PEGylated siRNA lipoplexes for silencing of BLIMP-1 in Primary Effusion Lymphoma: in vitro evidences of antitumoral activity

Daniela Belletti[1], Giovanni Tosi[1], Flavio Forni[1], Ivana Lagreca[2], Patrizia Barozzi[2], Francesca Pederzoli[1], Maria Angela Vandelli[1], Giovanni Riva[2], Mario Luppi[2], Barbara Ruozi[1]*

[1]Department of Life Sciences, University of Modena and Reggio Emilia, Modena, Italy.

[2]Department of Medical and Surgical Sciences, University of Modena and Reggio Emilia; Hematology Unit, AOU Policlinico, Modena, Italy.

***Corresponding Author:**

Prof. Barbara Ruozi
Department of Life Sciences
University of Modena and Reggio Emilia, Italy
Phone: +39 059 2058562
mail: barbara.ruozi@unimore.it

Keywords:
Primary Effusion Lymphoma, siRNAs anti-Blimp-1, post-pegylated lipoplexes, cytotoxicity.

ABSTRACT:

Silencing of the B lymphocyte-induced maturation protein 1 (Blimp-1), a pivotal transcriptional regulator during terminal differentiation of B cells into plasma cells with siRNAs is under investigation as novel therapeutic approach in Primary Effusion Lymphoma (PEL), a HHV-8 related and aggressive B cell Lymphoma currently lacking of an efficacious therapeutic approach.

The clinical application of small interfering RNA (siRNA) in cancer therapy is limited by the lack of an efficient systemic siRNA delivery system.

In this study we aim to develop pegylated siRNA lipoplexes formed using the cationic lipid DOTAP and DSPE-PEG_{2000}, capable to effectively stabilize anti Blimp-1 siRNA and suitable for systemic administration.

Two types of pegylated lipoplexes using a classic (C-PEG Lipoplexes) or a post-pegylation method (P-PEG-Lipoplexes) were formulated and compared in their physicochemical properties (size, zeta potential, morphology and structure) and efficiency on PEL cell lines.

A stable siRNAs protection was obtained with post pegylation approach (2% molar of DSPE-PEG_{2000} respect to lipid) resulting in structures with diameters of 300 nm and a complexation efficiency higher that 80% (0.08 nmol/10 nmol of lipid). *In vitro* studies on PEL cell lines suggested that empty liposomes were characterized by a low cell toxicity also after PEG modification (cell viability and cell density over 85% after treatment with 10 μM of lipid).

We demonstrated that P-PEG-Lipoplexes were able to significantly reduce the levels of BLIMP-1 protein leading to reduction of viability (less that 15% after transfection with 100 nM of complexed siRNAs) and activation of apoptosis.

In vitro efficiency encourage us to further test the *in vivo* potential of P-PEG-Lipoplexes in PEL therapy.

INTRODUCTION:

Increasing knowledge of the genetic basis of cancer has accelerated the application of siRNA, a small interfering double-stranded RNAs able to inhibit protein expression in a sequence dependent manner through degradation and/or blockage of the corresponding mRNA, as a new generation of cancer therapeutics [1]. However, the future of such gene-based therapeutics is dependent on achieving successful stability and delivery. To date, *in vivo* systemic delivery of siRNA-based therapeutics to tumor cells remains a challenge.

Accumulation of drugs after systemic administration in lymphoma as in solid tumors takes advantage of the increased vascular permeability of new angiogenic vessels, called enhanced permeability and retention (EPR) effect [2-3]. Unfortunately, siRNA is not stable in bloodstream as after injection it is subjected to a rapid enzymatic degradation by endogenous nucleases and siRNA is rapidly taken up by phagocytes and aggregated with serum proteins with a consequent short half-life (~ 0.3 h) [4]. Extensive research is ongoing regarding the design and assessment of non-viral delivery technologies for siRNA to treat a wide range of cancers [5-7].

Different types of polymeric and lipidic nanocarriers were designed to overcome this problems by protecting siRNAs and improving their uptake into the target cells; generally, these systems differ for efficiency and safety profile [8]. Cationic liposomes emerged as one of the most attractive carriers, able to electrostatically interact with siRNA molecules, forming stable lipoplexes that need to be optimized in term of size, surface charge and structure, in order to efficiently deliver gene material into cells [9-11].

Formulation of siRNA with positively charged vectors is used to neutralize the overall surface charge and thereby increase cellular internalization.

We have recently demonstrated efficient tumor cell apoptosis after intraperitoneally administration of siRNA/DOTAP lipoplex in a Primary Effusion Lymphoma (PEL) animal model [12]. PEL is a B-cell non-Hodgkin lymphoma involving the serous cavities invariably associated with Human HerpesVirus-8 (HHV-8) in which the standard chemotherapy is often inefficacious with consequent low prognoses (median survival time shorter than 6 months) [13].

Rapid uptake by reticuloendothelial system (RES), aggregation [14-16] and adverse effects related to the cationic charge (inflammatory toxicity, hepatotoxicity, leukopenia and thrombocytopenia) were described in literature [17-18]. This aspect could affect the possibility to administer cationic lipoplex by intravenous route (IV).

The improvement of lipoplex therapy under *in vivo* condition is largely dependent on the development of a strategy able to simultaneously mask the cationic charge, avoid RES uptake and increase the half life supporting the passive targeting into the tumor. This goal is typically achieved by the addition of a PEG moiety [19-22].

Unfortunately, the steric hindrance produced by the PEG molecules on the liposomal surface could affect the formation and the stabilization of lipoplexes as well as siRNA endosomal escape [23-26]. This discrepancy concerning pegylation in siRNA delivery systems is described as the "PEG-dilemma" [27].

Thus, the pegylation of siRNA lipoplexes should be carefully optimized. Different strategies to insert PEG on a lipidic construct were investigated. PEG lipo-polymer (CHOL-PEG or DSPE-PEG) directly incorporated into the lipidic layer or adsorbed on the surface are typically used. Alternatively, as innovative strategy the use of cleavable PEG was developed as this molecules were incorporated into the liposomes conferring them stealth propriety, to finally been removed once into the cell by response of local tumor environment (typically a reduction in pH) [28-29].

Notwithstanding interesting preliminary results were achieved, a higher depth investigation should be devoted both to the toxicity profile of those new molecules and to the tumor selectivity.

On the bases of these premises, this article aimed to focus the attention on technological optimization of pegylated cationic lipoplexes applicable for IV administration of anti-BLIMP-1 siRNAs, formed using DOTAP (cationic lipid recently approved for clinical trials [30]) and a FDA approved PEG lipid (DSPE-PEG). Two different approaches are described to insert PEG moieties on cationic surface of lipoplexes, namely the conventional (C-PEG) and the post-pegylation (P-PEG) process. Along with the physicochemical properties (size, zeta potential, morphology and structure) of the colloidal delivery systems, both siRNA loading and efficiency to mediate gene silencing in PEL cells were evaluated.

