Original Research Article

Hyaluronan grafted lipid-based nanoparticles as RNAi carriers for cancer cells

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A R T I C L E   I N F O

Keywords:
Hyaluronan
Lipid-based nanoparticles
RNAi
Immune response
Cancer cells

A B S T R A C T

RNA interference (RNAi), a natural cellular mechanism for RNA-guided regulation of gene expression could in fact become new therapeutic modality if an appropriate efficient delivery strategy that is also reproducible and safe will be developed. Numerous efforts have been made for the past eight years to address this challenge with only mild success. The majority of these strategies are based on cationic formulations that condense the RNAi payload and deliver it into the cell cytoplasm. However, most of these formulations also evoke adverse effects such as mitochondrial damage, interfering with blood coagulation cascade, induce interferon response, promote cytokine induction and activate the complement. Herein, we present a strategy that is devised from neutral phospholipids and cholesterol that self-assembled into lipid-based nanoparticles (LNPs). These LNPs were then coated with the glycosaminoglycan, hyaluronan (HA). HA-LNPs bound and internalized specifically into cancer cells compared with control, non-coated particles. Next, loaded with siRNAs against the multidrug resistance extrusion pump, p-glycoprotein (P-gp), HA-LNPs efficiently and specifically reduced mRNA and P-gp protein levels compared with control particles and with HA-LNPs loaded with control, non-targeted siRNAs. In addition, no cellular toxicity or cytokine induction was observed when these particles were cultured with human Peripheral Blood Mononuclear Cells (PBMCs). The HA-LNPs may offer an alternative approach to cationic lipid-based formulations for RNAi delivery into cancer cells in an efficient and safe manner.

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1. Introduction

The silencing of genes via small interfering RNAs (siRNAs) can affect the expression of virtually any protein in a cell, and thus this process can become a potential therapeutic modality to treat a variety of human diseases ranging from cancer and genetic disorders to viral infections. While siRNAs are routinely used for in vitro experiments to elucidate the activity of genes and proteins, there is no clinically approved product that utilizes siRNAs based therapeutics despite the great potential it holds [1,2]. Multiple tasks are needed in order for the RNAi molecules to act in the appropriate target cells. To fulfill some of these tasks, RNAi molecules (such as siRNAs or miRNAs) must be encapsulated in nano-scaled carriers that will be delivering it into the appropriate target cells in vivo [3]. It is the mission of the nanocarrier to protect the RNAi molecules from the hostile environment surrounding in the circulation and at the same time enabling it to enter the cell cytoplasm [4,5]. Today, most RNAi carriers are utilizing cationic molecules and are based on charge interactions with the negatively charged RNA. While these reagents work extremely well in vitro, in most types of cells, cationic molecules are non-natural, highly immunogenic and will result in dramatic adverse effects when introduced systemically. Some reported adverse effects of the cationic formulations include robust pro-inflammatory response, induction of interferon responsive genes, complement and lymphocytes activation, mitochondrial damage and interference with coagulation cascade. The mechanism underlining the robust inflammatory response was recently reported and agonizing Toll-like receptor 4 by the cationic formulations play a major role in this immune toxicity [6–10], as well as in evoking mitochondrial damage [11].

In order to enable safe in vivo therapeutic gene silencing without the robust pro-inflammatory response, we devised a non-cationic lipid transfection strategy based on the glycosaminoglycan, hyaluronan (HA) that is grafted on the surface of lipid-based nanoparti-

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cles (HA-LNPs). HA, a naturally occurring glycosaminoglycan is one of the major components of the extracellular matrix (ECM). It is found in many tissues such as skin, joint tissue (in synovial fluid) and eyes [12,13]. HA is known as a bioadhesive compound capable of binding with high affinity to both cell-surface and intracellular receptors, to ECM components and to itself. In cancer cells, binding of HA to its receptors is involved in tumor growth and spreading. CD44 regulates cancer cells proliferation and metastatic processes [14].

In addition, disruption of HA–CD44 binding was shown to reduce tumor progression. Administration of exogenous HA resulted in arrest of tumor spreading. HA is a non-toxic and non-immunogenic compound, already approved for use in eye surgery, joint therapy and wound healing. Coating small unilamellar liposomes with HA stabilizes these particles in a cycle of lyophilization and rehydration [15], provides selective targeting to tumors expressing the HA receptors [16–18], and presents a scaffold for conjugation of other ligands to the surface for further improving the selectivity to cell surface receptors [19].

HA-LNPs were originally developed as vehicles for delivering small molecules chemotherapeutics to target cells *in vivo* [17,18,20,21]. Herein, we report of a simple approach for delivering siRNAs into cancer cells using HA-LNPs that do not induce immune activation.

