Supplementary Information to:

A nuclease resistant oligonucleotide with cell penetrating properties.

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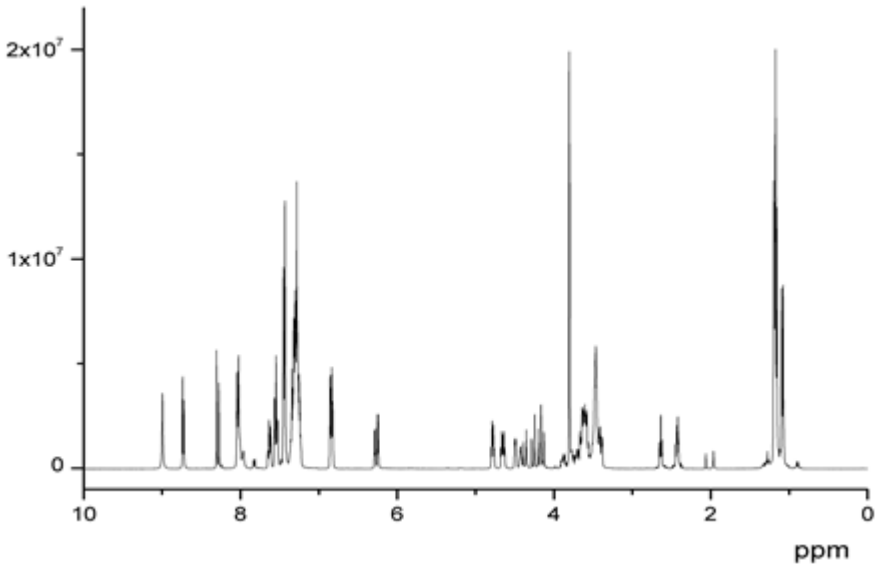
General materials and methods: All NMR-spectra were recorded using a Bruker AVANCE DRX-400 instrument (400.13 MHz in ^{1}H, 162.00 MHz in ^{31}P, and 100.62 MHz in ^{13}C) in solvents as specified. ^{13}C and ^{1}H chemical shifts are given in ppm. Most assignments in ^{1}H-NMR were made by standard ^{1}H-^{1}H-COSY. Silica gel column chromatography was generally carried out using Matrex silica, 60 Å (35-70 μm, Amicon). TLC-analysis was carried out on pre-coated plates, Silica Gel 60 F_{254} (Merck), with detection by UV-light and/or by charring with 8 % sulfuric acid in methanol. Solutions were concentrated under reduced pressure at temperatures not exceeding 40 °C. Reagents were of ordinary commercial grade and solvents of commercial p.a. grade. Solvents were dried, over 4Å (pyridine, CH_2Cl_2, and dioxane) or 3Å (methanol, ethanol and acetonirile) molecular sieves. THF was distilled at atmospheric pressure over $LiAlH_4$, prior to use. Toluene, cyclohexane and diethylether were dried over sodium wire. Reversed-phase HPLC-chromatography was accomplished on a Jasco apparatus. The UV detection was carried out at 254 nm. Mass analysis was performed on a Micromass LCT ESI-TOF mass spectrometer using leucine enkephalin as an internal lock mass standard. Oligonucleotides **dO1-dO4** were purchased from Scandinavian Gene Synthesis, except a second lot of **dO4** that was purchased from Eurogentec S.A. (Belgium). Compound **1** was synthesized as described earlier.[14] Oligonucleotides were analyzed in negative mode as solutions in acetonitrile/water, 1:1 (v/v).

5'-O-monomethoxytrityl-2'-O-(N-(trifluoroacetamidoethyl)-carbamoyl)methyladenosine (1). 5'-*O*-Monomethoxytrityladenosine (2.50 g, 4.77 mmol) was dried by evaporation of added dry tetrahydrofuran (THF, distilled from $LiAlH_4$ LAH)) and then dissolved in 200 ml dry THF. Potassium tertbutoxide (0.696 g, 6.20 mmol) was added and after 15 min allyl bromoacetate (1.12 g, 6.20 mmol). The reaction was stirred for 2 h and TLC showed that all of compound 1 was consumed. The THF was evaporated under reduced pressure and 100 ml EtOH (99.8% dried over 3Å molecular sieves) and ethylenediamine (2.87 g, 47.7 mmol) was added. The reaction mixture was stirred for 2 h and 100 ml dry THF was added and the reaction was left over night at room temperature. TLC showed complete conversion of the alkylated products. The solvent was evaporated and the excess ethylenediamine was removed by co-evaporation with added dioxane four times (4x15 ml). The solid material was dissolved in 200 ml dry CH_2Cl_2 and triethylamine (1.45 g, 14.31 mmol) and trifluoroacetic anhydride (TFAA, 1.65 g, 7.85 mmol) were added. TLC showed that the reaction was complete after 2 h at room temperature. The reaction mixture was washed with a total of 400 ml water/brine 1:1. The organic layer was dried with $MgSO_4$ and concentrated. The crude product was purified by column chromatography on silica using CH_2Cl_2/methanol (15:1 with 0.005% triethylamine) as eluent giving 2.7 g (77%) of 4 as a white foam. A slightly modified version of this reaction was performed with methyl bromoacetate instead of the allyl reagent, with the difference that the ethylenediamine was dried first by two times evaporation of added n-butanol and then three times with dioxane. The same product 4 was then obtained in a total yield of 72%. Elemental analysis: Calcd. For $C_{36}H_{36}N_7O_7F_3$: C, 58.77; H, 4.93; N, 13.33. Found: C, 58.57; H, 5.03; N, 13.16. ^{1}H-NMR ($CDCl_3$): 3.15-3.25 (m, 2H); 3.30-3.50 (m, 4H); 3.70 (s, 3H, CH_3-trityl); 4.10-4.15 (d, 2H, CH_2-carbamoyl); 4.15 (d, 1H, 4H'); 4.20 (m, 1H, 3H'); 4.40 (m, 1H, 2H'); 5.95 (s, 2H, NH_2); 6.10 (d, 1H, H1'); 6.75 (d, 2H, trityl); 7.10-7.40 (m, 12H, trityl); 7.90 (s, 1H, H2-base); 8.25 (s, 1H, H8-base).

