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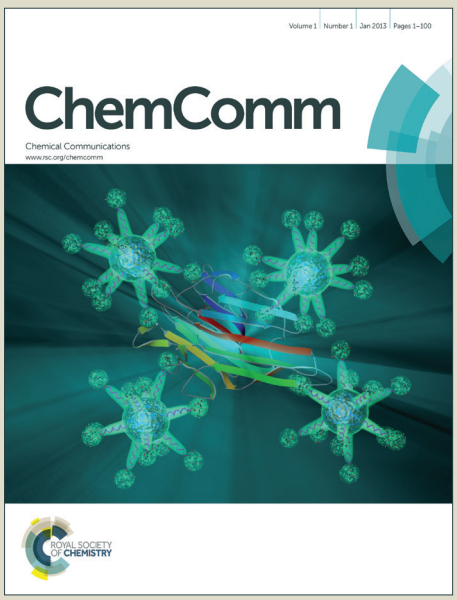

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Nuclease resistant oligonucleotides with cell penetrating properties

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2'-O-AECM modified oligonucleotides provide an unusual combination of remarkable properties. This includes the combination of high resistance towards enzymatic degradation with that AECM oligonucleotides appear to be spontaneously taken up into cells.

Synthetic oligonucleotides are of tremendous importance for the development of life science research, with modified oligonucleotides being used for diagnostics as well as means to treat patients with genetic disorders. The different therapeutic approaches, such as siRNA[1] (short interfering RNA) and antisense technologies[2], including development of artificial ribonucleases[3, 4, 5] and pre-mRNA splice-switching[6], are typically limited by e.g., lability of oligonucleotides in biological fluids and in particular by poor delivery to the site of action. Modified oligonucleotides are introduced to increase affinity to the target and resistance to degradation. Especially 2'-O-alkyl oligonucleotide modifications have been much investigated for use in biotechnology and/or therapy.[7] To modify the 2'-position has several advantages, including a typically quite straightforward synthesis that can result in a low cost of production. Many 2'-O-alkyloligoribonucleotide modifications give increased stability of duplexes with RNA.[8]

The 2'-O-carbamoylmethyl (CM) modification can give substantial stabilization of duplexes[9] and was recently also shown to be highly resistant to enzymatic degradation[10]. Of additional relevance is that 2'-O-aminopropyl-RNA having a positively charged ammonium group displays high resistance towards degradation by nucleases.[11] Oligonucleotides containing the N-(N, N-dimethylaminoethyl)-carbamoyl)methyl modification give duplexes with RNA that have somewhat higher melting points than for the corresponding DNA:RNA complexes, despite the apparent bulkiness of the substituent.[12] A combination of the features mentioned above, can be accomplished by further substitution of the carbamoylmethyl group to obtain the 2'-O-(N-(aminoethyl)carbamoyl)methyl (AECM) modification (Figure 1). This AECM modification should have even higher hydrogen bonding potential (e.g., with the hydration network) than the CM as well as possibility for electrostatic interaction with phosphodiester linkages. It has been reported that oligonucleotides containing a single 2'-O-(N-(aminoethyl)carbamoyl)uridine gave a substantial decrease in melting point of duplexes both with DNA and RNA.[13] Unconvinced by the data, and by the fact that the AECM has never been tested with a higher degree of modifications or as a fully modified oligonucleotides. We have pursued our work on the AECM modification show that duplex stability in the systems presented here (see below) are even somewhat stabilizing and this in combination with other properties makes the AECM modification highly interesting for further development.

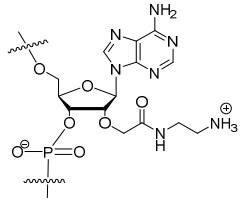

FIGURE 1. The 2'-O-(N-(aminoethyl)carbamoyl)methyl (AECM) modification.

In a recent study on the stability of an AECM modified dinucleotide towards basic conditions that may be used for deprotection, we also found an even higher resistance towards degradation by spleen and snake venom phosphodiesterases than for the CM modification.[14] We now report on synthesis of 2'-O-(N-(aminoethyl)carbamoyl)-methyladenosine containing oligonucleotides, which demonstrate a positive effect on the thermal melting of duplexes with RNA, resistance to degradation in human serum and cellular uptake without any additives, such as cationic lipids or cell penetrating peptides.