2. MATERIALS AND METHODS:

2.1 Materials

N-[1(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride salt (DOTAP, $C_{42}H_{80}ClNO_4$; Mol.Wt. 698.54 Da) was obtained from Avanti Polar-Lipids (Alabaster, AL, USA) and N-(carbonyl-methoxypolyethyleneglycol 2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine, sodium salt (DSPE-PEG_{2000}, $C_{133}H_{263}NO_{55}PNa$, Mol.Wt. 2779 Da)

was kindly grafted from Lipoid (Steinhausen, ZG, Switzerland). PRDM1/Blimp-1 stealth select RNAi was purchased from Invitrogen (Milan, Italy). It was composed of three non-overlapping sequences of 25nt with the following sense targeting sequence: $siRNA_a$ [5'-gcgacgaagccaugaaucucauuaa-3'], $siRNA_b$ [5'-ggccuuucaaaugucagacuugcaa-3'], $siRNA_c$ [5'-cagaacgggaugaacaucuacuucu-3]. Cell culture medium generally used for the culture of human normal and neoplastic leukocyte (RPMI 1640), fetal bovine serum (FBS), and phosphate buffered saline (PBS) pH 7.4 were purchased from Euroclone Celbio (Milan, Italy). 7-aminoactinomycin D (7AAD) was obtained from BD Biosciences (Mississauga, ON, Canada). Antibody for Blimp-1, C14A4 and βTubulin, 9F3 were purchased from Cell Signaling Technology (Danvers, MA, USA). RNAse free water was purchased from Sigma-Aldrich (Milan, Italy). A MilliQ water system, supplied with distilled water, provided high purity water (18 MΩ). All other chemicals were commercially obtained as reagent grade products and used without any further purification.

CRO-AP/3 and BCBL-1 cell lines were commercially provided by Leibniz Institute DSMZ (Braunschweig, Germany), DSMZ n° ACC 683; ACC 275 respectively.

2.2 Preparation of Liposomes (Liposome, C-PEG-Liposome, P-PEG-Liposome)

All liposomes were prepared by a thin layer evaporation method followed by an extrusion procedure.

Cationic liposome (Liposome) was formulated dissolving DOTAP (5 mg) in chloroform (2.5 mL). The solvent was removed by rotary evaporation (B-480 Büchi, Buchs, SG, Switzerland) at 20 ± 1°C for 1 h and 10 mbar followed by 3 h at 0.15 mbar. The dry lipidic film was then hydrated with 5 mL of RNAse free water preheated to 40 ± 2°C. The hydrated lipidic film was vortexed for 3 min (Zx3, VelpScientifica, Usmate, Italy) and warmed three times in a water bath at 40 ± 2°C for 3 min before to be extruded through a 200 nm polycarbonate filter (19 cycles).

Pegylation was introduced by adding a 2% molar of pegylated-lipid (DSPE-PEG_{2000}) respect to DOTAP, using two different procedures.

Conventional pegylated liposome (C-PEG-Liposome) was formulated using the above reported procedure simply mixing DOTAP (4.6 mg) with DSPE-PEG_{2000} (0.4 mg) in order to have a 2% molar of DSPE-PEG.

Post-pegylated liposome (P-PEG-Liposome) was formed incubating Liposome with preformed DSPE-PEG_{2000} micelles. Briefly, DSPE-PEG_{2000} micelles were formulated drying under vacuum a solution of 1 mg of DSPE-PEG_{2000} in chloroform (0.5 mL). The film

obtained was further dried in vacuum (10 mmHg) overnight and then rehydrated with water until reaching a 1 mM suspension. The micelles were submitted to three cycles of bath sonication (each cycle 2 min) and warmed in a water bath at 50 ± 2°C for 5 min. The suspension was then equilibrated overnight at 8°C under dark. To form P-PEG-Liposome, DSPE-PEG_{2000} micelles were diluted above CMC (20 µM) and mixed to Liposome in a water bath at 37 ± 1°C under gently agitation (2% molar of DSPE-PEG respect to DOTAP was used in this experiments).

A concentration of 5% molar of DSPE-PEG respect to DOTAP has also been tested in an attempt to optimize the formative parameters.

2.3 Preparation of Lipoplexes

All lipoplexes were formulated by using 1.5 mM liposome suspension and 4 mM siRNA solution working in low ionic strength (I<0.1 mmol/L; potassium acetate, HEPES-KOH at pH 7.4) to optimize complexation [31]. As previously reported [32, 33], the complexation ratio between siRNA and DOTAP was maintained at 1:100 molar (N/P ratio of 1/2).

To form cationic lipoplexes (Lipoplexes), an aliquot of liposome formulation containing 20 nmol of Liposome was added to 0.2 nmol of each siRNA duplex. Lipoplexes were simply formed by incubating for 10 min. at r.t. the mixture previously pipetted up and down 5 times. Lipoplexes were named as "Lipoplex(a)", "Lipoplex(b)" or "Lipoplex(c)" according to the duplex used ($siRNA_a$, $siRNA_b$ or $siRNA_c$).

Conventional pegylated lipoplex (C-PEG-Lipoplexes) were formed as previously described for Lipoplexes but using preformed pegylated liposome (C-PEG-Liposome). Also in this case, C-PEG-Lipoplexes were named as "C-PEG-Lipoplex(a)", "C-PEG-Lipoplex(b)" or "C-PEG-Lipoplex(c)" according to the duplex used ($siRNA_a$, $siRNA_b$ or $siRNA_c$).

Post-pegylated lipoplex (P-PEG-Lipoplexes) were formed starting from Lipoplexes. Lipoplexes were incubated with preformed DSPE-PEG_{2000} micelles in a water bath at 37 ± 1°C for 1 h under gently mixing, as described for P-PEG-Liposome. P-PEG-Lipoplexes were named as "P-PEG-Lipoplex(a)", "P-PEG-Lipoplex(b)" or "P-PEG-Lipoplexe(c)" according to the duplex used ($siRNA_a$, $siRNA_b$ or $siRNA_c$).

All lipoplexes were formulated in at least 2 lots and used for experiments within 1 h from the preparation.

A description of Liposomes and Lipoplexes composition is reported in supplementary, T1.

2.4 Physicochemical characterization of samples (Liposomes and Lipoplexes)

2.4.1 Photon correlation spectroscopy (PCS) Mean particle size (Z-Average), size distribution and polydispersivity index (PDI) were determined at r.t. in water (pH 6) or in cell cultured medium (RPMI added with FBS) using a Zetasizer Nano ZS (Malvern, UK). The results were expressed also as intensity distribution, i.e. the size below which is placed the 50% [D(50)] and 90% [D(90)] of all the particles. The zeta potential was measured in water (pH 6) at r.t. using the same equipment with a combination of laser Doppler velocimetry and phase analysis light scattering (PALS). Before the analyses, samples were diluted at 100 µg/mL of lipid.

2.4.2 Atomic force microscopy (AFM) The morphology of the samples was evaluated at r.t. with a Nanoscope IIIa microscope (Digital Instruments, Tonawanda, NY, USA) equipped with a commercial silicon tip-cantilever (tip diameter, 5–10 nm; stiffness, 40Nm^{-1}; resonance frequency, 150 KHz). The height and the phase imaging data were simultaneously acquired. All the samples were analyzed without dilution at a scan rate of 1 Hz over a selected area using freshly cleaved mica as the substrate. The force applied to the surface was roughly adjusted by the ratio between the engaged or set point amplitude (Asp) and the free air amplitude (A_0). Images were processed and analyzed by using a program obtained from Gwyddion [34].

