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Lipid Oligonucleotide Conjugates as Responsive Nanomaterials for Drug Delivery

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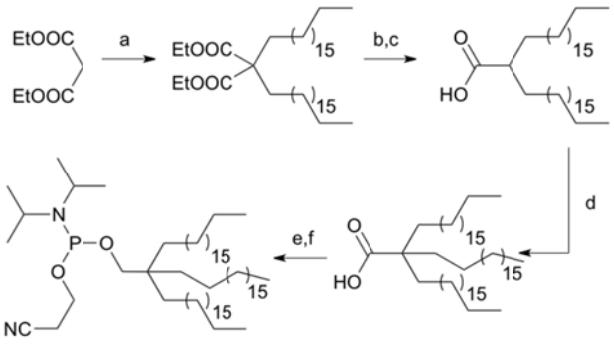
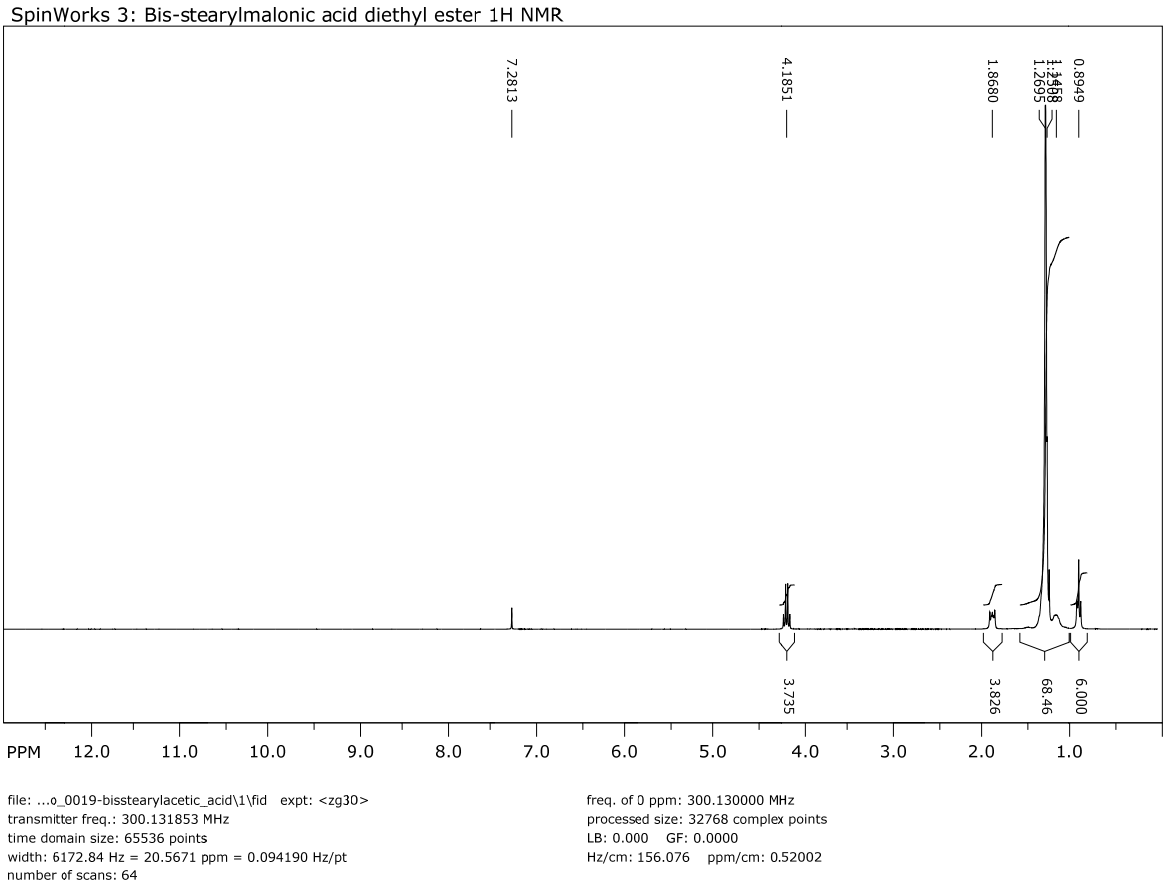
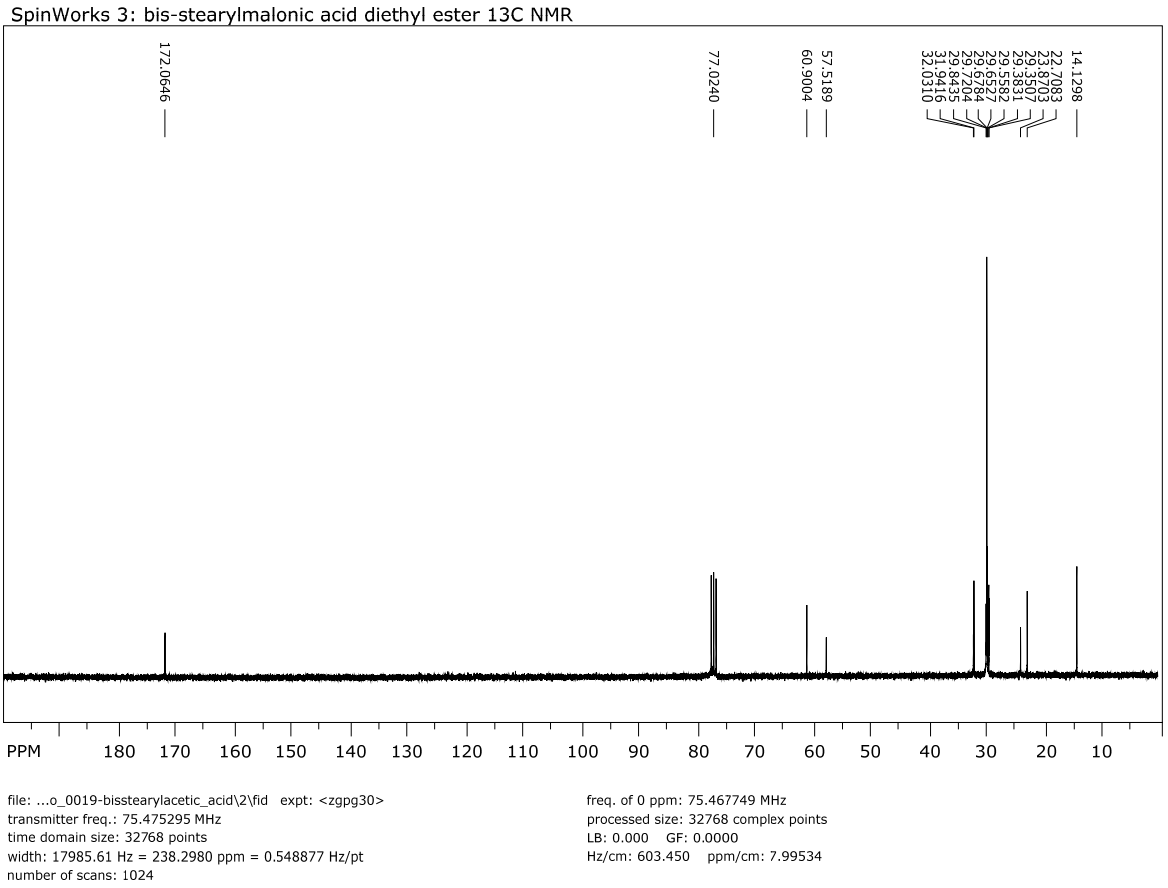
A/ Synthesis of the triple chain phosphoramidite 4:



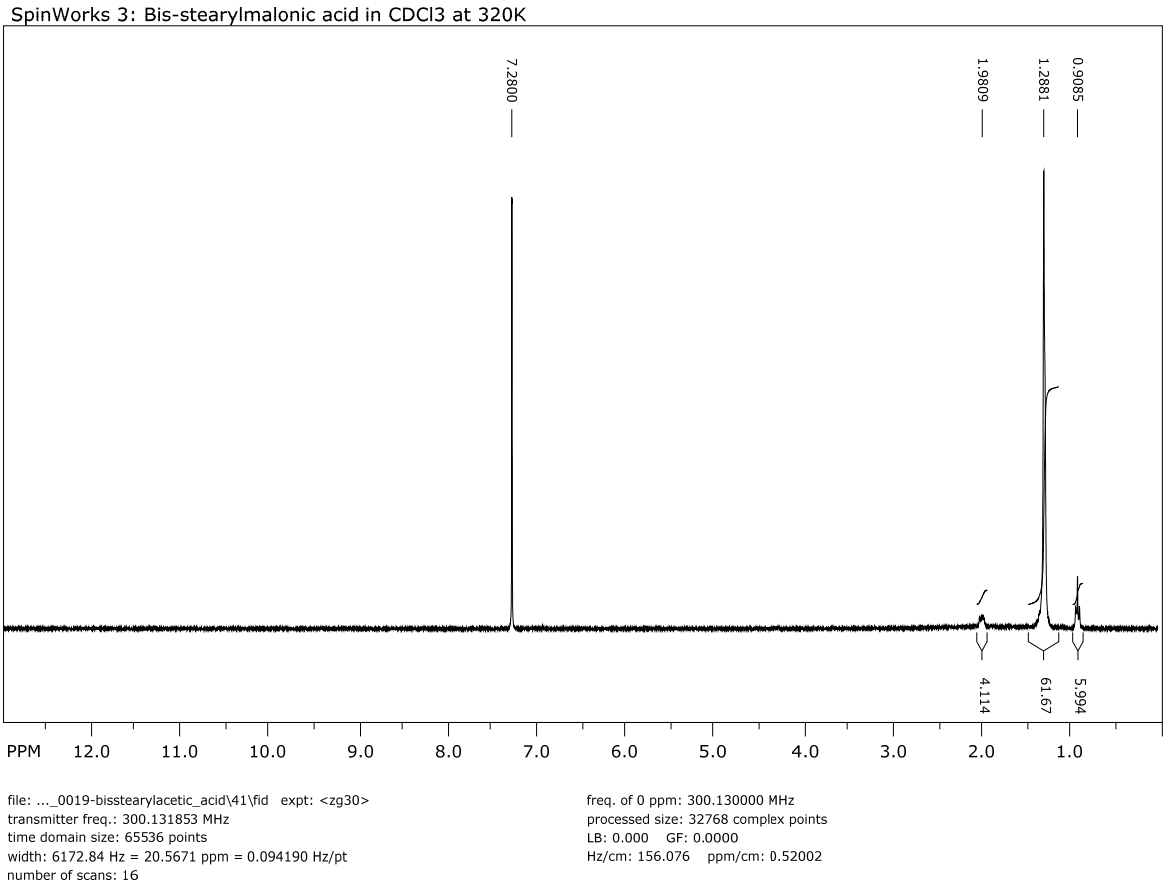
*A-1 Synthesis of 2,2-bis-stearyl acetic acid **2***

NaH (60% suspension in oil) (1.517 g, 37.9 mmol) was suspended under argon in 20 mL of dry DMF. Diethyl malonate (1.93 mL, 12.6 mmol) was gradually added at 0°C. After 15 mns, a solution of stearyl bromide (8.82 g, 26.5 mmol) in 20 mL of dry THF was added and the resulting mixture stirred at rt for 16h and 4h at 50°C. MeOH (10 mL) and acetic acid (2 mL) followed by 200 mL of cold water were then added. The aqueous phase was extracted with 3*75 mL of CH_2Cl_2. The collected organic layers were washed with brine and dried over Na_2SO_4. The resulting solid was recrystallized from iPrOH affording 8.85 g of a white solid.



