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Exploring the HYDRAtion method for loading siRNA on liposomes: the interplay between stability and biological activity in human undiluted ascites fluid

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Abstract

Delivery of small interfering RNA (siRNA) is recently gaining tremendous attention for the treatment of ovarian cancer. The present study investigated the potential of different liposomal formulations composed of (2, 3-Dioleoyloxy-propyl)-trimethylammonium (DOTAP) and 1, 2-Dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) encapsulating siRNA (hydration method) for their ability to knockdown luciferase (Luc) activity in human ovarian cancer SKOV-3 cells. Fluorescence Single Particle Tracking (fSPT) and fluorescence correlation spectroscopy (FCS) in human undiluted ascites fluid obtained from a peritoneal carcinomatosis patient revealed that cationic hydra-lipoplexes (HYDRA-LPXs) and HYDRA-LPXs decorated with stable DSPE-PEG (DSPE HYDRA-LPXs) showed high stability during at least 24 hours. HYDRA-LPXs decorated with sheddable C8 and C16 PEG-Ceramides (Cer HYDRA-LPXs) resulted in rapid and premature release of siRNA already in the first hours. Despite their role in preventing aggregation *in vivo*, liposomes decorated with stable PEG residues resulted in a poor transfection compared to the ones decorated with sheddable PEG residues in reduced serum conditions. Yet, the transfection efficiency of both Cer HYDRA-LPXs significantly decreased following 1 hour of incubation in ascites fluid due to a drastic drop in the cellular uptake, while DSPE HYDRA-LPXs are still taken up by cells, but too stable to induce efficient gene silencing.

Keywords: siRNA delivery, siRNA encapsulation, peritoneal metastasis, sheddable PEG, ascites

Introduction

Small interfering RNA (siRNA) therapeutics hold great potential for the treatment of different diseases, such as neurodegenerative pathologies, genetic and metabolic disorders and cancer [1, 2]. To ensure their therapeutic activity, siRNA-based medicines should be delivered into the cytoplasm of the target cell where the natural RNAi machinery could be engaged. Towards this goal, complexation or encapsulation of siRNA within nano-sized particles is being utilized to protect the siRNA from blood nucleases and other extracellular components with the aim to increase the fraction that enters into cells following administration. Apart from protection of the siRNA against nucleases , these nano-carriers should also (i) be resistant to the formation of large aggregates while circulating in the extracellular biofluids, (ii) prevent premature release of the siRNA before reaching the target cell [3], and (iii) (sufficiently) interact extracellularly with the plasma membrane and intracelluarly with the endosomal membrane, an interaction which is essential for therapeutic activity of siRNA [3, 4].

Over the last decade, both polymeric and lipid nanoparticles (LNPs) were employed for siRNA delivery [2, 5]. Due to the negative charge of the siRNA, cationic lipid-based and polymeric NPs are widely used to obtain spontaneous electrostatic interactions and protect the siRNA from degradation [6-8]. The most important difference between the lipid-based and polymeric vehicles is that the majority of the cationic polymers do not contain a hydrophobic tail, and thus are completely soluble in water. Also, cationic polymers can be synthesized in different molecular weights and shapes (linear versus branched) and can be more easily tuned with functional groups, to influence, for example, the intracellular trafficking of the vehicles and their biological activity [8, 9]. Generally speaking, LNPs contain a lipid bilayer-disrupting lipids that are activated by low endosomal pH, whereas polymers are very easy to modify and control their structure in a way that they become positively charged in acidic endosomes in order to enhance endosomal escape of siRNA [10].

Lipid nanoparticles (LNPs) have been extensively investigated as candidates for siRNA delivery in cancer therapy [11]. Currently, LNPs represent the most promising platform for systemic delivery of siRNAs, with lipid-based formulations in clinical trials for the treatment of different diseases [2, 5]. Despite major advances with LNPs for siRNA delivery, the main challenge remains their colloidal stability within the fluids of the body. In this context, grafting LNPs with polyethylene glycol (PEG) is the most common strategy to prevent aggregation and prolong their half-life in the circulation [12]. PEGylation, however, has been associated with poor biological activity of siRNA [12]. It is speculated that PEG chains significantly decrease the uptake of LNPs by cells and disrupt their interaction with the endosomal membrane [4, 13]. For instance, increasing the PEGylation of siRNA-lipoplexes (siRNA-LPXs) from 2 to 5 mol% dramatically diminished the siRNA gene silencing *in vivo* [14]. Furthermore, it has been postulated that multiple administration of stabilized PEGylated lipid particles (SPLP), induced a strong immune response against PEG, resulting in accelerated blood clearance [15-17]. Worth mentioning also is that PEG residues interfere with the electrostatic interactions between negatively charged siRNA and positively charged lipids resulting in a low complexation/encapsulation efficiency. Also, we observed that PEGylation leads to the rapid premature release of the siRNA from the surface of the liposomes in different biological fluids [18-20].

Therefore, when tailoring LNPs for *in vivo* siRNA delivery, a delicate balance between avoiding (i) aggregation, (ii) premature release, (iii) immune responses and realizing efficient intracellular release of siRNA in the cytoplasm of cells should be installed to overcome PEG-associated problems. In this respect, an interesting approach could be the use of 'exchangeable' PEG-derivatized lipids, also called "sheddable PEG", which diffuse out of LPXs upon contact with biological membranes, depending on the length of the acyl) chain of the lipid anchor.

Among the most used sheddable PEG-lipids are ceramides (Cer-PEG) and diacylglycerols (PEG-S-DAGs) [21, 22].