2. Materials and methods

2.1. Preparation of LNPs

Multilamellar vesicles (MLVs) composed of Pure Soybean phosphatidylcholine (Phospholipon 90G), which was a kind gift from Phospholipid GMBH (Germany). 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE) and Cholesterol (Chol) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). PC:Chol:DPPE were at mole ratios of 60:20:19.9, with the addition of 0.1% DPPE labeled with Rhodamine fluorophore (Invitrogen) were prepared by the traditional lipid-film method [15,17,18,22]. Briefly, the lipids were dissolved in ethanol, evaporated to dryness under reduced pressure in a rotary evaporator (Buchi Rotary Evaporator Vacuum System Flawil, Switzerland). Following evaporation, the dry lipid film was hydrated in 10 ml of HEPES (pH 7.4). This was followed by extensive agitation using a vortex device and a 2 h incubation in a shaker bath at 65 °C. The MLV were extruded through a Lipex extrusion device (Northern lipids, Vancouver, Canada), operated at 65 °C and under nitrogen pressures of 200–500 psi. The extrusion was carried out in stages using progressively smaller pore-size polycarbonate membranes (Whatman Inc., UK), with several cycles per pore-size, for achieving unilamellar vesicles in a final size range of ∼100 nm in diameter. Lipid mass was quantified as previously reported [19,22].

2.2. Surface modification of LNPs

High molecular weight Hyaluronan (HA), 700 KDa (Lifecore Biomedical LLCChaska, MN, USA) was dissolved in 0.2 M MES buffer (pH 5.5) to a final concentration of 5 mg/ml. HA was activated with EDC and sulfo-NHS at a molar ratio of 1:1:6. After 30 min of activation the LNPs were added and the pH was adjusted to 7.4. The solution was incubated at room temp (2 h). The free HA was removed by 3 cycles of repeated washing by centrifugation (1.3×10^5 g, 4 °C, 60 min). HA was quantified as previously demonstrated [19,22]. The final HA/lipid ratio was typically 75 μg HA/μmole lipid as assayed by ^{3}H-HA (ARC, Saint Louis, MI) with an average of 2% of the particles that had the HA decoration.

2.3. Particle size distribution and zeta potential measurements

Particle size distribution and mean diameter of the HA-LNPs and control, non-surface modified NPs (LNPs) were measured on a Malvern Zetasizer Nano ZS zeta potential and dynamic light scattering instrument (Malvern Instruments, Southborough, MA) using the automatic algorithm mode and analyzed with the PCS 1.32a. All measurements were done in 0.01 mol/l NaCl, pH 6.7, at room temperature.

2.4. siRNA encapsulation and quantification of the efficiency of entrapment

HA-LNPs or LNPs were lyophilized until complete water removal was ensured (48 h). The lyophilized particles were hydrated with DEPC-treated water with or without siRNAs. The particles were shaken gently for 30 min at room temperature. Unencapsulated siRNA was removed via ultra-fast centrifugation at 6.4×10^5 g, 4 °C, 20 min prior to adding the suspension to the cells.

siRNA encapsulation efficiency was determined by the Quant-iT RiboGreen RNA assay (Invitrogen) as previously described by us and others [19,23,24]. Briefly, the entrapment efficiency was determined by comparing fluorescence of the RNA binding dye RiboGreen in the LNP and HA-LNP samples, in the presence and absence of Triton X-100 [23]. In the absence of detergent, fluorescence can be measured from accessible (unentrapped) siRNA only. Whereas, in the presence of the detergent, fluorescence is measured from total siRNA [24] thus, the % encapsulation is described by the equation:

$$\% \text{ siRNA encapsulation} = [1 - (\text{free siRNA conc.}/\text{total siRNA conc.})] \times 100$$

2.5. Internalization assay using confocal microscopy

Internalization assay was performed in 24 well plates. 7.0×10^4 A549 or NAR cells were seeded on cover slips in RPMI medium, supplemented with antibiotics, L-Glutamine and 10% fetal calf serum (Biological industries, Beit Haemek, Israel). For nuclei staining, cells were stained with Hoechst (2.5 mg/ml stock, 1:10,000 in PBS) (33258, Sigma). For LNPs labeling, Lissamine™ Rhodamine B – 1,2-dihexadecanoyl-sn-glycero-3 DPPE phosphoethanolamine, triethylammonium salt (rhodamine DHPE) (L-1392, Invitrogen) was added during the particles preparation. the Cells were exposed to HA-LNPs or non-surface modified, LNPs (25 μg, 50 μl from the prepared LNPs stock solution) in medium without serum for a period of 1 h at 37 °C in a humidified atmosphere with 5% CO$_2$. Subsequently, the cells were washed twice using cold PBS, fixated with 4% Paraformaldehyde (PFA) and washed again with cold PBS. Membrane and nuclei staining were preformed after fixation. The cells were mounted by fluorescent mounting medium (Golden Bridge international, Mukilteo, WA, USA) and fluorescent was measured using Andor Spinning disc confocal microscope and the Meta 510 Zeiss LSM confocal microscope. Laser beams at 405 nm and 561 nm were used for UV and Rhodamine, fluorophores excitation, respectively. Serial optical sections of the cells were recorded for each treatment and the images were processed using Zeiss LSM Image browser software.

