Supporting information

Chemical conjugation of aptamer-sphingomyelin nanosystems and their potential as inhibitors of tumour cell proliferation in breast cancer cells

Jenifer García-Fernández[a], Laura Rivadulla Costa[a,b], Celia Pinto-Díez[c], M. Elena Martín[d], Víctor M. González[c,d], María de la Fuente Freire*[a,e]

Experimental Procedures

1. Preparation of fluorescently labelled nanoconjugates

Fluorescent labelled nanosystems were prepared analogous to description above. The fluorophore SM-TopFluor®, solubilized in ethanol, was added to the organic phase, and consequently injected into the aqueous phase for a final concentration of 4 µg/mL. In this way, SM-TopFluor® will be incorporated in the lipidic structure of the system.

2. Purification

With the aim of evaluating conjugation efficiency, different isolation procedures were tested in order to separate unconjugated aptamer from the conjugated fraction. After performing ultrafiltration using Amicon 100 KDa (Amicon Ultra-0.5 Centrifugal Filter Unit, Merck Millipore, Darmstadt, Germany), recoveries were too low (fractions over 30% were missing after collecting ultrafiltrate and fraction from the filter). Dialysis was then carried out and parameters like dialysis time and membrane cut-off were optimized to collect unconjugated aptamer with the highest efficiency. Dialysis membrane of 300 kDa (Spectrum™ Dialysis Devices Spectra/Por™Float-A-Lyzer™ G2, Thermo Fisher Scientific, USA) and 4 hours were set as optimum time.

3. Agarose gel

Agarose gel electrophoresis was performed to validate dialysis as isolation procedure for separate conjugated aptamers from non-conjugated formulation. For this purpose, agarose gel was prepared at a concentration of 3%. SYBR Gold staining 1X (Invitrogen™) was employed as fluorescent DNA marker and TrackIt™ Cyan/Orange Loading Buffer 1X (Thermo Fisher Scientific) was added to facilitate DNA loading and tracking of samples. Additionally, glycerol 1% was included in each well to increase the density of samples and prevent nanoemulsions to spread out of well. Tris-Acetate-EDTA Buffer (TAE) 1X was used as running buffer. Power supply was applied at 90-100V for 45 minutes. At the end, gel was exposed to UV light and the picture was taken with a ChemiDoc System (Bio-Rad Laboratories, Inc., California, USA).

4. Quantitative PCR protocol

Quantification of internalized aptamer in treated and non-treated MDA-MB-231 cells was performed by qPCR, including the following conditions:

Table S1. Component and corresponding information for qPCR performance.

PCR component	Concentration	Volume (µL)
AceQ® qPCR Probe Master Mix 2x	1x	5
Forward Primer (F3 10 µM)	0.2 µM	0.2
Reverse Primer (R3 10 µM)	0.2 µM	0.2

ROX Reference Dye 1 50x		1x	0.2
Template DNA	apMNK2F 10 µM *(calibration)*	10 – 0.01 fmol	2
	Samples	To determine	
dH_2O			2.4

Table S2. qPCR conditions settled in StepOnePlus™ Real-Time PCR System (Applied Biosystems™).

		Ramp (ºC/s)	Temperature (ºC)	Time
Hold	***Step 1***	>1.6	95	5’
PCR	***Step 1***	>1.6	95	15’’
	Step 2	<1.6	60	1’
Melt curve	***Step 1***	>1.6	60	1’
	Step 2	>0.3	95	15’’

*x40 cycles

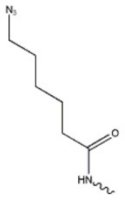
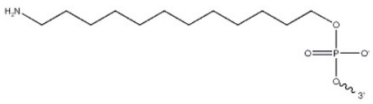
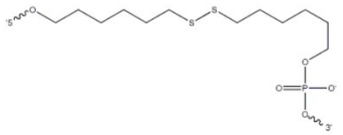
Characterization