Ovarian cancer leads to more than 140,000 deaths annually in women around the world [23]. In the majority of the cases, ovarian cancer often migrates to the peritoneal cavity forming fatal peritoneal carcinomatosis. Interestingly, intraperitoneal (i.p.) administration of different drugs has recently shown some advantages to treat peritoneal tumors when compared to intravenous administration, mainly due to high concentrations of drug at the tumor site following i.p. administration [24-26]. Also, the delivery of siRNA to ovarian cancer cells using different nano- sized carriers is recently gaining increasing attention in cancer therapy [27-30]. It has been reported that the co-delivery of chemotherapeutic agents with siRNA is an efficient strategy to enhance tumor killing effect and overcome resistance of cancer cells when compared to delivery systems carrying either siRNA or chemotherapeutics alone [31-33]. First of all, siRNAs can specifically silence cancer associated genes, causing less side effects in non-cancer cells. Moreover, siRNA is not limited to target receptors that are expressed on the surface of cancer cells, but can also silence genes associated with intracellular targets [34].

In this study, we investigate the suitability of different liposomes (cationic, PEGylated and grafted with diffusible Cer-PEG) composed of DOTAP and DOPE (as lipids) encapsulating siRNA within the aqueous core (prepared by the hydration method) [18, 35] to knockdown luciferase in the human ovarian cancer cell line SKOV-3. Initially we employ advanced fluorescence microscopy techniques, such as fluorescence correlation spectroscopy (FCS) and fluorescence single particle tracking (fSPT) [36, 37] to follow the release and aggregation of the LPXs in ascites fluid obtained from a peritoneal carcinomatosis patient. Additionally, we test the uptake, toxicity and silencing efficiency of all the formulations. We hypothesized that

liposomes encapsulating siRNA and decorated with diffusible Cer-PEG may be an attractive strategy to target tumors confined within the peritoneal cavity following i.p. adminstration, as they represent a fine balance between PEGylation and de-PEGylation [38].

Materials and Methods

Materials

(2,3-Dioleoyloxy-propyl)-trimethylammonium-chloride (DOTAP) and 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) were purchased from Corden Pharma LLC (Liestal, Switzerland).

1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol)-2000] (DSPE-PEG), N-palmitoyl-sphingosine-1-{succinyl[methoxy(polyethylene-glycol)2000]} (C16 mPEG 2000 Ceramide)**,** N-octanoyl-sphingosine-1-{succinyl[methoxy(polyethylene glycol)2000]} (C8 mPEG 2000 Ceramide) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Chloroform, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and sodium chloride (NaCl) were purchased from Sigma Aldrich (Bornem, Belgium).

Penicillin-Streptomycin (5000 U/ml), L-Glutamine (200 mM), 0.25% Trypsin-EDTA (1X) Phenol Red, McCoy's 5A (Modified), Opti-MEM® and 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine perchlorate (DID) (λex= 644 nm, λem= 665 nm) were purchased from Invitrogen (Merelbeke, Belgium). Luciferase Assay Substrate was purchased Promega (Madison, WI, USA). Fetal Bovine Serum (FBS) was purchased from HyClone® Thermo Scientific (Cramlington, UK). Passive Lysis Buffer and Luciferase Assay Kit were purchased from (Promega, Leiden, Netherlands).

Preparation and characterization of the HYDRA LPXs

Liposomes corresponding to 5 mM of DOTAP and 5 mM of DOPE lipids were prepared by mixing the appropriate amount of each lipid in a round bottomed flask before evaporation. PEGylated liposomes were prepared by adding the desired amounts of DSPE-PEG, or C8 Cer-PEG or C16 Cer-PEG dissolved in chloroform (corresponding to 5 mol% the total lipids) to

the lipids before evaporation. A lipid film was formed by rotary evaporation of the chloroform at 40°C.

To obtain the so named 'HYDRA lipoplexes', the dried lipid film was hydrated with a siRNA solution in HEPES buffer (20 mM, pH 7.4) resulting in LPXs with a charge ratio of ± 8. Finally, the obtained solution was sonicated using a probe sonicator (Branson Ultrasonics Digital Sonifier®,Danbury,USA).We showed previously that this method results in the encapsulation of 50% of the complexed siRNA inside the liposomes and 50% bound to the outer surface of the liposomes [35]. The average size and zeta potential of all formulations were measured using Zetasizer Nano-ZS (Malvern, Worcestershire, UK).

Fluorescence single particle tracking (fSPT)

Fluorescence Single particle tracking (fSPT) is a fluorescence microscopy technique which is used to characterize the diffusion of nanoparticles. Briefly, fSPT makes use a fast CCD camera and wide-field laser illumination to obtain movies of single, fluorescently labeled particles in biological media. The movies are then analyzed by using in-house image processing software [36], where the motion trajectories and subsequently the diffusion coefficient of each individual particle is calculated. Based on the trajectories of all the particles, it is possible to make a distribution of diffusion coefficients, which is then converted into size distribution using the Stokes-Einstein equation given the viscosity of the biofluid at which the experiment was performed is known. Finally, the distribution is refined by the maximal entropy method [36]. We showed previously that fSPT is ideally suited to characterize the size (and thus the extent of aggregation) of nanoparticles in biological fluids like human serum, ascites fluid, human plasma, and blood [3, 20, 36]. The main advantage of fSPT over the widely used sizing techniques such as Dynamic Light Scattering (DLS), is the ability to perform sizing measurements in undiluted biological fluids, without the influence of the proteins present in these fluids [3].

fSPT measurements were performed on the HYDRA-LPXs (Cationic, 5% DSPE-PEG, 5% C8 Cer-PEG, 5% C16 Cer-PEG) labeled with the lipophilic dye DID, that labels the lipid bilayer of the liposomes). LPXs were dispersed in biofluids as follows. First, formulations were diluted 400 times in HEPES buffer. Then 5 µl was added to 45 µl of biofluid (~90 vol% of ascites fluid), and incubated for 1, 2 and 3 h at 37°C in a 96-well plate (Greiner bio-one, Frickenhausen, Germany). fSPT-videos of the different formulations in the biofluids were recorded with the NIS Elements software (Nikon) driving the EMCCD camera (Cascade II:512, Roper Scientific, AZ, USA) and a TE2000 inverted microscope equipped with a 100_ NA1.4 oil immersion lens (Nikon) as previously described [3, 20] using the following values of viscosity at room temperature: 1.39 cP for human ascites fluid and 0.94 for HEPES buffer [3]. Human ascites fluid was obtained from a patient diagnosed with peritoneal carcinomatosis at the Medical Oncology Department of Ghent University Hospital (approved by the Ethics Committee of the Ghent University Hospital (no. 2013/589)).

