AS1411-decorated niosomes as effective nanocarriers for Ru(III)-based drugs in anticancer strategies. Riccardi, C., Fàbrega, C., Grijalvo, S., Vitiello, G., D'Errico, G., Eritja, R., Montesarchio, D. J. Mat. Chem. B, 6, 5368-5384 (2018). DOI: 10.1039/c8tb01563e

AS1411-decorated niosomes as effective nanocarriers for Ru(III)-based drugs in anticancer strategies

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Abstract: Niosomes are self-assembled vesicles made up of single chain non-ionic surfactants combined with appropriate amounts of cholesterol or other lipids, exploited as carriers for hydrophilic or lipophilic drugs. Compared to liposomes, niosomes are typically more stable, less expensive and, being generally obtained from synthetic surfactants, more easily derivatizable, providing vesicular structures with a higher versatility and chemical diversity. Herein, we investigated the physico-chemical and biological properties of niosomes loaded with two active ingredients, i.e. the nucleolipidic Ru(III)-complex HoThyRu, selected as an anticancer agent, and the nucleolin-targeting AS1411 aptamer, allowing selective recognition of cancer cells. The morphology, average size, zeta potential, electrophoretic mobility, and stability over time of the functionalized niosomes were analyzed using different biophysical techniques. These formulations, tested on both cancer and normal cells, showed promising antiproliferative activity on HeLa cells, with a higher efficacy associated with the nanosystems containing both AS1411 and HoThyRu with respect to the controls. In all the tested cell lines, AS1411 proved to markedly enhance the bioactivity of the Ru(III)-containing niosomes.

Introduction

Vesicles and other colloidal structures have been extensively studied in the last decades as effective platforms for drug delivery. Among these, liposomes, first described by Bangham in 1965¹ and since then widely

exploited as drug carriers, have been prepared from different phospholipids and analyzed in detail for a variety of applications.²⁻⁵ Though being biodegradable and non-toxic, phospholipids have thus far found quite limited clinical applications, essentially due to their intrinsically low physical and chemical stability. Therefore, also other amphiphilic molecules have been evaluated to obtain efficient vesicular carriers.⁶⁻⁸

In this frame, vesicles prepared from non-ionic surfactants,^{9,10} currently known as niosomes or NSVs (Non-ionic Surfactant Vesicles), have gained increasing attention as drug delivery systems, providing a cheaper and more stable alternative to phospholipids.^{4,8} In particular, the basic components of niosome formulations include non-ionic surfactants and lipids. In addition, also charged molecules can be added to better stabilize niosome vesicles and prevent their aggregation, such as diacetyl phosphate, phosphatidic acid, stearylamine and cetylpyridinium chloride.^{4,10,11} Although several preparation methods are reported in the literature,^{4,6,8,11-14} niosomes are generally obtained by hydration of films containing both a non-ionic surfactant and a lipid molecule, able to fully encapsulate the drug, simply dissolved or dispersed in aqueous solutions.^{4,12} Thus the drug is entrapped in a membrane due to the self-assembly of the surfactant + lipid molecules, generally organized in stable bilayers.¹⁵

The formation of stable vesicle-like structures depends on the chemical structure, geometry and hydrophilic-lipophilic balance of the components, as well as on the ratio between the lipid and the surfactant used.¹² The size of niosomes includes particle diameters ranging from nano- to submicrometers, and the formed vesicles can be unilamellar or multilamellar.^{8,13} Owing to their vesicle structure, niosomes are able to incorporate both hydrophilic and hydrophobic drugs, just as liposomes do.^{7,8,11,13} Over the past three decades, niosomes have been successfully used as drug carriers to overcome some major biopharmaceutical problems such as insolubility, side effects, and poor chemical stability of drugs.¹⁶ In fact, niosomes are able to protect the loaded drugs from premature degradation *in vivo* as well as from possible inactivation due to undesired immunological and pharmacological effects.^{16,17}

The high chemical stability of surfactants, compared with phospholipids, makes niosome purification, handling and storage much easier than those of conventional liposomes.^{18,19} In addition, surfactants can be easily prepared and chemically modified starting from different classes of molecules (such as amino acids, fatty acids, amides, alkyl esters and alkyl ether functional molecules).^{8,11,20-25} These allow a wide repertoire of different vesicular structures, whose properties can be finely tuned, thus also affecting the intrinsic pharmacokinetics and targeting ability of the drug.⁴ Notably, the niosome toxicity profile is generally considered safe because of the non-ionic nature of its basic components. In fact, non-ionic surfactants show a higher biocompatibility and a lower toxicity than their anionic, cationic or zwitterionic counterparts used in liposome preparation.⁶ In addition, they can be effectively provided by various administration routes, namely oral, ocular, parenteral and transdermal.²⁶

The first useful application of niosomes was reported in the 70's by researchers in the cosmetics industry.²⁷ Indeed, niosome vesicles showed also *in vitro* and *in vivo* efficacy in therapeutic applications, encapsulating a large variety of drugs including not only α -tocopherol,^{28,29} resveratrol,³⁰⁻³³ itraconazole,^{34,35} insulin,^{36,37} clarithromycin,¹⁷ doxorubicin,³⁸ paclitaxel,³⁹

glucocorticoids,¹⁵ enoxacin,⁴⁰ and acyclovir^{41–45} but also oligonucleotides⁴⁶ and other molecules,^{47–51} thus demonstrating their applicability in different fields such as anticancer,^{52,53} anti-inflammatory,^{15,54} antimycotic,^{34,35} antiviral,^{45,55} antioxidant,⁵⁶ and gene therapy^{57–60} (for applications of niosomes in therapy, see recent reviews^{4,6,12,13}).

In this work, with the aim of exploring niosome vesicles as versatile scaffolds for effective loading of multiple anticancer drugs to be used in combination therapies, we prepared a novel niosomal formulation starting from 2,3-bis(tetradecyloxy)propan-1-aminium chloride (Fig. 1a) and the non-ionic surfactant polysorbate 80 (Fig. 1b), optimizing a previously described system.^{58,61}

To test their efficacy as multifunctional carriers, these niosomal formulations have been here loaded with the anticancer nucleolipid-based Ru(III)-complex HoThyRu (Fig. 1c), previously studied in POPC and DOTAP-based liposomes^{62–65} and proved to show IC₅₀ values in the μ M range on human MCF-7 breast adenocarcinoma cells.

In the field of anticancer metal complexes, the design and development of adequate drug delivery systems for the transport of metal complexes is indeed a well-established approach to overcome their instability in aqueous solutions and their non-specific binding in biological systems. Besides the liposome-based systems, also mono- and dinuclear Ru(III)-complexes with the pyrrolidinedithiocarbamate ligand and the more sterically hindered carbazolidithiocarbamate ligand have been developed.⁶⁶

These approaches have been employed also for other metalbased complexes, such as gold, copper and silver.⁶⁷ Particularly, gold(III)-based anticancer peptidomimetics⁶⁸ and dinuclear gold(I) pyrrolidinedithiocarbamate complexes⁶⁹ have been prepared showing in all cases promising antiproliferative activity. Functionalized-nanostructured titanium oxide (TiO₂) as a carrier of copper complexes for cancer treatment showed high biocompatibility in glioma and melanoma cell lines.⁷⁰

In this context, the functionalization of niosomes with the nucleolipid-based Ru(III)-complex HoThyRu is certainly a valuable alternative to obtain an effective metallo-drug delivery.

Then, to ensure highly specific recognition towards cancer cells, the investigated niosomes have been decorated also with the G-quadruplex-forming aptamer AS1411 (for a recent review covering the state-of-the-art knowledge on AS1411, see P. J. Bates et al.⁷¹). This 26-mer G-rich oligonucleotide, with the sequence 5'-GGTGGTGGTGGTTGTGGTGGTGGTGG-3', entered in Phase II anticancer clinical trials, is able to selectively target nucleolin, a multifunctional protein involved in cell survival, growth and proliferation, overexpressed on the outer membrane of cancer cells. Therefore, in the context of tumor-selective delivery of therapeutic or imaging agents in cancer pathologies, recently AS1411 has been largely employed as a targeting agent, being used to decorate nanoparticle-based nanosystems for the specific recognition of cancer cells.⁷¹

Several AS1411-linked conjugates or nanoparticles have been successfully tested in vivo for transporting chemotherapeutic agents (such as paclitaxel,^{72–75} docetaxel,^{76–78} doxorubicin,^{79–88} and epirubicin),⁸⁹ small interfering RNA (siRNA)^{90,91} and therapeutic proteins.⁹² On the other hand, these systems have shown significant efficacy also for the in vivo delivery of imaging agents in PET, SPECT, CT, MRI and fluorescence analysis.^{79,93–103} AS1411-functionalized nanosystems showed in all cases a higher cancer cell

uptake and/or tumor accumulation compared to the same non-functionalized materials or the free drugs. Particularly, for small molecule-based chemotherapies, there is evidence that drug delivery via AS1411-functionalized nanoparticles can circumvent drug-resistance pumps,⁸⁶ pass the blood–brain barrier⁷⁶ and reduce toxicity to normal tissues.^{83,84,86}

Fig. 1 Molecular structures of (a) the cationic amino lipid, (b) the non-ionic surfactant polysorbate 80, mixed for the preparation of the herein studied niosomes and (c) the Ru(III)-complex HoThyRu, selected as anticancer agents.

Thus, in consideration of the great potential of AS1411 as a highly efficient targeting agent, niosome formulations loaded with both this aptamer and the Ru(III)-complex HoThyRu have been prepared to obtain effective delivery of the ruthenium-based drug to the target tumor cells. Preliminarily, several AS1411/niosome formulations with the oligonucleotide mixed at different ratios have been examined in order to determine the oligonucleotide/lipid ratio at which AS1411 can be considered fully incorporated within the niosome structure. Once established the optimal ratio, this was then used in all the successive assays with the niosomes containing both AS1411 and HoThyRu.

