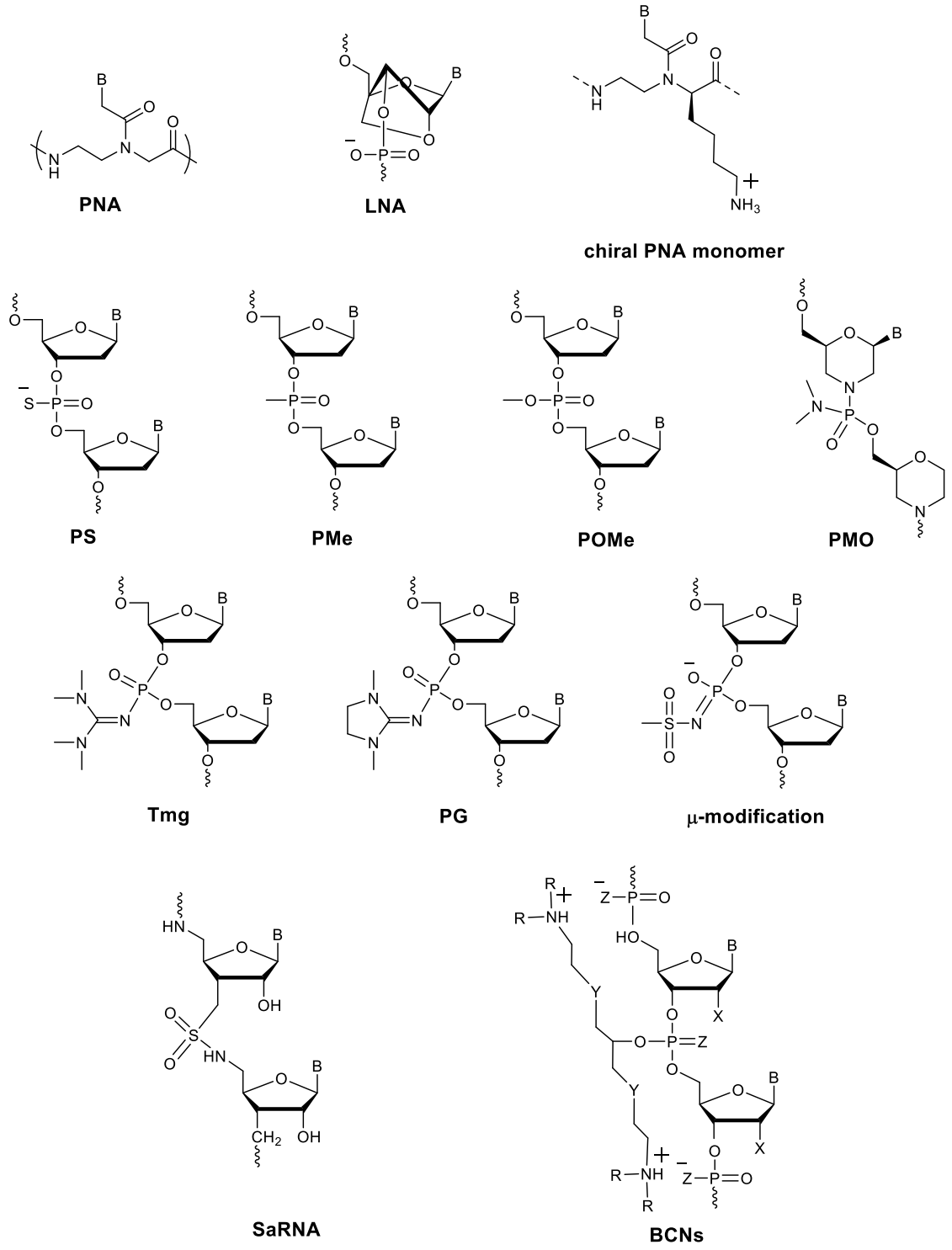
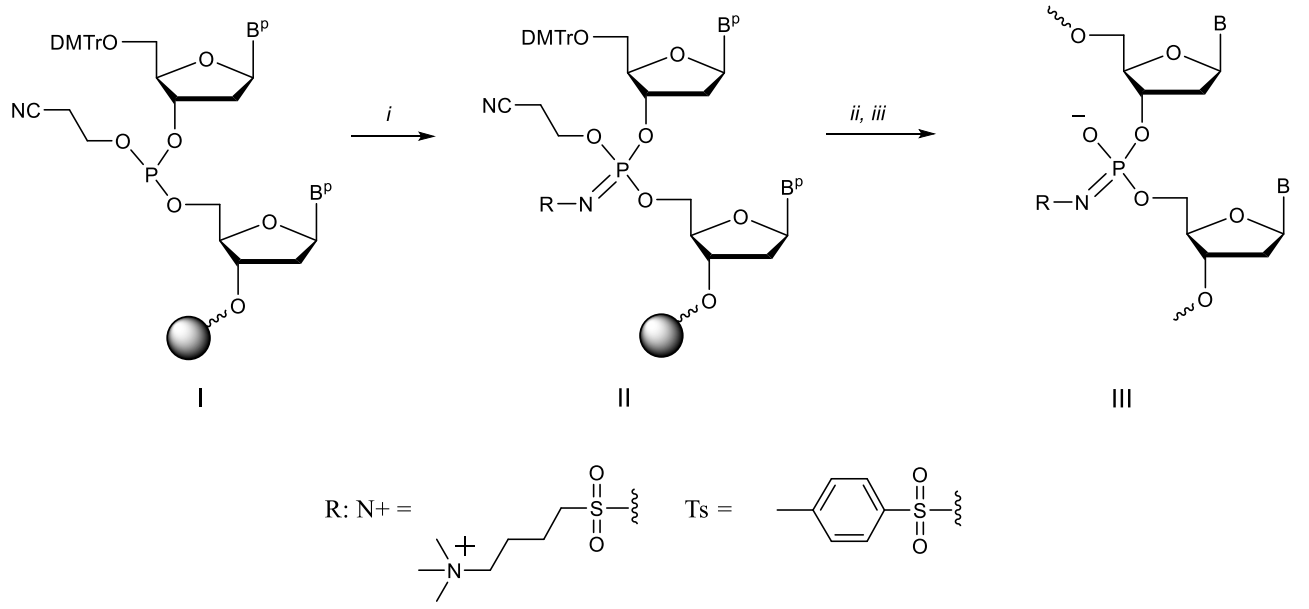


Figure 3. Structures of chemically modified ON analogues. Chiral PNA monomers were prepared by bonding a nucleobase to the α-nitrogen of *N*-(2-aminoethyl)-D-lysine. SaRNA stands for sulfonamide RNA; BCNSs stands for branched charge-neutralising sleeves, X is H or OMe, Z is O or S, Y is O or CH_2, and R is H or CH_3. B is a heterocyclic base.