Videos were recorded at room temperature (22.5 °C) with the NIS Elements software (Nikon) driving the EMCCD camera (Cascade II: 512, Roper Scientific, AZ, USA) and a TE2000 inverted microscope equipped with a 100_ NA1.4 oil immersion lens (Nikon).

Fluorescence Correlation Spectroscopy on HYDRA LPXs

FCS is a microscopy-based technique that monitors the fluorescence intensity fluctuations of molecules diffusing in and out of the focal volume of a confocal microscope [3]. When free fluorescently labeled siRNA molecules pass through the focal volume, a fluorescence baseline with an intensity that corresponds to the concentration of the free labeled siRNA molecules is obtained. When the siRNA is complexed/encapsulated within a carrier, the concentration of free siRNA drops and subsequently, a drop in the baseline intensity of the fluorescence signal occurs, accompanied with fluorescence fluctuations (i.e. peaks) each time a complex passes the focal volume. Release of siRNA, on its turn, results in an increase in the fluorescence baseline.

An important advantage of FCS is the low volume of samples needed to perform the experiments (~50 µl). Our group showed before that FCS is ideally suited to measure the amount of siRNA that is released/associated from/with nano-sized carriers in various types of biofluids [3,4,15]. FCS measurements were carried-out on HYDRA-LPXs containing 30% Cy-5 siRNA and 70% non-labeled siRNA, with a charge ratio of ± 8. 5 µl of LPXs were diluted to a final volume of 50 µl in respectively HEPES buffer or ascites fluid (~ 90 vol%) and FCS measurements were done (i) immediately after diluting the LPXs (in HEPES or ascites fluid), (ii) 1 h and (iii) 24 h after incubation with the biofluids at 37°C. FCS measurements were performed on a C1si laser scanning confocal microscope (Nikon, Japan), equipped with a time-correlated single photon counting (TCSPC) data acquisition module (Picoquant, Berlin, Germany). The laser beam was held stationary and focused through a water immersion objective lens (Plan Apo 60, NA 1.2, collar rim correction, Nikon, Japan) at ~ 50 µm above the bottom of the glass-bottom 96-well plate (Grainer Bioone, Frickenhausen, Germany), which contained the fluorescent LPXs. The 633 nm laser beam was used to record fluorescence intensity fluctuations using SymPhoTime (Picoquant, Berlin, Germany).

Cell culture

The human ovarian cancer cell line SKOV-3 which stably expresses firefly luciferase was used for *in vitro* experiments. Cells were cultured in McCoy's 5A medium supplemented with FBS, Penicillin-Streptomycin and L-Glutamine. Cells were cultured until 80% to 90% confluency and detached from tissue culture dishes with 0.25% trypsin. Cells were maintained in an incubator at 37°C in a humidified atmosphere with 5% CO_2.

Cell viability assay

The MTT assay was used as a measure of cell viability following incubation of the studied formulations with a final negative control siRNA concentration of 10, 15 and 20 nM. SKOV-3

cells were cultured on 24-well tissue culture plates (35,000 cells per well). On the next day, cells were incubated for 4 h with 500 µl Opti-MEM® containing the LPXs of interest or 30% of ethanol as positive control. Then, cells were washed and incubated with McCoy's 5A medium for an additional 24 h. Thereafter, 100 µl of MTT stock solution (5 mg/ml MTT in PBS) in McCoy's 5A was added to each well and incubated for 3 h. After aspirating the medium, 500 µl of DMSO was added to each well in order to dissolve the formazan crystals. The plates were covered with aluminum foil, placed on an orbital shaker for 10 min and the absorbance of the formed formazan crystals was determined at 590 nm with reference at 690 nm using a Wallac Envision™ multilabel reader (PerkinElmer, Zaventem, Belgium). Percentages of cell viability for each sample were calculated as follows: (absorbance of the sample/absorbance of the negative control) x 100%.

Internalization of siRNA into SKOV-3 cells

SKOV-3 cells were plated on 24-well plates (35,000 cells in each well) and allowed to grow in an incubator for 24 h. Cells were incubated with the formulations containing 10% Alexa Fluor-488 siRNA at a final siRNA concentration of 15 nM for 4 h at 37°C. At the end of the incubation, cells were washed extensively with warm growth medium and PBS, then detached using trypsin and analyzed by FACS (FACSCalibur Flow Cytometer, BD Biosciences, USA). Uptake experiments were done with LPXs administered in Opti-MEM® or with LPXs which were first incubated for 1 h in human ascites fluid, as previously described [20]. Uptake experiments in the ascites fluid were performed by incubating 300 µl of each of the studied formulations with 700 µl of ascites fluid for 1 h at 37°C. Then, 300 µl of the mixture were added in triplicates to 700 µl of Opti-MEM® in each well of a 24 well-plate. After 4 h of incubation at 37°C, cells were washed, detached and analyzed as described above.

Transfection efficiency

SKOV-3 cells were cultured on 24-well plates (35,000 cells/well) in 500 µl of medium containing 10% FBS 24 h prior to the transfection. Cells were incubated with HYDRA-LPXs at a final siRNA concentration of 15 nM respectively in Opti-MEM® or following 1 h of pre-incubation in ascites fluid as previously explained [20]. After 4 h incubation, the transfection medium was replaced by culture medium and cells were returned to the incubator for 24 h. Then, cells were lysed with Passive Lysis Buffer and analyzed for firefly luciferase expression using the luciferase assay kit (Promega). The bioluminescence (Relative Light Units, RLU) was measured using a GloMax Luminometer (Promega). The percentage of luciferase downregulation was determined by the following equation:

% transfection = 100 − (100 x RLU_{luc}/RLU_{ctrl}), where RLU_{ctrl} is the mean for control siRNA and RLU_{luc} is the mean for luciferase siRNA. Transfection experiments were performed in triplicates on three different days. For transfections in the ascites fluid, 300 µl of each HYDRA-LPX (of each formulation) were incubated with 700 µl ascites fluid and incubated for 1 h at 37°C. Thereafter, 300 µl of the mixture was added to 700 µl of Opti-MEM® in each well of a 24 well-plate. Then, the medium was replaced with growth medium and cells were returned to the incubator for 24 h, as described above.