The resulting formulations have been characterized using gel electrophoresis, zeta potential, dynamic light scattering (DLS), transmission electron microscopy (TEM), electron paramagnetic resonance (EPR), and ^{31}P nuclear magnetic resonance (NMR) measurements, and their antiproliferative activity has been evaluated in vitro on a selected panel of human cancer and healthy cells.

Experimental Section

Materials and general methods

All the reagents and solvents were of the highest commercially available quality and were used as received. The non-ionic surfactant polysorbate-80 (Tween-80), HEPES and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT reagent) were purchased from Sigma-Aldrich. The cationic lipid [2,3-di(tetradecyloxy)propan-1-aminium chloride] and the AS1411 oligonucleotide (5'-GGTGGTGGTGGTTGTGGTGGTGGTGG-3') were purchased as purified compounds from Sapala Organics Private Ltd (India) and Biomers (Germany), respectively. The scrambled 26-mer

oligonucleotide (5'-CCACCTTTTTTCCAGGGTATCCCAAG-3') was synthesized using an ABI 3400 DNA synthesizer and with the 1 μ mol scale synthesis and the standard protocols. RC membrane 0.45 μ m, 15 mm syringe, and non-sterile filters were purchased from Phenomenex. PBS buffer and Dulbecco's Modified Eagle's Medium (DMEM) were purchased from Gibco.

Gel reagents (acrylamide/bisacrylamide, APS, TEMED, and SYBERGreen) were purchased from Sigma-Aldrich.

Spin labeled 5- and 16-doxyl stearic acid probes (5-DSA and 16-DSA, respectively) were purchased from Sigma-Aldrich and stored at -20 °C in aqueous solutions.

HoThyRu was synthesized according to reported procedures,^{62,63} with minor modifications, as reported in the ESI† (Schemes S1 and S2). TLC analyses were carried out on silica gel plates from Macherey-Nagel (60, F254). Reaction products on TLC plates were visualized by using UV-light and then by treatment with an oxidant acidic solution (acetic acid/water/sulfuric acid, 10:4:5 volume ratio). For column chromatography purification, silica gel from Macherey-Nagel (Kieselgel 60, 0.063–0.200 mm) was used.

The identity of all the synthetic intermediates and the final compounds was confirmed by ¹H and ¹³C NMR spectroscopy, as well as by ESI-MS spectrometry, showing data in accordance with literature reports.^{62,63}

HeLa and HTB-38 cells were purchased from the American Type Culture Collection (Manassas, VA), while the HCC2998 cell line was kindly provided by Dr Diego Arango (Molecular Oncology Group; CIBBIM-Nanomedicine, Vall d' Hebron Institute of Research, VHIR, Spain). The HEK-293T cell line was kindly provided by Dr Montserrat Terrazas (MolecularModeling and Bioinformatics, Institute for Research in Biomedicine, IRB, Spain).

All the cell lines were grown in DMEM supplemented with 10% heat-inactivated fetal bovine serum FBS, 50 IU per mL penicillin and 50 μ g mL⁻¹ streptomycin. All the reagents for cell culture were purchased from Gibco Invitrogen. Cells were cultured at 37 °C in a 5% CO₂ humidified atmosphere.

UV spectra were obtained using a JASCO V-550 spectrophotometer equipped with a Peltier Thermostat JASCO ETC-505T. Zeta potential data were obtained using a Zetasizer NanoZS (Malvern Instruments). DLS measurements were obtained using a Zetasizer Nano ZS or Zetasizer Nano ZSP, as specified. EPR spectra were recorded on a 9 GHz Bruker Elexsys E-500 spectrometer. ³¹P NMR spectra were recorded on a Bruker WM-400 spectrometer using D₂O as a solvent. All the chemical shifts (δ) are expressed in ppm. 85% H₃PO₄ was used as the external reference, set at 0 ppm, for the ³¹P NMR spectra.

Cell viability by MTT assay was measured using an automated spectrophotometric plate reader, Glomax multi detection system (Promega).

Preparation of the AS1411 samples

Lyophilized AS1411 was dissolved in a known volume of Milli-Q water and its concentration was determined by UV analysis recording its absorbance in a 1 cm path length cuvette at 260 nm(85 °C) using a molar extinction coefficient of 281 700 cm⁻¹ M⁻¹, calculated for the unstacked oligonucleotide. The UV spectra were recorded in the range 220–380 nm

with a medium response, a scanning speed of 100 nm min^{-1} , a bandwidth of 2.0 nm and corrected by subtraction of the background scan. Then, AS1411 was diluted in PBS buffer from the stock solution. Before use, AS1411 was annealed by heating the sample for 5 min at 90°C and then allowing it to slowly cool to room temperature overnight. The annealed samples were then kept at 4°C until use.

Preparation of niosomes

Niosomes were prepared using a film-hydration method by mixing the cationic lipid 2,3-di(tetradecyloxy)propan-1-aminium chloride and the non-ionic surfactant polysorbate 80 in $3 : 1$ molar amounts, according to reported procedures with minor modifications.^{58,61}

In particular, 10 mg of 2,3-di(tetradecyloxy)propan-1-aminium chloride ($19.2 \mu\text{mol}$) and 8.4 mg of polysorbate 80 ($6.4 \mu\text{mol}$) were weighed in an Eppendorf tube and then dissolved in 1.0 mL of CHCl_3 . The dispersion was vortexed and sonicated until a clear suspension was observed. Thereafter the solvent was evaporated and the resulting crude was kept under vacuum at r.t. overnight. The dried lipid film was hydrated with 1.0 mL of the selected buffer (HEPES or PBS), heated at 60°C for 20 min , filtered through a $0.45 \mu\text{m}$ membrane filter and then kept at 4°C . The dispersion was vortexed and sonicated for 3 min before use.

For the preparation of the niosome_HoThyRu system, to a mixture containing 10 and 8.4 mg of the cationic lipid and polysorbate, respectively, 1.0 mg of the Ru complex HoThyRu ($0.74 \mu\text{mol}$) was added so as to obtain a niosome_HoThyRu formulation with 3% in mol of HoThyRu. The mixture of the three components was then dissolved in CHCl_3 and the desired system was obtained following the same procedure described above, except for the heating step, performed at 40°C for 10 min instead of 60°C , in order to prevent the possible degradation of the Ru(III) complexes.¹⁰⁴

These procedures allowed obtaining final niosomes with 25.6 mM concentration and niosome_HoThyRu formulations with the 0.74 mM Ru(III)-complex. The initial 25.6 mM stock solutions were then freshly diluted, as required for the measurements.

The AS1411/niosome and AS1411/niosome_HoThyRu formulations were obtained by adding the required amount of AS1411 to the niosome dispersion. The resulting mixtures were vortexed and sonicated for 2 min and finally incubated at 37°C for 30 min .

For gel electrophoresis, DLS and zeta potential assays, carried out to assess the optimal ratio in AS1411/niosome formulations, different mixtures ranging from 0 to $1 : 16$ N/P charge ratios [(no. of anionic phosphate groups)_{oligonucleotide} / (no. of cationic amino groups)_{cationic lipid}] were prepared. Once the $1 : 12$ N/P charge ratio was determined to be the optimal one successive assays were all performed using a $1 : 12$ N/P charge ratio between AS1411 and the niosome (with or without HoThyRu) corresponding to 0.3% in mol of AS1411 compared to the lipid. Considering that AS1411 has 25 negative charges and that the lipid has only one positive charge, the N/P ratio can be converted into an effective ratio in moles dividing it by 25 (see Tables S1 and S4 respectively for the concentration of niosomes in AS1411/niosome formulations with different N/P charge ratios and the concentration of all the components in the niosome formulations with the selected $1:12$ N/P charge ratio, ES1†).

Electrophoretic mobility shift assay

Defined volumes of a 0.5 μ M AS1411 solution were mixed with increasing amounts of niosomes (taken from a 25.6 mM stock concentration in HEPES) providing different AS1411/niosome formulations ranging from 0 to 1 : 16 N/P charge ratios (total volume of 30 μ L). The resulting samples were incubated at 37 °C for 30 min. These formulations were then analyzed using gel electrophoresis carried out on a 20% polyacrylamide gel at 150 V for 7 h at 25 °C in TBE buffer (Tris–Borate–EDTA, 1x). The gels were imaged using a Gene Genius Bioimaging system (Syngene), after staining with SYBRGreen solution.

Zeta potential and DLS measurements of AS1411/niosome samples at different N/P charge ratios

The zeta potential and DLS measurements of the AS1411/ niosome formulations at different oligonucleotide/lipid charge ratios were obtained using a Zetasizer Nano ZS (Malvern Instruments). For zeta potential analysis, defined volumes of a 0.5 μ M AS1411 stock solution were mixed with different concentrations of the niosomes (taken from a 25.6 mM stock concentration in HEPES) so as to provide 50 μ L of the AS1411/ niosome formulations with N/P charge ratios in the range 0–1 : 16.

All the measurements were performed at r.t. with a scattering angle of 173° and an equilibration time of 120 s, by diluting the prepared samples with water so as to obtain a final volume of 1.0 mL. The Z-average (radius expressed in nm) and polydispersity index (Pdl) values with associated standard deviations of three determinations were directly obtained from the measurements, fitting the correlation functions with the cumulant analysis algorithm as implemented by the Malvern software (ISO 13321).