2.6. Cytokines induction assay

The effect of HA-LNPs and control, LNPs on TNF-α, IL-10 and IL-6 cytokines secretion from human Peripheral Blood Mononuclear Cells (PBMCs) was tested. PBMCs were freshly isolated from 3 healthy human donors obtained from Tel Hashomer (Sheba) Blood Bank.

Whole blood was diluted with RPMI 1640 in a ratio of 1:2 (Whole blood). The diluted blood was gently overlaid onto 15 ml Ficoll (lymphoprep) (1:3 ratios). Gradient were centrifuged at 22 °C, 900 g, for 25 min. Opaque-Light PBMCs ring was removed from the interphase into a new tube. PBMCs were washed with RPMI 1640 and centrifuged 250 g for 10 min. PBMCs pellet was resuspended in approximately 80 ml PBMC growth medium. Cells were resuspended with PBMCs medium to a final concentration of 3×10^6/ml. For each well 1 ml of the tested groups- HA-LNPs or LNPs was added in a final concentration of 12.5 μg/ml. Lipopolysacharides (LPS) (L6529, sigma) was used as a positive control (100 ng/ml). PBS was used as a negative control. Plates were incubated at 37 °C humidified, 5% CO$_2$/air.

PBMCs were incubation with HA-LNPs or LNPs, for 2 and separately for 24 h. Upon incubation, cells were centrifuged at 500 g for 10 min and the supernatant was removed and stored in −80 °C freezer for quantification of IL-6, TNF-α and IL-10 secreted cytokines.

Human cytokines TNF-α, IL-10 and IL-6 levels were determined using Human IL-6 DuoSet ELISA kit (R&D Systems), IL-10 DuoSet ELISA kit (R&D Systems), and Human TNF-α DuoSet ELISA kit (R&D Systems),respectively according to the manufacture instructions.

2.7. Delivery of siRNAs into cancer cells

Cells were seeded into 6 wells cell culture plates at 0.1×10^6 cells/well in RPMI medium, supplemented with antibiotics, L-Glutamine and 10% fetal calf serum (Biological industries, Beit Haemek, Israel). 24 h Post seeding the medium was removed and replaced with RPMI only. NCI-ADR/Res (NAR) cells, which highly express the P-glycoprotein (P-gp) protein extrusion pump, were used as the cancer cell model. The cells were then treated with empty HA-LNPs or with HA-LNPs encapsulating p-gp or luciferase siRNAs. P-gp was chosen as a surogated marker to evaluate the delivery strategy. Four hours post incubation the medium was removed and the cells were washed and supplemented with complete medium. Three days after transfection the cells were split 0.2×10^6 cell/well into fresh 6 wells cell culture plate. Final siRNA concentration on the cells was 300 nM.

2.8. Quantification of mRNA levels by QPCR

The mRNA levels of P-gp (ABCB1 gene) in cells was quantified by real-time PCR. 6 days after transfection Total RNA was isolated using the EzRNA RNA purification kit (Biological industries, Beit Haemek, Israel), and 1 μg of RNA from each sample was reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA), Quantification of cDNA (5 ng to-

tal) was performed on the step one Sequence Detection System (Applied Biosystems, Foster City, CA), using syber green (Applied Biosystems). GAPDH was chosen as a house keeping gene.

For real time PCR the following primers were chosen
Primers for ABCB1 (P-gp):
Forward – ATT CCA CCC ATG GCA AAT TC
Reverse – GGA TCT CGC TCC TGG AAG ATG
Primers for GAPDH:
Forward – TCA GGG TTT CAC ATT TGG CA
Reverse – GAG CAT GGA TCG GAA AAC CA

2.9. Flow cytometry analysis

CD44 was surface labeled with a pan-CD44 monoclonal antibody clone IM7 and the staning was done as previously reported [22]. P-glycoprotein (P-gp) protein was labeled on the surface of non-permeabilized cells and quantified by Flow cytometry analysis. 7 days post transfection the cells were detached with trypsin EDTA and then resuspended in PBS 1X supplemented with 0.1% FBS (FACS buffer) containing an anti-Pgp polyclonal antibody 1:20 diluted, AB10333 (abcam) or an isotype-matched negative control 11-457-C100 (medimabs) for 30 min on ice. Cells were then washed 3 times with FACS buffer and incubated with a FITC-conjugated anti-rabbit antibody (1:400 dilution) for 30 min on ice in the dark. Samples were then collected and analyzed using a FACSscan CellQuest (Becton Dickinson, Franklin Lakes, NJ).). Ten thousand cells were analyzed at each experimental point. Data analysis was performed using FlowJo software (Tree Star, Inc. Oregon, USA).