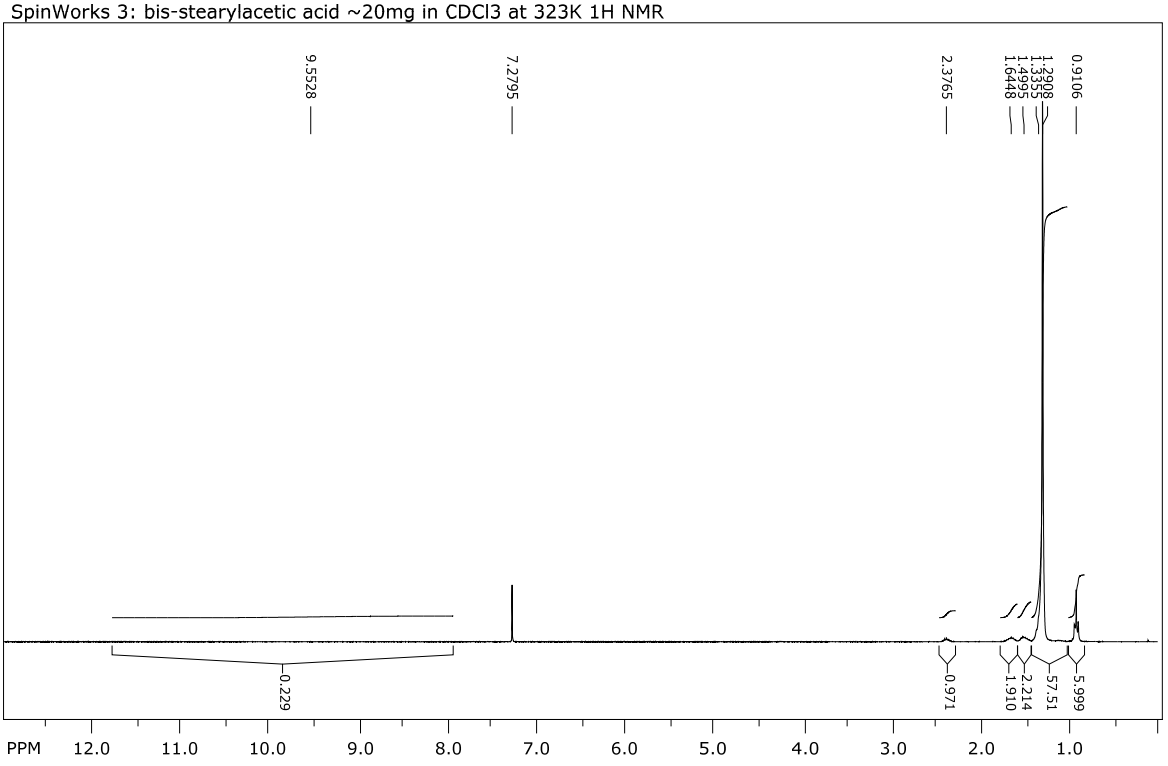


The obtained 2,2-bis-stearyl diethyl malonate **1** was added to a mixture of aqueous KOH (5 g in 100 mL water) and iPrOH (100 mL). The mixture was heated at 80°C for 16h and then diluted with water giving a slurry, which was then neutralized with 50mL of 47% aqueous H_2SO_4. The resulting white solid was filtered and washed with CH_2Cl_2 to afford 5.5 g (77% yield) bis-stearyl malonic acid. This acid was almost insoluble in dichloromethane or DMSO at room temperature and slightly more soluble in chloroform. Its solubility in chloroform greatly increases upon heating the solution above 40^{o}C.



Bis-stearyl malonic acid was decarboxylated in boiling decane under inert atmosphere, yielding the desired 2,2-bis-stearyl acetic acid **2**, which was purified by chromatography ($CHCl_3$ as eluent) to afford 5.5 g (77% yield) of the desired 2,2-bis-stearyl acetic acid **2**.
^{1}H NMR (300 MHz, 320K, $CDCl_3$) δ 2.34 (m, 1H, C**H**COOH), 1.63 (m, 3H), 1.50 (m, 3H), 1.48, 1.29 (m, 59H), 0.90 (t, J = 6.5Hz, 6H). ^{13}C NMR (75 MHz, 320K, $CDCl_3$) δ 177.20, 45.89 (CH), 32.68, 32.11, 29.88, 29.84, 29.81, 29.76, 29.73, 29.68, 29.65, 29.54, 27.65, 22.83, 14.25 (CH_3).

SpinWorks 3: bis-stearylacetic acid ~20mg in CDCl3 at 323K 1H NMR



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number of scans: 16

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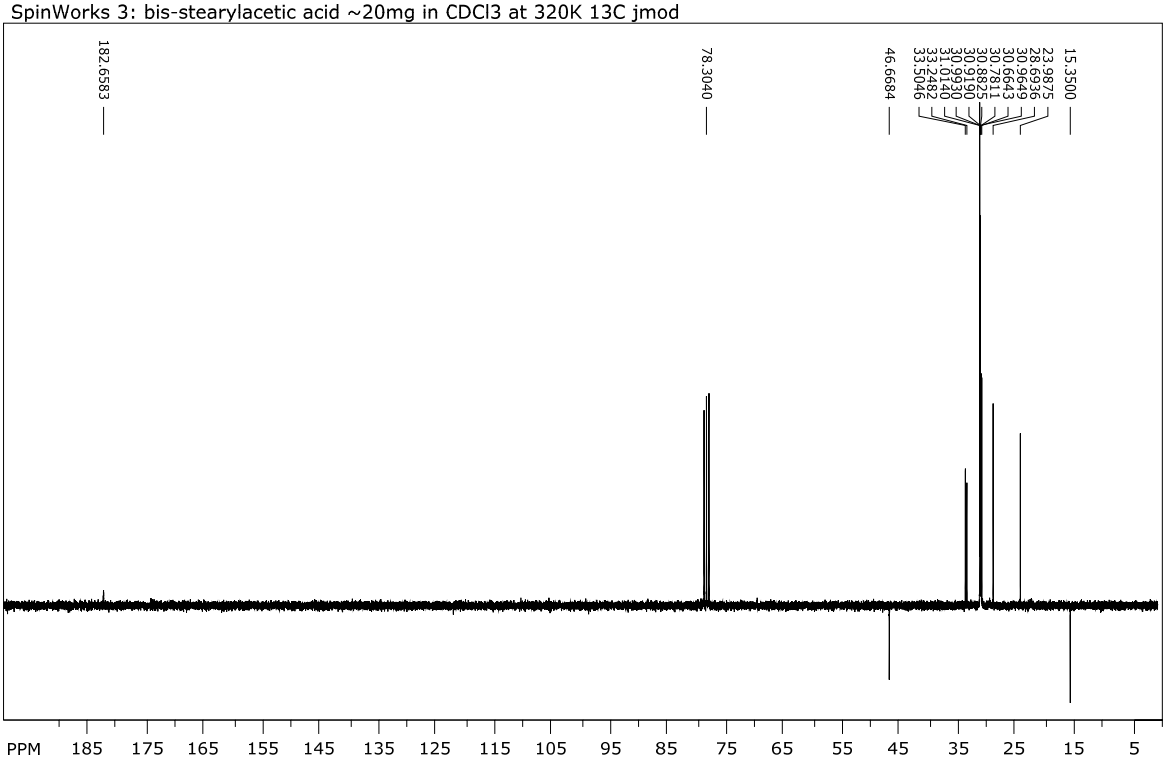
SpinWorks 3: bis-stearylacetic acid ~20mg in CDCl3 at 320K 13C jmod



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*A-2 Synthesis of 2,2,2-tris-stearyl acetic acid **3***

LDA (2M solution, 6.56 mL, 13.12 mmol) was slowly added to 2,2-bis-stearyl acetic acid **2** (3.21 g, 5.68 mmol) in 85 mL dry THF. The mixture was then heated at 50°C for 2h to dissolve all remaining solid materials. Stearyl bromide (7.29 g, 21.88 mmol) in 20 mL dry THF was added in portions to the reaction medium and the reaction stirred overnight at rt. The solvents were evaporated, 15 mL of aqueous 1M HCl solution was added. The aqueous phase was extracted with hexane followed by CH_2Cl_2. The collected organic layers were dried over Na_2SO_4. The crude solid was recrystallized from hexane followed by ethyl acetate to afford 3.17 g (68% yield) of pure **3**.

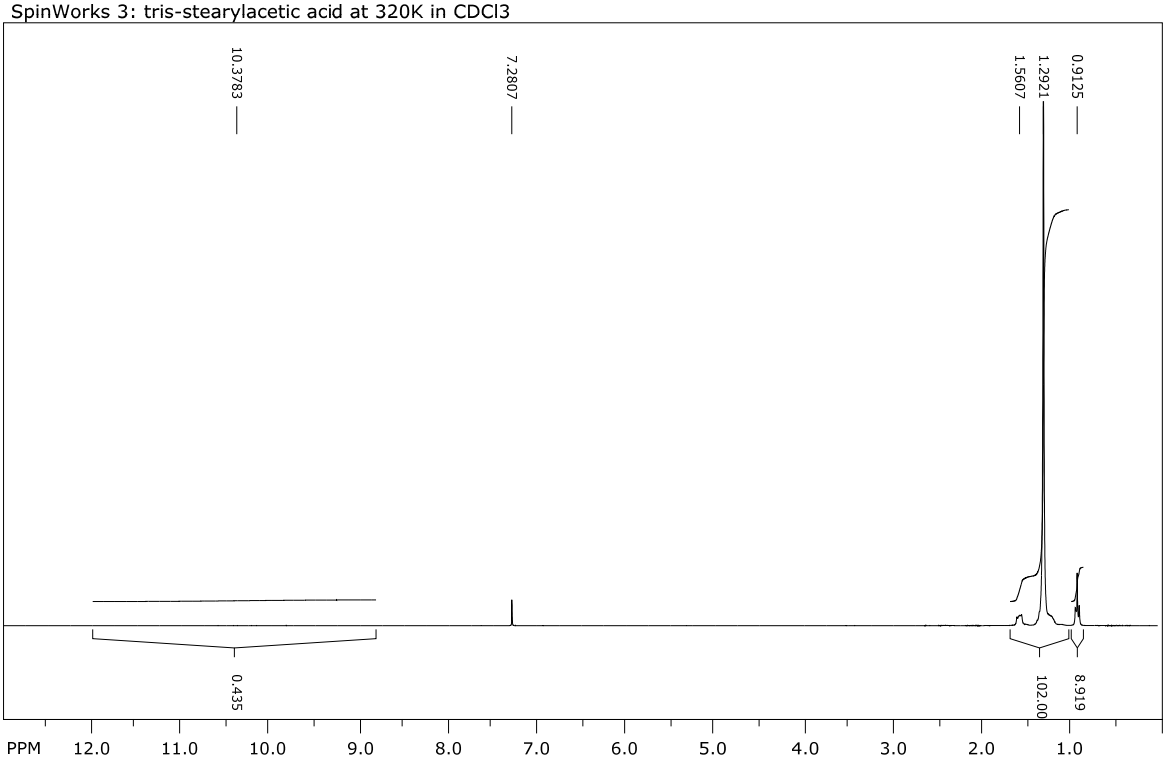
^{1}H NMR (300 MHz, $CDCl_3$) δ 1.52 (m, 6H, C**H$_2$**-C-COOH), 1.25 (m, 97H), 0.88 (t, J = 6.4 Hz, 9H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 184.24, 48.82, 34.33 (broad), 31.97, 30.18 (broad), 29.76, 29.70, 29.53, 29.41, 23.85 (broad), 22.73, 14.15 (CH_3).