For DLS analysis, AS1411 (1.0 μ M) was mixed with different concentrations of the niosome taken from the 25.6 mM stock solution in HEPES, so as to provide AS1411/niosome formulations ranging from 0 to 1 : 16 N/P charge ratios in a total volume of 0.50 mL. The measurements were carried out at r.t. with a scattering angle of 173° and an equilibration time of 120 s, without diluting the samples. The Z-average radius expressed in nm, and Pdl values with the standard deviation of three independent determinations were directly obtained from the measurements.

DLS-monitoring of the niosome stability over time

All the niosome formulations – prepared as described before – were analyzed using a Zetasizer Nano ZSP (Malvern Instruments) to determine their Z-average size, homogeneity and storage stability.

Firstly, the average particle size and Pdl values of freshly prepared samples at different concentrations (i.e., 17.5 mM, 3.5 mM and 175 μ M) were determined, obtained by diluting the initial 25.6 mM stock solution. The measurements were taken at r.t. with a scattering angle of 173° and with an equilibration time of 120 s. The Z-average radius expressed in nm, and polydispersity index (Pdl) values with associated standard deviations of three determinations were directly obtained from the measurements, fitting the correlation functions with the cumulant analysis algorithm as implemented by the Malvern software (ISO 13321).

Then, all the prepared formulations were stored at 4 °C and stability studies were carried out collecting the DLS data every week for one month with the same instrument setting. The analysis over time was carried out in

order to monitor the possible changes in size and/or distribution attributable to the vesicle aggregation phenomenon.

TEM morphology analysis

The morphology of the unfunctionalized niosomes with the cationic lipid and the polysorbate 80 in 3:1 molar ratio was evaluated by transmission electron microscopy (TEM) analysis. Briefly, 5 μL of the niosome sample (2.6 mM in PBS) were adhered onto glow discharged carbon coated grids for 120 s. The remaining liquid was removed by blotting onto filter paper by allowing evaporation. Then, staining with 10 μL of 2% aqueous phosphotungstic acid was performed for 120 s without removing the excess but allowing evaporation. The samples were observed under a microscope TEM FEI TECNAI G2 s-Twin, operating at an accelerating voltage of 120 keV in a bright-field image mode. Digital images were acquired using an Eagle 4K camera.

EPR measurements

The samples for EPR experiments were prepared as described above for the zeta potential and DLS analyses, including also 1 mol% (with respect to the lipid + surfactant) of 5-DSA or 16-DSA.

The capillaries containing the samples were placed in a standard 4 mm quartz sample tube containing light silicone oil for thermal stability. The temperature of the sample was regulated at 25 °C and maintained constant during the measurement by blowing thermostated nitrogen gas through a quartz Dewar. The instrumental settings were as follows: sweep width, 120 G; resolution, 1024 points; modulation frequency, 100 kHz; modulation amplitude, 1.0 G; time constant, 20.5 ms, and incident power, 5.0 mW. Several scans, typically 64, were accumulated to improve the signal-to-noise ratio.

The analysis of the EPR spectra allowed the determination of the order parameter, S , and the nitrogen coupling constant a_N' , which is an index of the micropolarity experienced by the nitroxide moiety.¹⁰⁵

NMR measurements

For NMR measurements, the dried lipid film, after CHCl_3 evaporation, was hydrated with 1 mL of D_2O ; the successive procedure was identical to the one described for the above measurements.

Cancer and normal cell cultures

HeLa (human cervix adenocarcinoma cells), HTB-38, HCC2998 (human colorectal adenocarcinoma cells) and HEK-293T (human embryonic kidney) cell lines were grown in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (50 units per mL) and streptomycin (50 $\mu\text{g mL}^{-1}$). All cells were cultured in a humidified 5% carbon dioxide atmosphere at 37 °C.

Cells were regularly passaged in order to maintain exponential growth. 24 h before the experiments, the cells were trypsinized and placed in a 96-well plate (100 μL per well). In particular, HeLa cells were seeded at a density of 1×10^3 cells per well; HTB-38 and HEK-293T were seeded at a density of 5×10^3 cells per well; finally, HCC2998 cells were seeded at a density of 1×10^4 cells per well, after ensuring that the seeding density was suitable to guarantee 80% confluence during experiment completion.

Analysis of cell viability by the MTT colorimetric assay

The cell viability of all the prepared niosome formulations and controls was investigated at different niosome concentrations – preparing the diluted samples from the initial stock solutions – in PBS using the MTT [1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan] colorimetric assay, following the procedure used by the National Cancer Institute *in vitro* Anticancer Drug Discovery Screen Program.¹⁰⁶

For each assay, the cells were seeded in 96-well plates in 100 μ L DMEM allowing growth for 24 h. Then the cells were treated with a range of concentrations (1.25 $\rightarrow$ 150 μ M, depending on the analyzed cancer cell line) of the samples dissolved in PBS and finally incubated for 72 h at 37 °C under 5%CO₂.

Cell viability was evaluated using the MTT assay procedure, based on the measurement of the level of mitochondrial dehydrogenase activity using MTT as the substrate. The assay is based on the redox ability of living mitochondria to convert dissolved MTT into insoluble purple formazan. Briefly, after the treatment, the medium was removed and the cells were incubated with 100 μ L per well of a MTT solution (0.25 mg mL⁻¹) for 2 h in a humidified 5% CO₂ incubator at 37 °C. The incubation was stopped by removing the MTT solution, then 100 μ L per well of DMSO was added to dissolve the formazan crystals, and the mixture was kept under stirring at r.t. for 15 min. Finally, the absorbance was determined at 570 nm using an automated spectrophotometric plate reader, Glomax multi-detection system (Promega). The experiments were repeated four times in triplicate (total n = 12). Percentages of the number of survival cells are reported as mean values $\pm$ SD.

All the data were normalized to PBS used as the control. The statistical significance of replicates was analyzed using Student's *t*-test with * $p \leq 0.1$, ** $p \leq 0.05$, and *** $p \leq 0.01$.

Results and Discussion

1. Preparation of the niosome vesicles

The herein studied niosome vesicles were prepared using a film-hydration method – according to reported procedures with minor modifications^{58,61} – by dissolving in CHCl₃ the cationic lipid 2,3-bis(tetradecyloxy)propan-1-aminium chloride (Fig. 1a) and the non-ionic surfactant polysorbate 80 (Fig. 1b) in 3 : 1 molar ratio. This molar ratio was selected after careful optimization, in terms of overall niosome size and stability over time, which was done on the basis of DLS and EPR analyses of a set of formulations including the two components in different molar ratios (data not shown). After solvent evaporation, the dried lipid film was hydrated with the selected buffer (PBS, 20 mM HEPES or D₂O, according to the experimental requirements), heated and filtered, finally obtaining the stock niosome formulation at 25.6 mM total concentration (expressed considering the sum of both the lipid and surfactant moles).

For the HoThyRu-containing niosome (here named niosome_HoThyRu) preparation, the nucleolipid Ru(III) complex (Fig. 1c) was dissolved in CHCl₃ along with the lipid and polysorbate so as to obtain a

mixture with HoThyRu at 3% in mol, and treated as described above. The amount of HoThyRu was chosen to obtain a well-defined ruthenium concentration in the formulation. Particularly, this was kept in the low mM range, i.e. at concentrations similar to or even lower than the ones analyzed in our previous studies,^{62,63} to verify if bioactivity enhancement could be produced by the niosome nanosystem vs. previously investigated nanocarriers. The AS1411-containing formulations (here named AS1411/niosome and AS1411/niosome_HoThyRu, respectively) were prepared by mixing a fixed amount of the oligonucleotide with increasing amounts of the niosome formulations, and finally incubating at 37 °C for 30 min. Thus a set of functionalized nanosystems were obtained with different negative/positive (N/P) charge ratios [corresponding to: (no. of anionic phosphate groups)/oligonucleotide/(no. of cationic amino groups)/cationic lipid].

2. Optimization of the AS1411/niosome formulation by gel electrophoresis, DLS and Z-potential assays

In preliminary experiments, various AS1411/niosome formulations with different N/P charge ratios were prepared and examined by gel electrophoresis in order to assess the ability of niosomes to incorporate AS1411, and particularly define at which oligonucleotide/ lipid ratio AS1411 can be considered fully inserted within the niosome structure. For this purpose, a fixed volume of a 0.5 mM AS1411 solution was mixed with increasing concentrations of niosomes, providing AS1411/niosome formulations ranging from 0 to 1 : 16 N/P charge ratios (see Table S1 for the actual concentration of the niosome components within the AS1411/niosome formulations at the different N/P charge ratios used in these experiments, ESI†). Analysis of the different niosomal systems was carried out using a 20% polyacrylamide gel, run under native conditions in TBE buffer (Fig. 2a). In a gel retardation assay, AS1411 showed bands with the same mobility as free AS1411 when formulated in niosomes with N/P ratio from 0 to 1 : 5 (lanes 2–5, Fig. 2a). In turn, it was sensibly retarded in niosomes from 1 : 8 N/P ratio on (lanes 6–10, Fig. 2a), indicating that no detectable amount of free AS1411 in solution was present in the latter samples, thus demonstrating the ability of niosomes to fully incorporate the aptamer within their structure.

AS1411/niosome formulations ranging from 0 to 1 : 16 N/P charge ratios were also analyzed by zeta potential and DLS measurements in order to determine their surface charge and overall size, respectively (Fig. 2b). The starting AS1411 concentration was 0.5 and 1 mM for the zeta potential and DLS analyses, respectively.

As depicted in Fig. 2b, the zeta potential results showed negative values for AS1411 alone (ca. 10 mV) and the AS1411/ niosome formulation at the highest oligonucleotide/lipid charge ratio here investigated (1 : 0.5), as expected due to the prevalence of the negative charges of the 26-mer oligonucleotide. Upon decreasing the N/P charge ratio, positive zeta potential values were obtained for the surface charge of the formulation. The first positive value (ca. +1 mV) was observed for the formulation with AS1411/niosome at 1 : 1 N/P charge ratio. Then, the zeta potential reached almost constant values starting from the AS1411/niosome sample with 1 : 8 N/P charge ratio, indicating an electrostatic stabilization level corresponding to ca. +10 mV (Fig. 2b and Table S2, ESI†).