2.4.3 Transmission electron microscopy (TEM) The structural assembly of lipidic structures was analyzed by using negative staining electron microscopy (NS-TEM). Briefly, a drop of sample was placed on a 200-mesh formvar copper grid (TABB Laboratories Equipment, Berks, UK) and allowed to adsorb. The water surplus was removed by a filter paper. Then, a drop of 2% (w/v) aqueous solution of uranyl acetate was added and incubated for 5 min. Finally the aqueous solution surplus was removed by a blotting paper and the dried sample was observed by TEM (model JEM 2010; JEOL, Peabody, MA, USA) operating at an acceleration voltage of 200 KV.

2.5. Evaluation of loading efficiency (complexation efficiency)

The complexation efficiency was evaluated using a 5% (w/v) agarose gel electrophoresis analysis. For all the samples, 0.1 nmol of each siRNA duplex were complexed to liposomes at 1:100 molar ratio (genetic material:cationic lipid) and run for 1 h at 100 V in TBE buffer

(0.045 M tris-borate, pH 8.3 and 0.001 M EDTA) along with different amounts of free siRNA. The "loading" buffer was added to each sample. The non-complexed siRNA, that migrates as free siRNA during the electrophoresis analysis, was visualized under UV light (302 nm) after staining with ethidium bromide. The non-complexed siRNA was then quantified by using gel quantification software (Quantity One, IV Version, BIORAD Laboratories, Hercules, CA, USA). Loading efficiency was calculated comparing the intensity of the band of non-complexed siRNA in the sample with those of free siRNA at three different concentrations (0.01, 0.05 and 0.1 nmol). Data were expressed as complexation efficiency applying the following formula:

Complexation Efficiency % = [(0.1 – non-complexed siRNA nmol) *100]/0.1

Assay was carried out twice for each preparation batch.

2.6 Cell experiments

2.6.1 In vitro cell line HHV-8 positive PEL cell lines (namely BCBL-1 and CRO-AP/3) were cultured in RPMI-1640 medium supplemented with 10% (v/v) of heat-inactivated fetal bovine serum (FBS), L-glutamine 1 mM and antibiotics (complete medium). Cell culture was maintained at 37°C in a humidified incubator with 5% CO_2 atmosphere.

Before the transfection experiments, density gradient separation procedure was used to isolate and separate viable cells and naturally apoptotic cells [35]. Briefly, the cell suspension was diluted by addition of an equal volume of 0.9% (w/v) NaCl aqueous solution. The dilute suspension was laid over a mixture of sodium diatrizoate (9.1%, w/v) and polysaccharide (5.7%, w/v) solution (density, 1.007 g/mL; osmolarity, 290 mOsm) and centrifuged at 1,800 rpm for 25 min at r.t.. After centrifugation, the viable cells formed a distinct band at the sample/solution interface, while apoptotic/dead cells and cell debris were concentrated on the bottom. The viable cells were removed from the interface, twice washed using culture medium and re-seeded. Using this technique, the negative controls showed very low levels of dead cells (PEL cell viability >95%).

2.6.2 Transfection experiments To carry out the transfection experiments, 1.25×10^5 cells were seeded in a total volume of 0.1 mL of complete culture medium (supplemented with 2.5% of FBS) in a 24 wells plate.

Initially, empty liposomes (Liposome, P-PEG-Liposome and C-PEG-Liposome) were added on cells at different concentrations (5, 10 and 20 μM) to identify the carrier concentrations without cytotoxic effects on the PEL cell line. Afterwards, complexes were separately formed

in function of the three siRNA [Lipoplex(a), Lipoplex(b), Lipoplex(c); C-PEG- Lipoplex(a), C-PEG- Lipoplex(b), C-PEG- Lipoplex(c); P-PEG- Lipoplex(a), P-PEG- Lipoplex(b), P-PEG- Lipoplex(c)]. The cells were transfected by the mix obtained combining each single type of complex that is generally defined Lipoplexes, C-PEG- Lipoplexes and P-PEG- Lipoplexes. As control, a combination of free siRNAs ($siRNA_a$, $siRNA_b$ or $siRNA_c$) was tested. Treated cells were re-incubated at 37 ± 0.1°C /CO_2 5%, adding 0.4 mL of complete medium (supplemented with 5% of FBS) after 1 h.

2.6.3 Analysis of cell cytotoxicity Cell viability and cell density were recorded by flow cytometry (FacsCalibur, BD Biosciences, CA, USA), 42 h after transfection. After this incubation time, the cells were gently re-suspended and analyzed without any treatment. The instrument was equipped with a 15mW 488nm air-cooled argon laser (for full details, please refer to the Supplementary Information – Methods).

Alternatively, PEL viability was assayed by staining with Annexin V/Propidium Iodide (PI) (Miltenyi Biotech, Bergisch Gladbach, Germany), in accordance with manufacturer's instructions (for full details, please refer to the Supplementary Information – Methods).

Data were analyzed by CellQuest (BD Biosciences) and Summit software (Dako Colorado, Fort Collins, CO, USA).

2.6.4 Western blot analysis As Blimp-1 mRNA and protein have half-lives of <2 and 4 hours respectively [35], level of protein was measured by Western blot analysis 15 h after lipoplexes transfection.

Total cell lysates were obtained through Mammalian Cell Extraction Kit (BioVision), performed according to the manufacturer's instructions. Protein content of the lysates was assessed by the Bradford assay and equalized in order to load 20 or 30 µg of protein per well. Proteins were separated by Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis (SDS-PAGE), under reducing condition, using 8% precast gels (Biorad). Gels were run until the protein marker (Biorad Precision Plus Protein Dual Color) reached the bottom of the gel, using a standard Tris-Glycine-SDS buffer. Proteins were then transferred onto nitrocellulose membranes in a standard transfer buffer with 20% methanol, using a wet blotting system. The resulting membranes were blocked in 5% milk (Biorad) and then incubated overnight at 4°C with antibodies specific to Blimp-1 (C14A4, Cell Signaling) and β-Tubulin (9F3, Cell Signaling). On the following day, the membranes were washed with TBS-Tween 20 and then incubated for 1 h with appropriate horseradish peroxidase-conjugated secondary antibodies

(donkey anti-rabbit IgG, Amersham Biosciences). After further washing out the immunoreactive solution, proteins were detected by the enhanced chemioluminescence system (ECL) (Biorad) and the chemiluminescent signal was capture using ChemiDoc XRS + Imaging system (Biorad). Band intensity of the target proteins was evaluated through the Image Lab 3.0 Software (Biorad). Target protein levels were normalized using β-Tubulin as loading control protein.

2.7 Statistical analysis

All data were expressed as averages with standard deviations. Mean comparisons were performed by the Student t-test. The difference was considered significant when the p-value was less than 0.05.

3. RESULTS

3.1. Physicochemical characterization of Lipoplexes, C-PEG Lipoplexes and P PEG Lipoplexes

Lipoplexes were chosen as references considering their efficiency in therapeutic siRNA delivery into PEL cells after intraperitoneally administration in PEL mice. Also the specificity of silencing mediated by siRNAs anti BLIMP-1 was previously supported by *in vitro* experiments by using a mock siRNA [12]. The Z-Average, PDI, zeta potential of the samples are reported in Table 1.