2.10. Statistical analysis

Differences between treatment groups were evaluated by one-way ANOVA with significance determined by Bonferroni adjusted *t*-tests.

3. Results

3.1. Particle size distribution and surface charge measurements

To investigate the cellular targeting of HA-LNPs as delivery vehicles for RNAi, we prepared two types of LNPs differing in their surface coating, namely hyaluronan (HA)-coated LNPs denoted HA-LNPs and non-coated, control particles, LNPs. The two types LNPs characteristics are summarized in Table 1.

LNPs composed of natural phospholipids (PC and DPPE) and cholesterol had a mean size of 138.87 ± 1.12 nm and had a mildly negative zeta potential of −8.89 ± 0.40 mV. Coating the LNPs covalently with of high molecular weight HA did not change the size distribution compared to non-coating particles, however, a significant decrease in the Zeta potential of the HA-LNPs, (e.g. −19.2 mV ± 0.76) was observed. The difference in Zeta potential between the two types of LNPs is attributed to the negatively charged carboxyl groups of the hyaluronan [15]. After characterizing the particles structure and composition we tested their capability to internalize into specific cancer cells.

3.2. HA-LNPs are specifically taken up by cancer cells

Almost all cancer cells highly express HA receptors, CD44 and CD168 [14,22]. To examine the uptake of HA-LNPs into cancer cells, we used the human lung adenocarcinoma A549 cell line as our *in vitro* model cells. We first tested the expression of CD44 in A549 cells by flow cytometry. Fig. 1a shows high CD44 expression on these cells. We then incubated HA-LNPs and separately LNPs with the cells and cells were washed as detailed in the experimental section. Confocal microscopy analysis was used to identify the

HA-LNPs inside the cells cytoplasm (Fig. 1b). The significant uptake is strongly correlated with the presence of HA on the particles surface. As opposed to the control, uncoated particles, LNPs (Fig. 1c), which demonstrated a weak cellular uptake. The significant difference between the two types of LNPs resides by the covalent coating with HA. CD44 is the surface receptor that binds HA and is over expressed on various cancer cells, including A549. We have previously shown that high molecular weight HA (700KDa and above) that is covalently attached to LNPs have high affinity to CD44 receptors by a surface plasmon resonance study [22].

3.3. HA-LNPs entrapping siRNAs against P-gp selectively silence P-gp in cancer cells

For examining the capability of HA-LNPs for carrying siRNAs and inducing gene silencing in cancer cells, we entrapped siRNAs against P-gp (served here as a surrogated marker) and removed the unentrapped siRNAs as described in the experimental section. The efficiency of encapsulation was 23.9% ± 2.5, thus, the total siRNA concentration that was entrapped in the particles was 70 nM. In order to probe for knockdown we utilized, as our model cells, the human ovarian cell line, NCI-ADR/Res (NAR), which are highly resistant to chemotherapeutics and express a high level of the P-gp extrusion pumps as part of their drug resistance mechanism. In addition, these cells highly express CD44 on its surface as we previously shown [22]. We quantified the knockdown level at the mRNA level, using real-time quantitative PCR (Fig. 2a), and at the protein level using flow cytometry analysis (Fig. 2b). QPCR was determined 6 days post transfection, flow cytometry analysis was preformed 7 days post transfection. HA-LNPs entrapping P-gp-siRNAs reduced mRNA levels of this gene by ∼50% and subsequently also decreased P-gp protein levels. As negative controls, we used free P-gp- siRNAs, LNPs entrapping P-gp siRNAs, and HA-LNPs encapsulating Luciferase-siRNAs, an irrelevant siRNA that was acting as a control for demonstrating the specificity of the P-gp siRNA. All controls did not reduce mRNA (Fig. 2a) or protein levels of P-gp, respectively.