This product proved especially reluctant to mass analysis using classical protocols (ESI in positive or negative mode, MALDI with ordinary organic matrices). In contrast, very clean mass spectrum was obtained with a DCTB matrix (trans-2-[3-(4-tert-butylphenyl)-2-methyl-2-propenylidene]malononitrile) doped with Ag^+ (or Na^+).

Procedure: Samples were dissolved in CH_2Cl_2 at 10 mg/ml. The DCTB matrix solution was prepared by dissolving 10 mg in 1 ml of CH_2Cl_2. A solution of cationisation agent (AgTFA, 10 mg/ml) was also prepared. The solutions were combined in a 10:1:1 volume ratio of matrix to sample to cationisation agent. One to two microliters of the obtained solution was deposited onto the sample target and vacuum-dried.

MS : $C_{56}H_{112}O_2$ (exact mass=816.87) : found 923.80 $[M+Ag]^+$, 1031.72 $[M-H+2Ag]^+$.

SpinWorks 3: tris-stearylacetic acid at 320K in CDCl3



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number of scans: 16

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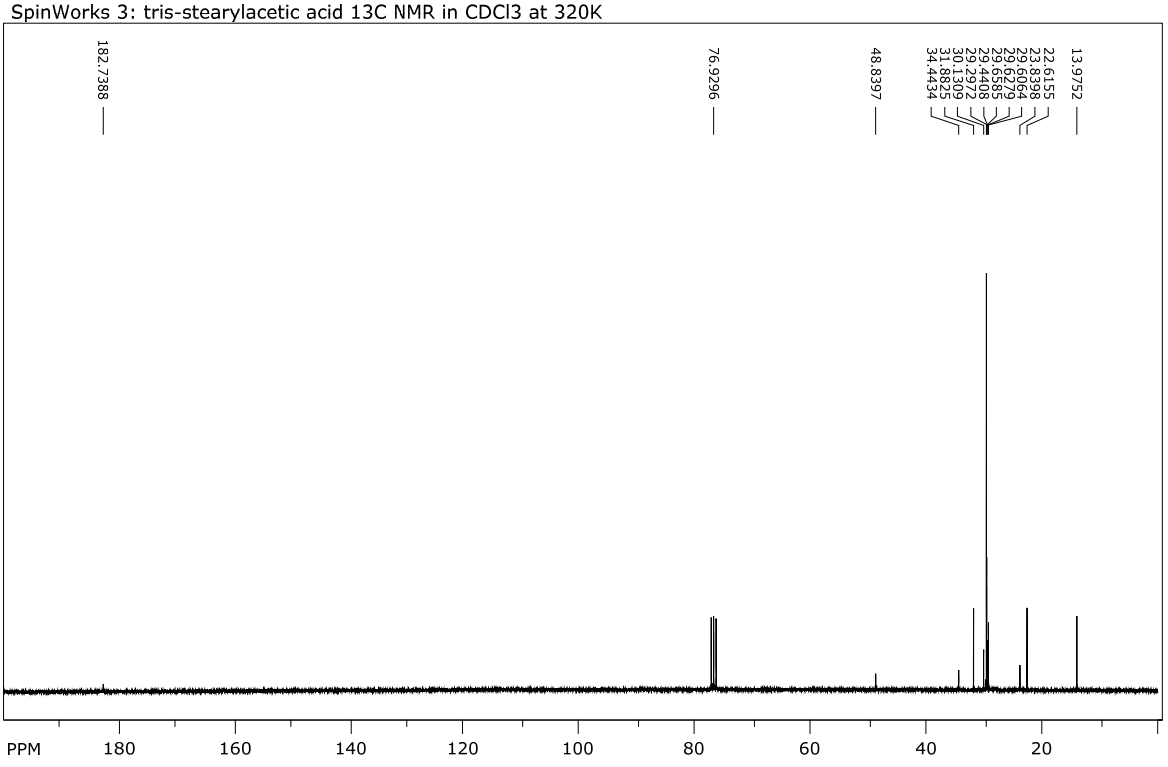
SpinWorks 3: tris-stearylacetic acid 13C NMR in CDCl3 at 320K



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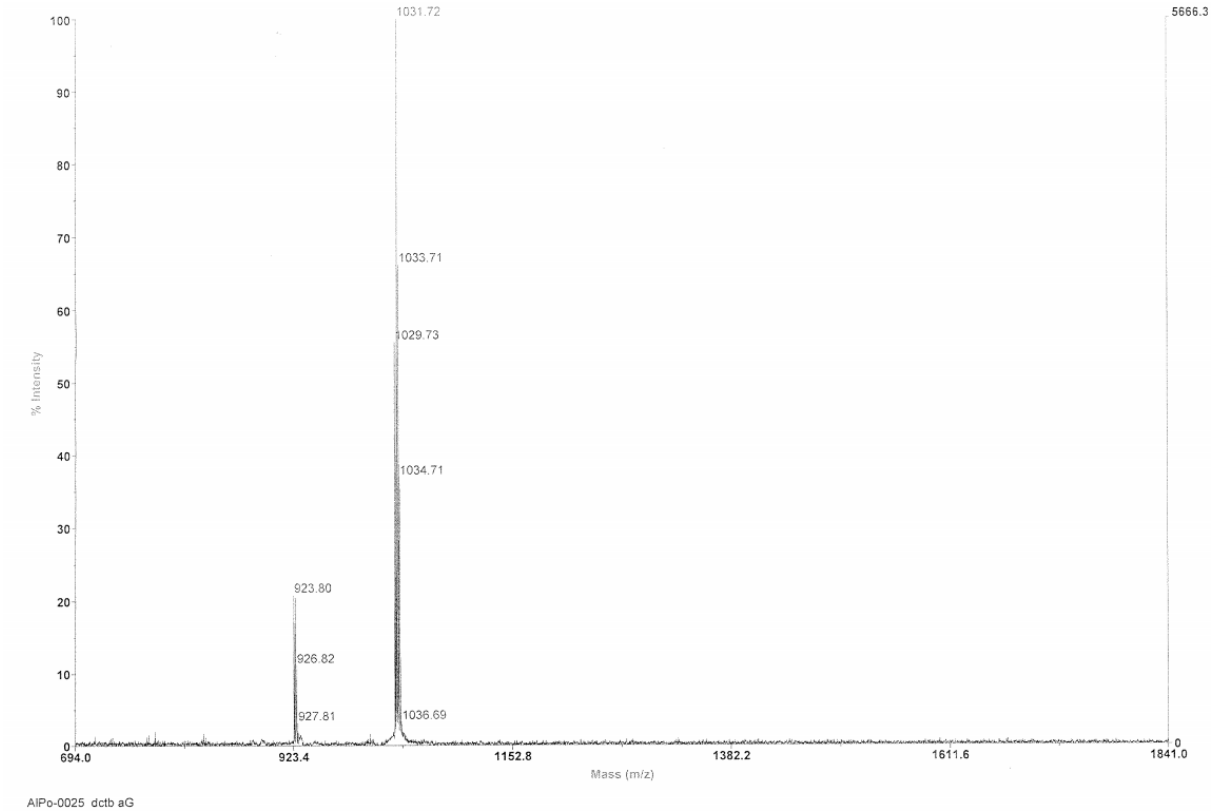


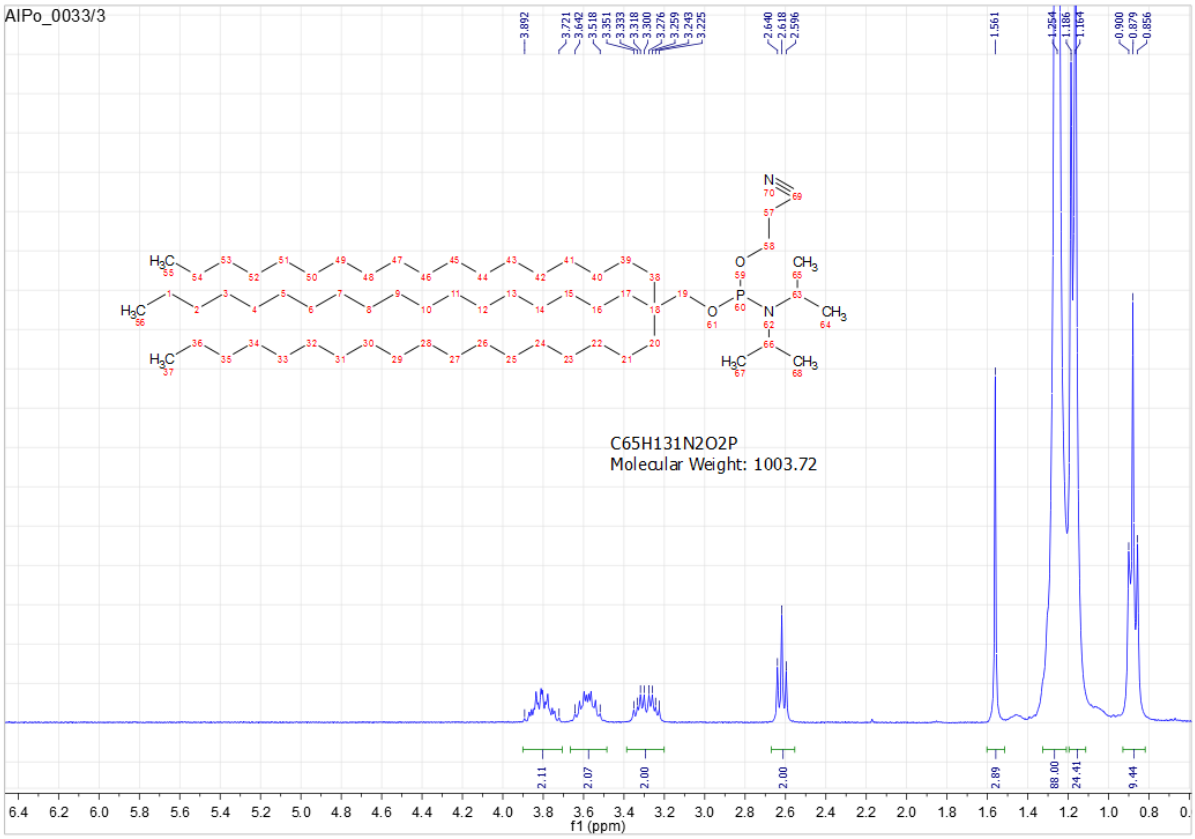
A-3 Synthesis of 2,2,2-tris-stearyl ethanol