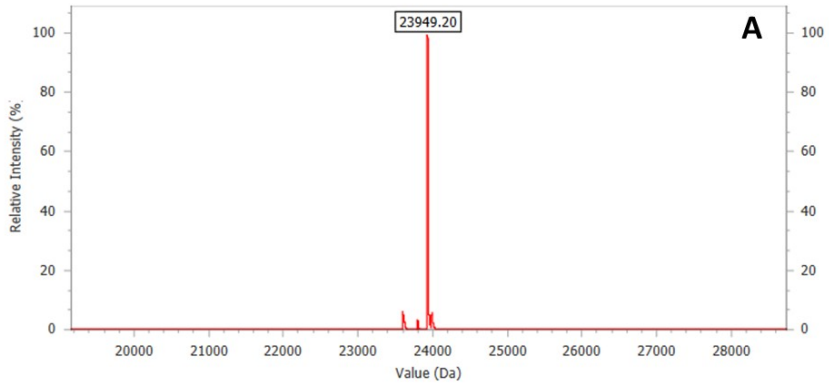
1. Characterization of aptamer modifications

a. Information about MNK2F’s modifications

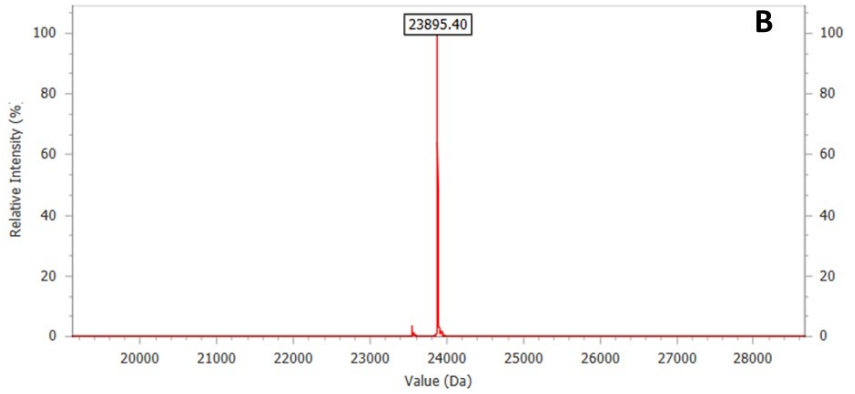
Synthesized aptamers were quality controlled via proprietary ESI-MS and quantified twice by UV spectrophotometry to provide yield measurements. Those and other data are collected in Table S3.

Table S3. Summary of aptamer MNK2F modifications including the azide, amino and thiol functional groups.

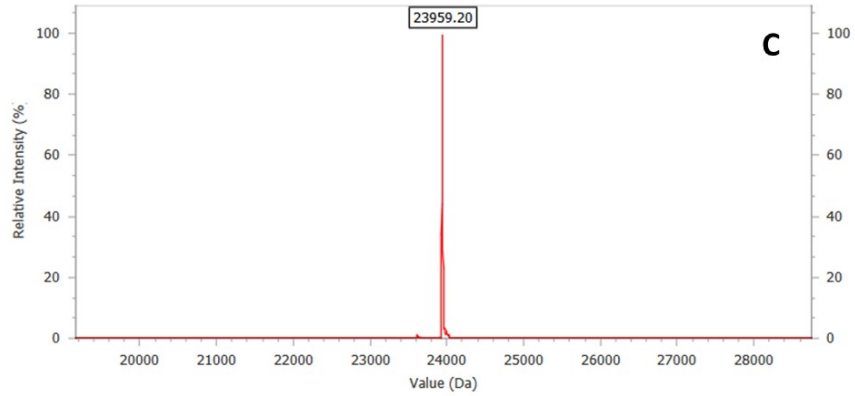
MODIFICATION	**AZIDE (NHS ESTER)**	**AMINO (MODIFIER C12)**	**THIOL (MODIFIER C6 S-S)**
MOLECULAR WEIGHT OF MODIFICATION	318.3 g/mol	263.3 g/mol	328.4 g/mol
STRUCTURE			
COMPLETE SEQUENCE	5’-Azide- GCG GAT GAA GAC TGG TGT GGG GTG GGC GGG CGG GGG TGG GGG TGG TAT GGC GCG TTG GCC CTA AAT ACG AGC AAC -3’	5’-AminoMC12- GCG GAT GAA GAC TGG TGT GGG GTG GGC GGG CGG GGG TGG GGG TGG TAT GGC GCG TTG GCC CTA AAT ACG AGC AAC -3’	5’-ThiolMC6-D- GCG GAT GAA GAC TGG TGT GGG GTG GGC GGG CGG GGG TGG GGG TGG TAT GGC GCG TTG GCC CTA AAT ACG AGC AAC -3’
LENGTH (NUMBER BASES)	75	75	75
GC CONTENT	66.7%	66.7%	66.7%
MELT TEMPERATURE	77 ºC	77 ºC	77 ºC
MOLECULAR WEIGHT	23949.5 g/mol	23894.5 g/mol	23959.6 g/mol
EXTINCTION COEFFICIENT	729600 L/(mol·cm)	729600 L/(mol·cm)	729600 L/(mol·cm)
NMOL/OD_{260}	1.37	1.37	1.37
MG/OD_{260}	32.83	32.75	32.84