Fig. 2 (a) 20% polyacrylamide gel electrophoresis under native conditions of AS1411/niosome formulations at different N/P charge ratios, run at 150 V at r.t. for 7 h. Lane 1: free AS1411 (0.5 mM); Lane 2 : 1 : 0.5; Lane 3 : 1 : 1; Lane 4 : 1 : 3; Lane 5 : 1 : 5; Lane 6 : 1 : 8; Lane 7 : 1 : 10; Lane 8 : 1 : 12; Lane 9 : 1 : 14; Lane 10 : 1 : 16 AS1411/niosome N/P charge ratios; (b) Z-average size (r. nm) and zeta potential (mV) values of AS1411/niosome formulations at different N/P charge ratios (red bars and blue squares, respectively) with the corresponding standard deviation (black error bar). The reported data are the average of three measurements (mean $\pm$ SD).

Concerning DLS analysis, as the N/P charge ratio diminished, lower Z-average size (Fig. 2b, red bars) and polydispersity index (Pdl, Table S3, ESI†) values were generally observed. Indeed, at 1 : 1 N/P charge ratio large particles were found, with a Z-average size of ca. 130 nm in radius, and this value decreased upon increasing the relative niosome amount within the formulation. Also in this case, constant values of ca. 80 nm in Z-average radius were obtained for the AS1411/niosome 1 : 10 and 1 : 12 N/P charge ratio formulations, also showing the lowest measured Pdl values (ca. 0.3, Table S3, ESI†). Upon further increasing the niosome amount keeping fixed the AS1411 content (1 : 14 and 1 : 16 N/P charge ratios), the Z-average size of the obtained nanosystems slightly decreased, even though with a concomitant increase in Pdl values (Table S3, ESI†).

Similar differences in the size of niosomes depending on the N/P charge ratios were already found in previous studies in which different DNA sequences were introduced within niosomal formulations.^{57,58,60} According to these data, the slight changes in size ranging from 0 to 1 : 16 N/P charge ratios could be explained as a fine balance between the compacting effect due to the internalization of the negatively charged oligonucleotides by the cationic lipid, reducing the particle size, and the space demanded by the lipid.

Hence, combining the results obtained from gel electrophoresis, zeta potential and DLS measurements, the optimal N/P charge ratio for the AS1411/niosome proved to be 1 : 12, which was therefore used in all the successive experiments (see Table S4 for the actual concentration of all the

components within the final formulations at the selected 1 : 12 N/P charge ratio, ESI†).

In particular, the 1 : 12 N/P charge ratio provided formulations with a Z-potential of ca. +12 mV, with satisfactory particle size and homogeneity parameters. Indeed, with these zeta potential values, the charged particles tend to repel each other being more stable in aq. solutions and less prone to aggregation.^{107,108} Additionally, positively charged nanosystems should also show enhanced interactions with the negatively charged cell surfaces, favouring cell uptake across the cellular membranes.¹⁰⁹

3. Characterization of niosome formulations by DLS, TEM, EPR, and NMR analysis

The studied niosomes (unfunctionalized niosomes, niosome_HoThyRu, AS1411/niosome and AS1411/niosome_HoThyRu formulations) were characterized by DLS, TEM, EPR and ³¹P NMR analysis.

DLS measurements were firstly used to determine the average particle size and homogeneity of the niosome formulations, also investigating the possible presence of HoThyRu in solution as free compound for the HoThyRu-containing systems. In addition, these measurements were also exploited to get information on the niosome stability over time, which is an important parameter to verify their possible storage in view of potential pharmacological applications. TEM microscopy was then exploited to get information on the overall shape, size and homogeneity of the unfunctionalized niosome formulation. EPR spectroscopy was here used to obtain information on the microstructural organization of the niosome bilayer and on the perturbations it experiences upon insertion of HoThyRu and/or AS1411, thus demonstrating their effective incorporation. ³¹P NMR analysis was used to confirm that no free oligonucleotide was present in the AS1411-containing niosome solution at the N/P charge ratio selected.

3.1. DLS particle size and stability measurements

All the niosome formulations - prepared as described above - were analyzed in a pseudo-physiological buffer, i.e. PBS, on a Zetasizer Nano ZSP (Malvern Instruments) in order to determine their Z-average size, homogeneity and stability over time. The latter parameter is a crucial factor to successfully develop an optimal dosage for effective biological applications.^{13,107}

First, their average particle size and Pdl were determined at r.t. on freshly prepared samples at three different concentrations, i.e. 17.5 mM, 3.5 mM and 175 μ M, obtained by diluting the initial 25.6 mM stock solution in PBS. Particularly, we selected a relatively high concentration (17.5 mM, also used for the EPR measurements, see section 3.3), a lower concentration (175 μ M, in the concentration range used for in vitro cell assays, see section 4) - and an additional, intermediate one (3.5 mM).

The HoThyRu concentration in the three mentioned formulations was 670, 135 and 6.7 μ M, respectively, for 17.5 mM, 3.5 mM and 175 μ M niosome formulations, while the amount of AS1411 was in turn 58, 12 and 0.58 μ M, always respecting the selected 1:12 N/P charge ratio (see Table S4, ESI†).

The size distributions by intensity of the freshly prepared formulations in PBS are depicted in Figure 3 for the highest - 17.5 mM - and the lowest -

175 μM - concentration analyzed, as representative examples. The related Z-average radius and Pdl values are reported in Table 1.

Figure 3. Size distribution by intensity of freshly prepared formulations at 17.5 mM (a) and 175 μM (b) niosome concentrations, reported as representative examples. For the corresponding concentration values of HoThyRu and AS1411 within the formulations, see Table S4 (ESI†).

Formulation	17.5 mM		175 μM	
	Z-average (r.nm) $\pm$ SD	Pdl $\pm$ SD	Z-average (r.nm) $\pm$ SD	Pdl $\pm$ SD
niosome	69.8 $\pm$ 0.3	0.44 $\pm$ 0.01	78.7 $\pm$ 0.8	0.39 $\pm$ 0.10
niosome_HoThyRu	56.8 $\pm$ 0.1	0.32 $\pm$ 0.01	58.0 $\pm$ 1.9	0.46 $\pm$ 0.12
AS1411/niosome	81.4 $\pm$ 0.6	0.32 $\pm$ 0.02	67.8 $\pm$ 0.5	0.22 $\pm$ 0.02
AS1411/niosome_HoThyRu	86.7 $\pm$ 0.6	0.32 $\pm$ 0.04	75.9 $\pm$ 0.5	0.39 $\pm$ 0.01

Table 1. Z-Average size (r., nm) and corresponding Pdl values for the freshly prepared formulations at 17.5 mM and 175 μM niosome concentration, in representative examples. The reported data are the average of three different measurements (mean $\pm$ SD).

The inclusion of HoThyRu and/or AS1411 within the niosomes was found to have an influence on the particle size. Indeed, the data reported in Figure 3 and Table 1 showed for all the formulations including the guest molecules a Z-average size value similar to that of the unfunctionalized niosomes (60-80 nm in radius), but with a narrower distribution, along with a reduced polydispersity index. Taken together, these results indicated that the functionalized niosomes, containing either AS1411 or HoThyRu or both, had greater homogeneity than the niosomes alone. Interestingly, all the

studied niosomes (unfunctionalized niosomes, niosome_HoThyRu, AS1411/niosome and AS1411/niosome_HoThyRu formulations) showed only small differences on varying the concentration.

Thereafter, these formulations were analyzed over time in order to evaluate the storage stability of the samples and also exclude possible long-term aggregation phenomena. To this aim, the formulations were stored at 4 °C for one month and analyzed every week at r.t. by DLS, in experiments carried out in duplicate.

Our findings indicated an overall stability of the niosomes in PBS, with essentially constant size at all the tested concentrations (Figure 4). A slight increase in the Z-average size was observed only for the niosome_HoThyRu system at the lowest concentration tested, which however already after one week reached a Z-average size around 100 nm, not undergoing further sensible changes for the successive weeks. It is worth highlighting that the studied systems are endowed with a size < 100 nm, even after a storage period, considered optimal for biomedical applications.^{110,111} Notably, the increase in size was not detected in the AS1411/niosome_HoThyRu formulation analyzed over time, suggesting that the oligonucleotide was somehow able to affect the compactness of the system. No relevant changes were found for the related Pdl values, confirming that these formulations also preserved a good homogeneity over time (see Table S5, ESI†).

Figure 4. Z-Average size (r_z , nm) reported as a function of time for all the formulations, as indicated on top of each panel. The formulations were analyzed at different niosome concentrations (17.5 mM, 3.5 mM and 175 μ M, represented with blue, red and green lines, respectively, with the associated standard deviation as black bars). The reported data are the average of three measurements in duplicate (mean $\pm$ SD).

In addition, DLS analysis was also used to evaluate the aggregation properties over time of HoThyRu alone, dissolved in PBS at the same concentration used in the niosome formulations. From our previous studies, the ability of nucleolipid-based Ru(III) complexes to form stable self-assembled aggregates in water was well known.^{62–64,104,112,113} In Figure S1 (ESI†) the DLS behaviour of HoThyRu, immediately after dissolution in PBS and after one week storage, is shown as a representative example at the highest concentration here analyzed. Freshly dissolved HoThyRu gave two distinct populations: one with *ca.* 25 nm radius, and another of higher size (*ca.* 120 nm in radius). Micelle-like structures could account for the first population, while the latter one, accounting for *ca.* 80% in intensity, can be attributed to liposome-like structures. However, when the system was left at 4 °C it underwent a structural re-organization giving, after one week, only the larger aggregates (Figure S1, ESI†). After one month, the size distribution intensity still showed only a single, narrow population of the same size, attributable to liposome-like aggregates (data not shown).