3.4. HA-LNPs do not induce a pro-inflammatory response in human PBMCs

One of the major hurdles in translating many of the novel delivery strategies for RNAi into new therapeutic modalities is unacceptable immunostimulation that may induce a robust pro-inflammatory response that might lead to cytokine storm and even to death. This hyper stimulation could be attributed to the RNAi payload, the nano-vehicle or the combination of the nano-vehicle that is entrapping the RNAi payload [9,25]. In order to evaluate the safety profile of HA-LNPs as a future drug delivery vehicle, an ex vivo cytokine induction study was performed using the human PBMCs, which examined the secretion of major inflammatory cytokines. The secretion level of three different inflammatory interleukins was tested using IL-6 and TNF-α as a model for the innate immune response and IL-10 as a model for the late immune response. The results are summarized in Tables 2 and 3. Neither the HA-LNPs nor the control, uncoated LNPs caused an elevate secretion of both innate and late cytokines response in two different time points, 2 h (Table 2) and 24 h (Table 3) post incubation

Table 1
Structural characterization of HA-LNPs and control, non-coated particles, LNPs.

Particle type	Lipid composition (% mole/mole)	Average size (nm)	Average Zeta potential (mV)
LNPs	Soy PC (60%), DPPE (20%) Cholesterol (20%)	138.87 ± 1.12	−8.89 ± 0.40
HA-LNPs	Soy PC (60%), DPPE (20%) Cholesterol (20%) and HA (50 µg/µmole lipid)	131.03 ± 1.42	−19.2 ± 0.76
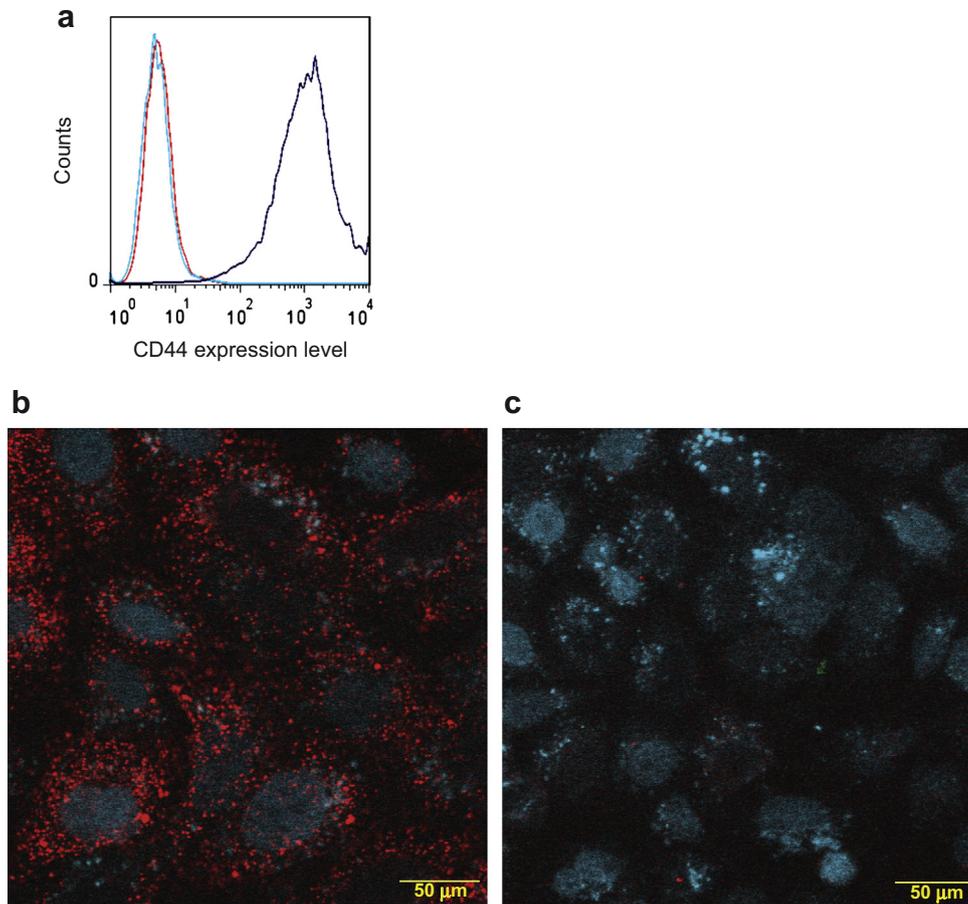
Fig. 1. HA-LNPs are specifically taken up by cancer cells. A representative histogram of CD44 expression in A549 cells is presented (a). Cells were stained with isotype control antibody (red curve), or with pan-CD44 clone IM7 (dark blue curve) as listed in the experimental section. Control, non-stained cells are also presented (light blue). Representative confocal images of HA-LNPs and control, non-coated LNPs internalizing into A549 cell line are presented (b and c). Cells were seeded onto 6 well plates. 25 µg of HA-LNPs (b), or control, non-HA-coated particles, LNPs (c) were added to the cells and incubated for 1 h at 37 °C. Hoechst reagent (H 33342) diluted 1:10,000 was used for nuclei staining. LNPs were pre-labeled with Rhodamine-DPPE to detect the LNPs intracellular pathway. The internalization was performed using a Zeiss confocal microscope (Meta 510). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