Sequence Name: MNK2F Azide
Sequence: 5'- /5AzideN/GCG GAT GAA GAC TGG TGT GGG GTG GGC GGG CGG GGG TGG GGG TGG TAT GGC GCG TTG GCC CTA AAT ACG AGC AAC -3'
Calculated Molecular Weight: 23949.5
Measured Molecular Weight: 23949.20



Sequence Name: MNK2F Amino
Sequence: 5'- /5AmMC12/GCG GAT GAA GAC TGG TGT GGG GTG GGC GGG CGG GGG TGG GGG TGG TAT GGC GCG TTG GCC CTA AAT ACG AGC AAC -3'
Calculated Molecular Weight: 23894.5
Measured Molecular Weight: 23895.40



Sequence Name: MNK2F Thiol
Sequence: 5'- /5ThioMC6-D/GCG GAT GAA GAC TGG TGT GGG GTG GGC GGG CGG GGG TGG GGG TGG TAT GGC GCG TTG GCC CTA AAT ACG AGC AAC -3'
Calculated Molecular Weight: 23959.6
Measured Molecular Weight: 23959.20

Figure S1. ESI-MS spectra of aptamer MNK2F modifications. A. MNK2F Azide; B. MNK2F Amino; C. MNK2F Thiol.

b. ESI-MS spectra of the three modifications

2. Characterization of aptamer nanoconjugates

a. Click reaction

The UV-Vis spectrum of the DSPE-PEG-DBCO can be recorded before the reaction to establish a baseline. Dibenzocyclooctyne (DBCO) reagents possess an embedded chromophore that allows for the simple and non–destructive spectroscopic identification of DBCO–containing compounds, and in this way, the progress of copper-free click chemistry ligation can be followed in real-time by simple UV-Vis spectroscopy. As the "click reaction" progresses, the signature of an absorbance band at 310 nm disappears as illustrated in Fig. 2 of the current manuscript (and as recorded elsewhere[1]). In addition, a band at 292 nm can also be used to monitor the course of the reaction, as it disappears once the group is conjugated. Furthermore, the triazole linkage created between the DBCO from the lipid and the azide group included in the 5' terminus of the aptamer has a characteristic absorption peak in the UV-Vis spectrum around 310-340 nm (depending on the specific molecular structure of the triazole compound) that can be used to confirm the covalent bond formation[2]. For this specific conjugate's spectrum, the absence of the bands at 292 and 310 nm confirm that the group is linked. A slight shoulder is observed at 312 nm, confirming the formation of the triazole bound as it is shown in Fig. S2.