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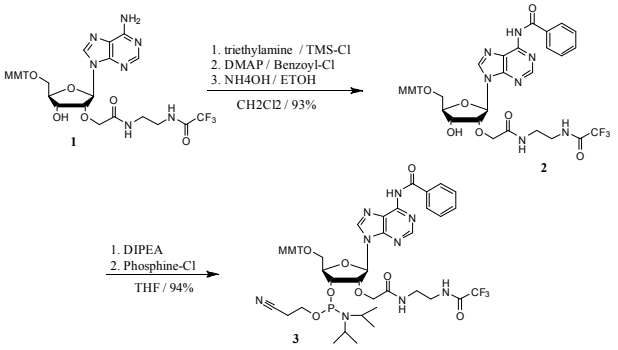
Synthesis of 2'-O-AECM adenosine derivative **1** was by a one-pot procedure where the precursor nucleoside (MMT-A) was alkylated with allyl bromoacetate, then treated with ethylenediamine and subsequently trifluoroacetylated[14]. **1** was then benzoylated at the N^6-position to produce the base-protected AECM nucleoside **2**. (Scheme 1).



SCHEME 1. Synthesis of the AECM-A phosphoramidite building block 3.

This material was further treated with 2-cyanoethyl-diisopropylchlorophosporamidite to give the AECM-A phosphoroamidate **3** (Scheme 1). Several oligonucleotides containing AECM modifications (Table 1, sequences **O1**, **O2**, **O3** and **O4**) as well as the corresponding oligodeoxynucleotides (Table 1, sequences **dO1**-**dO4**) were then prepared using machine assisted synthesis with phosphoroamidite chemistry. Aqueous concentrated ammonia give some amounts of hydrolysis of the AECM group[14] and therefore ethylenediamine (EDA) was used for complete deprotection of oligonucleotides.

TABLE 1. Sequences for evaluation of hybridization of AECM containing oligonucleotides.

Sequence no.	Sequence[a]
O1 and **dO1**	GGaCCGGaaGGTaCGaG
O2 and **dO2**	GaaGaaaGaGaGGaGG
O3 and **dO3**	CaaaGaaCaCCaG
O4 and **dO4**	aaaaaaaaaaaA

[a] In all sequences A, C, G , T= 2'-deoxyribonucleotides, In sequences **O1**-**O4** a = 2'-*O*-AECM-adenosine and in **dO1**-**dO4** a = 2'-deoxyadenosine.

Thermal melting, of duplexes of oligonucleotides **O1**-**O4** and **dO1**-**dO4** with complementary RNA as well as with complementary DNA, was then determined by use of UV spectroscopy. The resulting melting points (Tm values in Table 2) showed some sequence dependence but a general increase in melting of complexes with RNA (+0.5 to +2.3 degrees per modification) while in complexes with DNA there was little difference compared to a DNA/DNA duplex (-0.3 to +1.0 degrees per modification).

Thermal melting at different salt concentrations ranging from 0.05-0.50 M concentration of the complementary heteroduplex of Oligomer **O2** with complementary DNA and RNA vs the native DNA/DNA and DNA/RNA duplexes, respectively, was compared. That the slight Tm increase by incorporation of AECM modification with a complementary DNA at low salt in the duplex is absent at higher salt concentration (Figure 2, left panel) suggest that the stabilisation at 0.1 M concentration (Table 2) is mainly due to an electrostatic effect. With the duplex between the RNA complement and the AECM containing oligonucleotide **O2** the difference in thermal melting compared to with the non-modified oligonucleotide **dO2** at low salt largely remains also at higher salt concentration (Figure 2, right panel). This suggests that stabilisation of the duplex with RNA is not only an electrostatic effect as found with PNA[15] but that additional factors stabilize the duplex as was suggested for partially amide linked oligonucleotides[16].

TABLE 2. Thermal melting (*T*m) of duplexes between AECM containing oligonucleotides and complementary RNA and DNA. [a]

Sequence no.	*T*m (°C) RNA[a]	Δ*T*m (°C) per modification with RNA	*T*m (°C) DNA[a]	Δ*T*m (°C) per modification with DNA
dO1	58.4		61.8	
dO2	40.2		51.0	
dO3	38.6		47.0	
dO4	5.6		30.0	
O1	61.1	+ 0.5	60.2	- 0.3
O2	56.6	+ 2.3	60.0	+1.0
O3	44.6	+ 0.9	46.0	- 0.1
O4	25.3	+ 1.6	32.7	+0.2

[a] In 100 mM NaCl, 10 mM phosphate, 0.1 mM EDTA, pH 7 at 4 μM strand concentration

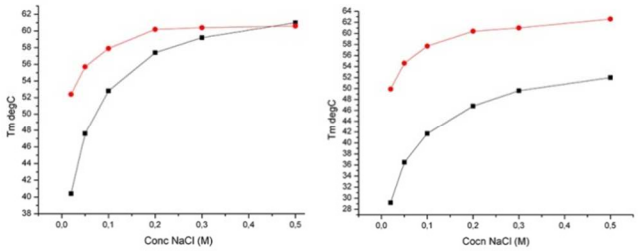


FIGURE 2. Salt dependence of the thermal melting points (Tm) for the duplexes of **O2** (red lines) and **dO2** (black lines) with complementary DNA (left panel) or RNA (right panel).