This analysis demonstrated the effective incorporation of HoThyRu in the niosome_HoThyRu formulations by confirming the absence of free Ru complex in the tested solutions. As illustrated in Figure 3a, niosome_HoThyRu at the highest analyzed concentration showed some micelle-like structures with size of *ca.* 14 nm in radius, which however disappeared with time. This population, remarkably, was never present at the lowest concentrations here tested, even in freshly prepared formulations (see Figure 3b, red line).

3.2. TEM morphology analysis

The morphology of the niosome formulation was analyzed also by TEM analysis. Typical obtained images are shown in Figure S2 (ESI†) (original magnification 29.000× and 43.000× respectively for panels a and b). Unfunctionalized niosomes, with the cationic lipid and polysorbate 80 mixed in 3:1 molar ratio, appeared approximately spherically-shaped and did not form very large aggregates under our experimental conditions. The niosome particles exhibited a good homogeneity, with size in the range 120-170 nm in diameter. These values are fully consistent with the data obtained by DLS measurements.

3.3. EPR measurements

Spin-label EPR spectroscopy measurements were used to get information on the microstructural organization of the niosomes, before and after the insertion of the guest molecules. Two amphiphilic spin-labels were alternatively inserted (1% in mol with respect to the niosome) in the studied systems: 5-doxyl stearic acid (5-DSA) and 16-doxyl stearic acid (16-DSA). The former bears the radical nitroxide group close to the hydrophilic moiety of the molecule, while in the latter one the reporter group is located at the end of the hydrophobic tail (see Figure S3 for the molecular structures of the two spin-labels, ESI†). Consequently, 5-DSA provides information on the bilayer region just below the interface with the aqueous medium, while 16-DSA gives information on the bilayer interior. The goal was to investigate the microstructural organization of the niosome formulations, particularly monitoring the loading of HoThyRu and/or AS1411 within the niosome bilayer and their ability in modifying - if any, and at what extent - its features.

The formulations were analyzed at 17.5 mM concentration with AS1411/niosome 1:12 N/P charge ratio, corresponding to *ca.* 670 μM in HoThyRu and *ca.* 60 μM in AS1411 content (see Table S4, ESI†). The chosen concentration for EPR measurements was determined by the sensitivity of the spin-labels. The spectra of 5-DSA and 16-DSA incorporated in freshly prepared niosome samples suspended in PBS buffer are depicted in Figure 5.

Figure 5. EPR spectra of 5-DSA and 16-DSA probes in (a) niosomes and AS1411/niosomes formulations (red and blue lines, respectively); (b) niosome_HoThyRu and AS1411/niosome_HoThyRu formulations (black and green lines, respectively).

	niosome		AS1411/niosome	
	S	a'_N	S	a'_N
5-DSA	0.65 ± 0.02	16.1 ± 0.1	0.59 ± 0.02	16.2 ± 0.1
16-DSA	0.43 ± 0.02	14.4 ± 0.1	0.41 ± 0.02	13.8 ± 0.1
	niosome_HoThyRu		AS1411/niosome_HoThyRu	
	S	a'_N	S	a'_N
5-DSA	0.48 ± 0.02	15.4 ± 0.1	0.47 ± 0.02	15.3 ± 0.1
16-DSA	0.40 ± 0.02	13.8 ± 0.1	0.39 ± 0.02	13.8 ± 0.1

Table 2. Values of the order parameter S and nitrogen hyperfine coupling constant a'_N of 5-DSA and 16-DSA probes in niosome, AS1411/niosome, niosome_HoThyRu and AS1411/niosome_HoThyRu formulations.

In all cases, the spectra showed an anisotropic line shape for both the 5-DSA and 16-DSA spin-label, as highlighted by the splitting of the high-field signal. The higher anisotropy observed for 5-DSA spectrum indicated a flexibility increase in the segmental chain mobility going from the polar head groups to the inner hydrophobic core, which is a characteristic hallmark of the fluid liquid-crystalline state of the bilayer.¹¹⁴

A quantitative analysis of the spectra was performed by determining the order parameter, S , and the nitrogen hyperfine coupling constant, a_N' , whose values are reported in Table 2. S is a measure of the local orientational ordering of the labeled molecule with respect to the normal to the bilayer surface, while a_N' is indicative of the micropolarity experienced by the spin probe.^{115,116}

The analysis of these values showed that the insertion of HoThyRu or AS1411 in niosome structures caused a decrease of the order parameter S , more evident for the 5-DSA than for the 16-DSA probe. Thus, the incorporation of the oligonucleotide or the nucleolipid Ru(III)-complex reduced the lipid packing density of the outer bilayer regions, close to the polar lipid/surfactant head groups. In turn, the a_N' parameter appeared only slightly affected by the addition of AS1411 or HoThyRu, with a marginal decrease of the local polarity.

These data provided clear evidence that the inclusion of either the oligonucleotide or the nucleolipid Ru(III)-complex, separately analyzed, perturbed the niosome structure, particularly reducing its fluidity. In both cases, these experiments nicely demonstrated their effective incorporation.

On the other hand, the addition of AS1411 to the niosome_HoThyRu formulation caused only slight changes in the S and a_N' values, suggesting that niosome microstructural organization, once altered by the presence of HoThyRu, was not significantly further perturbed by the successive insertion of the oligonucleotide. Indeed, experimental evidence of complete AS1411 association with the niosome_HoThyRu aggregates was provided by ³¹P NMR, as reported in the following subsection.

It is interesting to compare these results with those obtained for HoThyRu loaded in the bilayer of the previously investigated liposomes formed by the cationic lipid DOTAP (1,2-dioleoyl-3-trimethylammoniumpropane chloride). DOTAP liposomes showed a much more marked ordering gradient in going from the lipid polar head group to the bilayer interior; in the latter system, the insertion of the nucleolipid Ru(III) complex caused an increase of the order parameter, S , indicating a stiffening effect on the carbon atoms chain close to the outer surface.⁶³ Thus, our results reveal a different behaviour of niosomes with respect to classical liposomes as those formed by the lipid DOTAP: the former presents a much flatter order profile along the amphiphile chains, in that the polar head group region is more disordered and the bilayer inner core is more ordered. Moreover, the niosome bilayer microstructure responds to the incorporation of guest molecules by increasing the local disorder.

3.4. ³¹P NMR measurements and analysis of the overall features of the functionalized niosomes

With the aim of verifying whether AS1411 was effectively fully incorporated within the niosome_HoThyRu structure and not present as a free species in solution, ³¹P NMR spectra were recorded on freshly prepared AS1411/niosome_HoThyRu formulations using as a reference AS1411 alone

dissolved in D₂O at 50 μ M concentration, i.e. at a concentration very close to the one of the oligonucleotide in the niosome formulation with 1:12 N/P charge ratio. For this formulation, we used a niosome concentration of 15 mM, corresponding to ca. 570 μ M of HoThyRu (see Table S4, ES†), that are comparable to the concentrations investigated in the EPR analysis.

As reported in Figure 6, the ³¹P NMR spectrum of AS1411 alone showed, as expected, a single, narrow signal centered at around 0 ppm. Using the same acquisition time, AS1411/niosome_HoThyRu showed only a broad and very poorly resolved signal, slightly shifted to negative values. For this sample, acquisition times of 72 h were necessary to obtain a decent signal/noise ratio; also in these conditions, a unique, broad signal was apparent, indicating that AS1411 was completely incorporated in the niosome structure.

Taken together, the absence of a sharp signal, along with the presence of uniquely a broadened, upfield-shifted signal, demonstrated that, within the detection limit of the technique, no free oligonucleotide in solution was present in the tested formulation.

This result is also consistent with the gel electrophoresis data (see section 2), showing for the AS1411/niosome system that the oligonucleotide was fully incorporated in the niosome at the 1:12 N/P charge ratio. Thus, ³¹P NMR measurements confirmed that this N/P charge ratio, selected on the basis of our preliminary investigation, is suitable not only for the AS1411/niosome formulation but also for the system containing the Ru(III) complex HoThyRu.

Figure 6. ³¹P NMR spectra (161 MHz, D₂O) of 50 μ M AS1411 (a) and of AS1411/niosome_HoThyRu formulations at different acquisition times (b,c). The concentration of niosome formulations was 15 mM, corresponding to ca. 570 μ M of HoThyRu and 50 μ M of AS1411.

3.5. General summary of the niosome physico-chemical characterization results

The data obtained from this physico-chemical characterization define a quite clear picture of the microstructural features and stability of the here tested niosome formulations. Combined DLS and EPR measurements demonstrated that the studied niosomes were able to fully incorporate both the nucleolipid Ru(III) complex HoThyRu and the oligonucleotide AS1411, forming essentially homogeneous and monodisperse populations, present in pseudo-physiological solutions as single vesicle-like aggregates. The insertion of AS1411 and/or HoThyRu consistently perturbed the niosome formulations, as evidenced by the changes in their order parameter, S , derived from the EPR analysis, and in their Z -average size, obtained from DLS measurements.

DLS results also showed that only for freshly prepared niosome_HoThyRu samples, at the highest concentration analyzed, little amounts of free HoThyRu were present in solution, essentially forming micelles. However, this event was not observed in AS1411/niosome_HoThyRu formulations even at the highest tested concentrations. In turn, AS1411 was fully incorporated in the niosome aggregates, as confirmed by ^{31}P NMR analysis.