Synthesis of N^6-benzoyl-5'-O-(4-monomethoxytrityl)-2'-O-(2-N-trifloroacetamidoethyl)-carbamoylmethyl)adenosine 3'-O-[Cyanoetyl (N,N-diisopropylamino)phosphoramidite (3). Compound **1** (2.6 g, 3.53 mmol) was dried twice by evaporation of added acetonitrile (dried over 3 Å molecular sieves) and dissolved in 80 ml dry CH_2Cl_2. To the solution triethylamine (3.94 ml, 28.27 mmol) was added followed by trimethylsilyl chloride (TMS-Cl, 0.902 ml, 7.06 mmol). The reaction was left for 2 h and an additional portion of TMS-Cl (1.77 mmol) was added wherupon the reaction was left for 30 min. A catalytic amount of DMAP (20 mg) was added to the solution. The solution was chilled to –10 °C and benzoyl chloride (0.819 ml, 7.06 mmol) was added. The reaction was left to reach room temperature and after 3 h another portion of benzoyl chloride (1.77 mmol) was added and the reaction was thereafter left overnight. The solution was extracted with CH_2Cl_2 (200 ml) and washed twice with a mixture of (H_2O 50 ml / brine 50 ml / $NaHCO_3$, 0.1 M, 50 ml) then (H_2O 100 ml / brine 100 ml). The organic layer was dried over Na_2SO_4 and evaporated to give a pink foam. The crude product was dissolved in ethanol (60 ml, 99.5%) and ammonium hydroxide (12 ml, 30 %) and left for 1 h. Removal of solvent under reduced pressure yielded the crude product which was chromatographed on silica gel using CH_2Cl_2-MeOH (10:1 containing 0.05 % triethylamine) as eluent yielding **2** (2.75 g, 93 %) as a white foam (MS (ES-TOF) $[M-H]^+$ calcd for $C_{43}H_{39}F_3N_7O_8$ 838.2969; found 838.3000). 1.60 g, (1.90 mmol) of **2** was then dried twice by evaporation of added THF (distilled over $LiAlH_4$) and dissolved in dry THF (40 ml). Triethylamine (1.30 ml, 7.60 mmol) and then 2-Cyanoethyl *N,N*-diisopropylchlorophosphoramidite (0.848 ml, 3.8 mmol) were added dropwise with a syringe under nitrogen atmosphere during 2 min. The reaction mixture was left stirring for 30 min and then concentrated until 5 ml of solution remained. To the solution ethyl acetate (150 ml) was added. The crude solution was extracted twice with a mixture of (brine 50 ml/$NaCO_3$, 10 % (aq), 50 ml/H_2O 50 ml).

The organic layer was dried over Na_2SO_4 and removal of solvent under reduced pressure yielded the crude product as colorless foam. The crude product was dissolved in ethyl acetate (EtOAc, 10 ml) and was dropped with a syringe into hexane (180 ml) at –78 °C. After filtration the precipitate was collected and dried to give a white solid. The solid was chromatographed over silica gel using (EtOAc/MeCN 13:1 containing 1 % triethylamine) as eluent yielding **3** (as a mixture of the two phosphorus isomers in a 41 to 59 ratio) (1.86 g, 94 %) as white foam. ^{1}H-NMR ($CDCl_3$): 1.05-1.25 (m, 12H, CH_3-iPr); 2.30-2.65 (m, 2H, CH-iPr); 3.30-3.70 (m, 6H, 5H'+2*CH_2-ethylenediamine); 3.50-2.75 (m, 4H, CH_2-ethylene-CN); 3.75 (s, 3H, CH_3-trityl); 4.05-4.30 (m, 2H, CH_2-carbamoyl); 4.40-4.45 (m, 1H, 4H'); 4.55-4.65 (m, 1H, 3H'); 4.75 (m, 1H, 2H'); 6.25 (d, 1H, H1'); 6.80 (d, 2H, trityl); 7.25-7.60 (m, 17H, trityl + benzoyl); 8.25 (s, 1H, H2-base); 8.80 (s, 1H, H8-base). ^{31}P NMR ($CDCl_3$): 151 ppm (relative to external 85% H_3PO_4). MS (ES-TOF) $[M-H]^+$ calcd for $C_{52}H_{57}F_3N_9O_8P$ = 1039.40; found 1039.43.