with these particles. As a positive control, we used the Toll-like receptor 4 (TLR4) natural ligand, lipopolysaccharides (LPS) that secreted high levels of both TNFa and IL-6 already 2 h post incubation with increase after 24 h of exposure to the LPS. IL-10 was secreted after 24 h, as expected. The findings that HA-LNPs or LNPs do not stimulate the innate immune arm or the adoptive arm via an increase in the cytokine secretion levels are in a good agreement with our previous published work with murine immune cells [22].

4. Discussion

Although RNAi based gene silencing was discovered almost 15 years ago [26], therapeutic gene silencing is still not clinically approved. It is clear that the major challenge in achieving therapeutic knockdown depends on an efficient, reproducible and safe *in vivo* delivery strategy. The first stage in attempting *in vivo* gene silencing is encapsulating or attaching the RNAi cargo to a nanoscale carrier [2,3]. The carrier has the primary task of protecting the RNAi payload from degradation by serum nucleases and its fast removal from the circulation by the mononuclear phagocytic system (MPS) [2,5]. At the same time, the carrier must also be able to facilitate the entry of the RNAi payload (in the form of siRNA; miRNA mimic; or anti-miR) into a specific target cell, as naked RNAi molecules cannot cross the cell membrane without a special transfection reagent [2,4]. There are two basic criteria that are required from the RNAi carrier. The first, is enabling the RNAi carrier to efficiently reach and penetrate the target cell without affecting other cells, and release its payload in the cell cytoplasm. The second requirement is that the carrier and its cargo will not generate adverse effects such as general toxicity, mitochondrial toxicity, or a robust stimulation of the immune system [27]. An ideal carrier will be equipt with selective ligands that are highly or exclusively expressed on target cells and thus endowing the carriers with specific targeting capabilities. The major advantage of a targeted RNAi delivery strategy would be in maximizing the local concentration in the desired tissue therefore enhancing the silencing effects and preventing nonspecific RNAi distribution. This will also minimize adverse effects in bystandard cells [28].