The high concentrations explored by EPR and thus also by DLS measurements were dictated by the sensitivity of the spin-labeled probes, necessarily inserted at very low concentrations in the formulations to avoid significant alterations of the studied systems. These systems provided very interesting data on the intimate microstructure of the studied niosomes, however at concentrations far from those used in biological applications. For this reason, also lower concentrations were tested by DLS, which in all cases confirmed that the niosomes incorporating AS1411 and the nucleolipid Ru(III) complex provided stable nanosystems, with essentially constant size over time. Thus, the microstructural and stability analyses carried out on these niosome formulations fully validated them as suitable potential carriers for delivering drugs of different chemical nature.

4. In vitro bioactivity studies of the niosome formulations

Niosome formulations including the cationic lipid 2,3-di(tetradecyl)propan-1-amine were previously shown to be efficient transfecting agents for DNA plasmids in a variety of human cell lines, such as HEK293T, ARPE-19, MSC-D1,⁵⁹ as well as in vivo rat brain and retina models.^{58,60} Here, we analyzed for the first time the efficacy of these niosomes in delivering multiple therapeutic agents, i.e. an anticancer ruthenium complex in the presence of the nucleolin-targeting aptamer AS1411. The antiproliferative activity of the niosome formulations was evaluated on a selected panel of human cells including a human cervix adenocarcinoma (HeLa) and two colorectal cancer cell lines (HTB-38, HCC2998), as well as a non tumoral embryogenic human cell line (HEK293T). HeLa was selected as a relatively drug-sensitive cell line with high nucleolin content, already proved to be responsive to niosome formulations⁶¹ as well as to AS1411.⁷¹ In contrast, HTB-38 and HCC2998 colorectal cancer cell lines were selected being highly resistant even to very effective chemotherapeutic agents such as 5-fluorouracil and camptothecin.^{117,118} Finally, the embryogenic cell line HEK293T was selected

as non tumoral cell line, used also in previous studies as a control to verify the absence of toxicity for niosome formulations.⁵⁹

Figure 7. Cell viability assays on HCC2998 (a and b) and HTB-38 (c and d) cells, at the reported concentrations. Panels a and c show the three controls: HoThyRu (light green square), AS1411 (dark green circles) and unfunctionalized niosomes (orange triangles). Panels a and b show the bar graphs of the tested samples, as explained in the legend. Data are reported as mean values $\pm$ SD (error bar), for four independent experiments in triplicate ($n = 12$). All the data are normalized to PBS used as control. *corresponds to t-test results with: $*p \leq 0.1$, $**p \leq 0.05$, $***p \leq 0.01$ significantly different within each indicated pair.

The cytotoxic activity induced by niosome formulations was assessed analyzing a range of concentrations from $1.25 \mu\text{M} \rightarrow 150 \mu\text{M}$ using the standard tetrazolium-based (MTT) assay after 72 h of treatment. Three different niosome formulations carrying HoThyRu were prepared as described in the experimental section, i.e. niosome_HoThyRu,

AS1411/niosome_HoThyRu and scrambled/niosome_HoThyRu. The latter formulation was obtained following the same protocol used for the preparation of AS1411/niosome_HoThyRu, but replacing the AS1411 aptamer with a scrambled 26-mer oligonucleotide at the same concentration. This additional control was here introduced to verify that the activity of the AS1411-containing niosomes was due to the specific features of the nucleolin-targeting aptamer and not to unspecific effects, generically attributable to the presence of an oligonucleotide. The formulations added to the cell cultures provided a range of HoThyRu concentrations from 97 nM (1.25 μ M niosome) to 5.8 μ M (150 μ M niosome), along with a range of AS1411 or scrambled oligonucleotide concentrations from 8.3 nM (1.25 μ M niosome) to 500 nM (150 μ M niosome), as reported in Table S4 (ESI†).

Figure 8. Cell viability assays on HeLa (a and b) and HEK293T (c and d) cells, at the reported concentrations. Panels a and c show the three controls: HoThyRu (light green square), AS1411 (dark green circles) and unfunctionalized niosome (orange triangles). Panels b and d show the bar graphs of the tested samples, as explained in the legend. Data are reported

as mean values $\pm$ SD (error bar), for four independent experiments in triplicate ($n = 12$). All the data are normalized to PBS used as control. *corresponds to t-test results with: $*p \leq 0.1$, $**p \leq 0.05$, $***p \leq 0.01$ significantly different within each indicated pair

Firstly, MTT assays were carried out to investigate the cytotoxic response promoted by AS1411, HoThyRu and the unfunctionalized niosomes in all the tested cell lines (Figures 7, 8 and S4, ESI†). As expected, for the free AS1411 aptamer no relevant antiproliferative activity at the explored concentrations was revealed in all the tested cell lines. In the case of HoThyRu and the unfunctionalized niosome, a different behaviour was observed depending on the analyzed cell. While on both colorectal cancer cells the Ru(III)-complex showed no cytotoxicity, a slightly increased cytotoxic effect was observed, reaching a plateau around 20%, on HeLa and HEK293T cells at the highest studied concentration (5.8 μ M HoThyRu, which corresponds to the amount which was then incorporated in the 150 μ M niosome). This result could be reasonably explained considering that the Ru concentration in the tested formulations (see Table S4, ESI†) was deliberately taken below its IC_{50} values (7-15 μ M and 40-65 μ M), previously determined respectively for the Ru(III)-complex loaded in POPC or DOTAP-based liposomes on MCF-7 and WiDr cells, which are the human cancer cell lines most responsive to the Ru-based drugs we tested so far.^{62–65}

In turn, unfunctionalized niosomes gave limited reduction in cell viability (comprised in the 10-20% range) on HCC2998 and HEK293T cells, however their cytotoxic effect was not negligible on HeLa and HTB-38 cells, increasing in a concentration-dependent manner. The highest impact was found on the most sensitive HeLa cells, where cytotoxic effects were detected starting from 50 μ M. Interestingly, the colorectal cell lines showed a different behavior when treated with the niosomes, thus further proving their different resistance profile to drugs.¹¹⁸

Next, we evaluated the niosomes loaded with HoThyRu as antiproliferative drug (niosome_HoThyRu) in parallel with the combination HoThyRu/oligonucleotide, using in one system AS1411 as internalization enhancer (AS1411/niosome_HoThyRu), and in the other a scrambled oligonucleotide as a control (scrambled/niosome_HoThyRu). The concentrations used were selected so to minimize potential toxicity produced by the unfunctionalized niosomes in each type of cell line (Figures 7 and 8).

In general, the scrambled/niosome_HoThyRu nanosystem produced similar, essentially limited effects as niosome_HoThyRu, while a sensibly increased bioactivity was found for AS1411/niosome_HoThyRu, thus confirming that the observed cytotoxicity enhancement was due to the incorporation of AS1411 into the niosome. The cellular response obtained was cell-dependent, further demonstrating that the HCC2998 and HTB-38 colorectal cancer cell lines were more resistant to drug treatments than HeLa cells. However, in HCC2998 the niosome carrier enhanced the HoThyRu antiproliferative activity, producing a 25% cell viability reduction at 3.8 μ M ruthenium concentration and almost 50% cell viability reduction at 5.8 μ M (Figures 7a and 7b), i.e. under conditions at which the niosome per se gave no toxicity (Figure S4, ESI†). This active ruthenium concentration was well below the one previously reported in colorectal cancer cells for the same ruthenium complex (IC_{50} values 40-65 μ M

determined on WiDr cells), transported by liposome carriers.^{62–65} These two cells lines did not show a remarkably different response even upon treatment with the AS1411-containing niosomes (Figures 7b and 7d). On the contrary, the HeLa cervical cancer cell lines presented a 40% reduction in cell viability when treated with 7.5 μ M niosome_HoThyRu (388 nM in HoThyRu), which increased to almost 70% upon treatment with AS1411/niosome_HoThyRu under the same concentration conditions (Figures 8a and 8b).

Taken together, these results proved that our niosome formulations can be regarded as very interesting nanocarriers to transport anticancer drugs. First of all, it is noteworthy considering that upon treatment either with the niosomes or with HoThyRu alone at the highest tested concentrations (50 μ M in niosome), the healthy HEK293T cells maintained a 80% cell viability, while the treatment with AS1411 alone did not produce any detectable cytotoxic effect (Figures 8c and 8d). Secondly, on the very sensitive HeLa cells the incorporation in niosomes caused a strong enhancement of the effective ruthenium cytotoxicity since > 50% cell viability reduction was observed with the 25 μ M niosome_HoThyRu formulation, containing the Ru(III) complex at 0.95 μ M concentration. However, the most promising results were obtained with the AS1411/niosome_HoThyRu formulations, in all cases – even considering the poorly responsive HCC2998 and HTB-38 cancer cells - the most effective ones. The specific bioactivity of AS1411-decorated niosomes, nicely proved by the comparison with the same formulations incorporating a scrambled oligonucleotide, demonstrated that, even in very low amounts, this aptamer was able to sensibly enhance the antiproliferative activity of the Ru(III)-complex.

Conclusions

A crucial achievement in the construction of effective carriers for drug delivery is the design and preparation of highly integrated and multifunctional nanosystems, allowing possible applications extended also to “on-demand” release, specific tissue/cell type targeting, *in vivo* imaging and diagnosis. In this context, we have here explored the potential of novel niosomal formulations, obtained mixing the cationic lipid 2,3-bis(tetradecyloxy)propan-1-aminium chloride and the non-ionic surfactant polysorbate 80 in 3:1 ratio, to provide effective nanosystems for Ru(III)-based anticancer strategies. The here optimized niosomes gave monodisperse vesicle-like aggregates, with typical size of *ca.* 100 nm and stable upon storage for 1 month, potentially compatible with *in vivo* applications. These niosomes - loaded with the nucleolipid Ru(III)-complex HoThyRu and the nucleolin-targeting AS1411 aptamer - were characterized in terms of morphology, average size, Zeta potential, electrophoretic mobility, and overall stability using a variety of physico-chemical techniques. Particularly, our results also demonstrated that the studied niosomes were able to fully incorporate both the nucleolipid Ru(III) complex HoThyRu and the oligonucleotide AS1411 with no free molecules detectable in solution. The functionalization is driven by electrostatic interactions, in the case of the negatively charged oligonucleotide, and by a combination of hydrophobic and electrostatic interactions for the HoThyRu encapsulation.