Figure S1. Proton-NMR spectrum of compound 3



Synthesis of 2'-O-AECM modified oligonucleotides (O1-O4) and non-modified oligonucleotides (dO1-dO4). The oligonucleotides were synthesized on an Applied Biosystems 392A DNA/RNA synthesizer. Monomers for the solid-phase synthesis were dried in vacuo in the presence of CaH_2 prior to use. Oligonucleotides were assembled on pre-loaded CPG cartridges using 2-cyanoethyl phosphoramidite chemistry compound **4** and commercial reagents [5'-DMT-dN-3'-P(OCE)N*i*Pr_2] dN = dU, dA^{Bz}, dC^{Ac}, dG^{iPrPac}, in a 1.0 µmol scale using the manufacturer's protocols and 10 min coupling time. For the detritylation step after coupling with compound **4** an extra round of 1 minute acid treatment was used. After synthesis the resin was transferred from the cartridge to a tightly sealed container and treated with 2 ml ethylenediamine solution (20% in methanol) for 24 h at room temperature. The CPG-resin was then removed by filtration and washed with 4 ml methanol and 4 ml H_2O. The filtrate was evaporated under reduced pressure at 40°C and further dried by twice evaporating added ethanol (99.8 %. 3 ml). H_2O was added and the crude oligonucleotide product lyophilized. The oligonucleotides were then purified with RP HPLC using an ODS Hypersil (250 × 10 mm, 5µm) column. A flow rate of 4 ml/min and a temperature of 50 °C was used. First 100 % buffer A was flushed through for 3 min, then a linear gradient of 0-50 % buffer B over 40 min was used. Buffer A: 50 mM triethylammonium acetate in water (pH 6.5); Buffer B: 50 mM triethylammonium acetate (pH 6.5) in 50 % aqueous acetonitrile. The oligonucleotides were lyophilized three times before use and stored frozen. MS (ES-TOF) $[M-H]^-$ mass of the oligonucleotides: **O1**: $C_{187}H_{245}N_{86}O_{106}P_{16}^-$ = 5889; found, 5890; **O2**: $C_{178}H_{236}N_{89}O_{95}P_{14}^-$ =5576; found, 5577; **O3**: $C_{154}H_{211}N_{71}O_{83}P_{12}^-$ = 4756; found, 4758; **O4**: $C_{178}H_{251}N_{89}O_{87}P_{12}^-$ = 5401; found, 5402.

Synthesis of the fluorescein labelled 2'-O-AECMA$_{10}$ and dA$_{10}$ oligonucleotides (O5 and dO5). The oligonucleotides were synthesized on an Applied Biosystems 392A DNA/RNA synthesizer. Monomers for the solid-phase synthesis were dried in vacuo in the presence of CaH_2 prior to use. Oligonucleotides were assembled on pre-loaded CPG cartridges 3'-(6-Fluorescein)-CPG purchased from Glen Research, using 2-cyanoethyl phosphoramidite chemistry and compound **4** and N^6-benzoyl-5'-O-DMT-dA-3'-P(OCE)N*i*Pr_2 (also from Glen Research) respectively at 1.0 µmol scale using the manufacturers protocols (and 1 min additional acid treatment after coupling with **4**, as above) with 10 min coupling time. After synthesis the resin was transferred from the cartridge and treated with 2 ml ethylenediamine solution (20% in methanol) for 24 h at room temperature in a tightly sealed flask. The CPG resin was then removed by filtration and washed with approximately 4 ml methanol and 4 ml H_2O. The filtrate was evaporated under reduced pressure at 40°C and dried by twice evaporating added ethanol (99.8 %, 3 ml). H_2O was added and the crude oligonucleotides were lyophilized. The oligonucleotides were then purified with RP HPLC using an ODS Hypersil (250 × 10 mm, 5 µm) column. A flow rate of 4 ml/min and a temperature of 50 °C was used. First 100 % buffer A was flushed through for 3 min, then a linear gradient of 0-50 % buffer B over 40 min was used. Buffer A: 50 mM triethylammonium acetate in water (pH 6.5); Buffer B: 50 mM triethylammonium acetate (pH 6.5) in 50 % aqueous acetonitrile. The oligonucleotides were lyophilized three times before use and stored frozen. MS (ES-TOF) $[M-H]^-$ mass of the Oligos: dA_{10} calculated for $C_{128}H_{146}N_{51}O_{58}P_{10}^-$ =3637; found, 3637; 2'-*O*-$AECMA_{10}$: calculated for $C_{168}H_{226}N_{71}O_{78}P_{10}^-$ =4798; found, 4798.