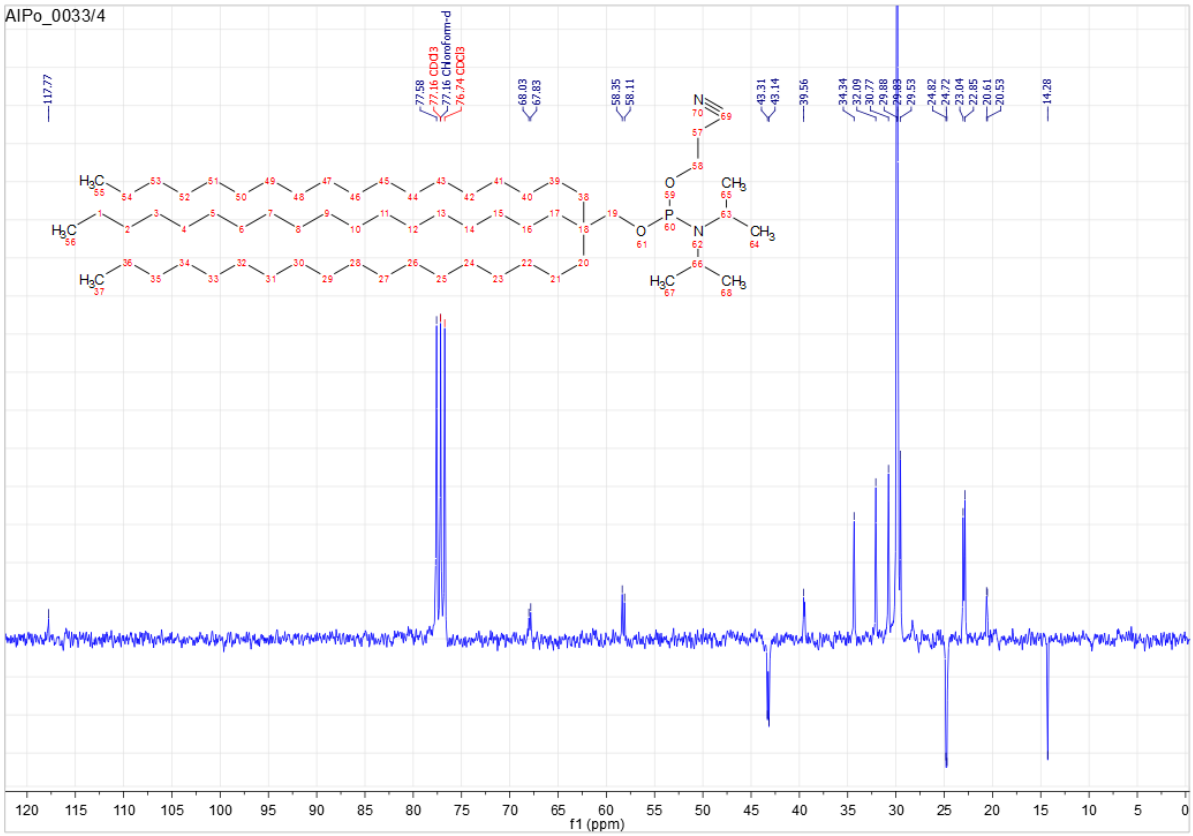
LAH (0.45 g, 11.85 mmol) was slowly added in portions to tris-stearyl acetic acid (2 g, 2.45 mmol) in dry THF (15 mL). The reaction mixture was stirred at 65°C for 12h. Water followed by aqueous 1M HCl were added. The aqueous phase was extracted with hexane. The collected organic layers were dried over Na_2SO_4. The crude was recrystallized from hexane followed by iPrOH to afford 1.40 g (71% yield) of 2,2,2-tris-stearyl ethanol.
^{1}H NMR (300 MHz, $CDCl_3$) δ 3.35 (s, 2H, C**H$_2$**OH), 1.25 (m, 94H), 1.16 (m, 12H), 0.88 (t, *J* = 6.5 Hz, 9H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 67.18, 39.47, 33.92, 31.95, 30.63, 29.73, 29.69, 29.39, 22.94, 22.72, 14.16 (CH_3).

*A-4 Synthesis of phosphoramidite **4***

2,2,2-tris-stearyl ethanol (330 mg, 0.41 mmol) was dried over P_2O_5 overnight before use. It was then dissolved in dry CH_2Cl_2 (5 mL) containing diisopropylethylamine (0.115 mL, 0.66 mmol). The phosphitylating reagent (0.12 mL, 0.53 mmol) was added to the reaction mixture. The reaction was stirred at rt for 3h and then quenched with saturated aqueous $NaHCO_3$ and extracted with CH_2Cl_2. The collected organic layers were washed with water and dried over Na_2SO_4. The resulting oil was chromatographed (Hex/EtOAc/TEA 40/2/1) to afford 0.38 g (92% yield) of pure **4** as colorless oil.
RM: this phosphoramidite is quite unstable on silica and must be chromatographed rapidly.

^{1}H NMR (300 MHz, $CDCl_3$) δ 3.81 (m, 2H, C**H$_2$**O CNE), 3.58 (m, 2H, iPr), 3.29 (ABX system, J_{AB} = 9.9 Hz, J_{AX} = 5.4 Hz, J_{BX} = 5.3 Hz, 2H, C**H$_2$**O), 2.62 (t, J = 6.6Hz, 2H, C**H$_2$**CN), 1.25 (m, 88H), 1.18-1.16 (m, 24H), 0.88 (t, J = 6.3Hz, 9H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 117.77 (CN) 67.91 (d, J = 15.0 Hz), 58.22 (d, J = 18.0 Hz), 43.22 (d, J = 12.8 Hz), 39.56, 34.34, 32.09, 30.77, 29.88, 29.83, 29.53, 24.77 (d, J = 7.5 Hz), 23.04, 22.85, 20.52 (d, J = 6.0 Hz), 14.28. ^{31}P NMR (122 MHz, $CDCl_3$) δ 150.56. MS (ESI, positive mode) $C_{65}H_{131}N_2O_2P$ (exact mass=1002.99): found 1003.1 $[M]^+$

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77.58
77.16 CDCl3
76.74 CDCl3
68.03
67.83
58.35
58.11
43.31
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39.56
34.34
32.09
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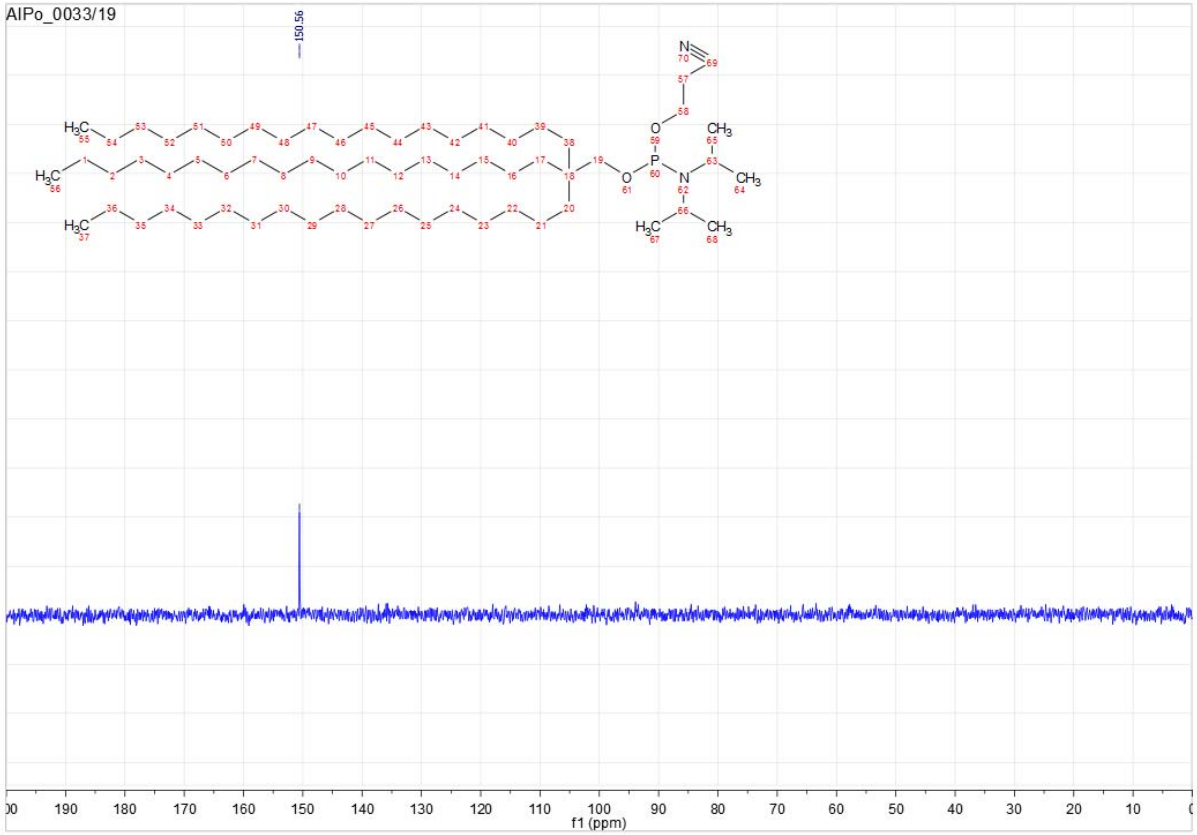
20.61
20.53
14.28
f1 (ppm)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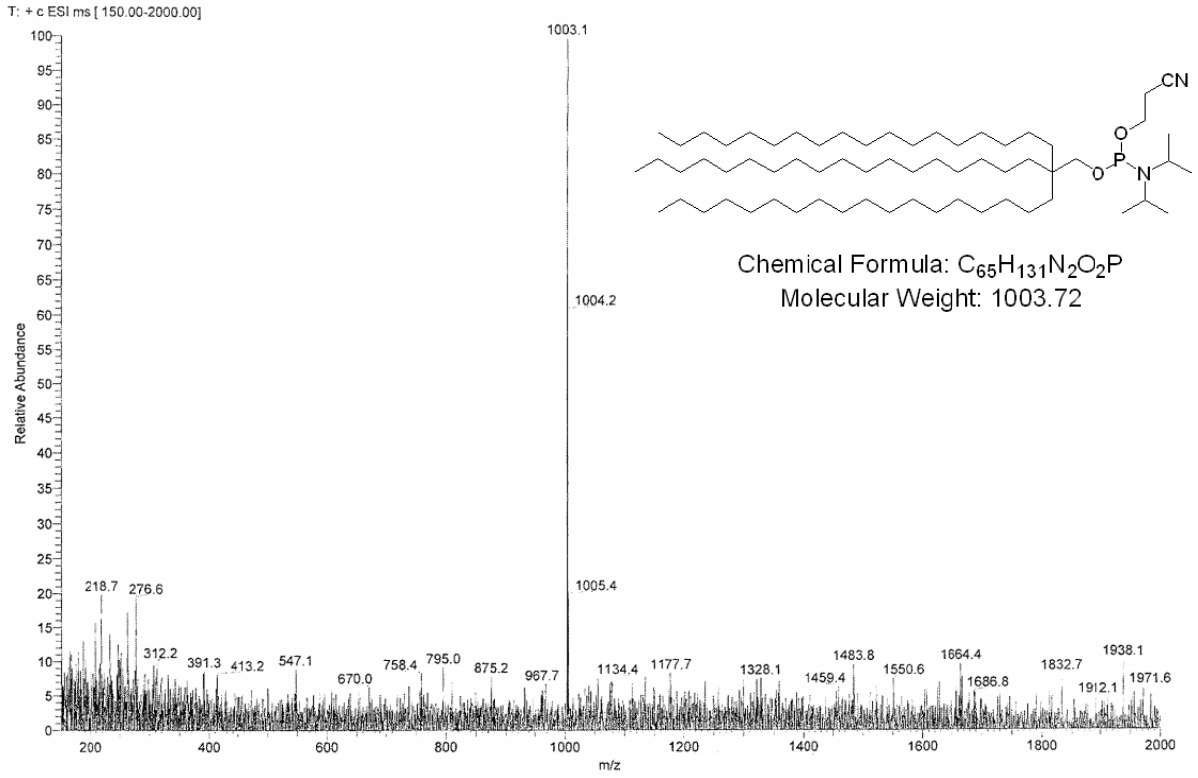


