Protocols

Neutral PEGylated liposomal formulation for efficient folate-mediated delivery of *MCL1* siRNA to activated macrophages

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ABSTRACT

Cationic liposomes are efficient vectors for systemic delivery of therapeutic small interfering RNA (siRNA), taking advantage of RNA interference (RNAi), a naturally occurring gene-silencing mechanism in mammalian cells. However, toxicity at high concentrations, short circulating half-lives and lack of specificity restrict their successful application in a wider scale. The purpose of this study was to evaluate the efficiency of neutral liposomes containing polyethylene glycol (PEG) to encapsulate siRNA in their aqueous core. This formulation will reduce drastically the toxicity associated to cationic liposomes by bringing surface charge to almost zero, increasing stealth degree and therefore circulation time. In this study, we evaluate the efficiency of folate-targeted liposomes for specific delivery of siRNA to activated macrophages, key effector cells in rheumatoid arthritis (RA) pathology which specifically express folate receptor β (FR β). Myeloid cell leukaemia-1 (Mcl-1) is a protein essential for synovial macrophage survival, since Mcl-1 suppression results in the induction of apoptosis. The effect of *MCL1* siRNA incorporated in liposomal formulation was assessed in primary human macrophages and successful inhibition of Mcl-1 expression was achieved. Here we show that the neutral liposomal derived from DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine) formulation developed is efficient to encapsulate *MCL1* siRNA and silencing gene expression in activated human macrophages.

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1. Introduction

RNAi is a naturally occurring gene-silencing mechanism that controls endogenous gene expression of in a sequence-specific manner at the messenger RNA (mRNA) level [1]. Double-stranded RNA is processed into small interfering RNA (siRNA), which is a 21 nucleotides long molecule, and then incorporated into a silencing complex which targets and silences mRNA with complementary sequences [2]. However, to achieve an *in vivo* application for siRNAs important issues still remain to be solved. Highly anionic

oligomeric siRNA molecules cannot cross the barriers of biological membranes and are prone to nuclease mediated degradation in cellular environments [3]. Therefore, there is the need to increase the efficiency of systemic delivery of siRNAs, which has been partially achieved by the development of novel delivery systems. Among these, the cationic liposomal vectors have received significant attention, although some disadvantages, including toxicity at higher concentrations, have restricted their widespread application [4].

For lipid-based non-viral vectors to be useful in nucleic acids delivery, they must fulfill several requirements [5]. Nucleic acid carriers should comprise a nucleic acid-containing core in a lipid envelope layer enabling intracellular delivery and processing. Also, a “stealth layer” should be incorporated to ensure biological stability reflected in adequate circulation times. Finally, the carriers should contain a targeting layer to enhance the particle uptake into specific target cells. It was demonstrated that mixing siRNA with

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preformed highly PEGylated cationic liposomes results in siRNA-liposome complexes that instantaneously release their siRNA when in contact with full human serum. Further characterization demonstrated that most of the siRNA was bound to the outer surface of the liposomes [6].

RA is a common chronic inflammatory rheumatic disease, affecting almost 1% of the world population [7]. This autoimmune disease is characterized by inflammation of the joints, resulting in synovial hyperplasia by infiltration of activated immune cells further leading to cartilage and bone destruction [8]. Macrophages are critical in the pathogenesis of RA [9] and resistance to apoptosis is an important factor to their persistence in the rheumatoid joint [10]. FR β , whose expression is particularly elevated in RA synovial macrophages [11], has been used as a target for immunotherapy in a number of clinical conditions. However, due to their intrinsic phagocytic capacity, it is challenging to achieve a macrophage-specific delivery.

In vivo utilization of siRNA as a therapeutic approach in rheumatoid diseases was previously demonstrated by electro transfer of siRNA targeting Tumor Necrosis Factor α (TNF- α) in arthritic joint tissue of mice and rats [12]. However, this local approach may be difficult to apply at the clinical setting [13]. Mcl-1, a highly regulated member of the anti-apoptotic B-cell lymphoma-2 (Bcl-2) family of proteins, is essential for synovial macrophage survival. It has been reported that Mcl-1 is highly expressed in the rheumatoid joint, particularly in macrophages [14]. Mcl-1 suppression results in the induction of apoptosis [15], suggesting that may be used as a potential therapeutic target in RA.