Recently, we synthesised a G-rich ON with all phosphates replaced by 4-(trimethylammonio)butylsulfonyl phosphoramidate group (N+, Scheme 1) that was designed to obtain formally charge-neutral zwitterionic N+TG_4T sequence.[67] The phosphate group was modified during the phosphoramidite solid-phase ON synthesis by Staudinger reaction of 3',5'-dinucleoside β-cyanoethyl phosphites (Scheme 1, I), with the designed sulfonyl azide as a replacement of the standard iodine/pyridine oxidation step. Each negatively charged phosphoramidate is neutralised by the positively charged quaternary ammonium group, providing a zwitterionic phosphate mimic. Resistance to enzymatic degradation, higher thermal stability, and a faster association that was independent of ionic strength was observed for this N+ modified G-quadruplex $(TG_4T)_4$. These properties encouraged us to evaluate N+ modification in the context of DNA duplexes and triplexes as well as to perform cell permeability studies. For comparison, we also evaluated properties of ONs modified with tosyl sulfonyl phosphoramidate (Ts-) that results in a negatively charged phosphate mimic.[61]



Scheme 1. Synthesis of ONs with Ts- and N+ modification using Staudinger reaction during solid-phase DNA synthesis. (*i*) 0.5 M TsN_3, MeCN, 37 °C, 30 min for Ts- modification; 0.7 M 4-(azidosulfonyl)-*N*,*N*,*N*-trimethylbutan-1-aminium iodide, DMF, 37 °C, 30 min for N+ modification; (*ii*) DNA synthesis; (*iii*) conc. aq. NH_3, 55°C, 12 h; B^p/B, protected/deprotected heterocyclic base.

Introduction of each N+ or Ts- modification creates a chiral center at the phosphorus atom resulting in a mixture of 2^n diastereomers, where n is the number of modified phosphate groups. Reverse-phase (RP) HPLC purification occasionally results in the separation of individual diastereomers (usually for ONs with a single modification), which supports the insignificant difference in the lipophilicity of the diastereomeric ONs.

We hypothesised N+ONs should hybridise with complementary single-stranded DNA (ssDNA) or RNA with higher affinity than native ONs, due to both a reduced charge repulsion between negatively charged phosphates and their thermal stability being less dependent on the ionic strength of the solution. Moreover, N+ONs carrying zwitterionic phosphates could lead to increased cell permeability.

For both N+ and Ts- modifications, we synthesised 14-mer ONs with either one, two, three, or four modifications introduced in various positions in the sequence. The thermal stability of a parallel DNA triplex, and duplexes of DNA and RNA formed with these ONs was then evaluated. Thermal denaturation experiments, nuclease resistance and cell permeability assays were also conducted to evaluate these chemically modified ONs.

Results

1. Synthesis and purification of modified ONs

4-(Azidosulfonyl)-*N*,*N*,*N*-trimethylbutan-1-aminium[68] and tosyl azide (*p*-toluenesulfonyl azide, TsN_3)[61] were synthesised as previously described and used for the automated synthesis of modified ONs (using automated DNA synthesizer). The N+ and Ts- ONs were cleaved from the solid support and deprotected using 28% NH_4OH, and initially purified using reverse-phase (RP) HPLC. However, separation of ONs with varying numbers of modifications was not ideal as there were only marginal changes in the retention time in RP-HPLC. Therefore, ion-exchange (IE) HPLC was used for purifying these ONs. Substitution of each phosphate with N+ modification, resulted in a shorter retention time (τ) in IE-HPLC ($\Delta\tau$ ~ -3 min/modification, Table 1). For Ts-modified ONs, incorporation of Ts- modifications, as a result of increased hydrophobicity, led to an increased retention time ($\Delta\tau$ ~ +1.5 – 2 min/modification, Table 1) compared to the native sequence. Composition of the ONs was confirmed by electrospray ionisation-mass spectrometry (ESI-MS) in the negative mode (Table 1). For clarity, we introduced the following nomenclature of the ONs synthesised. The prefix 5'- or 3'- with either N+ or Ts means that the first phosphate at the 5' or 3' end was modified; m-N+ or m-Ts- indicates that the named modification was incorporated in the middle of the sequence; 2N+, 3N+, 4N+ or 2Ts, 3Ts, 4Ts- indicates that two, three or four modifications were distributed evenly in the sequence.

Table 1. Name of the ONs synthesised, their sequence, retention time on ion-exchange column,[a] composition, and isolated yield.