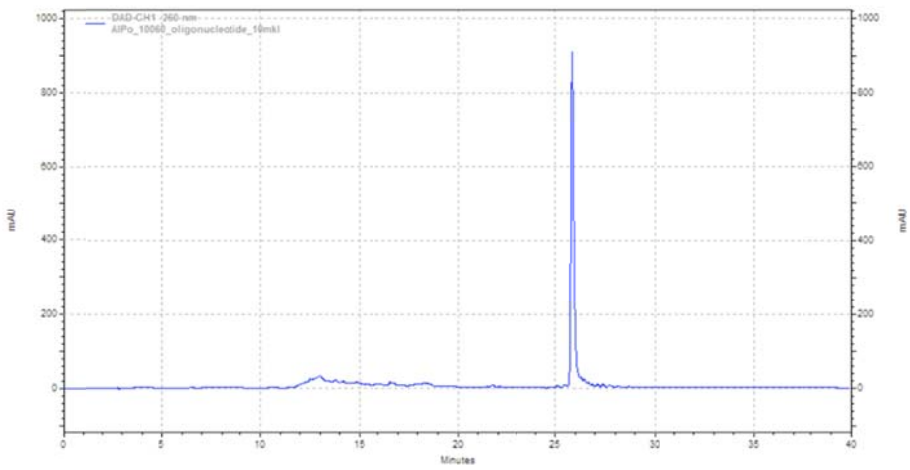
B/ Synthesis of the LONs (^{2}LON-T$_{15}$, ^{2}LON-A$_{15}$, ^{3}LON-A$_{15}$, ^{3}LON-T$_{15}$)

B/1 Synthesis of ^{2}LON-T$_{15}$, ^{2}LON-A$_{15}$

The protocol for the synthesis of these LONs can be found in reference: A. Gissot, C. Di Primo, I. Bestel, G. Giannone, H. Chapuis, and P. Barthélémy, *Chem. Commun.*, 2008, 5550.

- **^{2}LON-T$_{15}$**:

HPLC of purified oligonucleotide amphiphile *(C$_{15}$)$_2$U*-5'-5'-(dT)$_{15}$:*

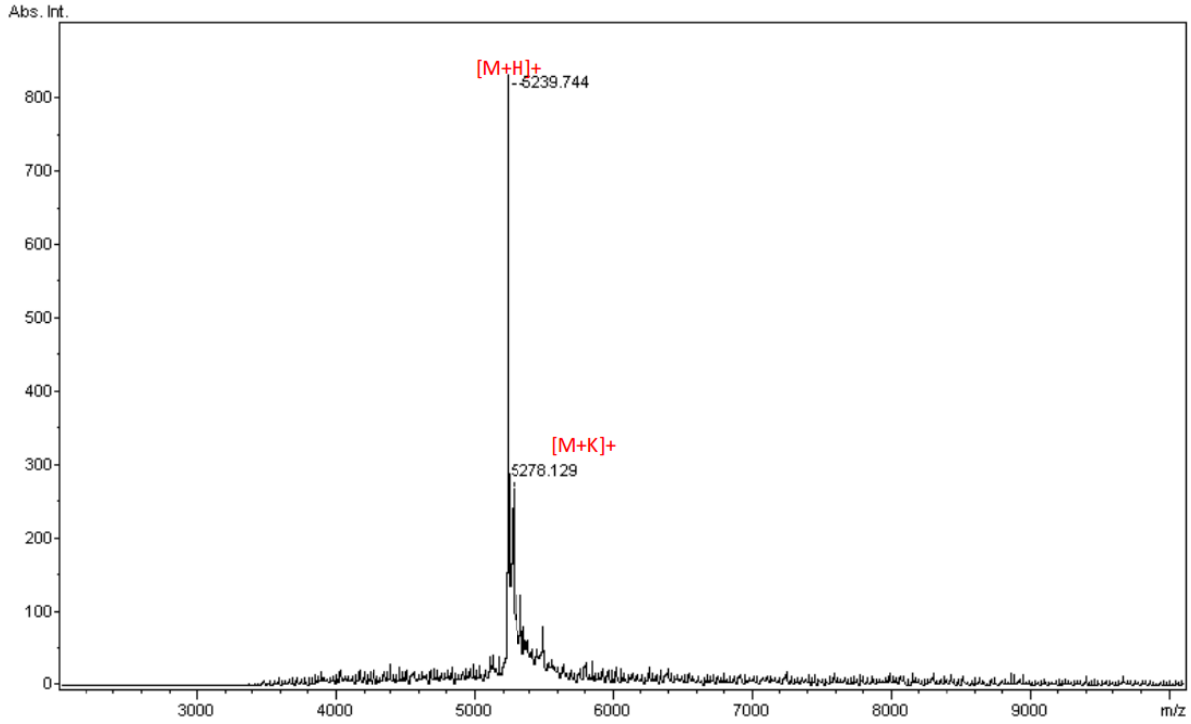


C$_4$-reverse phase HPLC, detection at 260 nm

$(C_{15})_2U$*-5'-5'-$(dT)_{15}$-3'-OH

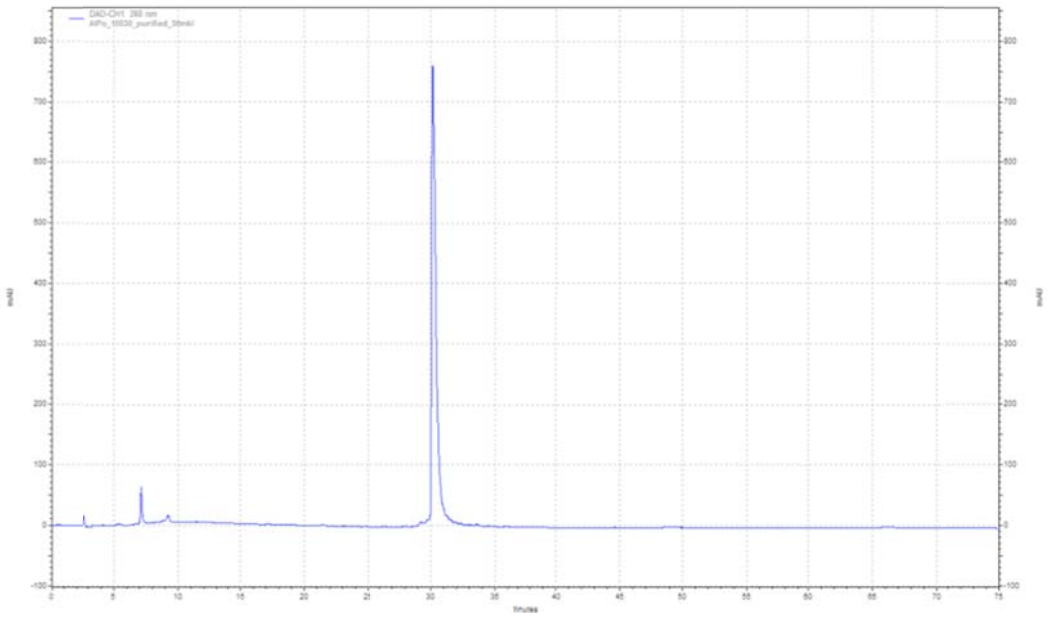
Formula : $C_{190}H_{267}N_{32}O_{111}P_{15}$; Formula Weight : 5240; Exact Mass : 5237.23

Mass-spectrometry (sample for SEM studies):



- **^{2}LON-A_{15}**:

HPLC of purified oligonucleotide amphiphile *$(C_{15})_2U$*-5'-5'-$(dA)_{15}$:*



C_4-reverse phase HPLC, detection at 260 nm

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B/2 Synthesis of ^{3}LON-T$_{15}$, ^{3}LON-A$_{15}$

Prior to use, the phosphoramidite **4** was dried over P_2O_5 overnight. It was then dissolved in dry CH_2Cl_2/CH_3CN 3/1 to a 0.1 M concentration. N-benzylthiotetrazole was used for activation of phosphoramidite prior to coupling. The phosphoramidite **4** (200 µL, 0.1 M sol.) was manually coupled last on the solid support with the activator and the phosphoramidite (0.25 mL) for 5 min. Deblocking and detachment from the solid support was achieved using 1 mL of a saturated aqueous NH_4OH/ethanol 3/1 (vol/vol) solution for 5 h at 55°C. The supernatant was collected and the CPG beads were washed 3 times with 0.25 mL of $EtOH/CH_3CN/H_2O$ 3/1/1 (vol). The solutions were pooled and evaporated (speed vac).
At this point, the crude **^{3}LON-T$_{15}$** and **^{3}LON-A$_{15}$** behave quite differently. While **^{3}LON-T$_{15}$** crude could be readily solubilized in 1 mL milliQ water, a heavy precipitate was obtained from **^{3}LON-A$_{15}$**.[1] **^{3}LON-T$_{15}$** was then purified on an analytical C_4-reverse phase HPLC using buffer A (0.1 M triethylammonium acetate, pH 7.0, 5 % acetonitrile) and buffer B (0.1 M triethylammonium acetate, pH 7.0, 80 % acetonitrile): gradient increase from 0 to 80% B in 10 mns and then keeping this ratio constant up to 50 mns. The LON eluted after ca. 42 mns under these conditions. Fractions containing the LON were pooled and evaporated to dryness. The LON was dissolved in water and dried again two times to remove all residual traces of buffer. Yield of final **^{3}LON-T$_{15}$** was excellent (40%). Yet, we found a tendency upon long storage (several weeks) for aqueous solutions of this LON toward the irreversible aggregation just like what was observed with **^{3}LON-A$_{15}$** right after synthesis. Accordingly, all paclitaxel loading experiments using this LON were conducted rapidly after its chemical synthesis.