This work aims to specifically apply PEGylated, DOPE-derived neutral liposomes, as delivery agent for siRNA to activated macrophages. Anchoring folate (or folic acid, FA) at the liposomal surface leads to the liposomes to be targeted to FR β expressed at the surface of macrophages. Locked nucleic acids (LNAs), chemically modified siRNAs, were used as an alternative to classic siRNA molecules, as LNAs have been demonstrated to be more stable and more resistant to degradation by RNases compared to other siRNA molecules [16]. *MCL1* LNA was used to silence the expression of this key gene in macrophage survival. Finally, the analysis of the effect of *MCL1* targeting by the LNA-loaded liposomes was performed by evaluating levels of Mcl-1 protein expression and apoptosis induction.

2. Materials and methods

2.1. Materials

DOPE and *N*-(carbonyl methoxypolyethylene glycol-2000)-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE-MPEG) were obtained from Lipoid GmbH (Germany). Cholesterol (CH) was obtained from Sigma (USA). LNAs labeled with fluorescein (FAM, λ_{ex} = 495 nm, λ_{em} = 520 nm) were purchased from Exiqon (Denmark). The sequences of LNAs were as follows: 5'- TTCCTGATGCCACCTT/36-FAM -3', for *MCL1* and 5'- GTGTAACACGTCTATACGCCCA/36-FAM -3', for negative control (NC). Throughout all experiments, RNase-free materials and conditions were carefully applied.

2.2. Preparation of LNA loaded liposomes

Liposomes composed of DOPE/CH/DSPE-MPEG (54:36:10, molar ratio) were prepared using HYDRA protocol [17]. Briefly, known amounts of DOPE, CH and DSPE-MPEG were dissolved in chloroform in a 50 mL round-bottom flask. The organic solvent was evaporated using a rotary evaporator. The resultant dried lipid film was dispersed in phosphate-buffered saline (PBS) buffer contain-

ing Folate-peptide (0.75 molar ratio) and 10 μ M LNAs. The lipid film with the LNA was allowed to hydrate overnight at 4 °C, resulting in LNA loaded liposomes. Thereafter, the mixture was extruded (extruder supplied by Lipex Biomembranes Inc.) through 200 nm and 100 nm pore size polycarbonate filters (Nucleopore) to form large unilamellar vesicles. After preparation, eventual free LNA was separated using Amicon Ultra Centrifugal Filter Units (100 kDa cut-off), which demonstrated have low binding LNA ability. The efficiency of encapsulation of LNA in the liposomal formulation was measured by fluorescence spectroscopy, through their FAM labeling. The possible interaction of LNA with FA-peptide was discarded by use of untargeted liposomes, with similar encapsulation efficiency.

2.3. Determination of the zeta potential and size distribution

The zeta-potential (ζ -potential) values and size distribution of liposomes were determined at pH 7.4 (PBS buffer) and 25.0 °C by electrophoretic laser Doppler anemometry and photon correlation spectroscopy (PCS), respectively, using a Malvern Zetasizer NS (Malvern Instruments). The lipid concentration was held constant at 400 μ M. The viscosity and refractive index values were 0.890 cP and 1.330, respectively.

2.4. Cell culture conditions

Human peripheral blood mononuclear cells were isolated from buffy coats of healthy blood donors from the Immunohaemotherapy Department of Hospital de São João (Porto, Portugal) by centrifugation over Lymphoprep (Axis-Shield), and monocytes were further purified by magnetic cell sorting using CD14 microbeads (Miltenyi Biotec). The ethics committee granted approval for the study and all volunteers gave their informed consent. Monocyte-derived macrophages were generated in RPMI 1640 (supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 U/mL penicillin and 100 μ g/ml streptomycin). M1 and M2 macrophages were obtained by differentiation of monocytes with granulocyte-macrophage colony-stimulating factor (GM-CSF, 20 ng/ml) or M-CSF (50 ng/ml) for 7 days, respectively. M1 macrophages were activated with lipopolysaccharide (LPS, 100 ng/ml) and interferon (IFN)- γ (25 ng/ml) and M2 macrophages were activated with interleukin (IL)-4 (20 ng/ml) or IL-10 (10 ng/ml) for 2 days. Raw 264.7 cells were routinely cultivated in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 100 U/mL penicillin and 100 μ g/ml streptomycin.