Tested on both cancer and normal cell lines, these niosome formulations showed increased antiproliferative activity when loaded with both the Ru(III)-complex and the AS1411 aptamer compared with all the tested controls. A valuable therapeutic window was found for the HeLa cancer cells: for concentrations up to *ca.* 3.5 μ M, the niosomes alone did not show appreciable cytotoxicity, whereas the functionalized niosomes produced effective antiproliferative effects. Lower antiproliferative activity was observed on both HCC2998 and HTB-38 colorectal cancer cell lines, generally poorly responsive to drug treatments and expressing very low contents of nucleolin. Notably, AS1411 – which *per se* did not cause any cytotoxic effect at all the tested concentrations – markedly enhanced the antiproliferative activity of the Ru-containing niosomes, demonstrating its precious activity as targeting agent.

To the best of our knowledge, niosomes were here unprecedentedly used to produce multifunctional nanosystems, incorporating multiple drugs with different chemical properties and functions. In this sense, our work can thus represent a valuable “proof-of-concept” proving the efficacy of niosomes in the construction of integrated, multifunctional platforms for anticancer strategies. Further studies, aimed at increasing the variety and complexity of these formulations and extend their application to the delivery of different anticancer drugs, for use in combination therapies, are currently underway in our laboratories.

Abbreviations

CT	computed tomography
DLS	dynamic light scattering
DMEM	Dulbecco's Modified Eagle's Medium
DOTAP	1,2-dioleoyl-3-trimethylammoniumpropane
DSA	doxyl stearic acid
EPR	electron paramagnetic resonance
FBS	fetal serum bovine
HTB-38	colorectal adenocarcinoma cell line
HCC2998	colorectal adenocarcinoma cell line
HEK293T	human embryonic kidney cell line
HeLa	human cervical cancer cell line
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
IC50	half-maximal inhibitory concentration
MCF-7	human breast adenocarcinoma cell line
MRI	magnetic resonance imaging
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NMR	nuclear magnetic resonance
NSVs	non-ionic surfactant vesicles
PBS	phosphate-buffered saline
PdI	polydispersity index
PET	positron emission tomography
POPC	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
ppm	parts per million
siRNA	small interfering RNA
SPECT	single photon emission computed tomography
TBE	tris-borate-EDTA

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

We are deeply grateful to dr. Rocco Di Girolamo, from the Department of Chemical Sciences, University of Naples Federico II, for his precious contribution for the TEM images acquisition. This work was supported by the Italian Association for Cancer Research (AIRC) (IG2015 n. 17037 to D. M.). C. R. thanks "Programma di scambi internazionali tra l'Università degli Studi di Napoli Federico II ed Università ed Istituti di ricerca stranieri per la mobilità di breve durata di docenti, ricercatori e studiosi". Financial support by the Spanish Ministerio de Ciencia e Innovación (MICINN) (Project CTQ2014-52588-R and CTQ2017-84415-R) and Generalitat de Catalunya (2017SGR114) are gratefully acknowledged. CIBER-BBN is an initiative funded by the Instituto de Salud Carlos III with assistance from the European Regional Development Fund.

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Graphical abstract

Schematic representation of the AS1411/niosome_HoThyRu formulations and of the thin film hydration method, used for their preparation accompanied by the chemical structures of all the nanosystem components.

Electronic Supplementary Information

AS1411-decorated niosomes as effective nanocarriers

for

Ru(III)-based drugs in anticancer strategies

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Synthesis and characterization of HoThyRu

Scheme S1. Synthetic procedure for the preparation of the nucleolipidic Ru(III)-complex HoThyRu. For all the synthesized compounds, the spectroscopic (¹H and ¹³C) and spectrometric (ESI-MS) data were in perfect agreement with literature data (G. Mangiapia, G. D'Errico, L. Simeone, C. Irace, A. Radulescu, A. Di Pascale, A. Colonna, D. Montesarchio and L. Paduano, *Biomaterials*, 2012, **33**, 3770–3782).

- **Synthesis of 3-(4-pyridylmethyl)-5'-O-(4,4'-dimethoxytriphenylmethyl)-thymidine (2)**

5'-O-(4,4'-dimethoxytriphenylmethyl)thymidine **1** (148 mg, 0.27 mmol) was dissolved in 6 mL of dry DMF. Cs₂CO₃ (531 mg, 1.63 mmol) and 4-(bromomethyl)pyridine hydrobromide (275 mg, 1.08 mmol) were then added to the reaction mixture, left at 60 °C under stirring. After 20 h, TLC analysis indicated the presence of the desired product and the reaction was quenched by removing the solvent under reduced pressure. First, a CH₂Cl₂/H₂O extraction was performed to remove the excess reagents. Then, the crude product was purified by chromatography on a silica gel column using AcOEt/MeOH (97:3, v/v, containing 2 % of TEA), as eluent, giving desired compound **2** in 67 % yield (114 mg, 0.18 mmol).

2: oil. *R_f* = 0.5 (AcOEt/MeOH, 95:5, v/v).

- **Synthesis of 3-(4-pyridylmethyl)-3'-O-oleyl-5'-O-(4,4'-dimethoxytriphenylmethyl)-thymidine (3)**

Alkylated compound **2** (114 mg, 0.18 mmol) was dissolved in 2 mL of dry CH₂Cl₂ and then DMAP (122 mg, 0.54 mmol), oleic acid (50.1 mg, 0.18 mmol) and DCC (111 mg, 0.54 mmol) were sequentially added. After stirring for 15 h at r.t., TLC analysis indicated the complete disappearance of the starting materials. Thus, the reaction mixture was concentrated under reduced pressure; the crude product was purified by chromatography on a silica gel column using *n*-hexane/AcOEt (7:3, v/v, containing 2 % of TEA) as eluent, providing desired compound **3** in 41 % yield (64.1 mg, 0.07 mmol).

3: oil. *R_f* = 0.3 (*n*-hexane/AcOEt, 7:3, v/v).

- **Synthesis of 3-(4-pyridylmethyl)-3'-O-oleyl-thymidine (4)**

Compound **3** (56.2 mg, 0.06 mmol) was dissolved in 4 mL of a 1 % TCA solution in CH₂Cl₂. Upon addition of the acid, the reaction mixture acquired an intense yellow-orange colour, typical of the trityl cation, and was left under stirring at r.t. After 1 h, TLC monitoring showed the complete disappearance of the starting compound. Thus the reaction was quenched by adding few drops of MeOH until complete decoloration, and finally TEA was added to neutralize the solution. Then the reaction mixture was concentrated *in vacuo* and purified by chromatography on a silica gel column eluted with CHCl₃/MeOH (99:1, v/v), giving the desired compound **4** in almost 71 % isolated yields (25.6 mg, 0.04 mmol).

4: yellow oil. *R_f* = 0.4 (CHCl₃/MeOH, 95:5, v/v).

- **Synthesis of 3-(4-pyridylmethyl)-3'-O-oleyl-5'-O-(benzyloxy)hexaethylene glycol acetyl-thymidine (5)**

To compound **4** (21.1 mg, 0.04 mmol), dissolved in 3 mL of dry CH₂Cl₂, DMAP (12.8 mg, 0.11 mmol), BnO-HEG acetic acid **8** (16.6 mg, 0.04 mmol) and DCC (21.7 mg, 0.11 mmol) were sequentially added, and the resulting reaction mixture was left under stirring at r.t.. After 15 h TLC analysis indicated the formation of a new product, so the solvent was removed under reduced pressure and the crude product was purified by chromatography on a silica gel column eluted with AcOEt, giving the desired compound **5** in 51 % yield (17.2 mg, 0.02 mmol).

5: oil. *R_f* = 0.4 (AcOEt/MeOH, 95:5, v/v).

- **Synthesis of HoThyRu**

Nucleolipid **5** (17.1 mg, 16.9 nmol) was dissolved in 2.5 mL of dry CH₂Cl₂ and then Na⁺ [*trans*-RuCl₄(DMSO)₂]⁻ (7.9 mg, 0.02 mmol) was added under stirring at 40 °C. After 15 h the reaction, monitored *via* TLC, indicated the complete disappearance of both starting materials and the concomitant formation of a new, more polar product. Thus, the solvent was removed under reduced pressure providing the target complex HoThyRu in almost quantitative yields (21.1 mg, 15.6 nmol).

HoThyRu: yellow oil. *R_f* = 0.2 (AcOEt/MeOH, 95:5 v/v).

Synthesis and characterization of BnO-HEG acetic acid

Scheme S2. Synthetic procedure for the preparation of BnO-HEG acetic acid (Bn = benzyl group). For all the synthesized compounds, the spectroscopic (¹H and ¹³C) and spectrometric (ESI-MS) data were in perfect agreement with literature data (G. Mangiapia, et al. *Biomaterials*, 2012, **33**, 3770–3782).

- **Synthesis of (monobenzyloxy)hexaethylene glycol (6)**

Hexaethylene glycol (2.0 g, 7.10 mmol) was dissolved in 8 mL of dry THF and NaH 60 % p.p. (170 mg, 4.25 mmol) and then benzylbromide (3.37 mL, 22.8 mmol) were sequentially added. The reaction mixture was stirred at r.t. for 12 h, then MeOH (1 mL) was added and the solvent removed *in vacuo*. The crude product was dissolved in CHCl₃, filtered on celite and then purified by chromatography on a silica gel column using AcOEt/MeOH (9:1, v/v) as eluent, yielding the desired compound **6** in 65 % yields (1.7 g, 4.60 mmol).