Thermal melting analysis of duplexes of modified non-modified oligonucleotides with complementary RNA and DNA. Absorbance vs. temperature profiles were measured at 260 nm on a Cary 300 UV/VIS dual beam spectrophotometer (Varian) equipped with a programmable thermo electrical temperature controlled 6×6 sample holder. Extinction coefficients were calculated by the nearest-neighbour approximation (J.D. Puglisi, I. Tinoco Jr, *Meth. Enzymol*. **1989**, 180, 304–325.). Melting temperatures were measured with 1:1 molar mixtures of oligonucleotide and the corresponding target RNA or DNA, each at a concentration of 4 μM, in a 10 mM phosphate buffer containing 100 mM NaCl and 0.1 mM EDTA at pH 7.0. Prior to every melting experiment, the phosphate buffer was degassed on an ultra sonic bath. The samples were rapidly heated to 90 °C, left for 5 min and then allowed to cool to 20 °C. After equilibration for 10 min at the starting temperature, the dissociation was recorded by heating to 90 °C at rate of 0.2 °C min^{-1}. The Varian Cary WinUV software, version 3 was used to determine the melting temperatures (T_m) from the derivatives of the experimental melting curves.

Circular dichroism (CD) spectroscopy of duplexes. CD spectra of **O2**/RNA complement, **O2**/DNA complement, **dO2**/RNA complement and **dO2**/DNA complement solutions from the thermal melting experiments above were obtained using a Jasco-810-150S CD spectrometer (Jasco, Japan). A quartz cell with 1 mm optical path was used. Spectra were recorded at 22 ºC between 200-300 nm with a bandwidth of 1 nm, a 2 s response time and a scan speed of 50 nm/min.

Serum stability test with modified and non-modified oligonucleotides. The fully AECM modified oligonucleotide **O4** (AECM-A_{12}-dA) and oligodeoxyribonucleotide **dO4** (dA_{13}) were dissolved in 0.05 mL water in an eppendorf tube and then 0.45 mL human serum (Sigma-Aldrich) was added. After mixing a 0.1 mL aliquot was immidiately withdrawn diluted with 0.9 mL buffer A and a 0.1 mL aliquot thereof immediately injected for HPLC-analysis. The remaining 90 % serum solution was incubated for 24 h at 37 °C wherupon another 0.1 mL aliquot was withdrawn and analyzed by HPLC (Figure 2). RP-HPLC analysis was performed using an ODS Hypersil (250 × 4.6 mm, 5 μm) column. A flow rate of 0.8 ml/min and a linear gradient from 50 mM triethylammonium acetate in water (pH 6.5) to 50 mM triethylammonium acetate (pH 6.5) in 25 % aqueous acetonitrile in 20 minutes was used. A second experiment of serum stability was done (see Figures S2 and S3) where the AECM modified oligonucleotide **O4** (AECM-A12-dA) and oligodeoxyribonucleotide **dO4** (dA13) at 15 μM final concentration were incubated in 90% human serum at 37 °C. Total reaction volume was 1 mL. Aliquots (50 μL) were removed after 0, 1, 3, 6, 12, 24 and 48 h, diluted with 200 μL of water and injected for HPLC-analysis. RP-HPLC analysis was performed using a Supelco Discovery® BIO Wide Pore C18-5 (250 × 4.6 mm, 5 μm) column. A flow rate of 0.8 ml/min and a linear gradient from 50 mM triethylammonium acetate in water (pH 6.5) to 50 mM triethylammonium acetate (pH 6.5) in 25 % aqueous acetonitrile in 20 minutes was used.

Oligonucleotide uptake by cells as visualized by confocal microscopy with modified oligonucleotides, compared with corresponding non-modified nucleotides. U2OS cells (human osteosarcoma cell line) grown at 37°C, 5 % CO_2 were plated onto cover slips in a 24 well plate in order to reach 80-90 % confluency the next day. Fluorescent labelled modified AECM-A_{10} (**O5**) and non-modified dA_{10} (**dO5**) oligonucleotides were diluted to specific concentrations in a final volume of 300 μl by using Opti-MEM. Cells were washed twice with PBS buffer and the diluted oligonucleotide was then added to the cells. After incubation, the cells were again washed 3 times with PBS buffer and further processed for confocal microscopy as follows: cells were fixed in 3.7 % paraformaldehyde for 25 min and washed three times with PBS buffer. The nucleus was stained with DRAQ5 (10 μM) for 4 min at 37 °C after which the cells were washed two times with PBS buffer. Finally the cell membrane was stained with WGA-Alexa555 (30 min incubation) after which the cells were again washed twice with PBS. Cells were then mounted with DAKO fluorescence mounting medium. Confocal microscopy was performed using a Zeiss LSM 510 microscope equipped with a Plan-Apochromat 63x/1.4 oil DIC objective and 0.8 aperture. Pictures were taken using a Z axis that crossed the middle plane of the cell.