2.5. RNA isolation, cDNA synthesis and quantitative RT-PCR

Total RNA was extracted using the TRIzol Reagent (Invitrogen) in conjunction with the PureLink RNA Mini Kit (Ambion), according to the manufacturer's instructions, and quantified with a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies). cDNA synthesis was performed with random hexamers and Superscript III following the manufacturer's instructions (Invitrogen). cDNA was then quantified in an iQ5 Real-Time PCR system (Bio-Rad) with iQ SYBR Green Supermix (Bio-Rad). Oligonucleotide primers used for quantitative Real-Time PCR (qPCR): forward, 5'- CAAAACGGGACTGGCTAGTAAA -3', reverse, 5'- CCTTCTAGGTCCTCTACATGGAAGA -3', for human *MCL1*, forward, 5'- TGTTCTTGTAAGGACGAAAC -3', reverse, 5'- TTAAGAGTCCCCTATTGCAC -3', for mice Mcl-1, and forward, 5'- TGCTATCCAGGCTGTGCTATCC -3', and reverse, 5'- CCAGGTCCAGACGAGGATG -3', for β -actin. β -actin gene was used as the internal reference. Data were analyzed using iQ5 Optical System software (Bio-Rad).

2.6. siRNA delivery

LNA transfection was performed using GenMute siRNA Transfection Reagent for Primary Macrophages (SignaGen) following the manufacturer's recommendations. Macrophage exposure to FR-targeting liposomes was performed as previously reported [18]. Briefly, cells were incubated with 100 nM of LNA-loaded liposomes in complete medium at 37 °C. After incubation, cells were washed twice with PBS (pH 7.4) and then resuspended in PBS for fluorescence-activated cell sorting (FACS) analysis or TRIzol Reagent (Invitrogen) for qPCR.

2.7. Western blot

Cells were collected, washed with PBS and resuspended in lysis buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA and 1% v/v: NP-40) containing 1 mM phenylmethylsulfonyl fluoride and complete protease inhibitor cocktail (Roche Diagnostics GmbH). After 30 min of incubation on ice, the suspensions were centrifuged and the supernatants were collected. Protein concentrations were measured by Bradford reagent (Sigma-Aldrich). Equal amounts of each protein sample were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane using iBlot Dry Blotting System (Invitrogen). The membranes were incubated at room temperature for 60 min in blocking buffer containing 5% fat-free milk and TBS-T (20 mM Tris-HCl pH 7.6, 137 mM NaCl, 0.1% Tween 20) and then probed overnight at 4 °C with primary mouse monoclonal antibodies specific for Mcl-1 (1:5000, Ab22, eBioscience) diluted in 3% fat-free milk in TBS-T. Following washes with TBS-T, membranes were incubated with goat anti-mouse IgG-HRP secondary antibody (1:25000, Santa Cruz Biotechnology, Inc.) diluted in TBS-T for 1 h at room temperature. Next, the membranes were washed again and submitted to WesternBright™ Quantum (Advanta) detection system for 5 min before being developed by chemiluminescence. In order to re-probe the membrane to analyze the expression of a constitutive protein (as loading control), membranes were submitted to a stripping protocol (Abcam®) with a mild stripping buffer, prepared according to manufacturer's instructions. After stripping, membranes were blocked again, incubated with mouse anti-human α -Tubulin antibody (1:30000, Sigma-Aldrich, clone B-5-1-2) or β -actin (1:1000, Abcam®), and then incubated with the secondary antibody and developed, as described before.

2.8. Apoptosis assay

The percentage of apoptotic cells was determined by Annexin V-FITC/Propidium Iodide staining according to the manufacturer's instruction (ExBio). Briefly, cells from each sample were washed in PBS and resuspended in Annexin V binding buffer (100 μ L). Annexin V-FITC (5 μ L) and Propidium Iodide (5 μ L) was added to each cell suspension, gently mixed and incubated for 10 min at room temperature. Finally, apoptotic (Annexin V^{pos}/PI^{neg}) and dead cells (double positives) were assessed by flow cytometry (BD FACS CANTO II).

3. Results and discussion

Although cationic liposomes are among the most popular nucleic acid delivery agents, some concerns remain regarding their safety for therapeutic use. In particular, toxicity of certain cationic lipid particles has been reported both *in vitro* and *in vivo*.

Additionally, it was demonstrated that simply mixing siRNA with preformed highly PEGylated liposomes does not avoid siRNA release into the serum and subsequent degradation by nucleases

Table 1

Physicochemical characterization of FR-targeting liposomal formulations based on DOPE/CH/DSPE-MPEG. Values represent the mean $\pm$ SD of 3 independent experiments.