6: oil. *R_f* = 0.5 (AcOEt/MeOH, 9:1, v/v).

- **Synthesis of tert-butyl (monobenzyloxy)hexaethylene glycol acetate (7)**

Alcohol **6** (155 mg, 0.42 mmol) was dissolved in 1 mL of dry THF and NaH 60 % p.p. (33.0 mg, 0.83 mmol) and *tert*-butyl bromoacetate (154 μL, 1.01 mmol) were

sequentially added. The reaction mixture was stirred at r.t. for 12 h, then few drops of MeOH were added and the solvent removed *in vacuo*. The crude product was dissolved in CHCl₃, filtered on celite and then purified by chromatography on a silica gel column eluted with AcOEt, yielding the desired compound **7** in 86 % yields (175 mg, 0.36 mmol).

7: oil. $R_f = 0.6$ (AcOEt/MeOH, 95:5, v/v).

- ***Synthesis of (monobenzyloxy)hexaethylene glycol acetic acid (8)***

Ester **7** (95.0 mg, 0.19 mmol) was dissolved in 1.5 mL of HCOOH and stirred at r.t. for 2 h. The solvent was then removed *in vacuo* and the residue was coevaporated three times with CHCl₃ (3 x 3 mL), yielding the desired compound **8** in almost quantitative yields (82.0 mg, 0.19 mmol).

8: oil. $R_f = 0.2$ (AcOEt/MeOH, 95:5, v/v).

Concentration of niosome vesicles in AS1411/niosome formulations at different N/P charge ratios ^a	
N/P charge ratio	niosome conc. (lipid + surfactant)
1:0.5	6.25 μ M
1:1	12.5 μ M
1:3	37.5 μ M
1:5	62.5 μ M
1:8	100 μ M
1:10	125 μ M
1:12	150 μ M
1:14	175 μ M
1:16	200 μ M

^a these values are referred to fixed AS1411 concentration (0.5 μ M).

Table S1. Concentration of niosome vesicles in AS1411/niosome formulations at different N/P charge ratios with fixed AS1411 concentration of 0.5 μ M, used in gel electrophoresis and Z-potential assays.

N/P charge ratio	Z-potential (mV) $\pm$ SD
AS1411	-10.0 $\pm$ 1.5
1:0.5	-9.3 $\pm$ 0.8
1:1	1.0 $\pm$ 0.1
1:5	4.3 $\pm$ 0.7
1:8	10.7 $\pm$ 1.2
1:10	7.9 $\pm$ 1.6
1:12	12.7 $\pm$ 0.9
1:14	10.4 $\pm$ 0.9
1:16	11.6 $\pm$ 1.6

Table S2. Zeta potential values (mV) of AS1411/niosome formulations at different N/P charge ratios. Data are reported as an average of three measurements (mean $\pm$ SD).

N/P charge ratio	Z-average (r. nm) $\pm$ SD	PdI $\pm$ SD
1:1	130 $\pm$ 20	0.39 $\pm$ 0.09
1:3	116 $\pm$ 2	0.36 $\pm$ 0.01
1:5	98 $\pm$ 4	0.40 $\pm$ 0.11
1:8	91.0 $\pm$ 0.2	0.27 $\pm$ 0.01
1:10	86.5 $\pm$ 0.1	0.27 $\pm$ 0.01
1:12	83.5 $\pm$ 0.5	0.34 $\pm$ 0.05
1:14	75.7 $\pm$ 0.1	0.35 $\pm$ 0.01
1:16	60.4 $\pm$ 0.6	0.38 $\pm$ 0.01

Table S3. Z-average size (r., nm) and associated polydispersity index (PdI) values determined for AS1411/niosome formulations at different N/P charge ratios. Data are reported as an average of three measurements (mean $\pm$ SD).

Concentration of niosome components in AS1411/niosome 1:12 N/P charge ratio formulations		
niosome	HoThyRu	AS1411
17.5 mM ^{a,c}	672 µM	58 µM
15 mM ^b	576 µM	50 µM
3.5 mM ^c	135 µM	12 µM
175 µM ^c	6.7 µM	585 nM
150 µM ^d	5.8 µM	500 nM
125 µM ^d	4.8 µM	416 nM
100 µM ^d	3.8 µM	333 nM
75 µM ^d	2.9 µM	250 nM
50 µM ^d	1.9 µM	166 nM
25 µM ^d	0.95 µM	83 nM
15 µM ^d	0.58 µM	50 nM
7.5 µM ^d	285 nM	33 nM
3.5 µM ^d	133 nM	16 nM
1.25 µM ^d	48 nM	8.3 nM

Table S4. Concentrations of niosome vesicles, HoThyRu and AS1411 in niosome formulations at the selected 1:12 oligonucleotide/lipid N/P charge ratio.

^a concentration used in EPR measurements;

^b concentration used in NMR measurements;

^c concentration used in DLS particle size and storage stability measurements;

^d concentration used in biological assays.

Figure S1. Size distribution by intensity of a 672 μM HoThyRu solution in PBS as a freshly prepared sample (up) and after one week storage at 4° C (down), reported as a representative example.

Figure S2. TEM images of unfunctionalized niosomes (cationic lipid and polysorbate 80 in 3:1 molar ratio); original magnification 29.000 $\times$ and 43.000 $\times$ respectively for panels **a** and **b**.

Figure S3. Molecular structures of **(a)** 5-DSA and **(b)** 16-DSA spin probes used in EPR measurements. DSA = doxyl stearic acid.

time (weeks)	17.5 mM		3.5 mM		175 μM	
	Pure niosome					
	Z-average (r. nm) ± SD	PdI± SD	Z-average (r. nm) ± SD	PdI± SD	Z-average (r. nm) ± SD	PdI± SD
0	69.8 ± 0.3	0.44 ± 0.01	62.9± 0.4	0.38 ± 0.01	78.7± 0.8	0.39 ± 0.10
1	85.7± 0.1	0.61± 0.10	65.9 ± 2.3	0.37 ± 0.01	79.4 ± 2.9	0.34 ± 0.02
3	86.5 ± 0.7	0.47 ± 0.03	69.0 ± 4.4	0.34 ± 0.03	84.5 ± 2.2	0.59 ± 0.11
4	74.7 ± 0.1	0.43 ± 0.02	79.5 ± 0.7	0.51 ± 0.06	76.9± 2.6	0.59 ± 0.12
	niosome_HoThyRu					
	Z-average (r. nm) ± SD	PdI± SD	Z-average (r. nm) ± SD	PdI± SD	Z-average (r. nm) ± SD	PdI± SD
0	56.8 ± 0.1	0.32 ± 0.01	76.1± 1.6	0.46 ± 0.02	56± 2	0.46 ± 0.12
1	52.9 ± 1.4	0.35 ± 0.07	87 ± 2	0.43 ± 0.03	107± 2	0.47 ± 0.03
3	54.8± 0.7	0.35 ± 0.01	68 ± 3	0.43 ± 0.03	93± 2	0.42 ± 0.02
4	59.1± 1.6	0.39 ± 0.01	103± 2	0.27 ± 0.03	106± 5	0.44 ± 0.02
	AS1411/niosome					
	Z-average (r. nm) ± SD	PdI± SD	Z-average (r. nm) ± SD	PdI± SD	Z-average (r. nm) ± SD	PdI± SD
0	81.4± 0.6	0.32 ± 0.02	66.5± 0.7	0.24 ± 0.01	67.8± 0.5	0.22 ± 0.01
1	85.6 ± 0.6	0.33 ± 0.01	77 ± 2	0.37 ± 0.01	68.3 ± 0.6	0.34 ± 0.02
3	80.6± 0.9	0.30 ± 0.03	68.3± 0.3	0.20 ± 0.01	72.9± 1.2	0.23 ± 0.01
4	87± 3	0.33 ± 0.01	80.2± 0.2	0.28 ± 0.01	75.9 ± 0.5	0.26 ± 0.01
	AS1411/niosome_HoThyRu					
	Z-average (r. nm) ± SD	PdI± SD	Z-average (r. nm) ± SD	PdI± SD	Z-average (r. nm) ± SD	PdI± SD
0	86.7± 0.6	0.32 ± 0.04	87± 5	0.37 ± 0.01	75.9 ± 0.5	0.39 ± 0.01
1	74.7± 0.4	0.26 ± 0.01	82.0 ± 1.3	0.36 ± 0.01	79.2 ± 0.3	0.28 ± 0.01
3	93.3 ± 0.6	0.38 ± 0.01	67.6 ± 0.4	0.21 ± 0.01	77.1 ± 0.1	0.30 ± 0.03
4	80.3 ± 0.1	0.29 ± 0.01	71.9 ± 0.1	0.26 ± 0.01	83.1 ± 0.7	0.36 ± 0.01

Table S5. Z-average size (r., nm) and corresponding PdI values, reported as a function of time for all the niosomal formulations (unfunctionalized niosomes, niosome_HoThyRu, AS1411/niosome and AS1411/niosome_HoThyRu) at different concentrations. Data are reported as an average of three measurements (mean $\pm$ SD).

Figure S4. Normalized cell viability for AS1411, HoThyRu and unfunctionalized niosome in the four indicated cell lines. Cells were treated with increasing concentrations of the three tested samples ranging from 1.25 to 150 μM . **a)** HEK293T, **b)** HeLa, **c)** HCC2998 and **d)** HTB-38. Data are reported as mean values $\pm$ SD (error bar), for four independent experiments in triplicate ($n = 15$). All data are normalized to PBS used as control.