	Sequence	Retention	Calculated	Observed	Isolated
ON1	5'-CCCCTTTCTTTTTT [c]	31.53	4121.7	-	
5'-N+ON2	5'-C_{N+}CCCTTTCTTTTTT	27.76	4296.7958	4297.7588 [d]	9 [h]
m-N+ON3	5'-CCCCTTT$_{N+}$CTTTTTT	27.43	4296.7958	4297.7455 [d]	11 [h]
3'-N+ON4	5'-CCCCTTTCTTTTT$_{N+}$T	27.74	4296.7958	4297.7466 [d]	20 [i]
2N+ON5	5'-C_{N+}CCCTTTCTTTTT$_{N+}$T	23.42	4473.9012	4473.8248 [d]	8 [h]
3N+ON6	5'-C_{N+}CCCTTT$_{N+}$CTTTTT$_{N+}$T	20.77	4649.0067	4650.1412 [d]	10 [h]
4N+ON7	5'-C_{N+}CCCT$_{N+}$TTCT$_{N+}$TTTT$_{N+}$T	17.09	4826.0965	4826.0516 [d]	23 [i]
4N+{FAM}	5'-C_{N+}CCCT$_{N+}$TTCT$_{N+}$TTTT$_{N+}$T{FAM}	- [j]	5396.4012	5396.1460 [e]	20
5'-Ts-ON8	5'-C_{Ts}CCCTTTCTTTTT	32.80	4272.7151	4274.6664 [d]	4 [h]
m-Ts-ON9	5'-CCCCTTT$_{Ts}$CTTTTTT	32.75	4272.7151	4273.4728 [d]	5 [h]
3'-Ts-	5'-CCCCTTTCTTTTT$_{Ts}$T	32.81	4272.7151	4273.4694 [e]	8 [h]
2Ts-ON11	5'-C_{Ts}CCCTTTCTTTTT$_{Ts}$T	35.67	4425.7398	4427.7401[e]	6 [h]
3Ts-ON12	5'-C_{Ts}CCCTTT$_{Ts}$CTTTTT$_{Ts}$T	36.76	4578.7646	4578.6076 [f]	30 [i]
4Ts-ON13	5'-C_{Ts}CCCT$_{Ts}$TTCT$_{Ts}$TTTT$_{Ts}$T	40.07	4731.7893	4731.7620 [g]	39 [i]
4Ts-	5'-C_{Ts}CCCT$_{Ts}$TTCT$_{Ts}$TTTT$_{Ts}$T{FAM}	- [j]	5301.2398	5302.8380 [e]	26 [j]
m-	5'-CCCCTTTCTTT$_{N+}$TTT	27.50	4296.7958	4296.7520 [d]	19 [i]
m-	5'-CCCCTTT C_{N+}TTTTTT	27.43	4296.7958	4296.7300 [d]	16 [i]
m-	5'-CCCC$_{N+}$TTTCTTTTTT	27.45	4296.7958	4296.7440 [d]	17 [i]
m-	5'-CC$_{N+}$CCTTTCTTTTTT	27.62	4296.7958	4296.7350 [d]	21 [i]
3N+ON18	5'-C_{N+}CCCTT$_{N+}$TCTTTTT$_{N+}$T	20.44	4649.0067	4649.937 [d]	17 [i]

[a] IE-HPLC was performed on IE-column (TSKgel Super Q-5PW) using a gradient of NaCl concentration (0 → 0.5 M) in 20 mM Tris-HCl, 1 mM EDTA, pH 9.0 over 30 min. [b] Based on ESI-MS in the negative mode; [c] Obtained from Integrated DNA Technologies; [d] Calculated for $[M -6H]^{6-}$; [e] for $[M -7H]^{5-}$; [f] for $[M +K^{+} -9H]^{8-}$; [g] for $[M -4H]^{4-}$. ESI-MS spectra are provided in Supporting Information.

[h] Synthesised in 1 µmol scale following procedure described previously,[61] purified by IE-HPLC followed by desalting.

[i] Synthesised in 1 µmol scale following modified procedure described previously,[68] purified by IE-HPLC followed by desalting.

[j] Synthesised in 3 – 4 µmol scale, purified by 20% denaturing PAGE (7M urea), followed by extraction from the gel and desalting.

The solubility of the ONs was not influenced by the introduction of Ts- and N+ modifications, as the purified, desalted and lyophilised ONs were fully dissolved in 50 µL H_2O. Ts-modified ONs have previously been shown to marginally destabilise duplexes with complementary DNA and RNA.[61] The chemical stability of 5'-N+ON2 at various pH (5.5, 7.0, and 8.5) was evaluated by incubation in 10 mM Na-phosphate buffer (140 mM NaCl, 0.1 mM Na_2-EDTA) at 50 °C for 24 h. No degradation was observed according to IE-HPLC analysis (see Fig. S15 in the Supporting Information, SI), which ensures that N+ modified ONs will be chemically stable during evaluation of thermal stability of complexes with complementary DNA and RNA.

Thermal denaturation experiments

Thermal stability of antiparallel ON/RNA and ON/DNA duplexes as well as parallel DNA triplexes was assessed in thermal denaturation experiments and the results are summarised in Table 2.

Table 2. T_m [°C, ± 0.5 °C] Data for triplex and duplex melting, taken from UV melting curves (λ = 260 nm).

Entry		Antiparallel duplex			Triplex[c]	
		RNA[a]		DNA[b]		
		pH 7.0	pH 5.0	pH 7.0	pH 5.0[d]	pH 6.0
1	**ON1**	46	48	50	45	28
2	**5'-N+ON2**	53 (+ 7.0)	44 (- 4.0)	51 (+ 1.0)	40 (- 5.0)	25 (- 3.0)
3	**m-N+ON3**	47 (+ 1.0)	43 (- 5.0)	48 (- 2.0)	55 (+ 10.0)	28
4	**3'-N+ON4**	58 (+ 12.0)	46 (- 2.0)	52 (+ 2.0)	56 (+ 11.0)	29 (+ 1.0)
5	**2N+ON5**	53 (+ 7.0)	44 (- 4.0)	52 (+ 2.0)	56 (+ 11.0)	28
6	**3N+ON6**	41 (- 5.0)	45 (- 3.0)	51 (+ 1.0)	48 (+ 3.0)	< 15
7	**4N+ON7**	55 (+ 9.0)	48 (0.0)	51 (+ 1.0)	48 (+ 3.0)	28
8	**5'-Ts-ON8**	54 (+ 8.0)	39 (- 9.0)	46 (- 4.0)	51 (+ 6.0)	24 (- 4.0)
9	**m-Ts-ON9**	44 (- 2.0)	31 (- 17.0)	37 (- 13.0)	51 (+ 6.0)	< 15
10	**3'-Ts-ON10**	57 (+ 11.0)	44 (- 4.0)	49 (- 1.0)	54 (+ 9.0)	27 (- 1.0)
11	**2Ts-ON11**	56 (+ 10.0)	43 (- 5.0)	51 (+ 1.0)	53 (+ 8.0)	25 (- 2.0)
12	**3Ts-ON12**	38 (- 8.0)	40 (- 8.0)	45 (- 5.0)	49 (+ 4.0)	< 15
13	**4Ts-ON13**	53 (+ 7.0)	39 (- 9.0)	44 (- 6.0)	47 (+ 2.0)	20 (- 8.0)
14	**m-N+ON14**	54 (+ 8.0)	-[f]	51 (+ 1.0)	-	-
15	**m-N+ON15**	56 (+ 10.0)	-	51 (+ 1.0)	-	-
16	**m-N+ON16**	54 (+ 8.0)	-	55 (+ 5.0)	-	-
17	**m-N+ON17**	56 (+ 10.0)	-	52 (+ 2.0)	-	-
18	**3N+ON18**	54 (+ 8.0)	-	51 (+ 1.0)	-	-

[a] RNA sequence for antiparallel duplex formation is **ON19**: 3'-rGGGGAAAGAAAAAA; C = 1.0 µM of each strand in 20 mM sodium cacodylate, 100 mM NaCl, 10 mM $MgCl_2$, pH 7.0. T_m values for ON/RNA duplexes were confirmed by CD melting experiments (Fig. S6, S7 and Table S2 in SI).