LNA	Z-average (nm)	Polydispersity index (Pdl)	ζ -Potential (mV)
–	122.1 $\pm$ 2.44	0.048 $\pm$ 0.019	–0.945 $\pm$ 1.18
MCL1	120.9 $\pm$ 2.53	0.050 $\pm$ 0.017	–0.993 $\pm$ 1.24

[17]. To be optimally protected siRNA molecules need to be encapsulated inside the core of highly PEGylated liposomes, as siRNAs that are bound to the outer surface are almost instantly released in serum through competition with ubiquitous negatively charged serum components. Efficient gene silencing has been shown by liposomes made of the neutral DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine) without surface modification to improve stealth or targeting, being the siRNA incorporated into DOPC [19,20].

We have previously described an innovative strategy for targeted liposomal delivery that uses a hydrophobic fragment of a surfactant protein conjugated to a folate moiety and a linker, the SP-DS3 peptide [21]. We have shown that this is more efficient than other classical systems using the PEG linker [21]. The novel liposomal constructs present high stealth degree, achieved with 10% DSPE-MPEG. Moreover, they present low cytotoxicity (at least until 750 μ g/ml and 72 h, Fig. S1) [21] and are able to deliver drugs to activated macrophages while not affecting normal cells, both *in vitro* and *in vivo* [18]. We measured the uptake of these FR-targeting liposomes in the monocytic cell line THP-1 with and without overexpression of human FR β . Liposomes with FA were highly internalized by THP-1 cells retrovirally transformed with FR β in comparison with the wild-type THP-1 cells, which weakly express FR β , that showed minimal uptake [18]. Furthermore, in comparison to liposomes without FA, targeted liposomes strongly accumulated in the joints of arthritic mice [18].

We propose to use DOPE-derived neutral PEGylated liposomes containing FA ligand at their surface, to promote specific MCL1 LNA delivery in activated macrophages, as a potential tool for treating RA.

3.1. Characterization of liposome encapsulating LNA

Size and surface charge are important characteristics in the successful development of vectors for systemic delivery. The size distribution of LNA-loaded liposomes was determined by dynamic light scattering (DSL) measurements. The results indicated that the size distribution pattern of liposomes was not influenced by encapsulation of MCL1 LNA (similar results obtained to NC LNA, data not shown), as in both cases a size of approximately 120 nm was observed (Table 1). Furthermore, both liposomal formulations exhibited a narrowed size distribution (polydispersity index <0.1), which is very favourable for the purpose of our work (Table 1). The ζ -potential (electrical potential at the surface of the hydrodynamic shear around the colloidal particle) was also evaluated. The surface potential of the particles cannot be measured directly, thus the ζ -potential is usually determined as a characteristic parameter for the particle charge. Similar to empty liposomes, the formulation prepared with LNA presented a ζ -potential close to zero (Table 1), suggesting that LNAs are encapsulated in the aqueous interior of liposomes, not altering the formation of neutral liposomes. These physicochemical characteristics of liposomal formulations remain unaltered at least until 16 weeks, suggesting a high stability of these liposomes in terms of size distribution.

Surprisingly, the encapsulation efficiency of $\approx$ 100% for MCL1 LNA in neutral liposomes indicates that, by hydrating the lipid film with a concentrated solution containing the LNA (HYDRA protocol), the negative charges on the LNA come into contact with the

Fig. 1. *MCL1* expression levels in macrophages. Primary human macrophages were obtained by differentiation of monocytes with GM-CSF or M-CSF for 7 days followed by 48 h activation with different stimuli. Relative mRNA expression levels were quantified by RT-qPCR and normalized for β -actin. Data show the mean + standard deviation for at least three independent experiments. Comparisons were performed using an unpaired two-tailed *t*-test (* $p < 0.05$ and ** $p < 0.005$).

neutral charge of all liposomal formulation, prior to liposome formation. Indeed, as soon as the liposomes form closed vesicles, the LNA will not be able to penetrate the lipid bilayers anymore. This alternative to the classic protocol of mixing siRNA with preformed liposomes allows a good distribution of the negatively charged LNA over all liposomal formulation, because the contact between them occurs before and during the formation, and the final closing of the liposomal membranes. The HYDRA protocol allows a distribution of the LNA molecules through lipid film in the round-bottom flasks, enabling a much larger contact area. The total encapsulation of siRNA molecules inside the core of the highly PEGylated liposomes