Additional oligonucleotide cellular uptake study by confocal laser scanning microscopy, using the CellMask™ membrane dye (see Figure S4 and S5). Human osteosarcoma U2OS cell were maintained and cultivated in Dulbecco's Modified Eagle's medium plus (10% FBS at 37ºC, 5% CO_2 and 95% humidity). The cells were seeded at a density of $2x10^4$ cells per well in a 175μm glass bottom 96-well plate (Greiner Bio-One) the day before, in order to be 80% confluent the next day. The fluorescein labelled AECM-A_{10} (**O5**) and and non-modified dA_{10} (**dO5**) oligonucleotides were diluted to 8μM in warm Opti-MEM (LifeTechnologies) and added to cells after medium removal. After 8h of incubation the cells were processed for the microscopy as follow: the medium was removed, cells washed once with warm Opti-MEM and stained with a 6.25μg.mL^{-1} solution of CellMask™ deep red membrane stain (LifeTechnologies) in Opti-MEM, 10 minutes, 37ºC. After staining, cells were fixed with warm 4% paraformaldehyde pH7.4 in Opti-MEM for 5 minutes at 37ºC, followed by 3 times washing with PBS. The cells were left in PBS and imaged immediately.
Confocal laser scanning microscopy was performed in an Inverted Nikon A1R+ Confocal Microscope (Nikon Corporation, Japan) with Apo 60x oil λS DIC N2 objective (NA 1.4, refractive index 1.515) and galvano scanner. Pictures were acquired with the NIS-Elements Advanced Research Software (Nikon Corporation, Japan) using a pinhole size of 39.6 μm and Ti ZDrive performed the Z stack bottom-to-top of 59 steps with 0.2μm/step.

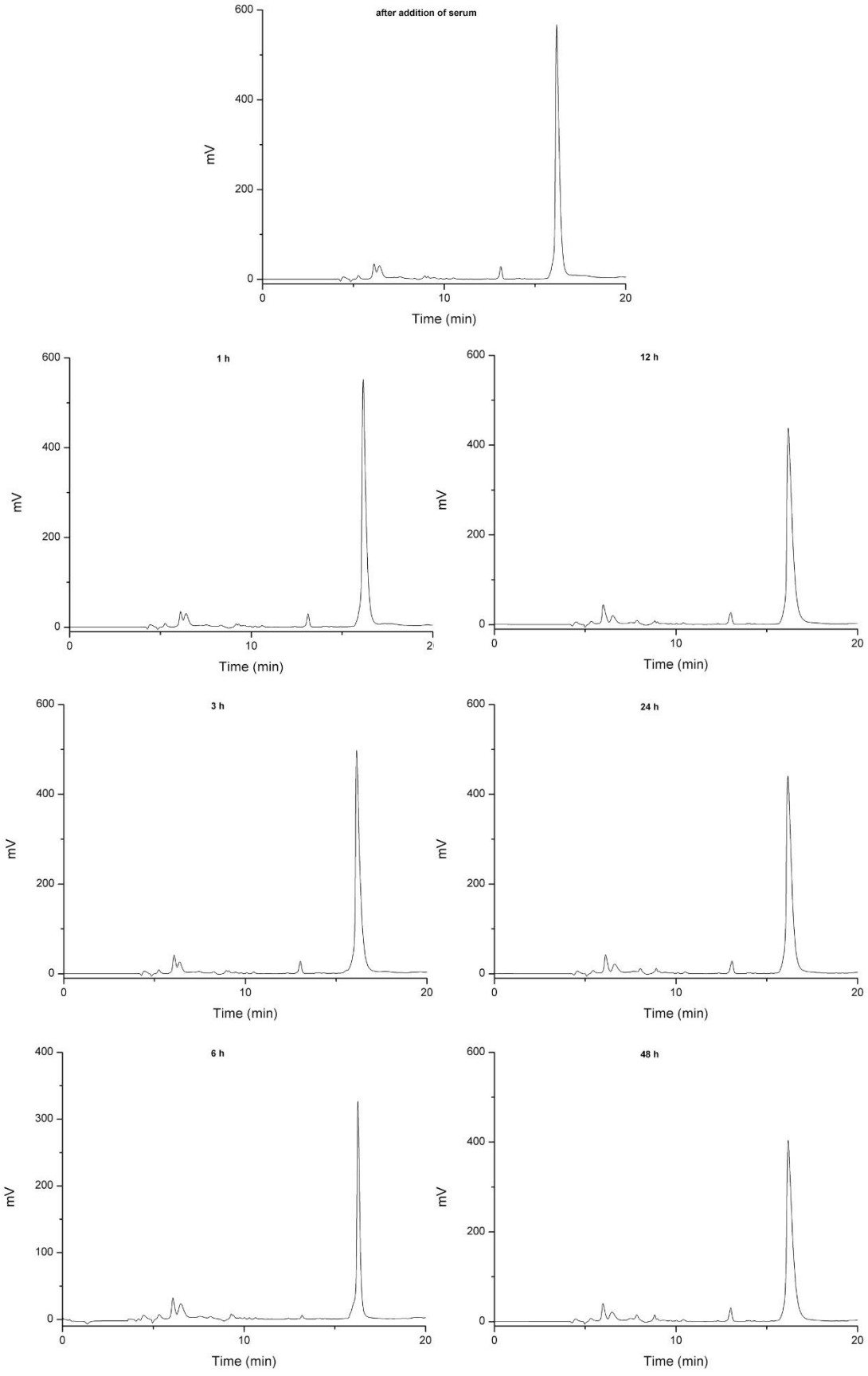


Figure S2. Chromatograms showing the stability of a fully modified AECM oligonucleotide (AECM-A12dA) in 90% human serum at different times of incubation up to 48 h.