- **^{3}LON-T$_{15}$**

Crude mixture (MS):

Although we are well aware MALDI-TOF analyses are not quantitative, MS of the crude seems to indicate that the single coupling of the phosphoramidite **4** to the polyT chain on the solid support worked well (the peaks at 4504 $(M+H)^+$, 4524 $(M+Na)^+$ and 4542 $(M+K)^+$ correspond to unmodified dT_{15}).

[1] No desired **^{3}LON-A$_{15}$** could be detected by MS in the supernatant. In addition, several attempts to heat and bath-sonicate in water or phosphate buffer the precipitate failed to detect any trace of the LON according to MS.

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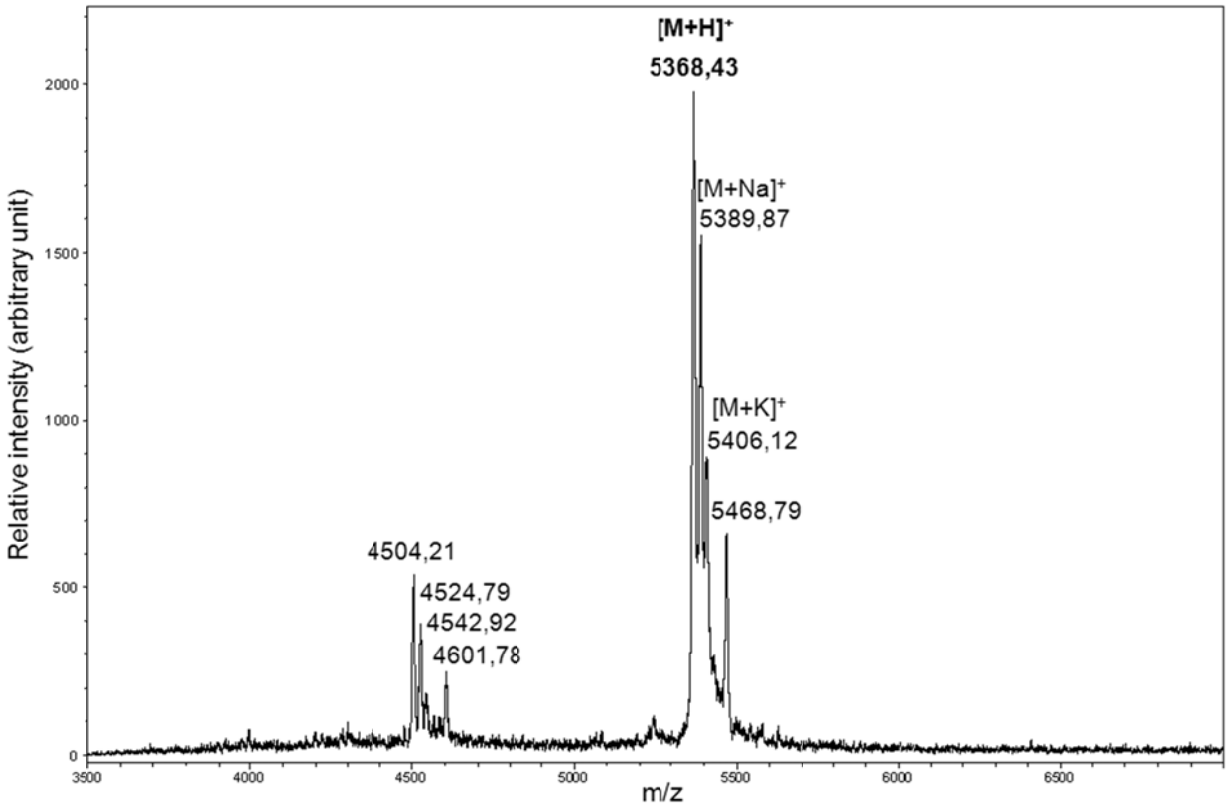
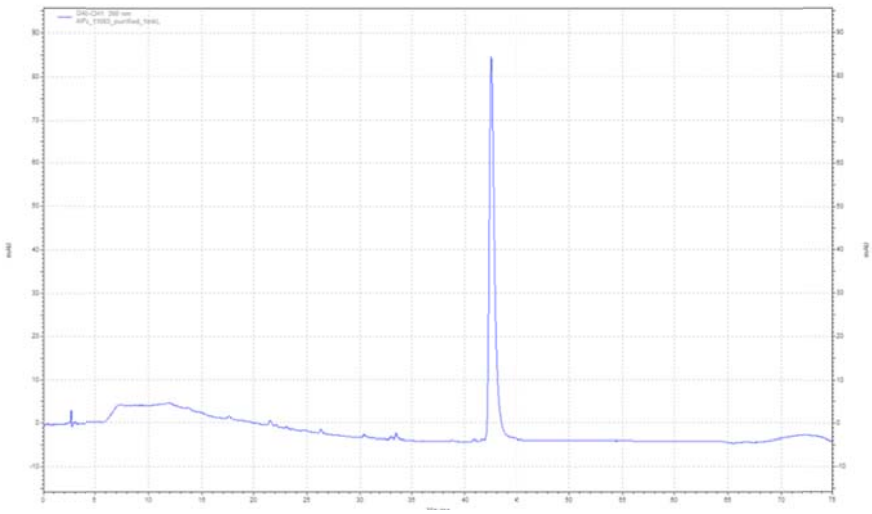


HPLC of purified oligonucleotide amphiphile *$(C_{18})_3CCH_2$-5'-$(dT)_{15}$*:



C_4-reverse phase HPLC, detection at 260 nm

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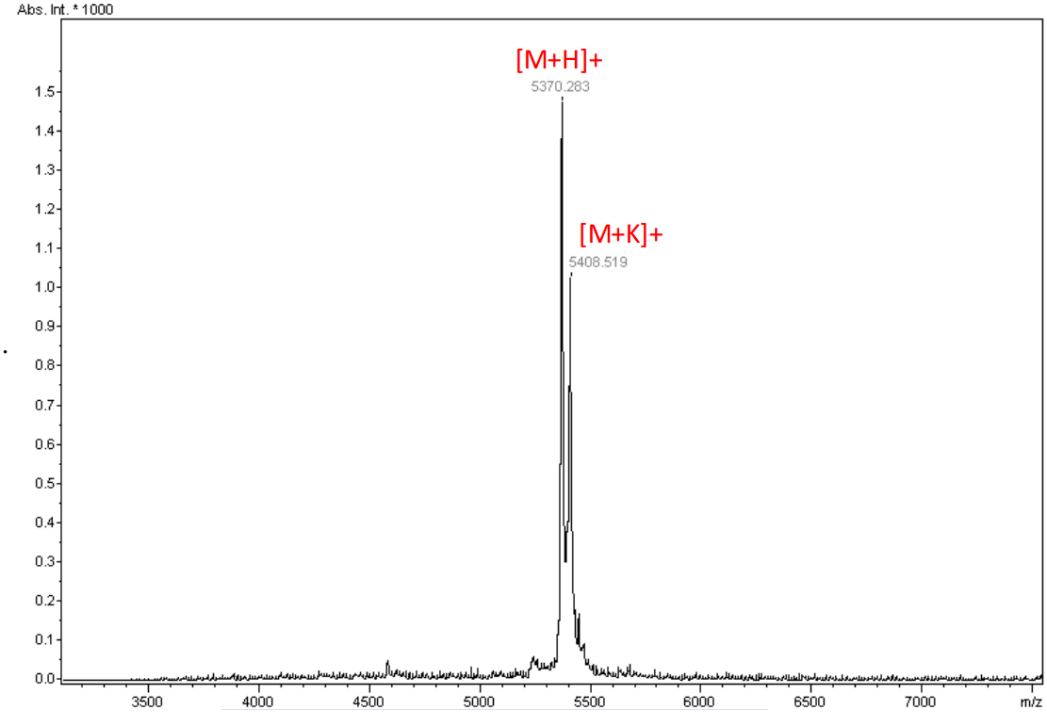
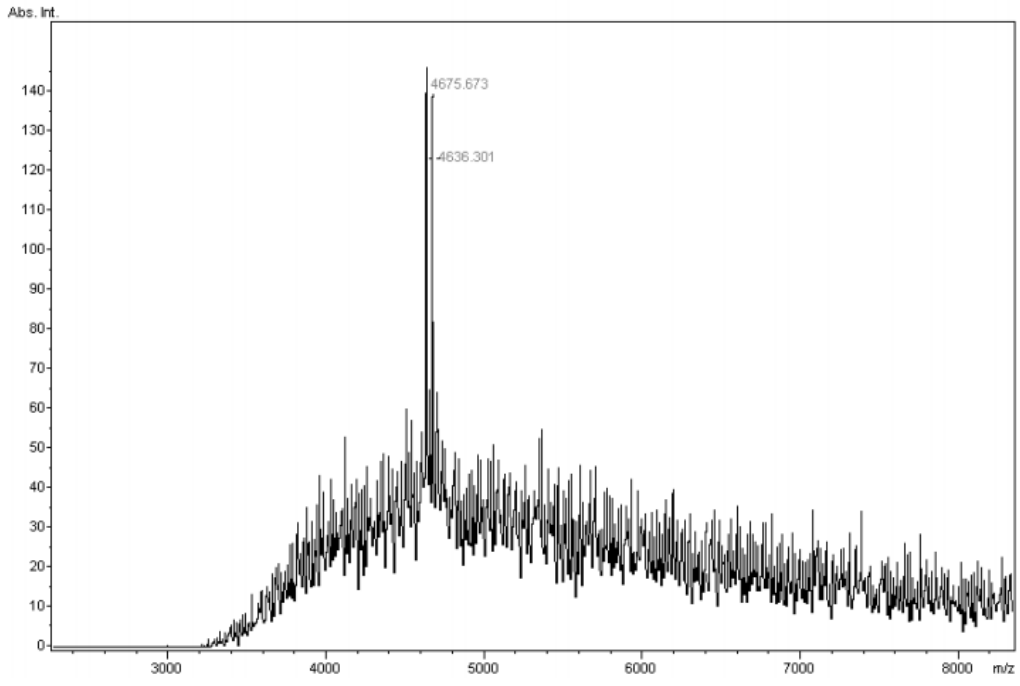
$(C_{18})_3CCH_2O$-PO_2-O-5'-$(dT)_{15}$-3'-OH
Formula: $C_{206}H_{309}N_{30}O_{106}P_{15}$ Formula weight 5366; Exact mass 5363.58



^{3}LON-A_{15}

Crude mixture (MS):

$(C_{18})_3CCH_2O$-PO_2-O-5'-$(dA)_{15}$-3'-OH.
Formula: $C_{206}H_{294}N_{75}O_{76}P_{15}$; Formula weight 5502; Exact mass: 5498.75
Crude product was submitted for mass-spectrometry.



The peaks at 4636 $(M+H)^+$ and 4675 $(M+K)^+$ correspond to unmodified dT_{15}.

C/ Size of LON molecules

The molecule of ^{2}LON-A_{15} on the figure 2 was constructed in MarvinSketch (MarvinSketch 5.9.0 and MarvinSpace 5.9.0 software) and optimized in Dreiding force field, implemented in the program. Then several torsion angles were manually tweaked in MarvinSpace to maximally elongate the chain.