[b] DNA sequence for antiparallel duplex formation is **ON20**: 3'-GGGGAAAGAAAAAA; C = 1.0 µM of each strand in 20 mM sodium cacodylate, 100 mM NaCl, 10 mM $MgCl_2$, pH 5.0 and pH 7.0.

[c] C = 1.5 µM of **ON1—13** and 1.0 µM of each strand of dsDNA (**D1**:3'-CTGCCCCTTTCTTTTTT/ 5'-GACGGGGAAGAAAAAA) in 20 mM sodium cacodylate, 100 mM NaCl, 10 mM $MgCl_2$, pH 5.0, 6.0 and 7.0; duplex T_m = 56.5 °C (pH 5.0), 58.5 °C (pH 6.0) and 57.0 °C (pH 7.0). Triplex formation was confirmed by size-exclusion HPLC (SE-HPLC) in sodium cacodylate buffer (pH 5.0 and pH 6.0, Fig. S14 in SI), no triplex was formed at pH 7.0.

[d] T_m for triplex melting was determined by subtraction of duplex melting curve from overlaid melting curve (Fig. S5 in SI).

[f] not performed

Sequences possessing a different number of N+ and Ts- modifications were studied initially in an antiparallel duplex formed with complementary RNA and compared with the corresponding antiparallel DNA duplexes at pH 7.0. Apart from ONs possessing modifications in the middle of the sequence (entries 6, 9 and 12 in Table 2), stabilised ON/RNA duplexes were obtained for both N+ONs (ΔT_m = +1 – +12 °C, entries 2 – 7, Table 2) and Ts-ONs (ΔT_m = +7 – +11 °C, entries 8 – 13, Table 2). The highest thermal stabilisation against RNA induced by a single modification was observed for ONs with one modification at 3'- end (ΔT_m = +12 °C and +11 °C for N+ and Ts- modifications, respectively). Corresponding antiparallel DNA duplexes were less thermally stable with ΔT_m = -1 – +2 °C. The same trend was seen for ONs with a modification at both the 5'- and 3'- ends: ON/RNA duplexes were more stable (ΔT_m = +7 °C for **2N+ON5** and ΔT_m = +10 °C for **2TS-ON11**) than corresponding antiparallel DNA duplexes (ΔT_m = +2 °C for **2N+ON5** and ΔT_m = +1 °C for **2TS-ON11**). Thermal stability of ON/RNA duplexes was not improved by increasing the number of N+ or Ts- modifications in the ONs.

For Watson-Crick type duplexes, both the N+ and Ts- modifications destablised the DNA duplex at pH 5.0. The destablising effect was more pronounced for Ts- than for the N+ modification (ΔT_m = -17 – -4 °C for Ts- and -5 – 0 °C for N+ modifications, respectively). For antiparallel ON/DNA duplexes formed at pH 7.0, when comparing ONs with the same number of modifications, N+ modifications led to higher T_m values than Ts-ONs. Incorporation of three and four Ts- moieties led to a further decrease in T_m whereas corresponding N+ ONs did not disrupt duplex thermal stability. These results indicate that the N+ and Ts- modifications can be viewed as RNA-like

modifications, because their use in ONs led to higher ΔT_m values for ON/RNA than for ON/DNA duplexes.

The same modified sequences (**ON1** – **13**) were also studied in a pH-dependent Hoogsteen-type base pairing towards the duplex **D1** forming a parallel triplex[69] . As can be seen in Table 2, all parallel triplexes formed at pH 5.0 were more thermally stable than at pH 6.0 and no triplex was formed at pH 7.0, which is consistent with the trend for parallel triplexes based on CT-TFOs.[70] Some fluctuations were observed for Hoogsteen-type triplexes formed by N+ONs. Modification at 5' end destabilised triplexes at both pH 5.0 and 6.0 (ΔT_m = -5 °C and -3°C, respectively, Table 2). All other N+ONs formed more stable triplexes with **D1** at pH 5.0, while marginal changes were observed for triplexes at pH 6.0 except for **3N+ON6** with three modifications that did not form a triplex. Incorporation of Ts- modifications led to stabilised Hoogsteen-type triplexes at pH 5.0 (ΔT_m = +2 – +9 °C, Table 2), whereas triplexes at pH 6.0 were less stable (ΔT_m = -1 – -8 °C). For Ts-ONs with the modification in the center of the sequence (**m-Ts-ON9** and **3Ts-ON12**), no triplex formation was observed at pH 6.0/room temperature. These results show that Hoogsteen-type triplexes with single N+ or Ts modifications at the 3'end are more thermally stable at the 5' end, and that increasing the number of modifications showed no advantage for T_m of parallel triplexes.

A position-dependent influence of the N+ and Ts- moieties on T_m is suggested by the less thermally stable duplexes formed by the ONs with a single modification in the middle of the sequence (in TC motif) compared to native DNA. We synthesised another set of N+ONs, with single (**ON14 – 17**, entries 14 – 17, Table 2) and triple modifications

(**ON18**, entry 18, Table 2) that had no modifications in the center of the sequence and evaluated the thermal stability of their antiparallel duplexes formed with complementary RNA and DNA at pH 7.0. Results in Table 2 show that **ON14 – 17** form more stable duplexes with RNA (ΔT_m = +8 – +10 °C) and DNA (ΔT_m = +1 – +5 °C). It is interesting that sequences with the N+ modification in the CT motif (**m-N+ON15** and **m-N+ON16**) did not destabilise the antiparallel duplexes unlike the N+ modification in the TC motif (**m-N+ON3** and **3N+ON6**). One possible reason for this position-dependent influence of the N+ and Ts- moieties on duplex stability might be due to a propeller twist[71] in the TC dinucleotide interfering with the N+ and Ts- moieties and destabilising the DNA and RNA duplexes.