Cationic liposome (Liposome) and $siRNA_a$ formed stable and monodisperse (PDI of about 0.05) complex with a Z-Average of about 300 nm. Both $siRNA_b$ and $siRNA_c$ rearranged with Liposome forming complexes with higher mean diameter (Z-Average up to 400 nm) and polidispersivity index (PDI>0.1) if compared with Lipoplex(a). Generally, zeta potential of Liposome (about 42 mV) strongly decreased after complexation (about 20mV). The electrostatic interaction between siRNA and Liposome induces the reorganization of lipids around the genetic material masking the positive charge of cationic lipids.

By AFM observation, Lipoplexes appeared as compact lipid-based structures with irregular shape and surface (Figure 1,a). Applying TEM analysis (Figure 1,b), Lipoplexes showed a complicated reorganization of lipids around the genetic materials while starting liposomes appeared as homogeneous vesicles delimited by a contrasted layer (Figure 1,c). No free duplex was detectable by both the microscopic analyses.

Conventional pegylated lipoplexes (C-PEG-Lipoplexes), appeared as large structures with a wide size distribution and a surface charge proximally to the neutrality (see Table 1). Starting conventional pegylated liposome (C-PEG-Liposome) was stable without remarkable difference in the particle size (about 140 nm with a PDI value near 0.1) respect to the cationic liposome. Moreover, zeta potential appeared only partially reduced by the presence of PEG moieties on the surface (Table 1). Upon mixing with siRNA, the particle size of pegylated complexes increased from 130 ± 15 nm of C-PEG-Liposome to 1,300 ± 200 nm, 2,500 ± 450 nm and 1,400 ± 230 nm for C-PEG-Lipoplex(a), C-PEG-Lipoplex(b) and C-PEG-Lipoplex(c), respectively. The PDI (>0.3) of these formulations suggested the presence of a heterogeneous population. Dimensional distribution (Figure S1) identified a bimodal formulation characterized by two defined and narrow peaks: the first at low values is typical of un-complexed pegylated liposomes while the second, higher than 1 μm, is probably associated with irregular aggregates between C-PEG-Liposome and siRNA. zeta potential significantly decreased from 33±1 mV (C-PEG-Liposome) to about 6±1 mV for all the three C-PEG-Lipoplexes. These findings suggested the presence of adsorbed siRNA on the liposomal surface. According with the PCS analysis, both AFM and TEM observations confirmed well detectable morphological differences between Lipoplexes and C-PEG-Lipoplexes. C-PEG-Lipoplexes were characterized by the aggregation of C-PEG-Liposome around the siRNAs molecules, forming complicated complexes in which both genetic and un-formed materials were detectable (Figure 1,d and 1,e). The presence of non-complexed and docked siRNA fragments on liposomal surface suggested an unstable complexation.

Similarly to cationic lipoplexes (Lipoplexes), post-pegylated lipoplexes (P-PEG-Lipoplexes) were stable and monodisperse structures with the size ranging from 300 to 420 nm, and a low PDI value (< 0.1). DSPE-PEG_{2000} was insert on preformed complexes (Lipoplexes) under conditions that allow the transfer of lipo-polymer from micelles to the lipoplexes (37°C, under agitation); after the incubation zeta potential values resulted lower than those of un-modified Lipoplexes and with narrow range (from 10 to 12 mV), suggesting the presence of an homogeneous PEG coverage of the complex surface. AFM images (Figure 1, g) reported compact structures with symmetrical shape and regular surface with only few aggregates. TEM images (Figure 1,h) well identified the architecture of P-PEG-Lipoplexes, i.e. a) high electron dense core of preformed Lipoplexes, in which the lipidic lamellae typical of liposomes and the encapsulated siRNAs (indicate by arrows) were identifiable; b) the laminar

texture of the outer layers that is the evidence of a compact coverage characterized by a bright contrast due to the pegylation [31].

	Samples	Z-Average [nm]	(PDI)	D50 [nm]	D90 [nm]	Zeta potential (mV)
Liposome		145±15	0.09±0.01	165 ±10	250±12	42±5
Cationic lipoplexes	**Lipoplex (a)**	300±15	0.05±0.01	310±10	425±17	19±1
	Lipoplex(b)	390±25	0.13±0.02	410±15	5oo±20	20±1
	Lipoplex (c)	450±30	0.11±0.01	450±10	570±13	17±1
C-PEG-Liposome		130±15	0.11±0.01	140±10	250±8	33±1
Conventional pegylated lipoplexes	**C-PEG-Lipoplex (a)**	1,300±200	0.31±0.11	1000±30	2400±80	6±1
	C-PEG-Lipoplex (b)	2,500±450	0.48±0.22	>2000	>4000	5±1
	C-PEG-Lipoplex (c)	1,400±230	0.57±0.13	1340±15	>4000	6.2±1
P-PEG-Liposome		140±8	0,11±0.03	150±3	235±9	27±4
Post-pegylated lipoplexes	**P-PEG-Lipoplex (a)**	320±30	0.06±0.01	320±5	430±7	12±1
	P-PEG-Lipoplex (b)	350±35	0.04±0.01	345±8	435±10	10±
	P-PEG-Lipoplex (c)	410±55	0.17±0.02	410±10	550±10	12±1

Table 1: P*hysicochemical and technological parameters of liposomes and lipoplexes. Values are the mean ±S.D. (n = 9). All the increments in mean diameter of lipoplexes resulted significant respect to liposomes. Differences between the complexes generated starting from liposome formulations and three different siRNA (Lipoplex(a), Lipoplex(b), Lipoplex(c) and P-PEG-Lipoplex(a), P-PEG-Lipoplex(b), P-PEG-Lipoplex(c)) resulted not significant accordingly to Student t-test*