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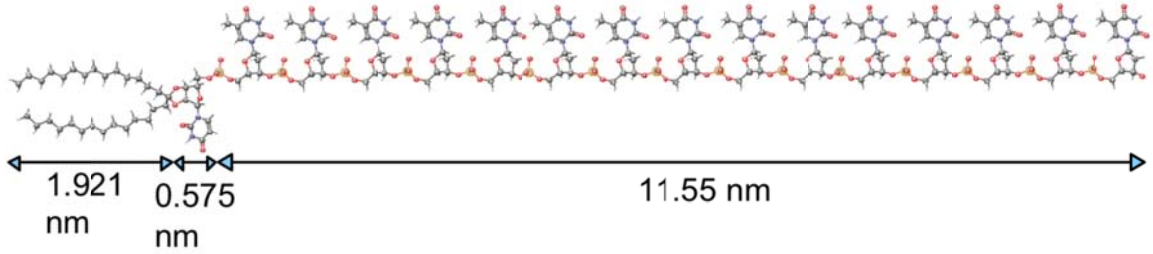

Maximal elongation of a whole ^{2}LON-T_{15} molecule : 1.921+0.575+11.55 = 14.046 nm.

For ^{3}LON molecules the hydrophobic chain lenth can be assessed as 0.15+0.1265*(17+2) + 0.1175= 2.671 nm where (17+2) numbers reflect stearyl chains attached to ethyl fragment in beta-position, and 0.1175 nm is the length of C-O bond projection. O-P bond was not taken into the calculations to compensate its absence at the 3'end of the oligonucleotide chain.

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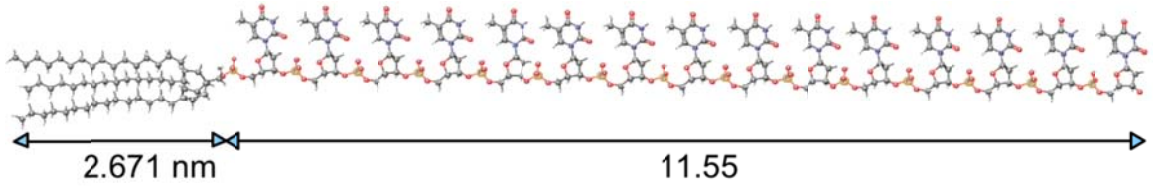
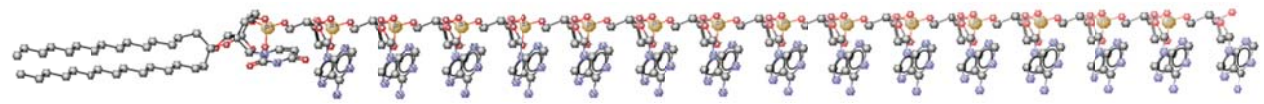


The maximal elongation of ^{3}LON-T_{15} will not exceed 14.221 nm.



3D model of maximally elongated ^{2}LON-A_{15} **Error! Bookmark not defined.Error! Bookmark not defined.**molecule Its maximal elongation is assessed as 14.046 nm, equivalent to ^{2}LON-T_{15} molecule.

D/ Physico chemistry

Particle size and zeta determination. Particle zeta and size were determined using a Zetasizer 3000 HAS MALVERN. Experiments were realized with samples containing different concentration of LONs dissolved in deionized water or phosphate buffer. Measurements were performed at 25°C. LON concentrations are indicated in tables below.

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Oligonucleotide $(dT)_{15}$. Non-modified oligonucleotide $(dT)_{15}$ (for comparison) was dissolved in water. Solutions of different concentrations were prepared (1.4 μM; 12 μM; 112 μM and 687 μM). DLS studies did not show any aggregates for this non-modified oligonucleotide.

Size of LONs

Particle size and zeta determination. Particle zeta potentials and sizes were determined using a Zetasizer 3000 HAS MALVERN. Experiments were conducted with different concentration of LONs dissolved in deionized water or 10 mM phosphate buffer pH 7, 100 mM NaCl. Measurements were performed at 25°C.
Non-modified oligonucleotide $(dT)_{15}$ (for comparison) was dissolved in water. Solutions of different concentrations were prepared (1.4 μM; 12 μM; 112 μM and 687 μM). No aggregates for this non-modified oligonucleotide were found.

Size of LONs

Entry	Concentration, μM in pure water	Mean Intensity, diameter. nm
^{2}LON-T_{15}	800	8.26
^{2}LON-A_{15}	50	16.34
^{3}LON-T_{15}	5	15.37

Impact of ionic strength on the size

Size of micellar aggregates of **^{2}LON-T_{15}** in phosphate buffers of different concentrations (**^{2}LON-T_{15}** = 409.25 μM)

Buffer concentration, M	0	0.01	0.1
Diameter, nm	9.13	11.13	14,1

Impact of temperature on the size of 2LON-T15 in PBS 0.1M pH 7

Concentration (μM)	T °C	PdI	Mean Intensity, diameter. nm
400	25	0.260	14.1
400	35	0.250	14.2
400	45	0.243	13.7
40	25	0.350	15.4
40	35	0.460	14.8
40	45	0.423	15.0

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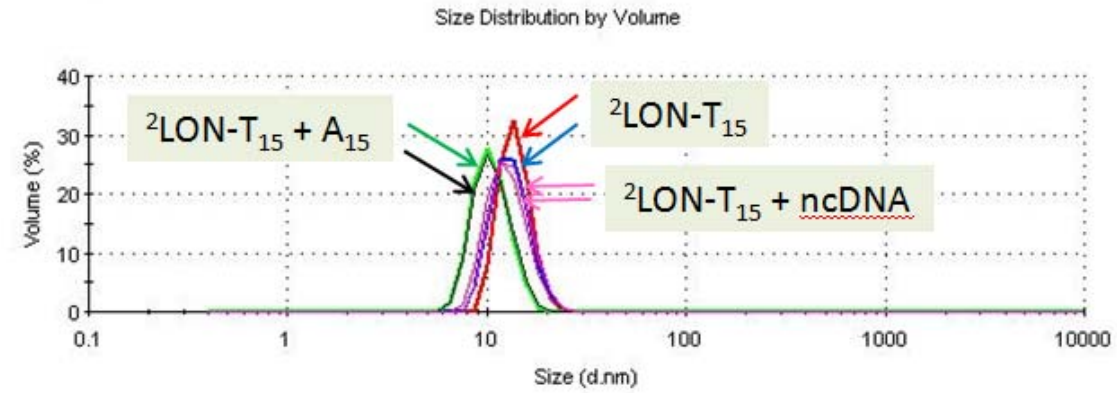
Representative DLS results (analyzed by volume and in the absence of paclitaxel) obtained for the different LON systems investigated in Figure 4:

		Diam. (nm)	% Volume	Width (nm)
Z-Average (d.nm): 16.87	Peak 1:	13.46	99.9	3.012
PdI: 0.330	Peak 2:	275.5	0.1	78.18
Intercept: 0.916	Peak 3:	0.000	0.0	0.000
Result quality : Good				

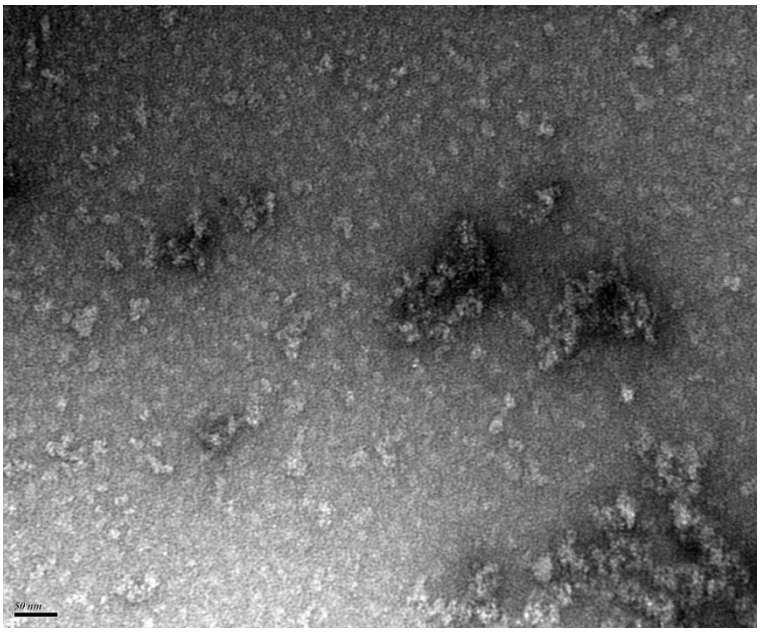


E/ Electron microscopy (TEM, SEM)

See the experimental section for details on the preparation of the samples for TEM and SEM analyses.