Table 3. T_m [°C] and thermodynamic data at 298K for antiparallel duplexes at different NaCl concentrations, taken from UV melting curves (λ = 260 nm)[a]

	Antiparallel	NaCl	T_m (°C) [b]	ΔH [c] (kJ/mol)	$T\Delta S$	ΔG_{298}
ON/DNA	ON1/ON20	25	19	-430 (±20)	-400 (±20)	-30 (±28)
		50	37	-400 (±9)	-350 (±8)	-50 (±12)
		100	50	-368 (±8)	-305 (±7)	-63 (±10)
	4N+ON7/ON20	25	41 (+ 22.0)	-388 (±9)	-334 (±8)	-54 (±12)
		50	44 (+ 7.0)	-322 (±15)	-266 (±14)	-56 (±20)
		100	51 (+ 1.0)	-320 (±8)	-260 (±7)	-60 (±10)
	4Ts-ON13/ON20	25	29 (+ 10.0)	-382 (±14)	-340 (±14)	-42 (±19)
		50	34 (- 3.0)	-372 (±12)	-326 (±11)	-46 (±16)
		100	45 (- 5.0)	-354 (±7)	-297 (±7)	-57 (±10)
ON/RNA	ON1/ON19	25	44	-390 (±10)	-332 (±9)	-58 (±13)
		100	46	-306 (±17)	-248 (±17)	-58 (±24)
	4N+ON7/ON19	25	45 (+ 1.0)	-419 (±7)	-359 (±6)	-60 (±9)
		100	55 (+ 9.0)	-329 (±13)	-264 (±12)	-66 (±17)
	4Ts-ON13/ON19	25	38 (- 6.0)	-420 (±20)	-370 (±20)	-50 (±28)
		100	53 (+ 7.0)	-407 (±13)	-337 (±12)	-70 (±17)

[a] 1 µM of each strand in 20 mM sodium cacodylate buffer (pH 7.0, supplemented with 25, 50 or 100 mM NaCl, respectively).

[b] T_m values are reported with ± 0.5 °C uncertainties as determined from several experiments. Values in brackets are ΔT_m values calculated as T_m (sample) - T_m (unmodified duplex) at the same salt concentration.

[c] Thermodynamic parameters are calculated as described in SI (see also Fig. S8 – S13) at 298K, errors were calculated as described in reference[72].

Next, we evaluated the binding affinity of N+ and Ts- modified ONs for complementary DNA and RNA at different salt concentrations (25, 50 and 100 mM NaCl, Table 3). It has been reported that the thermal stability of DNA duplexes decreases as salt concentrations are reduced due to the increased electrostatic repulsion between negatively charged phosphates.[73] Native DNA duplex **ON1**/**ON20** showed a decline in T_m values from 50 to 37 °C and to 19 °C when the NaCl concentration was decreased

from 100 to 50 mM and to 25 mM, respectively (Table 3). A similar trend was observed for Ts-modified ONs as the backbone is still negatively charged. In contrast, for N+ONs the decrease in T_m with decreasing NaCl concentration was not as significant as for negatively charged ONs, and the T_m value at 25 mM salt was 20 °C higher than the T_m for the unmodified duplex, and 12 °C higher than the Ts- modified duplex. However, such behavior was not as noticeable for the duplexes formed with complementary RNA. The T_m value of the control ON/RNA duplex (**ON1/ON19**) decreased by 2 °C when the NaCl concentration reduced from 100 to 25 mM, whereas the T_m for the N+ON/RNA duplex (**4N+ON7**/**ON19**) decreased by 10 °C, although it was still more thermally stable than the control ON/RNA duplex. In contrast, the duplex formed by Ts-ON and RNA (**4Ts-ON13**/**ON19)** was destabilised (ΔT_m = -6 °C) at lowest salt concentration tested.

We analysed melting profiles and obtained thermodynamic parameters of the duplexes at different salt concentrations assuming that there is no change in the heat capacity (ΔC_p = 0).[74] As the thermal stability of DNA duplexes increased with increasing concentrations of salt, we expected a favorable ΔH of duplex formation at higher salt concentration. However, in all cases studied, ΔH was less favorable at 100 mM than at 25 mM NaCl. Recently, similar observations have been reported for DNA modified with methyl phosphotriester linkage (POMe, Figure 3) using isothermal titration calorimetry (ITC) measurements which provides ΔH values directly.[58]

For the native DNA duplex (**ON1/ON20**), the more favorable ΔH at the low salt concentration (25 mM NaCl) was deprived by an even higher entropy penalty leading to a loss in ΔG ($\Delta\Delta G_{298}$ = 33 kJ/mol), thus lowering the T_m value at 25 mM NaCl. For

the unmodified RNA duplex (**ON1/ON19**), $\Delta\Delta H$ between 25 mM and 100 mM NaCl is 84 kJ/mol, and the corresponding entopic factor $\Delta(T\Delta S)$ is 84 kJ/mol. As a result, changes in ΔG_{298} were negligible as reflected by the small decrease in the T_m value (ΔT_m = 2 °) when the salt concentration was reduced from 100 mM to 25 mM NaCl.

For N+ or Ts- modified ONs, ΔH for ON/DNA duplexes was less favorable at the same salt concentration than for the unmodified duplex whereas $T\Delta S$ was more favorable. According to Kuo *et al*.,[58] an increase in salt concentration stabilises the DNA duplex by reducing the entropy costs of duplex formation rather than by reducing the strand charge repulsion. This reduction of entropy costs for duplex formation is due to the endothermic release of DNA-hydrating ordered water molecules into the bulk solvent. Since the introduction of the N+ modification compensated for the negative charge on the DNA backbone, the change in entropy costs for N+ON/DNA between 100 mM and 25 mM NaCl solutions was less than that for the unmodified duplex ($\Delta(T\Delta S)$ = -74 kJ/mol for N+ON/DNA *versus* -95 kJ/mol for the native DNA duplex, respectively). A similar trend was seen for the Ts- modified ONs ($\Delta(T\Delta S)$ = -43 kJ/mol), but the change in ΔH between 25 mM and 100 mM NaCl was the lowest for duplexes with DNA ($\Delta\Delta H$ = 28 kJ/mol). This indicates that the hydrophobicity of Ts results in less water molecules involved in the formation of H-bonds with dsDNA. However, it does not improve the interaction between two DNA strands possibly due to the large size of Ts moiety.