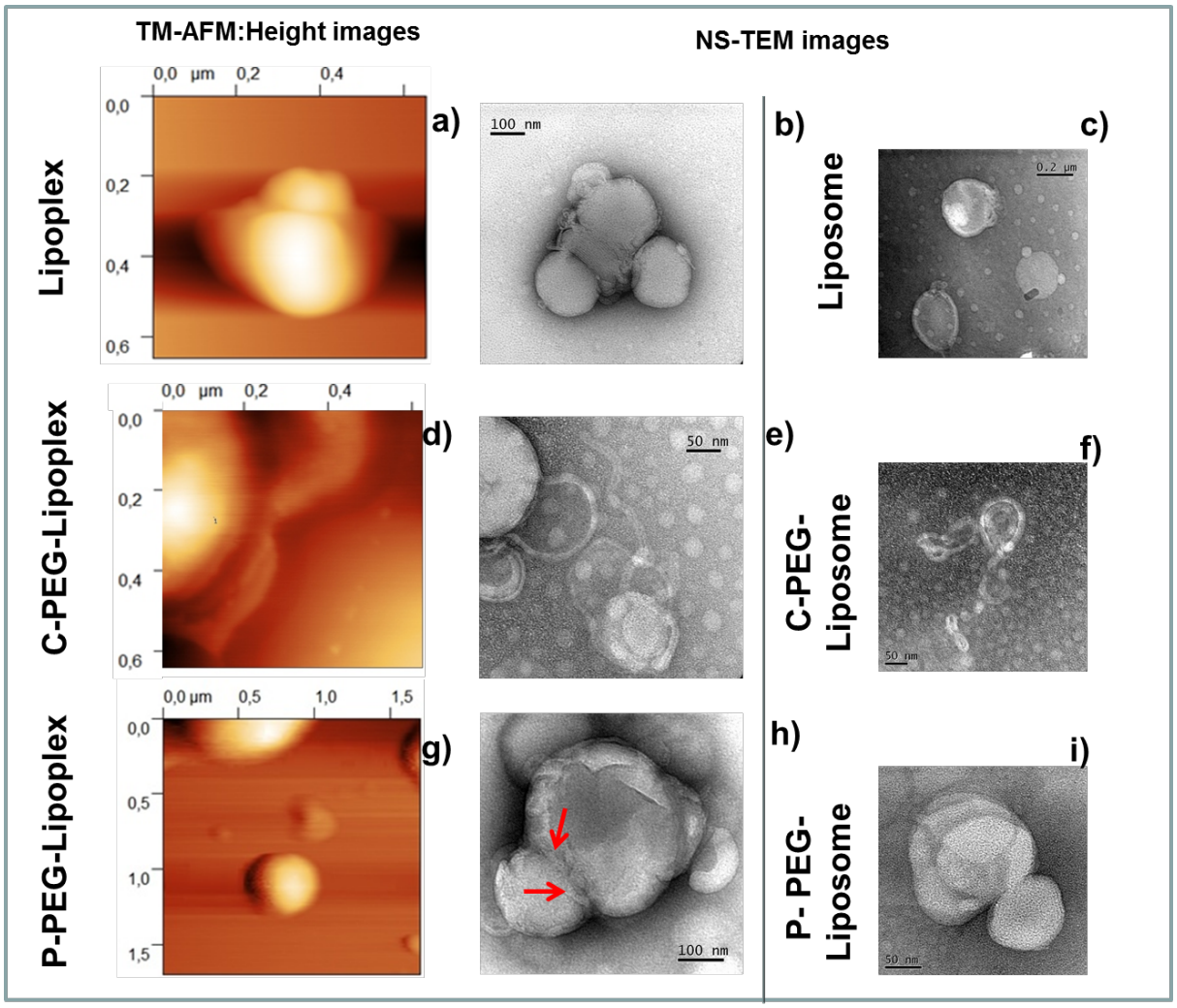

Fig.1: Morphological characterization of samples. *AFM height images of a) Lipoplex(a), d)C-PEG- Lipoplex (a) and g) P-PEG- Lipoplex (a). TEM images of b) Lipoplex (a), c)Liposome, e)C-PEG-Lipoplex(a), f)C-PEG-Liposome, h)P-PEG-Lipoplex, i) P-PEG-Liposome.*

3.2. Influence of PEGylation on siRNAs complexation capacity.

Lipoplexes, C-PEG-Lipoplexes and P-PEG-Lipoplexes obtained by using $siRNA_a$, $siRNA_b$ and $siRNA_c$ were subjected to agarose gel electrophoresis to detect non-complexed siRNA and to measure the complexation efficiency of the carriers.

Figure 2 reports the runs of free siRNAs, Lipoplexes, C-PEG-Lipoplexes and P-PEG-Lipoplexes and the quantification of the non-complexed siRNA.

Free siRNA (0.1 nmol; control) and the non-complexed siRNA migrated as a single bright band wholly detectable at the end of the run after ethidium bromide reaction (Figure 2).

siRNA were almost completely taken up in Lipoplexes (Figure 2). Only slight shadows related to non-complexed active were detectable at the end of the run. Also the quantification assay demonstrated that the efficiency of complexation was greater than 90% for each

complex analyzed. Classic pegylated lipoplexes (C-PEG-Lipoplexes) did not form stable complexes with siRNA as proven by the great amount of un-bound siRNA migrated during the analysis. Complexation efficiency was, in all cases, less than 15%. Differently, post-pegylated lipoplexes (P-PEG-Lipoplexes) were able to stabilize high amount of siRNA as revealed by the faint brightness of bands of the related non-complexed siRNA. The complexation of $siRNA_a$ and $siRNA_b$ was close to 80% while that of $siRNA_c$ decreased to about 70%.

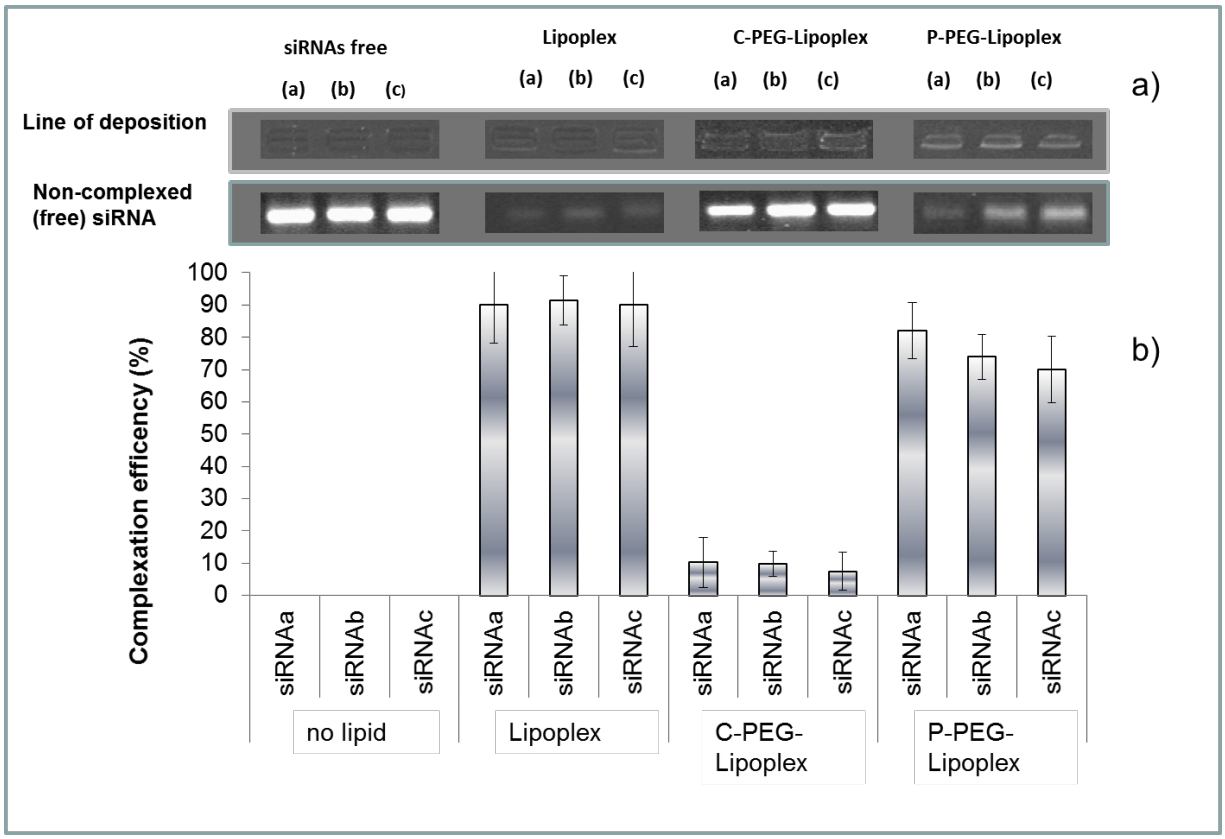

Fig 2: Complexation efficiency of lipoplexes: *a) agarose gel electrophoreses of lipoplexes; for each line the same amount (0.1nmoles) of $siRNA_a$ (lane a), $siRNA_b$ (lane b) and $siRNA_c$ (lane c) free and complexed, were loaded. Only non-complexed siRNA resulted able to migrate thought the gel being visible at the end of the run. b) quantification of complexation efficiency by Quantity One software. Data represent mean value of three independent experiments performed with different lipidic formulations.*