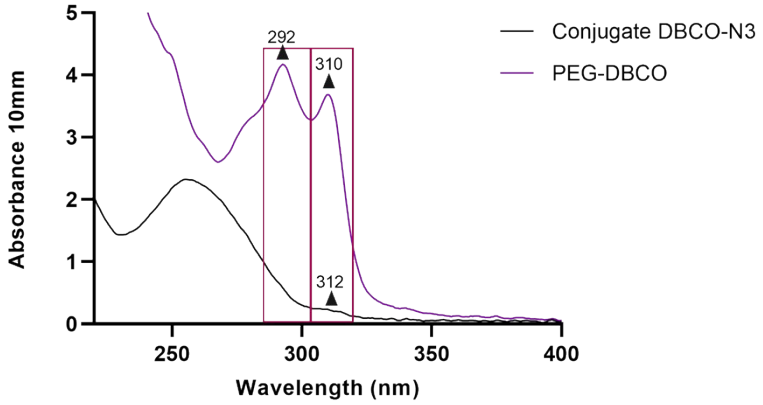


Fig. S2. UV-Vis spectrum characterization of PEG-DBCO reagent and aptamer conjugate DBCO-N3. Characteristic bands at 292 and 310 nm of DBCO decay once it is conjugated to azide. A shoulder band at 312 nm in the aptamer conjugated could be attributed to the formation of the triazole linkage.

b. Thiol-Maleimide

In the case of the thiol-maleimide conjugate, it is possible to follow the progress of a reaction between thiol and maleimide group by UV-Vis spectroscopy. This technique provided qualitative/quantitative information on yield/time evolution by monitoring the progressive decrease of the maleimide peak at 293 nm, associated with the corresponding loss of conjugation between the two carbonyl groups and the C=C unsaturation separating them, which accompanies the formation of the adduct[3]. This effect can be observed in Fig. S3, where maximum peak at 293 nm decays once PEG-maleimide is thiol-conjugated.

c. Carbodiimide

Agarose gel electrophoresis confirmed the conjugation between the nanoemulsion and the aptamer by

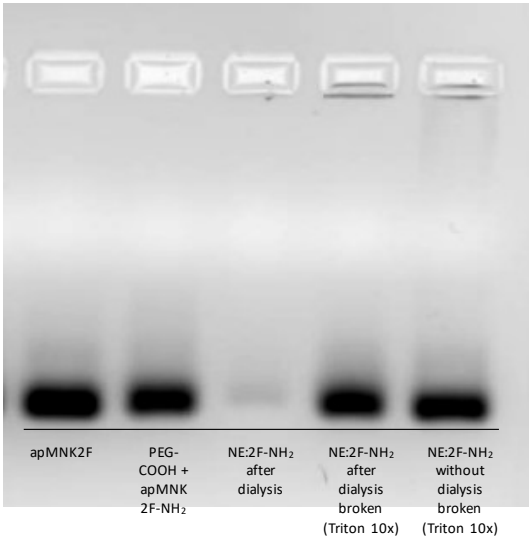


Fig. S4. Agarose gel electrophoresis to illustrate covalent conjugation between functionalized-PEG and modified-aptamer. The absence of the characteristic aptamer band in the well named as "NE:2F-NH2 after dialysis" confirms the formation and isolation of the nanoconjugate.

carbodiimide reaction. The presence of a band confirms the presence of the free aptamer or the conjugate lipid-aptamer before nanoemulsion formulation (indicated as PEG-COOH + apMNK2F-NH_2 in

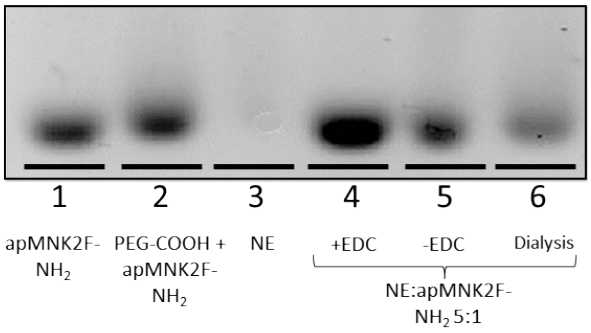


Fig. S4). On the contrary, the absence of a band confirms the formation and isolation of the nanoconjugate. Moreover, the appearance of the band after Triton-X incubation indicates the aptamer release from the nanoconjugates with and without dialysis (Figure S4). The EDC presence was also