To better understand the structure of the heteroduplex that is formed, with respect of the influence of the 2'-O-AECM modification, the **O2** and **dO2** duplexes with the corresponding RNA and DNA were studied by CD spectroscopy (Figure 3). The shape of the CD curves for AECM containing oligonucleotide duplexes with both DNA and RNA gave more pronounced negative bands at ca 210 nm and more pronounced positive bands around 270 nm which are typical of A-type helices[17]. This suggests that the incorporation of AECM modifications pushes the duplex to adopt an A-conformation which is also consistent with the higher melting points.

For potential use in oligonucleotide therapy it is crucial that the degradation is not too rapid. A dinucleotide with the AECM vicinal to the internucleosidic phosphodiester linkage is more resistant than most other 2'-modifications to the 3'- and 5-exonucleases/phosphodiesterases from snake venom and bovine spleen.[14] It may also be highly important that the oligonucleotide can survive transport in the blood stream. To investigate this we subjected the fully modified oligonucleotide

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04 to incubation in human serum (90 %). Under these conditions the corresponding native DNA oligonucleotide is completely degraded but the AECM modified oligonucleotide appears to be completely stable (Figure 4, additional data for treatment with serum is shown in the ESI).

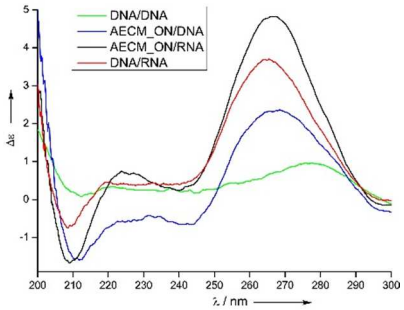


FIGURE 3. CD spectra of the duplexes of **O2** (blue and black lines) and dO2 (green and red lines) with complementary DNA or RNA.

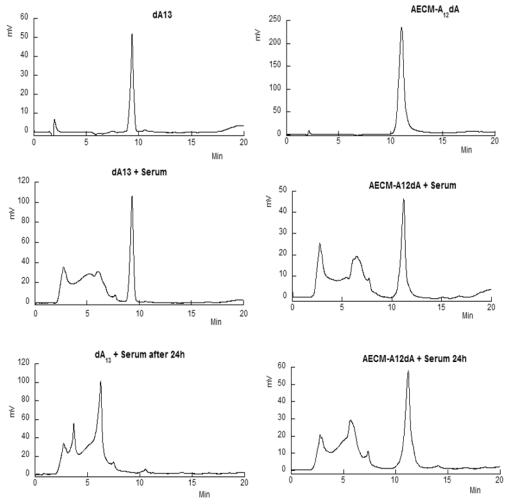
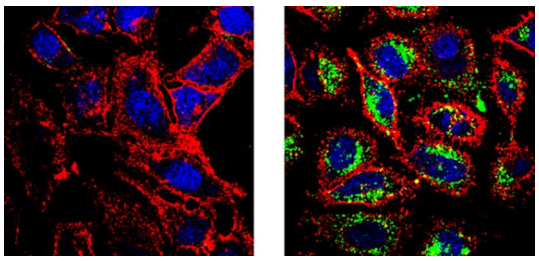


FIGURE 4. Chromatograms showing the stability of a fully modified AECM oligonucleotide (AECM-A12dA) in 90% human serum (right panel) under conditions where a native DNA oligonucleotide (dA13) is completely degraded (left panel). Top panels: oligonucleotide; central panels: with serum; bottom panels: After 24h incubation in serum.

It is highly unusual that oligonucleotides are taken up by cells to any substantial degree without the addition of transfection agents with only a couple of examples suggesting that this is possible[18, 19]. In that a number of positive charges are included in the structure, one could consider AECM oligonucleotides to somewhat (crudely) resemble an oligonucleotide-cell penetrating peptide (CPP) complex[20]. Since CPPs can enhance oligonucleotide uptake[21] it seemed that it would be interesting to see how a fully AECM modified oligonucleotide behaves with respect to cellular uptake.

In order to do this, AECM (**O5**) as well as native oligonucleotides (**dO5**) labelled with fluorescein were synthesized. U2OS cells were then treated with the respective oligonucleotide in the absence of any transfection agents and subjected to analysis by confocal microscopy (Figure 5). A quite remarkable difference between the modified and native oligonucleotide was found. It appears as if there is massive uptake of the AECM containing oligonucleotide after the relatively short incubation time of 8 h whereas the



corresponding native DNA oligonucleotide is not visibly taken up at all (Figure 5, additional confocal images are included in the ESI).