3.3. Toxicity of lipidic carriers

The cell density and the cell viability over the values of 90% and 80%, respectively, were maintained until the concentration of 10 µM of cationic lipids transfected by using cationic liposome (Liposome) or pegylated liposomes (C-PEG-Liposome or P-PEG-Liposome). No differences were observed changing the modality of PEG insertion (Figure 3).

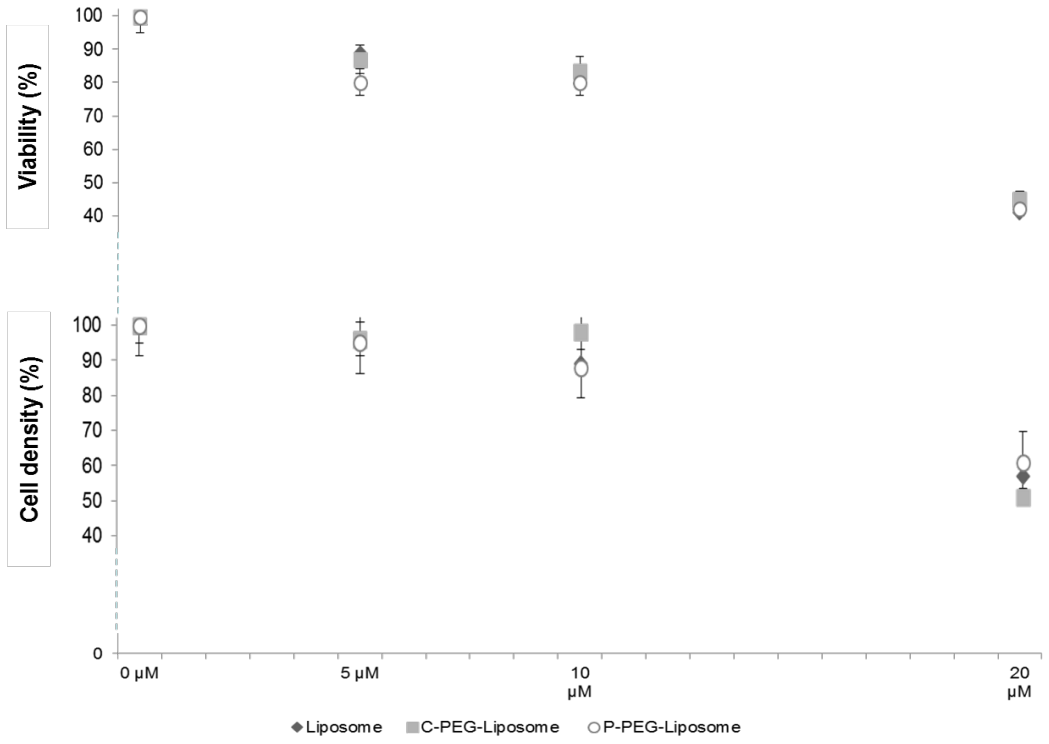

Fig 3: Toxicity of liposomal formulation: *a) % of viability and b) % of cell density of BCBL-1 after treatment with Liposome (♦), C-PEG-Liposome (■) and P-PEG-Liposome (○). Values were normalized respect to un-treated cells that were considered as 100% of viability and cell density (0 µM refers to negative control that was treated with only water).*

3.4. siRNA delivery by Lipoplexes

To evaluate the transfection activity and the therapeutic efficiency of lipofection approach, both Blimp-1 silencing level and antineoplastic effects were measured BCBL-1 cells (Figures 4 and 6). To validate the efficiency of our systems, data related to the transfection experiments of 100 nM of siRNA complexed performed on another PEL cell line, CRO-AP/3, are reported in supplementary file (Figure S5).

No significant effect was observed after transfection of all free carriers (Liposome, C-PEG-Liposome or P-PEG-Liposome) and after treatment with siRNAs free at 100 nM, which was the maximal concentration of siRNAs transfected.

Initially, un-modified lipoplexes (Lipoplexes) were evaluated. Testing two different concentrations (50 and 100 nM) of complexed siRNA, corresponding to 5 and 10 µM of lipid, respectively, a cytotoxic dose-dependent effect associated with a Blimp-1 level reduction was measured. Cell density decreased to 52 ± 3% and 14 ± 9% after 50 nM and 100 nM of siRNA complexed in Lipoplexes, respectively (Figure 4,b). Moreover, the cell viability decreased until to 32 ± 1% for the higher siRNA concentration (100 nM).

Reduction in cell viability was confirmed by the increase in Annexine V and PI positive cells indicating a high level of cells in end stage apoptosis or dead (Figure 4,c).

As evident in Figure 4,a, the reduction of Blimp-1 level was about 20% and 45% after transfection with 50 nM and 100 nM of siRNAs complexed, respectively.