Statistical analysis

Statistical analysis on the transfection data (Figure 5) was performed using GraphPad Prism 6. Data are presented as means ± SD. Statistically significant differences were calculated by using an analysis of variance (ANOVA) at a 0.05 significance level, followed by Sidak's post-test. For each formulation, transfection experiments carried out in Opti-MEM® were compared to these in ascites fluid.

Results

Characterization of the studied LPXs

Table 1 shows the size and zeta potential of the different used formulations. In the hydration method, formulations were prepared by hydrating the lipid film with siRNA directly. This results in liposomes in which the siRNA is also encapsulated inside the aqueous core of the resulting LPXs, as well as being complexed to the outer surface of the liposomal formulations. As depicted in **table 1**, all the studied formulations resulted in nano-sized vesicles, as determined by dynamic light scattering (DLS). When compared to empty liposomes, the size increased for all formulations when LPXs were formed, except for the 5% C16 Cer HYDRA-LPXs.

As expected, both PEGylation and the addition of siRNA influenced the charge of the liposomes. Introducing PEG chains on the surface of the liposomes results in shielding of the positive charge from about 56 mV for the cationic liposomes to about 17-20 mV for the PEGylated ones. Encapsulation of siRNA resulted in a further decrease of the charge, except for the 5% C16 Cer HYDRA-LPXs.

Aggregation of the formulations in undiluted ascites fluid

In our previous study we have shown that DLS is not an ideal technique for characterizing aggregation of nanoparticles in biological fluids, simply due to scattering that results from e.g. the proteins in the biofluids [3]. fSPT has proven to be superior over DLS for this purpose [3, 36]. Figure 1a shows the size distributions of the HYDRA-LPXs as measured by fSPT. In agreement with the DLS outcomes in Table 1, HYDRA-LPXs have an average size around 100 nm when measured in HEPES buffer (Figure 1a, black curve). Following 1 h of incubation in ascites fluid, the size distribution is shifted to the right (red curve), with a peak diameter around 200 nm. Upon longer times in ascites fluids, aggregation of the HYDRA-LPXs

seemed somehow to continue, resulting in HYDRA-LPXs with a peak diameter of 300 nm (Figure 1a, blue curve). After 3 h of incubation, the size distributions did not further change, indicating the aggregation reached an equilibrium already after 2 h (Figure 1a, green curve). Next, we were interested if PEGylation would further inhibits the aggregation of HYDRA-LPXs. Two types of PEGylation were tested, namely the incorporation of respectively stable, non-exchangeable DSPE-PEG chains, and exchangeable PEG-Ceramides. In the case of the DSPE-PEG HYDRA-LPXs (Figure 1b), initially a minor aggregation was observed following incubation in ascites fluid when compared to the distribution in HEPES buffer (~ 150 nm). Then, the PEGylated complexes remained stable, resulting in particles of about 250 nm in diameter. For the 5% C8 Cer HYDRA-LPXs, the size in HEPES buffer was around 100 nm (Figure 1c) and increased to 200 nm after 1 h of incubation in ascites fluid. Surprisingly, further incubation in ascites fluid for 2 and 3 h resulted again in smaller 5% C8 Cer HYDRA-LPXs of 150 nm and less. The SPT data in Figure 1D for the C16 Cer HYDRA-LPXs exhibit a similar behavior to the C8 Cer HYDRA-LPXs, with an equilibrium reached after 1 h of incubation in ascites fluid, and a peak diameter of about ~250 nm. Taken together, all the formulations seemed sufficiently stable in the ascites fluid: no micrometer-sized aggregates were observed and the size of all LPXs remained sufficiently small to allow endocytosis by cells.

Release of siRNA from the formulations in undiluted ascites fluid

As demonstrated previously, FCS is a suitable method to follow the release of siRNA from different types of formulations in undiluted biological fluids [3, 19, 20]. Figure 2 displays the complexation efficiency of the studied formulations. HYDRA-LPXs show a high complexation efficiency with about 80% of the siRNA complexed with the liposomes immediately after preparation in HEPES buffer (Figure 2, white bars) and during 24 h (grey and green bars). Following 1 h of incubation (Figure 2, blue bars), a burst release was observed

leading to about 40% complexed siRNA (60% of free siRNA). This release is highly likely ascribed to the siRNA which is bound on the surface of the liposomes and not actually encapsulated inside. No substantial further release was noted after 24 h of incubation in ascites fluid, demonstrating that eventually about 35% of the siRNA was encapsulated within the aqueous core of the liposomes (magenta bars). PEGylation clearly influenced the complexation efficiency and release profile of the studied formulations. For DSPE HYDRA-LPXs, only 50% of the siRNA is complexed immediately after preparation, suggesting that PEGylation lowers the siRNA complexation efficiency (white bars). This 50% of siRNA, however, can be retained in the DSPE HYDRA-LPXs for the complete 24 h incubation period and is therefore most likely encapsulated inside the liposomes (Figure 2, magenta bars). For the Cer HYDRA-LPXs, the effect of PEGylation was even more pronounced, with 75% and 85% of free siRNA (e.g. uncomplexed) as such in HEPES buffer for the 5% C8 Cer and 5% C16 Cer HYDRA-LPXs respectively. Following 24 h of incubation in ascites fluid, both Cer HYDRA-LPXs resulted in around 90% of free siRNA (Figure 2, magenta bars).