Fig. 2. Uptake of FR-targeting liposomes loaded with *MCL1* LNAs by FR β -expressing macrophages. Macrophages were obtained by incubation with 50 ng/ml M-CSF for 7 days followed by 1 day of activation with 20 ng/ml IL-4. These FR β -expressing macrophages were incubated with 100 μ g/ml of liposomes (FAM-conjugated) for 15, 30 and 60 min at 37 °C. Internalization of particles was analyzed by ImageStream multispectral imaging flow cytometry as previously described [18] (Fig. S2). Data show the mean + standard deviation of two independent experiments.

avoid their released into the serum and subsequent degradation by nucleases.

3.2. *MCL1* expression in macrophages

MCL1 is expressed in macrophages and is essential for their survival [15]. Macrophages M1 (classical, inflammatory) and M2 (alternative, anti-inflammatory) are referred as the two extremes of a spectrum of possible macrophage activation status [22]. We evaluated the effect of macrophage differentiation on *MCL1* expression by qPCR quantification in M1 and M2 macrophages developed

Fig. 3. Silencing of *MCL1* gene in RAW 264.7 and FR β -expressing macrophages. (A) RAW 264.7 cells and monocyte-derived macrophages activated with IL-4 were transfected with 100 nM *MCL1* LNA or with a negative LNA control (NC). In parallel, cells were incubated with the FR-targeting liposomes containing NC LNA and *MCL1* LNA. *MCL1* gene expression levels were assessed by qPCR and normalized with β -actin expression, 6 h post-transfection with the respective LNAs or incubation with the FR-targeting. (B) Basal levels of *MCL1* expression were assessed in RAW 264.7 cells and FR β -expressing macrophages by RT-qPCR and normalized for β -actin. Data show the mean + standard deviation of two independent experiments. Comparisons were performed using an unpaired two-tailed *t*-test (* $p < 0.05$).

Fig. 4. Apoptosis levels in FR β -expressing macrophages. Macrophages were transfected with 30 nM *MCL1* LNA or with a negative LNA control (NC) using GenMute. (A) The protein levels were analyzed 48 h after transfection and (B) the percentage of apoptotic and dead cells was analyzed 72 h after transfection. (C) Macrophages were incubated with liposome containing 100 nM of *MCL1* LNAs (FA-Lip + *MCL1*) or with a NC LNA (FA-Lip + NC). Percentages of apoptotic and dead cells were determined after 48 and 72 h. Data show the mean + standard deviation of three independent experiments. Comparisons were performed using an unpaired two-tailed *t*-test (***p* < 0.005 and ****p* < 0.001).

in culture. These were obtained by differentiating primary human monocytes into macrophages using GM-CSF or M-CSF, respectively.

After 7 days, GM-CSF-differentiated macrophages were activated with LPS and IFN- γ (simultaneously), while M-CSF-differentiated macrophages were also activated with IL-4 and IL-10 for 48 h *MCL1* mRNA is shown to be highly expressed after activation with LPS and IFN- γ , while in IL-4 or IL-10 activated macrophages the expression is low (Fig. 1). As the acquisition of folate uptake ability is promoted by M-CSF and maintained by IL-4 [23] and only M-CSF differentiated macrophages express high levels of FR β , IL-4 stimulated macrophages were chosen and used in subsequent studies.

3.3. *MCL1* silencing in macrophages

Previous results shown that liposomes with FA were highly internalized by THP-1 cells (human monocytic cell line) that specifically express FR β [18]. To evaluate the efficiency of therapeutically relevant FR-targeting LNA-loaded liposomes, we targeted FR β -expressing macrophages (M-CSF + IL4) with LNAs against *MCL1*. These LNA containing FR-targeting liposomes are efficiently internalized by FR β -expressing macrophages as $\approx$ 60% of macrophages internalize liposomes after 1 h of incubation (Fig. 2).