Today, most transfection agents are constructed from various cationic molecules. Lipid-based schemes are composed of different cationic lipids with helper lipids such as DOPE and represent common transfection reagents [29]. Liposomes-bearing cationic phospholipids or synthetic cationic polymers interact with the cell membrane in an undefined mechanism (probably via a temporal nick in the cell membrane; fusion; or via surface phospholipid receptors that internalized the entire particle or part of the payload) and deliver its payload into the cell cytoplasm. These strategies have been extensively used to deliver different types of oligonucleotides (plasmid DNA, anti-sense, mRNA, siRNAs, miRNA mimic and anti-miRs) [30]. When cationic liposomes interact with negatively charged oligonucleotides they form amorphous particles known as lipoplexes. Lipoplexes will protect oligonucleotides
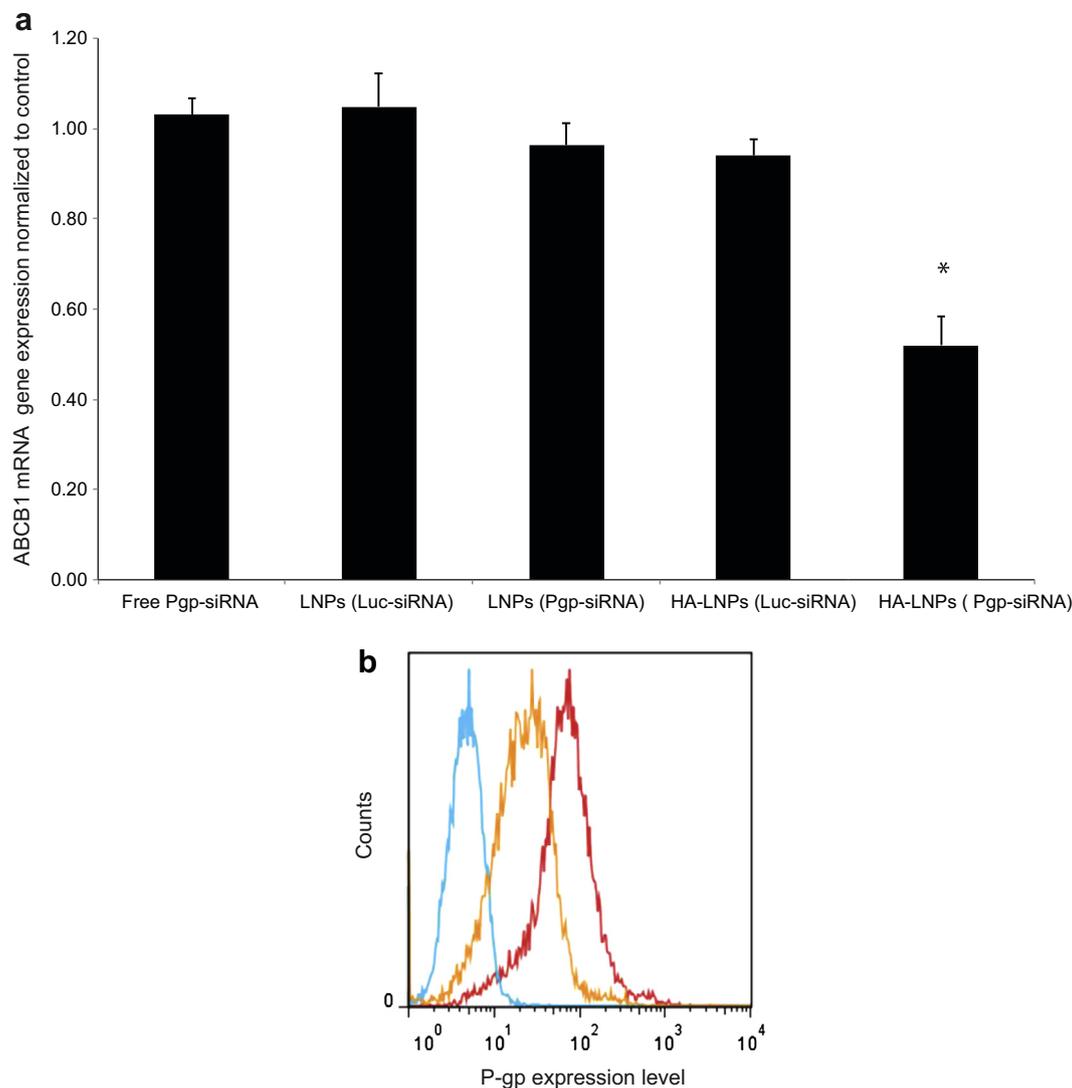
Fig. 2. HA-LNPs entrapping siRNAs selectively silence P-gp in cancer cells. Transfection was performed 24 h post cell seeding as detailed in the experimental section. The cells were treated with free P-gp-siRNA, LNPs entrapping luciferase-siRNA or P-gp-siRNA which served as controls or with HA-LNPs encapsulating P-gp-siRNA or luciferase-siRNA. 6 days post transfection the cells were analyzed for mRNA levels using real time PCR (a); *denoted $p < 0.001$. P-gp protein level was assayed using flow cytometry. A representative histogram is presented (b). Red curve: basal P-gp level in the cells; orange curve: P-gp level 7 d post transfection, treatment with HA-LNPs (P-gp-siRNA); and light blue – isotype control (p-gp – isoclass matched) antibody. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
Effect of HA-LNPs and control, non-coated – LNPs on the secretion of TNF-α, IL-10 and IL-6 in human PBMCs 2 h post incubation.

Treatment	TNF-α (pg/ml)	IL-10 (pg/ml)	IL-6 (pg/ml)
Negative control (PBS)	N.D.[*]	N.D.[*]	N.D.[*]
Positive control (LPS 1 μg/ml)	840.41 ± 67.01	N.D.[*]	20860.75 ± 2974.03[**]
LNPs	N.D.[*]	N.D.[*]	N.D.[*]
HA – LNPs	N.D.[*]	N.D.[*]	N.D.[*]

Values are expressed as means ± S.E.M. Significantly different: $P < 0.01$.
[*] N.D. – not detectable.
[**] Upper limit of quantification.

Table 3
Effect of HA-LNPs and control, non-coated – LNPs on the secretion of TNF-α, IL-10 and IL-6 in human PBMCs 24 h post incubation.

Group	TNF-α (pg/ml)	IL-10 (pg/ml)	IL-6 (pg/ml)
Negative control (PBS)	N.D.[*]	N.D.[*]	N.D.[*]
Positive control (LPS 1 μg/ml)	1094.86 ± 68.69	2228.29 ± 61.01	21976.11 ± 983.48[**]
LNPs	N.D.[*]	N.D.[*]	N.D.[*]
HA-LNPs	N.D.[*]	N.D.[*]	N.D.[*]

Values are expressed as means ± S.E.M. Significantly different: $P < 0.01$.
[*] N.D. – not detectable.
[**] Upper limit of quantification.

from enzymatic degradation and deliver these molecules into cells by interacting with the negatively charged cell membrane [31]. While using cationic liposomes is an extremely efficient delivery strategy for *in vitro* gene silencing in most type of cells, when injected into a vertebrate they cause severe adverse effects that prevent their use as effective *in vivo* carriers. These adverse effects may include blood clotting [8], mitochondrial toxicity [11] immunostimulation [32] and complement activation [33].