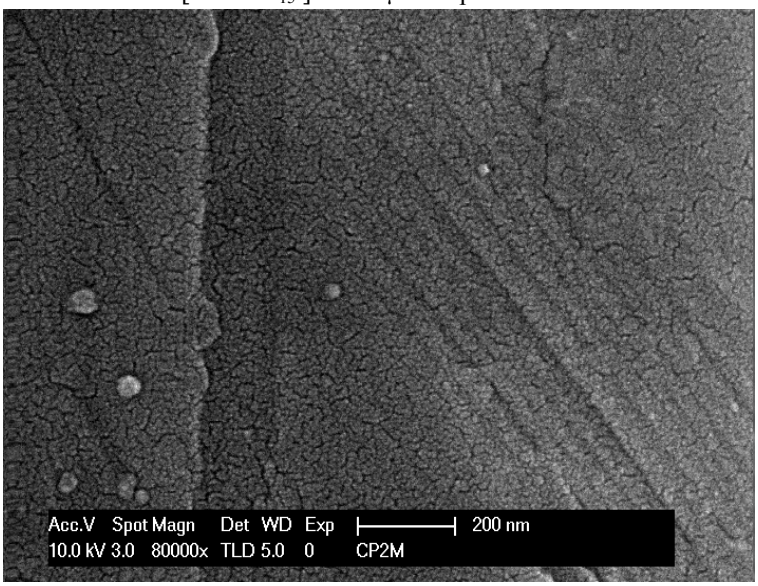
1) ^{2}LON-T$_{15}$

[^{2}LON-T$_{15}$] = 290 µM in pure water



TEM picture

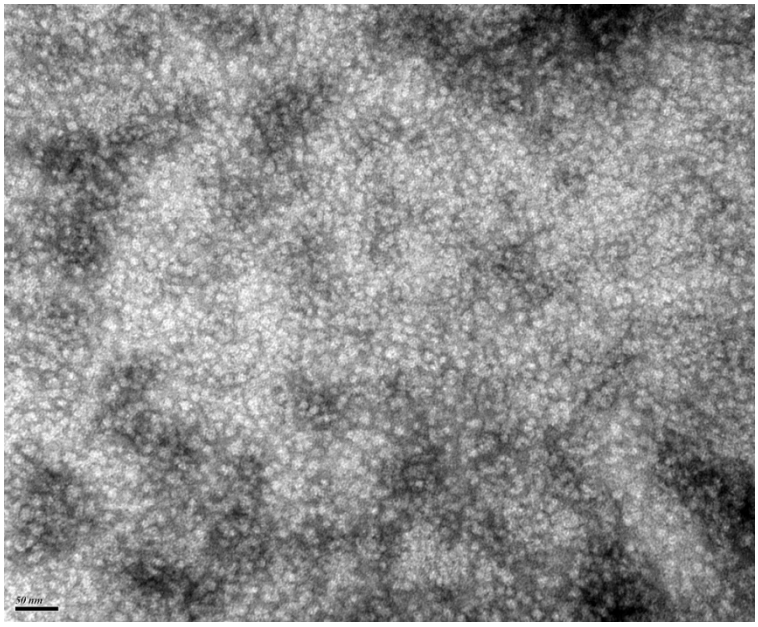
[^{2}LON-T_{15}] = 520 μM in pure water



SEM picture

2) ^{2}LON-A_{15}

[^{2}LON-A_{15}] = 40 μM in pure water



TEM picture

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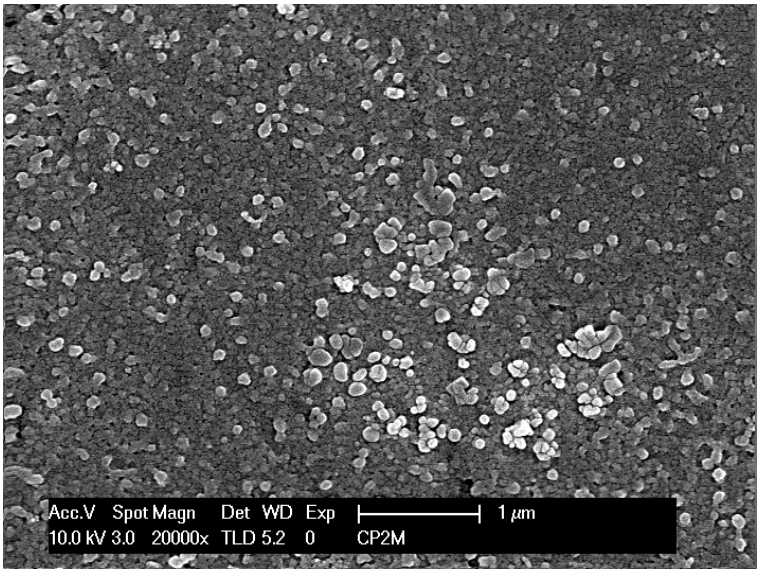
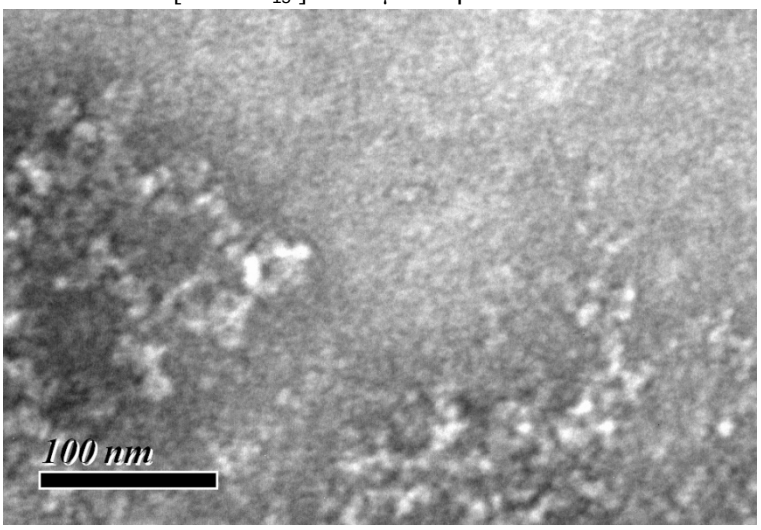
[^{2}LON-A_{15}] = 38 μM in pure water



SEM picture

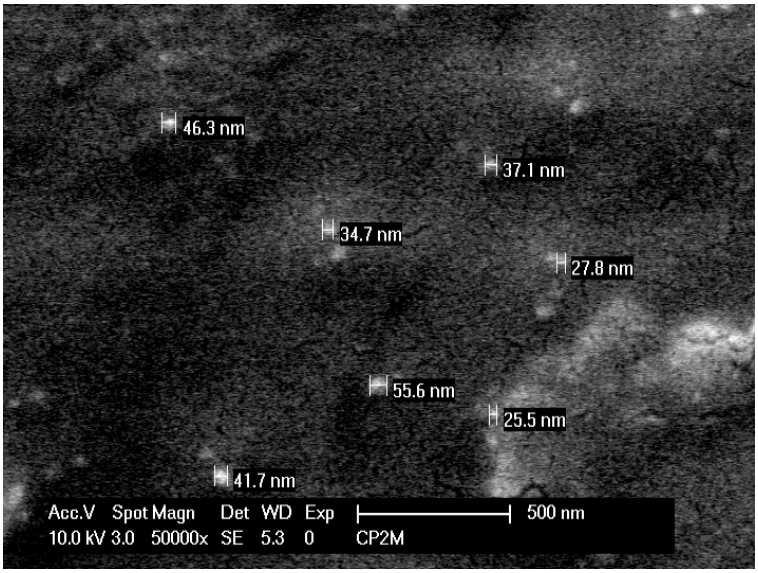
3) ^{3}LON-T_{15}

[^{3}LON-T_{15}] =150 μM in pure water



TEM picture

[^{3}LON-T_{15}] =380 µM in pure water

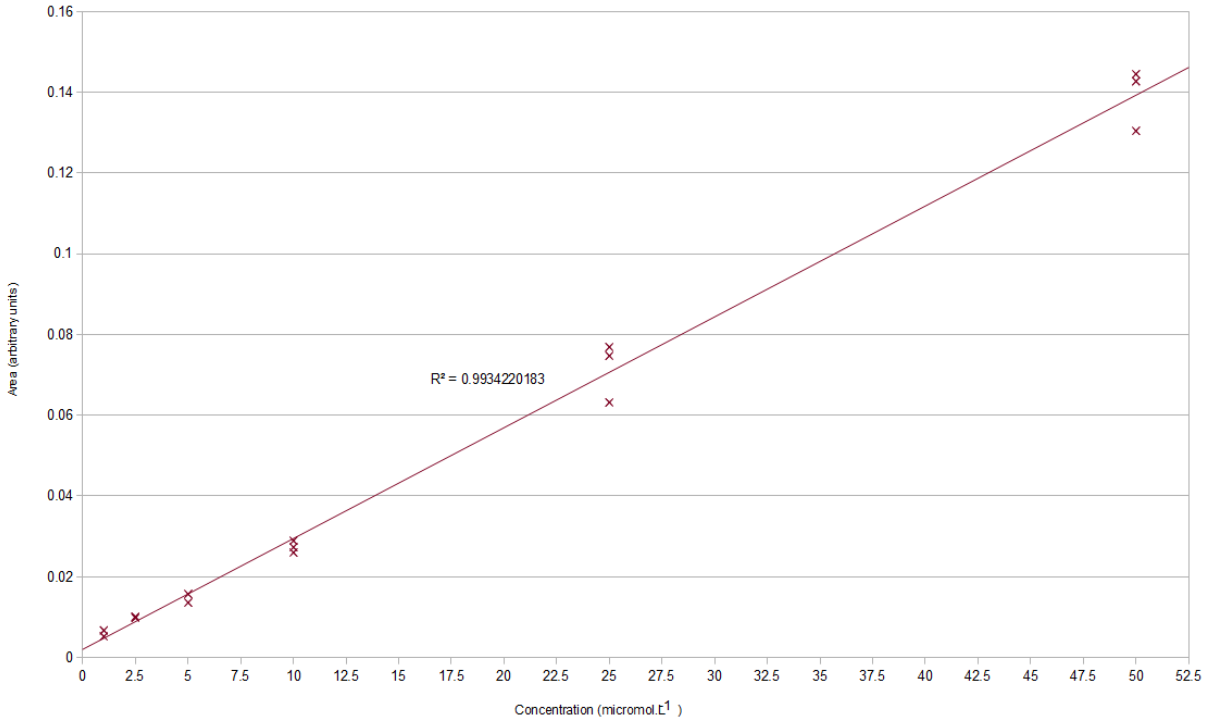


SEM picture

F/ Solubilization of paclitaxel in aqueous solutions of LONs:

F-1 Calibration curve for the determination of paclitaxel concentrations

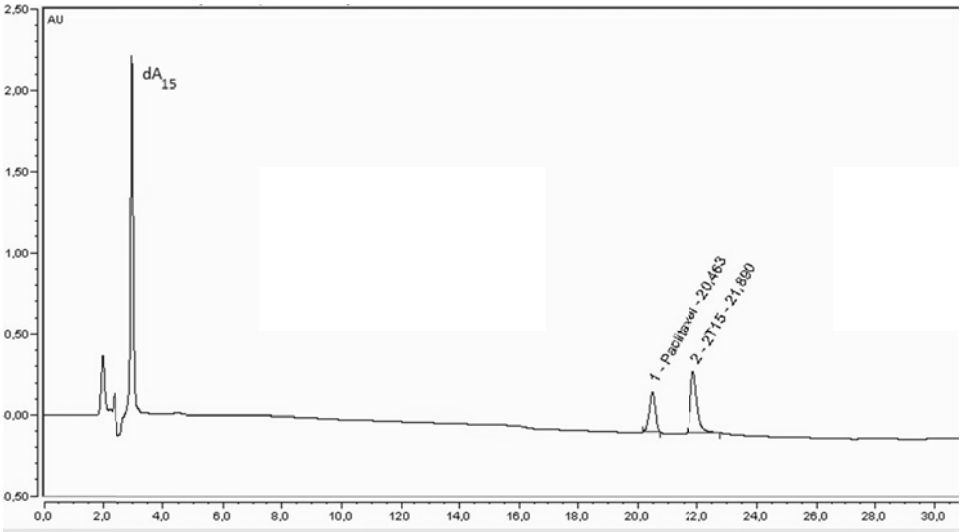
This curve was obtained after injection of a fixed volume (80 µL) of known paclitaxel concentrations (0.5; 1; 2.5; 5; 10; 25 and 50 µM) in aqueous acetonitrile. Samples were injected in triplicate to obtain the mean value for the peak area.



The peak area was translated into the quantity of paclitaxel following the equation:

n (paclitaxel) = (peak area-0.000125)/0.03396875 nmoles

F-2 Representative HPLC chromatogram of paclitaxel solubilization by a mixture of dA_{15} and 2LON-T_{15} oligonucleotides



No.	Peakname	Ret.Time min	Area AU*min	Amount	Type	Height AU	Rel.Area %	Resolution
1	Paclitaxel	20,463	0,0557	n.a.	BMB*	0,242	36,18	3,78
2	2T15	21,890	0,0982	n.a.	BMB*	0,371	63,82	n.a.
Total:			0,1538	0,0000		0,614	100,00	

C_4-RP-HPLC, detection at 227 nm. Elution conditions (eluent A (V/V): 5% CH_3CN / 95% 0.1 M TEAA pH 7; eluent B: 80% CH_3CN / 20% 0.1 M TEAA pH 7): isochratic (16% of B) for 2 mns, 16->90% of B after 28 mns and back to 16% of B