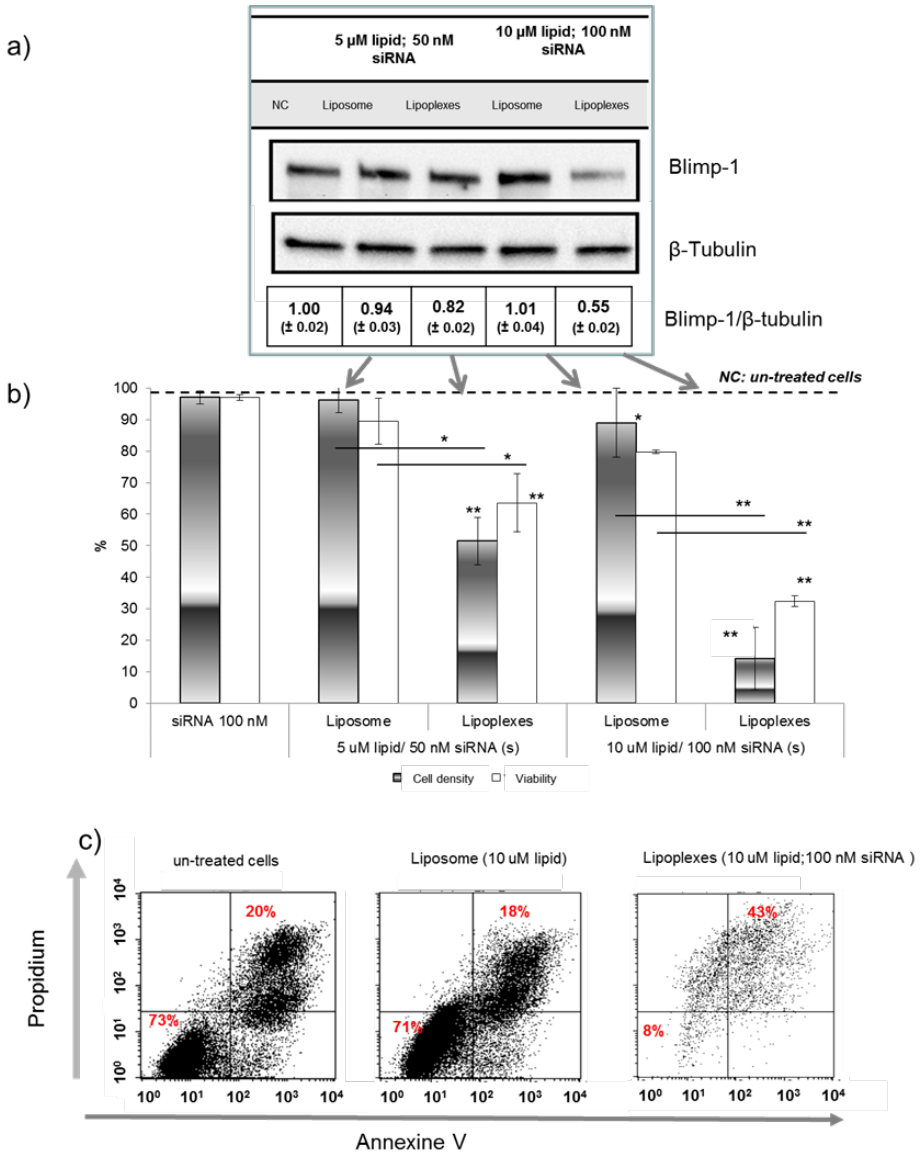

Fig. 4: siRNAs transfection in BCBL-1 mediated by Lipoplexes*: a) Western blot reports the Blimp-1 silencing mediated by Lipoplexes using β-tubulin as control. Protein levels in un-treated cells were compared to that observed after different concentrations of siRNAs (50 nM and 100 nM) transfected by Lipoplex (complex) and measured 15 hours after transfection. Treatment with Liposome without siRNA is also reported. Data represent mean values of three independent experiments. b) % of both cell density (gray bars) and viability (white bar) recorded 48 hours after treatment with siRNA alone, Liposome and Lipoplexes at different concentrations of siRNA transferred (50 and 100 nM). Results are normalized to control represented by un-treated cells maintained in the same cellular condition.*

Data represent mean values of three independent experiments, performed in triplicate wells for each condition.

Statistical analyses was performed:

** p<0.05 vs siRNA non-complexed; ** p<0.02 vs siRNA non-complexed. Bars reported the analyses compared to the negative control Liposome*

c) Annexine/Pi analyses: cells in the lower left quadrant (Annexin V−/PI−) are viable, those in the lower right quadrant (Annexin V+/PI−) are early apoptotic and those in the upper right quadrants (Annexin V+/PI+) are late apoptotic or necrotic.

The inability to protect and stabilize siRNAs during complexation made inefficient the transfection mediated by complexes prepared with conventional pegylated liposome (C-PEG-Lipoplexes) (Figure 5).

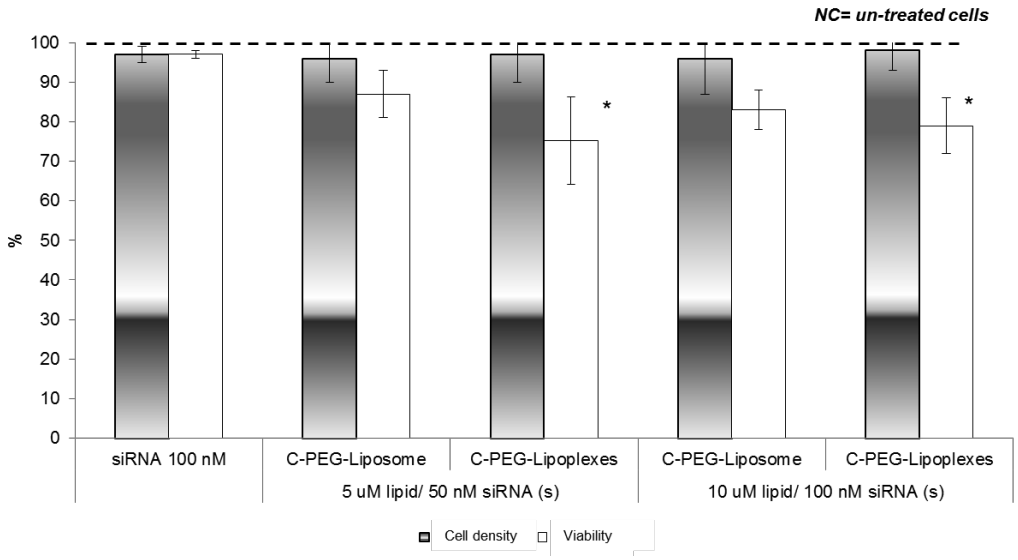

Fig. 5: siRNAs transfection in BCBL-1 mediated by C-PEG-Lipoplexes*: % of cell density (gray bars) and viability (white bar) of cells treated with siRNAs alone or C-PEG-Liposomes and C-PEG-Lipoplex at different concentrations of siRNAs transferred (50 and 100 nM). Results are normalized to control represented by un-treated cells maintained in the same cellular condition. Data represent mean values of three independent experiments, performed in triplicate wells for each condition.*

*Statistical analyses was performed: * $p<0.05$ vs siRNA non-complexed; ** $p<0.02$ vs siRNA non-complexed*

On the contrary, the efficiency of siRNA-mediated gene silencing after transfection by using post-pegylated lipoplexes (P-PEG-Lipoplexes) was confirmed both by cytometric and western blot analysis.

P-PEG-Lipoplexes produced a significant cytotoxic effect both in BCBL-1 and CRO-AP/3 cell lines (Figure 6,b and Supplementary Figure S5).

In details, after treatment with P-PEG-Lipoplexes, BCBL-1 cells underwent to a massive apoptosis being positive to Annexine V/PI as reported in Figure 6,c. A dose-dependent reduction of cell density (from 56±7 % to 26±7% after treatment with 50 and 100 nM of siRNAs respectively) and cell viability (from 64±4% to 36±8% after treatment with 50 and 100 nM of siRNAs respectively) was observed (Figure 6,b).

Also in this case, the quantification of Blimp-1 silencing reveled a reduction of the protein level, which resulted approximately 10% and 40% respect to un-treated cells, using 50 nM and 100 nM of siRNAs complexed, respectively (Figure 6,a).