In order to address this problem we have devised a simple strategy based on HA-coated lipid-based-nanoparticles (HA-LNPs), which is not based on cationic molecules but rather on neutral

phospholipids that entrap siRNAs much like a small molecule drugs or peptides. HA-LNPs are composed of two equally important elements. One is the core of the system, which are the nano-scale particles. Constructed from natural phospholipids with cholestrol it does not account as a foreign material to immune cells and therefore does not trigger an immune response. The second component is the HA coating. HA is a naturally-occurring high molecular glycosaminoglycan composed of the repeating disaccharide β1, 3 N-acetyl glycosaminyl-β1, and 4 glucuronide. It exists in living systems in both free and complexed forms [34]. HA provides two important properties to this carrier. One is preventing the recognition of the HA-LNPs as stealth material. This prevents the removal by the MPS as well as possible recognition by innate immune cells. In addition, the HA coating acts as a targeting molecule. Many cancer cells over express HA receptors, CD44 and CD168 in addition to other HA-binding proteins [14,35,36].

In order to determine that our improved HA coating method effectively covers the LNPs we tested the size and surface charge of LNPs versus HA-LNPs. Our results show that LNPs coated with 2% HA do not change significantly in size but substantialy change in their net charge, indicating that the particles are effectively covered with HA.

A receptor – mediated internalization approach might be a way for efficiently introducing RNAi molecules into the cell cytoplasm without alerting the immune system. We tested the *in vitro* targeting capabilities of HA-LNPs with fluorescently labeled LNPs. With the aid of confocal microscopy we showed (Fig. 1b) that HA-LNPs effectively bound and internalized into A549 cells, a cell line that expresses a high amount of CD44 (Fig. 1a). In contrast LNPs, which lacks the HA coating did not bind or accumulate in A549 cells (Fig. 1c). These results demonstrate the importance of the HA coating and emphasize HA as a cell penetrating agent, helping carriers to efficiently internalize into cells highly expressing its receptor(s).

We next tested this strategy with functional siRNA (against the multidrug resistant extrusion pump, P-gp). We assayed the knockdown effect at the mRNA level using real time PCR and the protein level using flow cytometry analysis. We chose the ABCB1 gene that encodes the P-gp as a surrogated marker to assess the delivery strategy. The results of our experiments demonstrate that HA-LNPs loaded with P-gp- siRNA showed selective knockdown at the mRNA level (Fig. 2a) and subsequently reduced the expression of the protein in the NAR resistant cells (Fig. 2b).

Interestingly, we discovered that the mRNA reduction was apparent only 6 days after transfection. Transfection with commercial siRNA transfection agents (e.g. lipofectamine) resulted in mRNA reduction after 2 days followed by a reduction in protein levels (data not shown). This result could indicate that unlike cationic transfection agents that mediate an endosomal escape, the siRNA, which is entrapped in the HA-LNPs undergoes a slow release process from the endosome that takes several days. The effective dose that the cells received was 70nM siRNAs assuming encapsulation efficiency of 24% (after the removal of unencapsulated siRNAs). This hypothesis might be supported by the relatively high concentration of siRNA used,which was needed in evoking gene silencing. It is conservable that most of the siRNAs that entered the cells remained trapped in the endosomes and were not recognized by Ago2 protein complex and activated via the RNA Induced Silencing Complex (RISC) to induce gene silencing.

As HA-LNPs were generated to become a safe alternative to cationic liposomes we tested if HA-LNPs are able to stimulate cytokine release in human PBMCs. The experimental results (Tables 2 and 3) showed that the HA-LNPs and its siRNA cargo did not evoke cytokine production (IL-6, TNF-α and IL-10) and thus we argue that they will not induce cytokines when injected systemically.

To conclude, here we have shown that HA-LNPs could be a safer alternative for commercializing cationic transfection reagents for targeting cancer cells. HA-LNPs do not trigger cytokine release from human PMBC's and therefore should not educe an immune response in the body. At the same time, the HA coating confers specific targeting properties to the HA-LNPs and ensures that HA-LNPs will only affect specific cells. Taken together, our results show that the strategy of HA-LNPs is a promising delivery approach for safe delivery of RNAi in inducing gene silencing in cancer cells.

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