Cytotoxicity of the studied formulations

The toxicity of the formulations was assessed on SKOV-3 human ovarian cancer cells using a MTT assay. As can be seen in Figure 3, the formulations did not exhibit any severe decrease in the metabolic activity of the cells, with maximum 20% mortality for the highest siRNA concentrations of 20 nM. A decrease in the cell viability was observed with increasing siRNA concentrations. To stay within the non-toxic range of concentrations, we decided to perform uptake and transfection experiments with a final concentration of 15 nM.

Cellular Uptake and transfection efficiency of the formulations by SKOV-3 cells

To evaluate the ability of the different formulations to knockdown the expression of a specific gene, SKOV-3 cells stably expressing luciferase were incubated with the formulations containing siRNA against luciferase (luc siRNA). To verify the knockdown specificity, luc siRNA formulations were compared to the same formulations loaded with a scrambled negative control siRNA.

We have recently proven the importance of performing uptake experiments of formulations carrying siRNA in the relevant biofluid [20]. Uptake is a key feature in siRNA delivery, since naked siRNA cannot internalize into cells due to its negative charge and hydrophilicity. To test whether the formulations are capable of delivering siRNA into cells, we incubated SKOV-3 cells with fluorescently labeled LPXs and followed their uptake with flow cytometry. The outcomes of the uptake experiments shown in Figure 4 indicate that all the formulations are internalized into SKOV-3 cells at 37°C when incubated in serum reduced media (i.e. Opti-MEM$^{®}$), represented by high percentage of positive cells (Figure 4, white bars). Following 1 h of incubation in ascites fluid (Figure 4, grey bars), however, all the formulations completely lose their ability to be taken up by cells, except for the DSPE-HYDRA LPXs where cellular uptake still occurs.

Figure 5 depicts the transfection efficiency of the studied formulations in Opti-MEM$^{®}$ (white bars) and following 1 h of incubation in ascites fluid (grey bars). In Opti-MEM$^{®}$, it can be seen that only DSPE HYDRA-LPXs resulted in a very poor transfection efficiency (37%). All other formulations demonstrated high and significant downregulation of 70% for the HYDRA-LPXs, 73% for the 5% C8 Cer HYDRA-LPXs and 80% for the 5% C16 Cer HYDRA-LPXs. Following incubation in ascites fluid, however, all the formulations lost their ability to silence luciferase, when compared with the situation in Opti-MEM$^{®}$.

Discussion

Choice of the study

The final goal of this study is to assess the suitability of liposomal-based formulations loaded with siRNA via the hydration method and possessing different surface modifications in undiluted ascites fluid obtained from a peritoneal carcinomatosis patient for siRNA delivery. As a follow up of our previous study [3] and towards enhancing the stability of LPXs and maximizing their biological activity, we focused on two main aspects: 1) the siRNA loading method and 2) the PEGylation strategy. On the level of loading, we used the hydration method [18, 35], while on the level of PEGylation we decided to evaluate the stable DSPE-PEG, diffusible C8 and C16 Cer-PEG. The different PEG-chains were chosen to understand whether Cer-PEGylation can still play a role in preventing aggregation in the extracellular IP fluid, while the diffusion of the PEG-chains out of the liposomal formulations upon interaction with biological membranes would enhance the cellular uptake and endosomal escape on the intracellular level. In this context, Cer-PEG with shorter C8 acyl chain lipids (C8) seems to be best candidates for local intraperitoneal delivery, due to the rapid exchange rate of the Cer-PEG from the surface of the liposomes, compared to the longer acyl chains (C20) [39]. Additionally, C8 Cer-PEG were successfully exploited to deliver plasmid DNA for regional gene therapy [40].

Influence of PEGylation on encapsulation efficiency

It is of interest to point out the differences between the hydration technique used in this study and the widely used complexation technique. With the conventional complexation technique, liposomes are prepared by hydrating the lipid film with a buffer solution. Then, LPXs are prepared by the simple mixing of siRNA and the preformed liposomes, taking

advantage of the electrostatic interactions between the negatively charged siRNA and the cationic liposomes. In this case the siRNA binds on the surface of the liposomes, and is not encapsulated into the aqueous core. We have previously demonstrated, however, that the surface bound siRNA in this classic complexation protocol is easily displaced when the liposomes are dispersed in biofluids such as human serum or ascites fluid, especially when PEGylated liposomes are used [3].

We hypothesized that when using the hydration protocol [35], which ensures an even distribution of the negatively charged siRNA over the inner and outer core of a cationic lipid, the premature release of siRNA in the ascites fluid could be overcome [35]. Indeed, only the surface bound siRNA is expected to be released from the LPXs in the biofluids, while the siRNA encapsulated in the aqueous core is protected from premature release. Figure 2 demonstrated that this hypothesis was valid to some extent. We have previously shown that 5% DSPE-PEG LPXs prepared with the classic complexation method, release the majority of the complexed siRNA immediately after incubation with ascites fluid [3]. For DSPE HYDRA-LPXs, however, 50% of the siRNA is still encapsulated within the liposomes following 24 h of incubation in ascites fluid (Figure 2). This agrees with our assumption that with the hydration method, the siRNA within the aqueous core of the liposomes is protected from the external environment, and thus the negatively charged proteins (mainly albumin) which compete for binding to the liposomes. Also the non-PEGylated HYDRA-LPXs retain about 35-40% of the complexed siRNA within the aqueous core after 24 h of incubation in ascites fluid, following the initial burst release of the siRNA which is complexed on the surface of the liposomes and exposed to the proteins in the ascites fluid. Unfortunately, the high stability of the HYDRA-LPXs and DSPE HYDRA-LPXs was not observed for the formulations containing C8 and C16 Cer-PEG chains. Both formulations resulted in the formation of complexes with a low complexation efficiency of siRNA. Also, the majority of siRNA that was complexed, is released

almost immediately following incubation in ascites fluid. This rapid and premature release most likely stems from a re-organization on the level of the lipid bilayer upon introducing the C8 and C16 Cer-PEG chains within the lipid bilayer, resulting in a smaller aqueous core and consequently lower complexation efficiency where most of the siRNA is free or attached to the surface of the complexes.