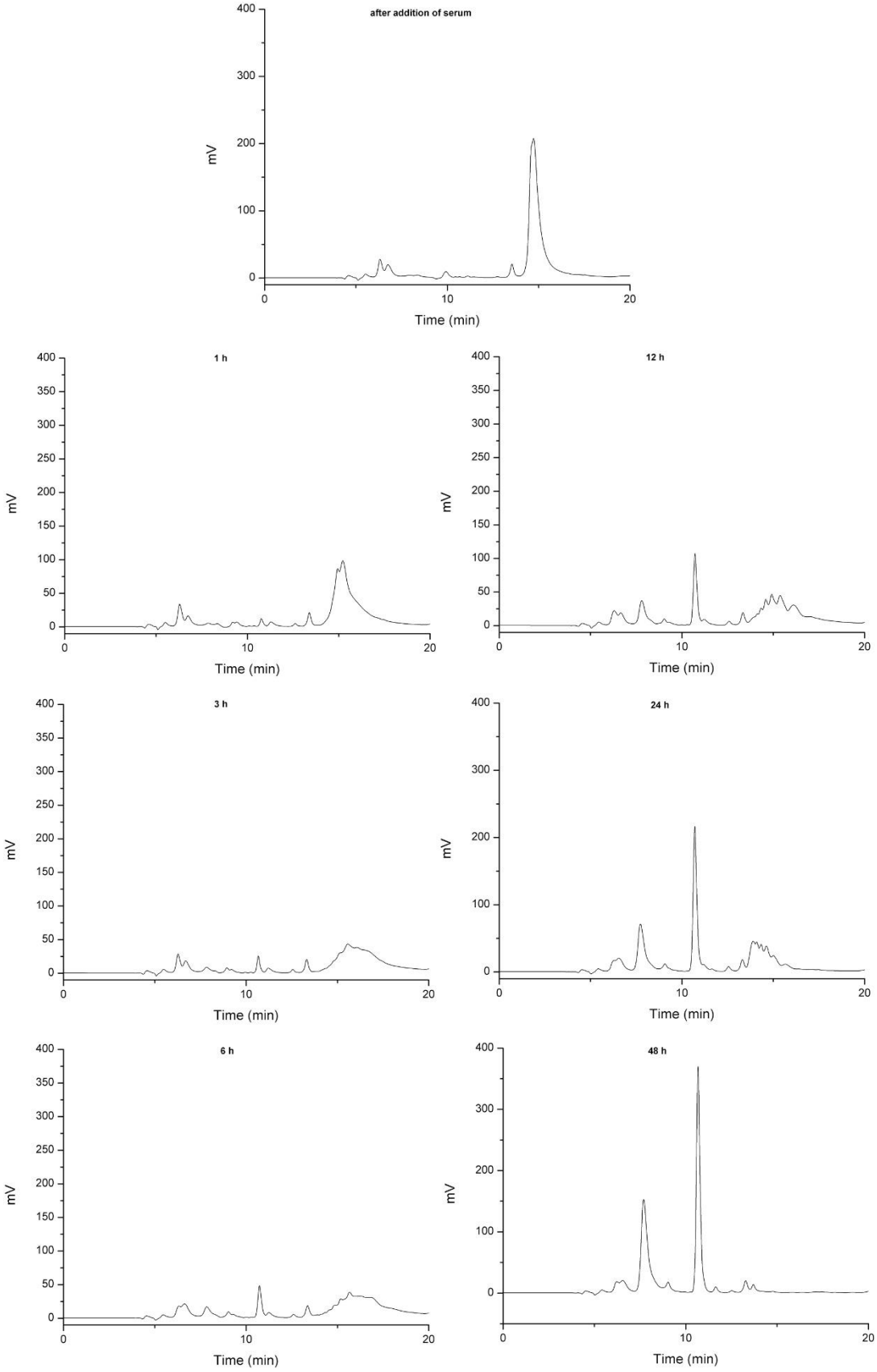
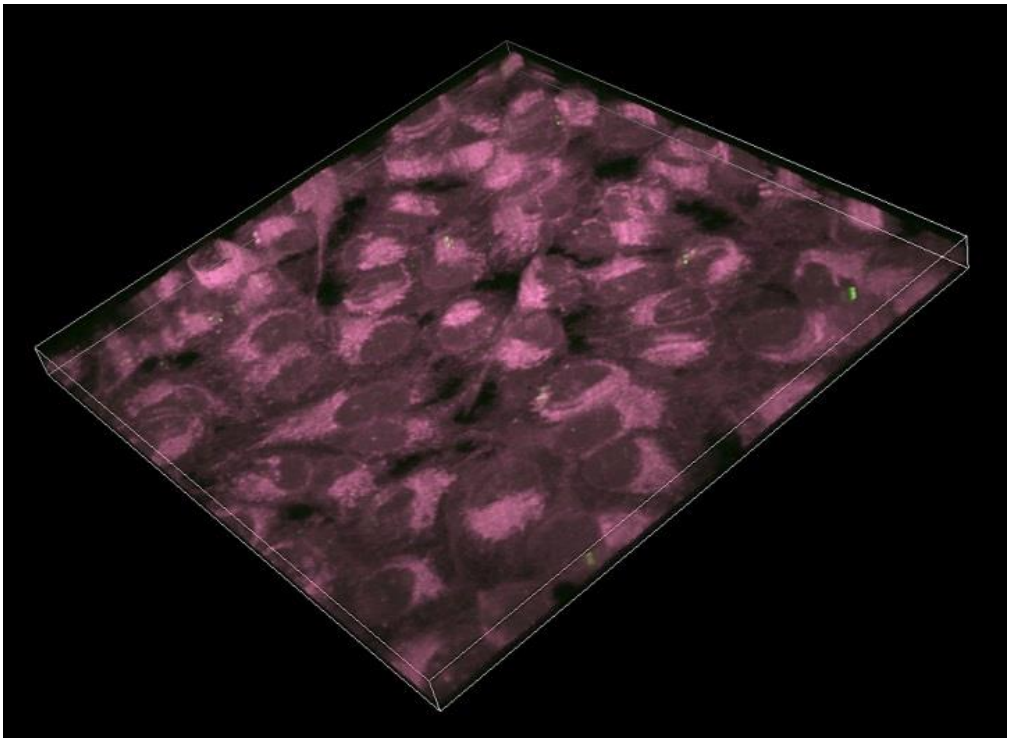