The release and effect of the LNA from these new liposomes were then tested in primary human macrophages expressing FR β and the murine macrophage-like RAW 264.7 cells that express FR α [24]. This cell line was shown to functionally bind and internalize a folate-based nanoparticle evaluated as a therapeutic device for rheumatoid arthritis [25]. Expression of *MCL1* and the silencing efficiency of the newly developed liposomal formulation was analyzed by qPCR (Fig. 3A). To assess the effect of the LNA on *MCL1* expression in macrophages, LNAs were transfected using the GenMute reagent (SigmaGen) and the silencing efficiency quantified by qPCR, in comparison to a negative LNA control (no homology to any known microRNA or mRNA sequences in mouse, rat or human). As expected, transfection of the LNA against the anti-apoptotic *MCL1* gene significantly reduced *MCL1* mRNA levels in both cell types ($\approx$ 60%). Similarly, although to a lower degree, *MCL1* mRNA levels were decreased approximately 40% and 30%, in RAW 264.7 and macrophages treated with FR-targeting liposomes, respectively. These differences in *MCL1* silencing may be due to the high stability of liposomes and the controlled release of the LNA in *ex vivo* systems. We also observed that although *MCL1* mRNA levels are increased in murine RAW 264.7 cells in comparison to human primary macrophages (Fig. 3B), *MCL1* silencing is achieved in both cell types.

3.4. In vitro apoptosis levels in macrophages

Mcl-1 protein expression in macrophages was examined after treatment with *MCL1* LNA. The results show a strong decrease in Mcl-1 protein expression (90%; Fig. 4A) indicating that the *MCL1* silencing effect is more clearly observed at the protein levels than at the mRNA level ($\approx 60\%$; Fig. 3A). More importantly, the decrease in Mcl-1 expression led to $\approx 70\%$ of dead cells after 72 h of transfection (Fig. 4B). As Mcl-1 is an anti-apoptotic member of the B-cell lymphoma 2 (Bcl-2) family of apoptosis-regulating proteins, these results clearly show that *MCL1* silencing is functionally relevant.

Levels of apoptosis were then analyzed in FR β -expressing macrophages incubated with LNA-loaded liposomes (Fig. 4C). These macrophages did not show significant higher levels of apoptosis when incubated for 48 and 72 h with 100 nM of Mcl-1 LNA-loaded liposomes (corresponding at ~ 264 $\mu\text{g/ml}$ liposomes). Nonetheless, an increase in the number of dead cells was observed for *MCL1* LNA-loaded liposomes in comparison to the control, in particular at 72 h. The lower levels of apoptosis remain even after incubation with 200 nM of *MCL1* LNA-loaded liposomes (data not shown). Due to lower levels of apoptosis, Mcl-1 protein expression was not analyzed in macrophages after incubation with *MCL1* LNA-loaded liposomes.

The differences observed between free *MCL1* LNA and loaded liposomes are most probably due to the slow release of the LNA into the cells, from the very stable liposomes. The lower degree of *MCL1* silencing induced by LNA-loaded liposomes may not sufficiently reduce the Mcl-1 protein levels to trigger apoptosis. As previously reported, *MCL1* downregulation may not sufficient *per se* cause apoptosis, but instead, it sensitizes cells to apoptosis [26]. Of note, *MCL1* LNA and LNA-loaded liposomes are uptaken by the FR β -expressing macrophages via different pathways, possibly resulting in different levels of silencing and apoptosis. While *MCL1* specific inhibition should induce apoptosis in a Mcl-1-dependent pathway, the cells may also be protected by other anti-apoptotic BCL-2 family members [27]. Furthermore, Mcl-1 is a short-lived protein that undergoes a complex regulation, including transcriptional and translational control, as well as the involvement of different E3 ligases and deubiquitinating enzymes [28].

4. Conclusion

In conclusion, here we show that the HYDRA protocol enables encapsulation of *MCL1* LNA into FR-targeted, highly PEGylated and neutral liposomal formulation to an extent that attains a maximal encapsulation efficiency of nearly 100%. This protocol is a laboratory-scale method that is a simple and inexpensive alternative to the classic protocol of mixing LNA with preformed liposomes, but with much higher LNA encapsulation efficiency. We show that it was possible to use a DOPE-derived neutral liposomal formulation and thereby reduce drastically the toxicity associated to cationic liposomes (Fig. S1). This neutral liposomal formulation ensures that siRNA molecules are encapsulated inside the core of the highly PEGylated liposomes. In this way, there is no siRNAs bound to the outer surface of liposomes, which would be instantly released in serum through competition with ubiquitous negatively charged serum components and subsequent degraded by nucleases. Furthermore, we also demonstrate that these *MCL1* LNA liposomes are efficiently uptaken by FR β -expressing primary macrophages.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.colsurfb.2017.04.023>.

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