Influence of stability on biological activity

While extracellular stability is one of the major concerns when designing siRNA delivery systems, the internalization and intracellular interactions with the different organelles are key factors to ensure maximal biological activity. The size and the charge of the studied formulations (Table 1) suggest that these complexes can cross biological membranes and deliver the siRNA into cells (Figure 4). Also, all PEGylated formulations used in this study succeeded in preventing the aggregation of the liposomal formulations for at least 3 hours when incubated in ascites fluid (Figure 1). Therefore, each PEGylation strategy seems suitable to stabilize the formulations and to assure the presence of nano-sized formulations for a sufficiently long time to warrant cellular uptake. Following internalization, endosomal escape is essential to ensure delivery of siRNA in the cytoplasm and to induce efficient silencing of the target gene [4]. For the HYDRA-LPXs and Cer HYDRA-LPXs, substantial luciferase inhibition was observed when the incubation took place in Opti-MEM® (Figure 5, white bars), except for the DSPE HYDRA-LPXs formulation. In the case of the HYDRA-LPXs, the cationic charge facilitates the interaction and destabilization of the endosomes, leading to fusion and eventually release of the siRNA into the cytosol. The fusion is further enhanced when a fusogenic "helper lipid" is incorporated within the liposomal formulation, such as DOPE in this case. It has been shown that upon acidification of the endosomal compartment DOPE triggers a conformational change from the stable lamellar phase into the highly unstable inverted

hexagonal phase, which is thought to destabilize the endosomal membrane and release the cargo into the cytosol [41] . This conformational transition is not possible in the DSPE HYDRA-LPXs [42]. This explains the relatively low transfection efficiency of this formulation when compared with other formulations (Figure 5).

When PEGylation is performed with sheddable Cer-PEG instead of stable DSPE-PEG chains, it can be seen that both Cer-PEG HYDRA-LPXs result in high transfection efficiency. The transfection efficiency of the Cer-PEG formulations is highly likely ascribed to the "de-PEGylation" that occurs upon interaction of the Cer-PEG formulations with the cell membrane [42], and consequently, similar interaction with the endosomes as the one described above for the HYDRA-LPXs is expected.

Since the final goal of this study is to determine the biological activity of the studied formulations in a relevant undiluted biofluid, we tested the transfection efficiency of all the formulations following incubation in ascites fluid obtained from a peritoneal carcinomatosis patient. (Figure 5, grey bars). We are convinced that undiluted ascites fluids better resembles the *in vivo* peritoneal situation, not only in terms of stability, but also biological activity [20]. The drop in the transfection efficiency observed upon incubation in ascites fluid can be discussed on two levels: 1) cellular uptake of the studied formulations and 2) intracellular trafficking of the DSPE HYDRA-LPXs specifically following internalization. In particular, the HYDRA-LPXs and Cer HYDRA-LPXs are not internalized into SKOV-3 cells following 1 h of incubation in ascites fluid (Figure 4). Consequently, the cargo (i.e. siRNA) is not delivered into the cytoplasm of the cell where it should be further processed by the RNAi machinery, leading eventually to the absence of biological activity depicted in Figure 5.

Based on the stability data in Figure 1 and Figure 2, DSPE HYDRA-LPXs are protected from aggregation in the presence of ascites fluid and are able to keep the siRNA encapsulated for a sufficient long time to allow internalization into the cells. Therefore, the poor transfection

efficiency observed for the DSPE HYDRA-LPXs is mainly due to the poor interaction with the endosomes, as also observed for the transfections in Opti-MEM®. The poor transfection efficiency of the DSPE-PEG formulation stands in line with recently published data, where DOTAP/cholesterol pDNA LPXs with 2% DSPE-PEG diminished the transfection compared to the non-PEGylated LPXs *in vitro* [43]. Additionally, DOTAP DOPE LPXs loaded with survivin siRNA and grafted with 1% DSPE-PEG successfully downregulated the activity of survivin in cultured cells, but lost its activity in peritoneal tumors *in vivo* [33].

Taken together, the findings of this study provide an evidence of the sensitive interplay that exists between extracellular stability, cellular uptake and biological activity when developing nano-sized siRNA formulations in protein-rich biological fluids. An optimal formulation designated for *in vivo* applications should fulfill all the requirements of colloidal stability, interaction with biological barriers, and intracellular trafficking in the relevant biofluid. One should keep in mind that formulations that show good stability and transfection characteristics in protein-free conditions such as Opti-MEM®, do not necessarily translate in potent formulations when transfections are being performed in the relevant biological fluids. For the formulations tested in this study, cellular uptake was the bottleneck for obtaining efficient gene knockdown, whereas poor intracellular processing was most probably the reason for the low gene knockdown observed with the only formulation taken up by the cells following incubation in undiluted ascites fluid (i.e. DSPE HYDRA-LPX).The cellular uptake in this case was not a problem, since this formulation exhibited low transfection efficiency (Figure 5) even in protein-free conditions (i.e. Opti-MEM®), where efficient internalization occurred.

Conclusions

In this study we tested the stability of different liposomal formulations for i.p. siRNA delivery based on the hydration technique and different types of PEGylation in human undiluted ascites

fluid, as well as, the transfection efficiency in the SKOV-3 ovarian cancer cell line. We hypothesized that liposomes encapsulating siRNA and grafted with Cer-PEG chains would be an ideal platform for i.p. siRNA delivery. Nonetheless, our findings revealed that these carriers are not taken up by SKOV-3 cells in protein-rich conditions (i.e. ascites fluid). On the contrary, liposomes encapsulating siRNA and coated with stable DSPE-PEG are endocytosed by SKOV-3 cells in protein-rich conditions, but are associated with poor biological activity highly likely due to insufficient intracellular processing.