For duplexes of N+ and Ts- ONs formed with complementary RNA, both ΔH and $T\Delta S$ terms were more negative at the same salt concentration than those for the unmodified ON/RNA complex, which is the opposite to duplexes formed with DNA. This led to

even larger enthalpy-entropy compensation at the medium salt concentration (100 mM NaCl). When the NaCl concentration decreased from 100 mM to 25 mM, even though the reduction of entropy costs for Ts-ON/RNA duplex was minimal for the RNA ($\Delta(T\Delta S)$ = -33 kJ/mol), the loss in ΔG for Ts-ON/RNA duplexes was larger than that of unmodified and N+ON/RNA duplexes ($\Delta\Delta G_{298}$ = 0, 6, and 20 kJ/mol for unmodified DNA/RNA, N+ON/RNA, and Ts-ON/RNA, respectively). This resulted in an unstable Ts-ON/RNA duplex at 25 mM NaCl. However, it should be noted that a significantly improved ΔH for Ts-ON/RNA duplex in comparison with the native ON/RNA at 100 mM NaCl (ΔH = -407 kJ/mol *versus* -306 kJ/mol, respectively) which is accountable for more favourable ΔG_{298} and higher T_m value.

2. Evaluation of N+ and Ts- modified ONs towards enzymatic digestion

Nuclease resistance of the modified ONs was evaluated using snake venom phosphodiesterase (phosphodiesterase I, Sigma) and compared to the unmodified sequence **ON1**. Under the conditions used in this experiment, **ON1** was completely degraded within 30 min (Fig. 4). Both N+ and Ts- modified ONs showed ~~enhanced~~ nuclease resistance when modifications were present at the 3'-end and /or in the middle of the sequence. A single N+ or Ts- modification at the 5'-end of the ON did not provide protection against phosphodiesterase I. However, resistance of the modified ONs towards phosphodiesterase increased with number of modifications present. N+ONs, with the same number of modifications, showed higher resistance to nuclease degradation than Ts-ONs. For example, 92.0 ± 1.8 % of **4N+ON7** remained intact,

whereas only 54 ± 3 % of **4TS-ON13** was intact after 120 min of enzymatic digestion (Fig. 4, see also Fig. S16 in SI). N+ONs possessing more than four modifications showed full enzymatic resistance after 120 min.[68]

A) B)

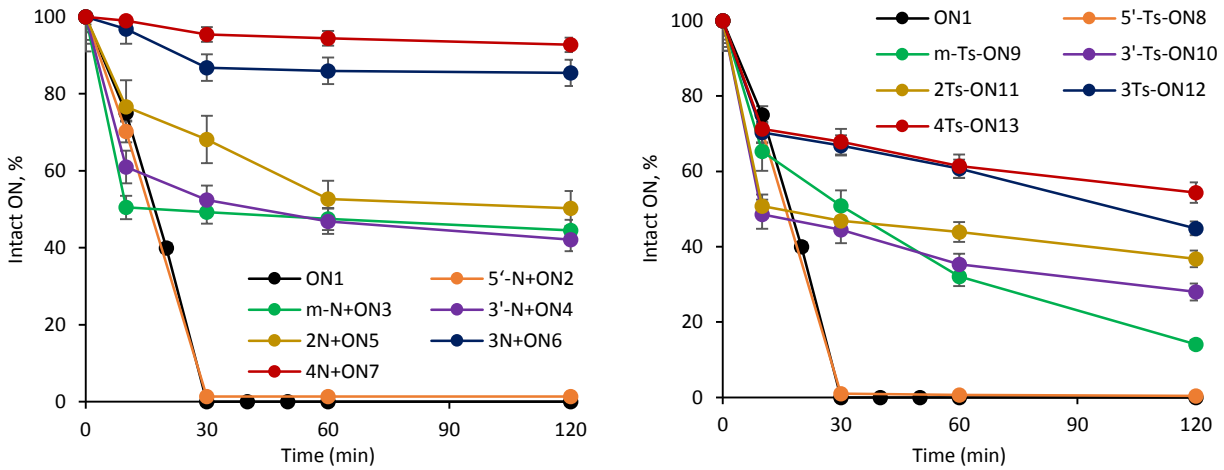


Figure 4. Percentage of intact ONs in 120 min. A) N+ONs; B) Ts-ONs. Percentage of intact ONs was determined by the ratio of full-length ONs at individual time in comparison with the sample at 0 min.

3. Cell permeability assay

The cellular uptake of two modified ONs synthesised possessing four N+ or Ts-modifications and a fluorescent label (6-FAM) at the 3'-end (Table 1, 4N+{FAM} and 4Ts-{FAM}, respectively) by NIH3T3 mouse fibroblasts was tested. The ONs were incubated with asynchronously growing NIH3T3 fibroblasts for 12 hours before the cells were processed for fluorescent confocal microscopy.