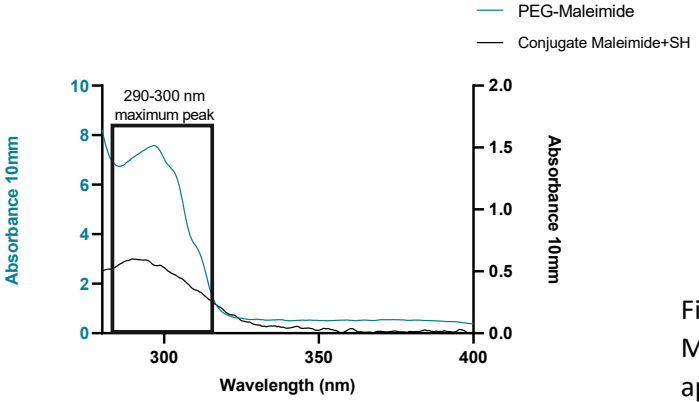


Fig. S3. UV-vis spectra of DSPE-PEG-Maleimide and its corresponding aptamer conjugate Maleimide-SH.

assessed, and agarose gel electrophoresis was employed to evaluate its reversibility effect in the conjugation if it was not removed by Amicon ultrafiltration (Fig. S5).

Fig. S5. Agarose gel electrophoresis to follow carbodiimide reaction and assess EDC effect on the resulting conjugate. 1. ApMNK2F-NH_2 – characteristic band of the free aptamer; 2. PEG-COOH + apMNK2F-NH_2 – characteristic band of the lipid-aptamer conjugate; 3. NE – characteristic band (absence) of the blank nanoemulsion (VE:SM:PEG); 4. 5. and 6. NE:apMNK2F-NH_2 5:1 – characteristic bands of the aptamer-conjugated nanoemulsion when: EDC is not removed (4. +EDC); after Amicon ultrafiltration to remove EDC (5. -EDC); after dialysis purification (6. Dialysis). Strong intensity of the band in well 4 compared to weak band in well 5 reveals the reversibility effect of the EDC when it is not removed from the reaction medium. Additionally, only a weak shadow is noticed after purification by dialysis is performed, proving that the aptamer is all conjugated to the nanoemulsion and no free aptamer is detected.