Conflict of interest – the authors declare no conflict of interest.

References

[1] M.R. Lares, J.J. Rossi, D.L. Ouellet, RNAi and small interfering RNAs in human disease therapeutic applications, Trends Biotechnol, 28 (2010) 570-579.

[2] A. Wittrup, J. Lieberman, Knocking down disease: a progress report on siRNA therapeutics, Nat Rev Genet, 16 (2015) 543-552.

[3] G.R. Dakwar, E. Zagato, J. Delanghe, S. Hobel, A. Aigner, H. Denys, K. Braeckmans, W. Ceelen, S.C. De Smedt, K. Remaut, Colloidal stability of nano-sized particles in the peritoneal fluid: towards optimizing drug delivery systems for intraperitoneal therapy, Acta Biomater, 10 (2014) 2965-2975.

[4] T.F. Martens, K. Remaut, J. Demeester, S.C. De Smedt, K. Braeckmans, Intracellular delivery of nanomaterials: How to catch endosomal escape in the act, Nano Today, 9 (2014) 344-364.

[5] R. Kanasty, J.R. Dorkin, A. Vegas, D. Anderson, Delivery materials for siRNA therapeutics, Nature Materials, 12 (2013) 967-977.

[6] C.K. Chen, C.H. Jones, P. Mistriotis, Y. Yu, X.N. Ma, A. Ravikrishnan, M. Jiang, S.T. Andreadis, B.A. Pfeifer, C. Cheng, Poly(ethylene glycol)-block-cationic polylactide nanocomplexes of differing charge density for gene delivery, Biomaterials, 34 (2013) 9688-9699.

[7] D.C. Forbes, N.A. Peppas, Polycationic Nanoparticles for siRNA Delivery: Comparing ARGET ATRP and UV-Initiated Formulations, Acs Nano, 8 (2014) 2908-2917.

[8] C.H. Jones, C.K. Chen, A. Ravikrishnan, S. Rane, B.A. Pfeifer, Overcoming Nonviral Gene Delivery Barriers: Perspective and Future, Mol Pharmaceut, 10 (2013) 4082-4098.

[9] A. Elouahabi, J.M. Ruysschaert, Formation and intracellular trafficking of lipoplexes and polyplexes, Mol Ther, 11 (2005) 336-347.

[10] S.K. Samal, M. Dash, S. Van Vlierberghe, D.L. Kaplan, E. Chiellini, C. van Blitterswijk, L. Moroni, P. Dubruel, Cationic polymers and their therapeutic potential, Chem Soc Rev, 41 (2012) 7147-7194.

[11] C. Wan, T.M. Allen, P.R. Cullis, Lipid nanoparticle delivery systems for siRNA-based therapeutics, Drug Delivery and Translational Research, 4 (2014) 74-83.

[12] L.C. Gomes-da-Silva, N.A. Fonseca, V. Moura, M.C.P. de Lima, S. Simoes, J.N. Moreira, Lipid-Based Nanoparticles for siRNA Delivery in Cancer Therapy: Paradigms and Challenges, Accounts of Chemical Research, 45 (2012) 1163-1171.

[13] S. Mishra, P. Webster, M.E. Davis, PEGylation significantly affects cellular uptake and intracellular trafficking of non-viral gene delivery particles, European Journal of Cell Biology, 83 (2004) 97-111.

[14] A. Santel, M. Aleku, O. Keil, J. Endruschat, V. Esche, G. Fisch, S. Dames, K. Loffler, M. Fechtner, W. Arnold, K. Giese, A. Klippel, J. Kaufmann, A novel siRNA-lipoplex technology for RNA interference in the mouse vascular endothelium, Gene Therapy, 13 (2006) 1222-1234.

[15] A. Judge, K. McClintock, J.R. Phelps, I. MacLachlan, Hypersensitivity and loss of disease site targeting caused by antibody responses to PEGylated liposomes, Mol Ther, 13 (2006) 328-337.

[16] T. Tagami, K. Nakamura, T. Shimizu, T. Ishida, H. Kiwada, Effect of siRNA in PEG-coated siRNA-lipoplex on anti-PEG IgM production, Journal of Controlled Release, 137 (2009) 234-240.

[17] T. Tagami, K. Nakamura, T. Shimizu, T. Ishida, H. Kiwada, Effect of siRNA in PEG-Coated siRNA-Lipoplex on the Anti-PEG IgM Production as Induced by the PEG-Coated siRNA-Lipoplex, Mol Ther, 17 (2009) S253-S253.

[18] K. Buyens, S.C. De Smedt, K. Braeckmans, J. Demeester, L. Peeters, L.A. van Grunsven, X.D. du Jeu, R. Sawant, V. Torchilin, K. Farkasova, M. Ogris, N.N. Sanders, Liposome based systems for systemic siRNA delivery: Stability in blood sets the requirements for optimal carrier design, Journal of Controlled Release, 158 (2012) 362-370.

[19] K. Buyens, B. Lucas, K. Raemdonck, K. Braeckmans, J. Vercammen, J. Hendrix, Y. Engelborghs, S.C. De Smedt, N.N. Sanders, A fast and sensitive method for measuring the integrity of siRNA-carrier complexes in full human serum, Journal of Controlled Release, 126 (2008) 67-76.

[20] G.R. Dakwar, K. Braeckmans, J. Demeester, W. Ceelen, S.C. De Smedt, K. Remaut, Disregarded Effect of Biological Fluids in siRNA Delivery: Human Ascites Fluid Severely Restricts Cellular Uptake of Nanoparticles, ACS Applied Materials & Interfaces, 7 (2015) 24322-24329.

[21] E. Ambegia, S. Ansell, P. Cullis, J. Heyes, L. Palmer, I. MacLachlan, Stabilized plasmid-lipid particles containing PEG-diacylglycerols exhibit extended circulation lifetimes and tumor selective gene expression, Biochimica Et Biophysica Acta-Biomembranes, 1669 (2005) 155-163.