FIGURE 5 Confocal microscopy images of U2OS cells treated with 8μM of fluorescein-labelled non-modified native oligonucleotide, dA10 (**d05**, left panel) or fully AECM modified oligonucleotide, AECM-A10, (**O5**, right panel) for 8 h and then washed before being processed for the microscopy (cell membranes were stained with WGA-Alexa555). The figure shows the central plane of the cell in the Z-axis.

It is interesting to note that the pattern of the labelled **O5** has a spotted appearance. This could indicate that most of the material is still in endosomes which is typical also for cell penetrating peptides (CPPs)[20,22]. If this is the case, it is not unlikely that, as for CPPs[20,22], some amount of material can escape the endosomes. 2'-O-AECM modified oligonucleotides display several remarkable properties.

The resistance towards degradation by phosphate cleaving enzymes when the AECM-modification is vicinal to the phosphate[14] is even higher than with the 2'-O-carbamoylmethyl[11] or the 2'-O-[2-(N-methylcarbamoyl)ethyl][23] modifications that already are remarkably stable 2'-modifications. Although typically only tested with snake venom phosphodiesterase (SVPD), 2'-O-modifications with alkylammonium substitutions all seem to be highly stabilizing towards nuclease degradation. Examples of this are 2'-O (3-aminopropyl)[24], 2'-O-(3-(N, N,-dimethyl-amino)propyl[25], 2'-O-aminohexyl (with spleen phosphodiesterase)[26], 2'-O-(N-(4-aminobutyl)carbamoyl[27], and range of different 2'-O-aminoalkyls combined with a phosphorothioate backbone[28].

Despite the positive charge, most of the above modifications were in general reported to give a drop in Tm for hybridisation with a complementary RNA, although the 2'-O-aminopropyl gave about the same Tm as the reference[24] and the 2'-O-(3-(N, N,-dimethyl-amino)propyl gave the same Tm when many subsequent modification were inserted and +1.2 °C per modification when only four modification were spread out in the sequence[25]. 2-O-carbamoylmethyl substitutions with no charge and only methyl[29] or in particular with no substitution[9, 10] on the nitrogen seem to give higher Tm values with complementary RNA but are less resistant to nucleases than the charged aminoalkyl 2'-modifications[9, 14, 29]. With a ΔTm/modification being about 1-2 °C higher compared to the other 2'-modifications with charged aminoalkyl groups mentioned above, the AECM group seem to give somewhat higher stability of duplexes with RNA. Apart from resistance to

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SVPD and Spleen PDE we show here that AECM oligonucleotides are also stable in human serum.

To our knowledge, none of the other mentioned aminoalkyl 2'-modifications have been shown to promote cellular uptake. The pKa value of the protonated amino group of the 2'-substituent is certainly lower than for most other mentioned aminoalkyl group. If this has a beneficial effect for uptake and/or thermal stability of duplexes is unknown and perhaps cellular uptake was never investigated for the other aminoalkyl 2'-modifications.

AECM modified oligonucleotides appear to be "cell penetrating oligonucleotides (CPO's)", i.e. entering cells without the aid of any additive transfection agent or CPP. There are certainly a number of studies needed ahead, to fully realize the potential of AECM-oligonucleotides, including functional assays in cells, perhaps enhancement of endosomal escape by additional entities and/or modifications etc. However, when taking all properties together it seems clear that oligonucleotides carrying the AECM modification are highly interesting for further investigations on their potential in oligonucleotide therapy.

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Notes and references

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Electronic Supplementary Information (ESI) available: Material and methods, experimental details from synthesis, thermal melting, CD spectroscopy, degradation studies in serum and cellular uptake studies. See DOI: 10.1039/c000000x/

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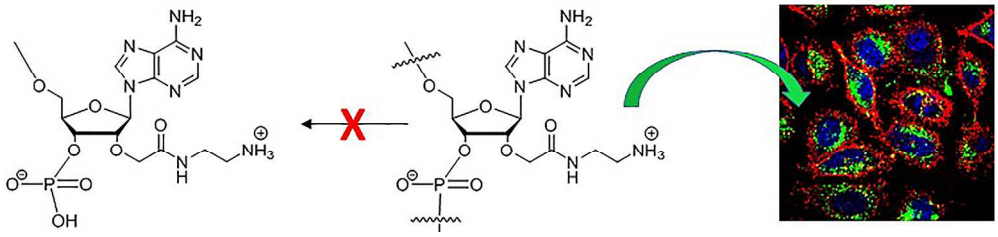

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