Figure S3. Chromatograms showing the degradation of a native DNA oligonucleotide (dA13) in 90% human serum at different times of incubation up to 48 h.

A



B

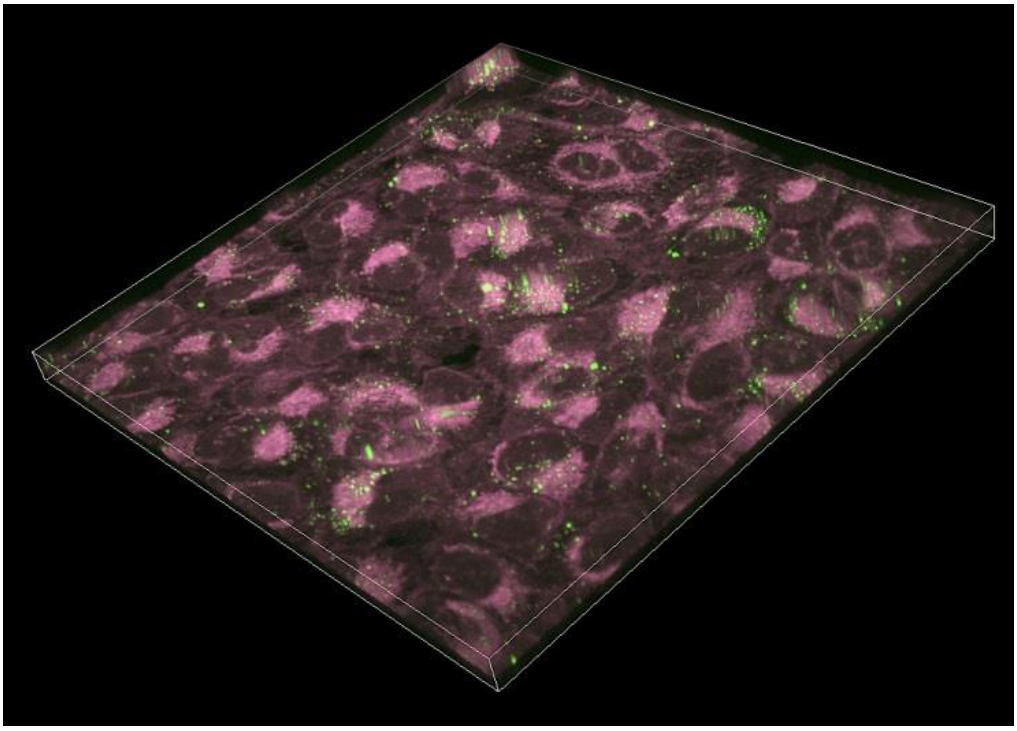
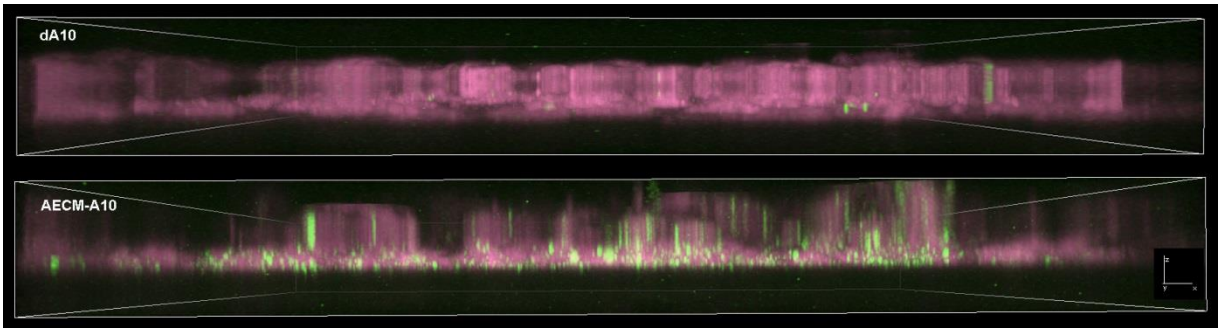