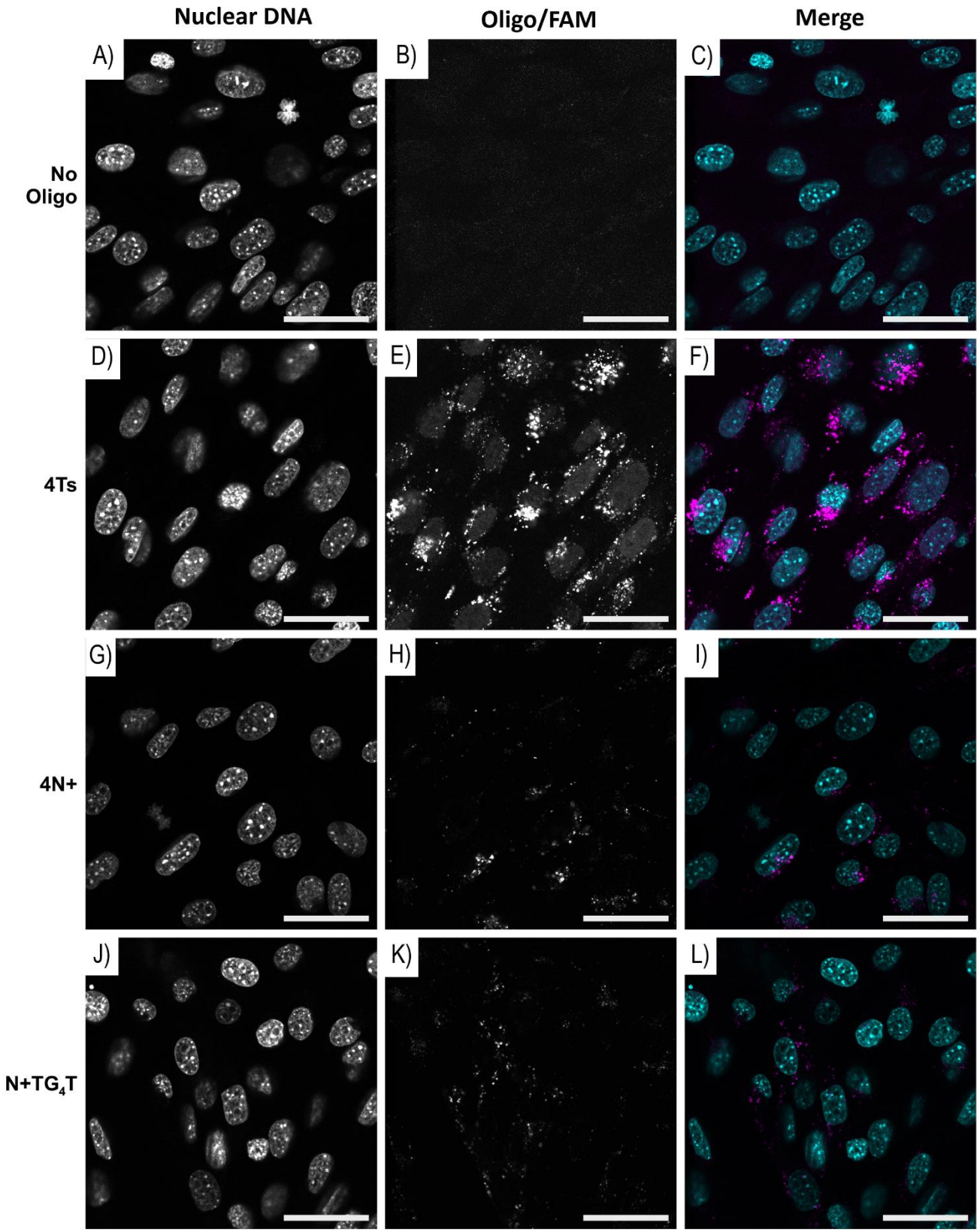


Figure 5. Representative images of mouse NIH 3T3 fibroblasts incubated with either (A-C) No Oligo or 20 μM of (D-F) 4Ts (G-I) 4N+, and (J-L) N+TG_4T FAM labelled ONs for 12 hours and then nuclear DNA stained with Hoechst 3342 and imaged with scanning confocal microscope. Individual panels are shown for each section: DNA in the nucleus stained by Hoechst 3342, Oligo/FAM, and Merge where pseudo-coloured panels are overlaid, nucleus (cyan) and ON (magenta). Scale bar: 40 μm.

Confocal sections that dissect through of the nucleus were collected. Figure 5 shows that FAM-labelled Ts- and N+ modified DNAs are concentrated in vesicles (small foci in FAM panel) that accumulate around the edge of the nucleus. Interestingly, the Ts-modified oligo has also diffused through the nucleus as indicated by the colocalisation of the ON (Fig. 5E) with the nuclear DNA (Fig. 5D). This is in contrast to the lack of colocalisation of the FAM signal with the nuclear DNA in the negative control (No Oligo) and for the N+ modified oligos (Fig. 5B, H and K, respectively). Confocal sections that dissect the nucleus were collected to ensure the FAM-ON imaged were at the edge of the nucleus and not on the cell surface. In addition, as shown in Figure 6, staining of the cell membrane confirmed that the Ts ON foci are present within the cytoplasm.

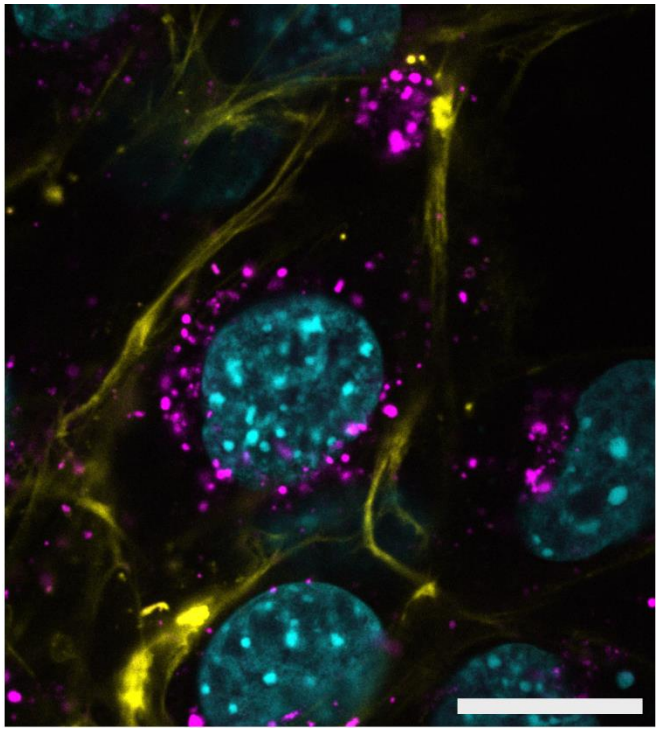


Figure 6. Representative confocal microscopy section showing the FAM vesicles within the cell membrane. Mouse NIH 3T3 fibroblasts were incubated with 20 µM of the FAM labelled 4Ts ON for 12 hours and then stained with Hoechst 3342 and CellBrite Fix 640 to identify the nuclear DNA and the cell membrane respectively. Overlaid pseudo-coloured panels of a section are shown: DNA of the nucleus (cyan), 4Ts-FAM (magenta), cell membrane (yellow). Scale bar: 20 µm.