In vitro studies

1. Flow cytometry analysis

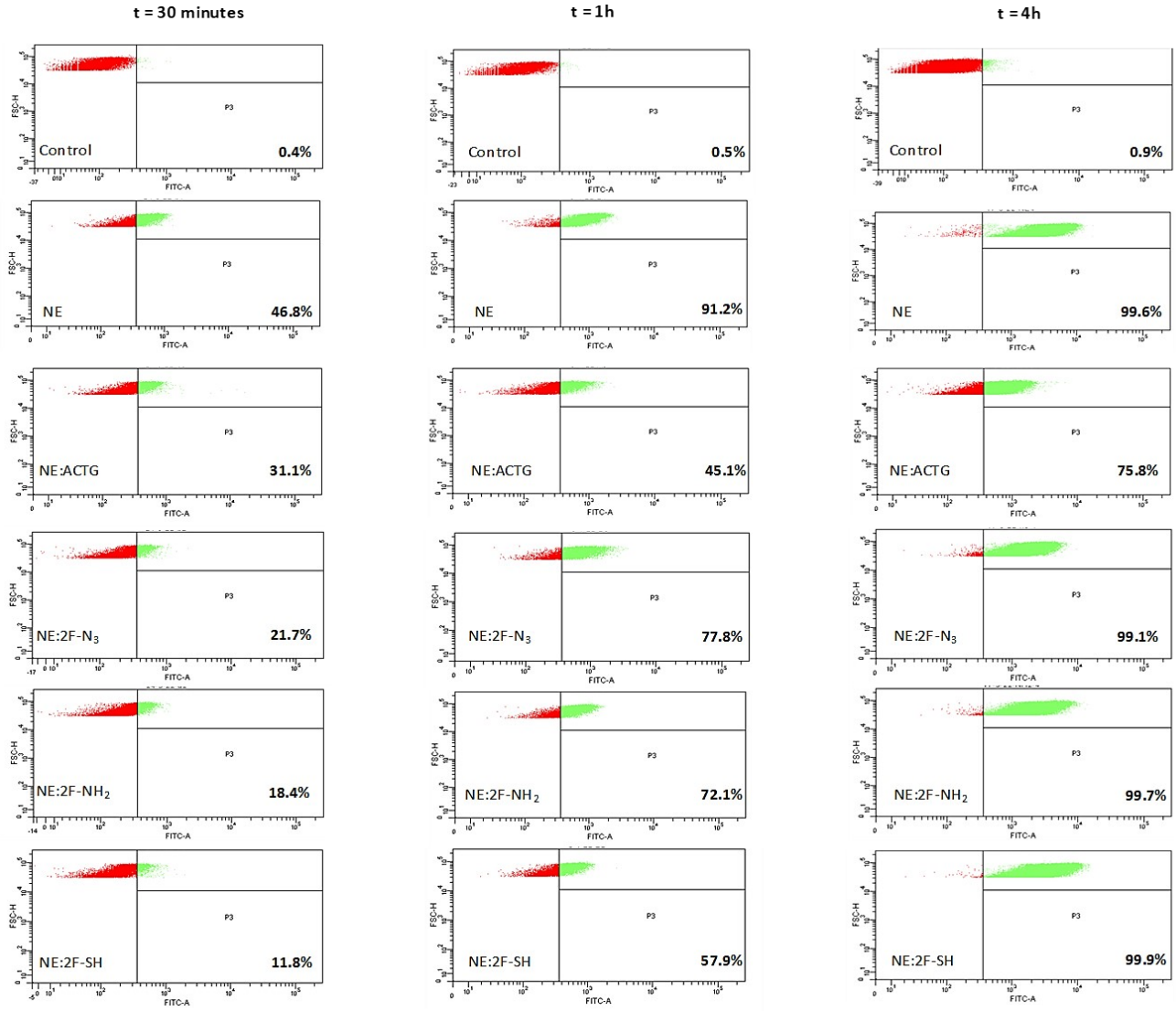


Fig. S6. Flow cytometry analysis showing percentage of cells internalizing Top Fluor®-SM nanosystems over time (30 minutes, 1 hour and 4 hours).

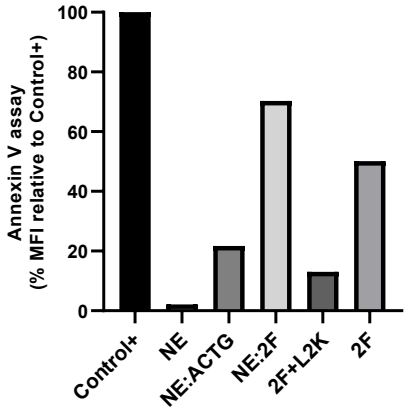


Fig. S7. Quantification of Annexin V assay for apoptosis detection by measuring Mean Fluorescence Intensity using Fiji Image J software. Data were represented in terms of %MFI relative to

2. Annexin V assay

REFERENCES

1 S. M. Hodgson, 2017.

2 J. Dommerholt, F. P. J. T. Rutjes, F. L. Van Delft, M. Vrabel, T. Carell and F. V. Nl, *Top Curr Chem*, DOI:10.1007/s41061-016-0016-4.

3 A. Gandini, D. Coelho, M. Gomes, B. Reis and A. Silvestre, *J Mater Chem*, 2009, **19**, 8656–8664.