Figure S4. Confocal laser scanning microscopy images of U2OS cells exposed to fluorescein-labelled A) non-modified dA_{10} (**dO5**) *and B)* AECM-A_{10} (**O5**) *for 8 h at 8μM using the CellMask™ dye to stain membrane structures.* The fluorescein-labelled oligonucleotides are visualized in green and membranes in pink. Images represents the xyz view from all z-sections merged into one single image.

A



B

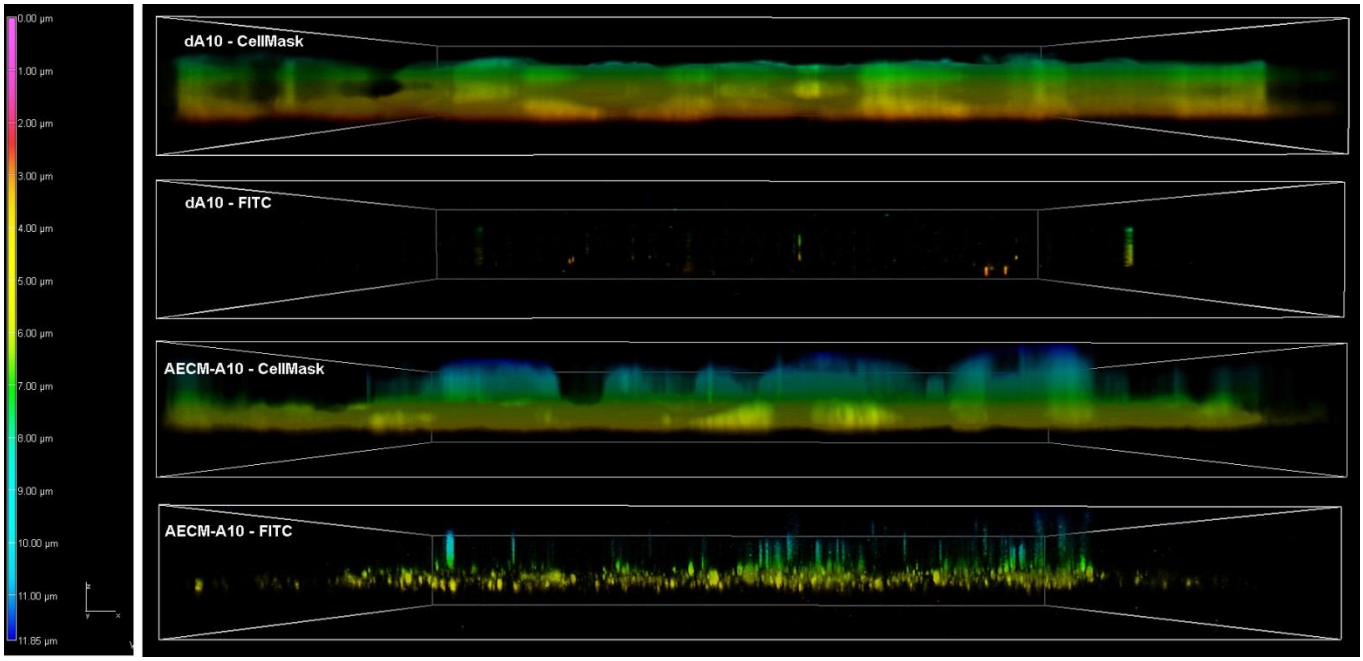


*Figure S5. The xz planes from confocal laser scanning microscopy images of U2OS cells exposed to dA10 (**dO5**) and AECM-A10 (**O5**) for 8 h at 8μM.*
A) Images represent the xz plan from Figure S4, showing that almost no uptake is seen of dA10 (upper panel) and that the uptake of AECM-A10 (lower panel) is mainly in the cells. The fluorescein-labelled oligonucleotides are visualized in green and cell membranes in pink.
B) Images represent the fluorescence distribution along the Z-axis from separate single channels (CellMask for cell membranes; FITC for the fluorescent dA10 and AECM-A10 oligonucleotides) of the images presented in A. The color scale represent the Z axis, where pink is 0 (bottom cell) and dark blue 11μm (top cell). The fluorescence from the AECM-A10 labelled oligonucleotide is mainly seen at about 4-6 μM which corresponds to the centre of the cells.

Figure S6. RP-HPLC Chromatograms of crude deprotected AECM-oligonucleotides ***O1-O4****.*