Discussion

Chemical modification provides an effective and efficient way of obtaining therapeutic antigene/antisense agents based on the nucleic acid scaffold. To regulate gene expression, chemically modified ONs need to be able to penetrate the cell membrane, resist nuclease degradation, not be toxic to the cell, and importantly, bind sequence-specifically to target DNA or RNA with high affinity.[75] As the electrostatic repulsion between negatively charged phosphates is considered to be one of the factors that determines thermodynamic stability of nucleic acid secondary structures, neutral or positively charged ON analogues should bind more tightly with complementary DNA or RNA. Several studies have focused on the introduction of positively charged groups to a nucleobase,[76-77] a sugar[78-79] or the DNA backbone[80-82] leading to formation of more stable duplexes and triplexes.[83] Introduction of SaRNA (Fig. 3) monomers to replace the phosphodiester backbone led to charge-neutral sulfonamide antisense oligonucleotides (SaASOs), which resulted in a slight destabilisation DNA-RNA duplex compared to a DNA-DNA duplex.[65] In contrast, incorporation of BCNS (Fig. 3) groups into the DNA backbone led to self-neutralising ONs that did not induce a change of T_m when binding complementary DNA sequences,[66] regardless of the number of BCNS incorporated. This is in line with our results where increasing the number of N+ or Ts-modifications showed no benefit in T_m values of the antiparallel duplexes formed with complementary RNA or DNA.

In order to introduce SaRNA monomers into an RNA backbone, a 14-step-synthesis of phosphoramidite for the SaRNA-TT dinucleotide was required. Similarly, incorporation of BCNS groups on a DNA backbone requires the synthesis of thymidine and 2'-OMe-uridine phosphoramidites bearing BCNS groups comprising nine and ten synthetic steps, respectively. In comparison, the synthesis of a N+ monomer requires only four synthetic steps starting from commercially available 1,4-butane sultone without any silica gel purification during the synthesis. The incorporation of the N+ modification onto DNA is performed during DNA synthesis instead of a standard oxidation step, which significantly simplifies synthesis. Moreover, N+ and Ts- modifications can be introduced into any position in the sequence, which is not the case for SaRNA and BCNS nucleic acid analogues.

Unlike PNAs and some with BCNS groups, N+ and Ts- modified ONs demonstrated excellent chemical stability and solubility in buffer solutions. The presence of the N+ modification enhanced the stability of parallel triplexes at pH 5.0. Ts-modification also stabilises parallel triplexes at pH 5.0 but the stability decreased as the number of Ts-moieties incorporated increased. Apart from ONs with N+ or Ts-modifications right in the middle of the sequence, both types of modified ON hybridised to complementary RNA with higher thermal stability than with DNA, indicating that N+ and Ts-modifications can be used in antisense strategies. This is in contrast with reports that ONs with Ts- groups destabilised duplex formation with complementary RNA (ΔT_m =

-1.6 – -1.2 °C/modification),[61] which suggests that the effect of Ts-modification on T_m is dependent on the sequence.

The thermal stability of duplexes formed by N+ONs and their complementary DNA sequence was less dependent on the ionic strength, which was predicted for zwitterionic nucleotides that can bind to natural DNA at low ionic strength as well or better than natural DNA.[84] Similar results have been reported for ONs with BCNS groups: the T_m of 2'-OMe duplexes increased with an increasing number of BCNS groups at low ionic strength (25 mM HEPES buffer, pH 7.3). When binding to complementary RNA sequences, such behavior was not as noticeable. It has been reported in the past that T_m values for ON/RNA duplexes are less sensitive to changes in ionic strength than ON/DNA duplexes.[85] The Ts-modification stabilised duplex formation with RNA at 100 mM salt, but destabilised the RNA duplex at a low salt concentration.

Thermodynamic analysis of melting curves revealed that the N+ modification stabilises the duplex with DNA due to a significantly reduced loss in entropy but stabilises duplex formation with RNA because of the improved enthalpy at the same salt concentrations. A similar trend for ΔH and $T\Delta S$ is observed for the Ts-modification compared to native DNA.

In line with the recent report,[58] the loss of thermodynamic stability of the native DNA duplex at low salt concentrations was caused by a large entropic penalty that was not compensated for by the improved enthalpy. For the native RNA duplex, the entropic penalty and improved enthalpy cancelled each other out resulting in similar thermodynamic stability in the presence of 25 and 100 mM NaCl.

The polyelectrolyte ion condensation theory can be used to explain how N+ modification stabilises duplex formation: for natural DNA, the double-helical form has a higher charge density in comparison with the single-stranded form. During denaturation, due to the reduction in charge density a portion of the counterions bound to DNA are lost to the bulk solvent. For a DNA duplex with one zwitterionic strand, the charge density of duplex and single stranded states is balanced, and only a fraction of the counterions should be lost during denaturation.[84,86] As a result, the thermal stability of zwitterionic N+DNA duplexes are less dependent on the ionic strength. This is in line with our thermodynamic analysis that the dsDNA having N+ modifications showed less entropy costs when the ionic strength changed.

Native DNA and RNA sequences are highly susceptible to nuclease degradation within the cell. Modifying the phosphate group reduces the possibility of enzymatic digestion which will be useful for cellular applications of N+ and Ts-modified ONs. The introduction of just a single N+ and Ts- modification at the 3'-end, but not the 5'-end, results in the modified ONs being resistant to enzymatic digestion by snake venom phosphodiesterase I.

The FAM labelled ONs were shown to penetrate cells without the use of a transfection reagent. While a small amount of the 4N+{FAM} ON had entered the cell after 12 hours, it was the Ts-ONs that displayed a high level of cellular and nuclear uptake. These results indicate that ONs with phosphate modifications such as N+ or Ts- might be

suitable tools for the application of DNA and RNA vaccines,[87] for the treatment of cancer,[88] infectious diseases,[89] and neurological disoders.[90]

Conclusion

ONs possessing N+ and Ts- modifications have good aqueous solubility and chemical stability, which allowed the assessment of these modifications in the context of DNA triplexes and duplexes. The presence of N+ or Ts- modifications on the internucleotidic phosphates enhanced the binding affinity of the ONs for complementary RNA and increased their resistance to digestion by phosphodiesterase I. Fluorescently labelled Ts-ONs penetrate the cell and enter the nucleus while N+ONs remain trapped in vesicles in the cytoplasm. These properties make N+ and Ts- modified ONs promising candidates for cell-based applications.

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Supporting Information includes charachterisation of modified ONs, description of analysis of melting curves and determination of thermodynamic parameters, evaluation of stability of 5'N+ON2 in the buffer and triplex formation using SEC-HPLC,

description of enzymatic digestion protocol as well as details of cell culture and permeability assays.

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