[22] M.S. Webb, D. Saxon, F.M.P. Wong, H.J. Lim, Z. Wang, M.B. Bally, L.S.L. Choi, P.R. Cullis, L.D. Mayer, Comparison of different hydrophobic anchors conjugated to poly(ethylene glycol): Effects on the pharmacokinetics of liposomal vincristine, Biochimica Et Biophysica Acta-Biomembranes, 1372 (1998) 272-282.

[23] J. Hunn, G.C. Rodriguez, Ovarian Cancer: Etiology, Risk Factors, and Epidemiology, Clinical Obstetrics and Gynecology, 55 (2012) 3-23.

[24] W.P. Ceelen, M.F. Flessner, Intraperitoneal therapy for peritoneal tumors: biophysics and clinical evidence, Nature Reviews Clinical Oncology, 7 (2010) 108-115.

[25] P. Zahedi, J. Stewart, R. De Souza, M. Piquette-Miller, C. Allen, An injectable depot system for sustained intraperitoneal chemotherapy of ovarian cancer results in favorable drug distribution at the whole body, peritoneal and intratumoral levels, Journal of Controlled Release, 158 (2012) 379-385.

[26] G.R. Dakwar, M. Shariati, W. Willaert, W. Ceelen, S.C. De Smedt, K. Remaut, Nanomedicine-based intraperitoneal therapy for the treatment of peritoneal carcinomatosis - Mission possible?, Adv Drug Deliv Rev, (2016).

[27] R.D. George, S.C.D.S. Stefaan, R. Katrien, Intraperitoneal nonviral nucleic acid delivery in the treatment of peritoneal   cancer, Intraperitoneal Cancer Therapy, CRC Press2015, pp. 359-371.

[28] M.S. Goldberg, siRNA Delivery for the treatment of ovarian cancer, Methods, 63 (2013) 95-100.

[29] H.J. Kim, A. Kim, K. Miyata, K. Kataoka, Recent progress in development of siRNA delivery vehicles for cancer therapy, Adv Drug Deliv Rev, 104 (2016) 61-77.

[30] S.J. Lee, M.J. Kim, I.C. Kwon, T.M. Roberts, Delivery strategies and potential targets for siRNA in major cancer types, Adv Drug Deliv Rev, 104 (2016) 2-15.

[31] M. Creixell, N.A. Peppas, Co-delivery of siRNA and therapeutic agents using nanocarriers to overcome cancer resistance, Nano Today, 7 (2012) 367-379.

[32] M. Saraswathy, S.Q. Gong, Recent developments in the co-delivery of siRNA and small molecule anticancer drugs for cancer treatment, Mater Today, 17 (2014) 298-306.

[33] J. Wang, Z. Lu, B.Z. Yeung, M.G. Wientjes, D.J. Cole, J.L.S. Au, Tumor priming enhances siRNA delivery and transfection in intraperitoneal tumors, Journal of Controlled Release, 178 (2014) 79-85.

[34] D. Bumcrot, M. Manoharan, V. Koteliansky, D.W.Y. Sah, RNAi therapeutics: a potential new class of pharmaceutical drugs, Nature Chemical Biology, 2 (2006) 711-719.

[35] K. Buyens, J. Demeester, S.C. De Smedt, N.N. Sanders, Elucidating the Encapsulation of Short Interfering RNA in PEGylated Cationic Liposomes, Langmuir, 25 (2009) 4886-4891.

[36] K. Braeckmans, K. Buyens, W. Bouquet, C. Vervaet, P. Joye, F. De Vos, L. Plawinski, L. Doeuvre, E. Angles-Cano, N.N. Sanders, J. Demeester, S.C. De Smedt, Sizing Nanomatter in Biological Fluids by Fluorescence Single Particle Tracking, Nano Letters, 10 (2010) 4435-4442.

[37] K. Braeckmans, K. Buyens, B. Naeye, D. Vercauteren, H. Deschout, K. Raemdonck, K. Remaut, N.N. Sanders, J. Demeester, S.C. De Smedt, Advanced fluorescence microscopy methods illuminate the transfection pathway of nucleic acid nanoparticles, Journal of Controlled Release, 148 (2010) 69-74.

[38] S.D. Li, L. Huang, Stealth nanoparticles: High density but sheddable PEG is a key for tumor targeting, Journal of Controlled Release, 145 (2010) 178-181.

[39] J.J. Wheeler, L. Palmer, M. Ossanlou, I. MacLachlan, R.W. Graham, Y.P. Zhang, M.J. Hope, P. Scherrer, P.R. Cullis, Stabilized plasmid-lipid particles: construction and characterization, Gene Therapy, 6 (1999) 271-281.

[40] Y.P. Zhang, L. Sekirov, E.G. Saravolac, J.J. Wheeler, P. Tardi, K. Clow, E. Leng, R. Sun, P.R. Cullis, P. Scherrer, Stabilized plasmid-lipid particles for regional gene therapy: formulation and transfection properties, Gene Therapy, 6 (1999) 1438-1447.

[41] I.S. Zuhorn, U. Bakowsky, E. Polushkin, W.H. Visser, M.C.A. Stuart, J.B.F.N. Engberts, D. Hoekstra, Nonbilayer phase of lipoplex-membrane mixture determines endosomal escape of genetic cargo and transfection efficiency, Mol Ther, 11 (2005) 801-810.

[42] F.X. Shi, L. Wasungu, A. Nomden, M.C.A. Stuart, E. Polushkin, J.B.F.N. Engberts, D. Hoekstra, Interference of poly(ethylene glycol)-lipid analogues with cationic-lipid-mediated delivery of oligonucleotides; role of lipid exchangeability and non-lamellar transitions, Biochemical Journal, 366 (2002) 333-341.

[43] J.L. Betker, J. Gomez, T.J. Anchordoquy, The effects of lipoplex formulation variables on the protein corona and comparisons with in vitro transfection efficiency, Journal of Controlled Release, 171 (2013) 261-268.