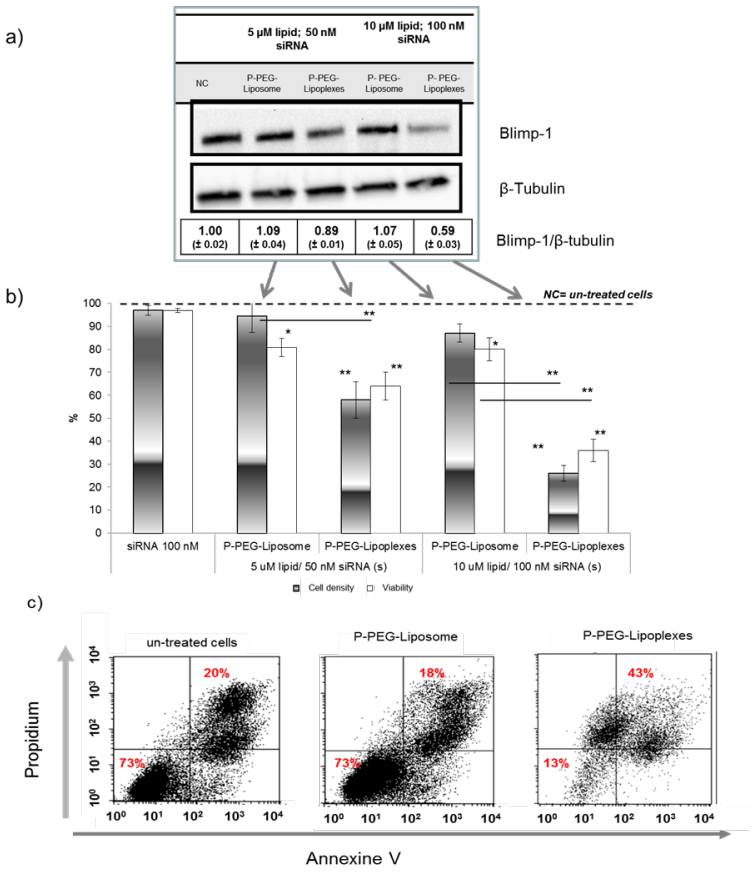

Fig 6: siRNAs transfection in BCBL-1 mediated by P-PEG-Lipoplexes*: a) Western blot reports the Blimp-1 silencing mediated by P-PEG-Lipoplexes using β-tubulin as control. Protein levels in un-treated cells were compared to that observed after different concentration of siRNAs (50 nM and 100 nM) transfected by P-PEG-Lipoplexes (complex), measured at 15 hours after transfection. Treatment with P-PEG-Liposome without siRNAs as reported. Data represent mean values of three independent experiments. b) % of both cell density (gray bars) and viability (white bar) 48 hours after treatment with siRNAs alone , P-PEG-Liposome and P-PEG-Lipoplexes at different concentrations of siRNAs transferred (50 and 100 nM). Results are normalized to control represented by un-treated cells maintained in the same cellular condition. Data represent mean values of three independent experiments, performed in triplicate wells for each condition. Statistical analyses was performed: * p<0.05 vs siRNAs non-complexed; ** p<0.02 vs siRNAs non-complexed. Bars reported the analyses compared to the negative control Liposome*

c) Annexine/Pi analyses: cells in the lower left quadrant (Annexin V−/PI−) are viable, those in the lower right quadrant (Annexin V+/PI−) are early apoptotic and those in the upper right quadrants (Annexin V+/PI+) are late apoptotic or necrotic.

Interestingly the transfection ability of both Lipoplexes and P-PEG-Lipoplexes did not seemed to be related to the presence of serum. The cytotoxicity resulted substantially unchanged after transfection at high FBS concentrations, thus supporting the in vivo translatability (see supplementary S2).

Taking advantage of the greater transfection ability of P-PEG-Lipoplexes made with 2% molar of DSPE-PEG_{2000}, high amount of DSPE-PEG was also tested, formulating lipoplexes with 5% molar of DSPE-PEG_{2000} (5% P-PEG-Lipoplex).

Despite of a high complexation efficiency (value in the range of 80-90%, Figure S3,a), the deposition of 5% molar of DSPE-PEG_{2000} micelles on preformed Lipoplexes decreased the efficiency to transfect siRNAs (Figure S3,b). Particularly, the same concentration of siRNAs transfected using P-PEG-Lipoplexes with a 5% of PEG coverage did not produce the same silencing obtained with complexes pegylated with a 2% PEG.

Cells maintained high cell density (over 70% or over 40%) after the transfection of 50 nM or 100 nM of siRNAs in 5% P-PEG-Lipoplexes, respectively. This data appeared attenuate if compared with the transfection mediated by 2% P-PEG-Lipoplexes (56% and 26% respectively).

At the same manner, the effect on cells viability of siRNAs transfected by 5% P-PEG-Lipoplexes was attenuated if compared with 2% P-PEG-Lipoplexes (the value ranged between 50 and 60% for all the concentrations of the siRNAs used) probably by the presence of highly concentrated coverage.

4. Discussion

Recently, experimental evidences suggested Blimp-1 as one of the possible target protein in PEL treatment. Indeed, the silencing of this transcriptional factor, that plays an important role during plasmacytic differentiation and is highly expressed both in normal and transformed plasma cells [36], seems to induce an important therapeutic response against PEL and other lymphoproliferative diseases [37].

The transfection of anti Blimp-1 siRNAs mediated by cationic lipidic complexes (Lipoplexes) was associated to a rapid induction of apoptosis and cytotoxic effect in PEL cell line. Those results were also confirmed during *in vivo* studies on PEL murine model in which we have observed a significant improvement in overall survival after intraperitoneally administration of Lipoplexes [12].

Lipoplexes clearly described the new entities formed between siRNAs and liposomes. Complexation of siRNAs with lipids transformed unilamellar cationic liposomes into supramolecular assemblies. These articulated concentric multilamellar structures can be compared to the well-known ‘‘sandwich’’ structures [33,38], in which siRNA molecules resulted un-detectable because they are efficiently stabilized between the lipidic layers.

The *in vivo* destiny of cationic complexes after IV administration is largely described, these structures rapidly interact with proteins leading to the formation of a dense coverage (protein corona) able to drive the biological features (increasing the RES uptake), promote the aggregation and influence both the geometry and the packaging of gene material into the complex [39].

Upon incubation with high percentage of FBS at 37°C, an increase of medium diameter (Z-Average) of Lipoplexes occurred within a few min, suggesting serum proteins coating the complex or penetrating into the complex to various extents (see Supplementary, figure S3,a). Of note, as reference, Lipoplexes incubated in the absence of serum did not show any aggregation or significant changes in size measures. Notwithstanding the interaction with serum components that lead to a size increase, the transfection efficiency of Lipoplexes was conserved also at high amount of FBS in medium (up to 10%, see supplementary Figure S2) suggesting the maintenance of the siRNA-lipids packaging.

In order to stabilize lipidic complexes we have investigated the PEGylation procedure that is commonly applied to extend the systemic circulatory lifetime without both toxicity and induction of immune response [40].

Preformed pegylated liposome resulted unable to form stable complexes with siRNAs. PEG creates an hydrophilic barrier that hampered the penetration of gene material through lipid bilayers, thus in C-PEG-Lipoplexes, siRNAs molecules resulted prevalently exposed on the surface of lipidic structures, unprotected and easily degradable from serum.

To avoid interference of PEG during the lipoplexes formation, a post pegylation strategy was used. In this case, siRNAs were previously mixed with Liposome assuring an efficient electrostatic interaction leading to siRNA stabilization into cationic core, then, incubation with DSPE micelles occurred. The insertion of poly(ethylene glycol) derivatized phospholipid into pre-formed liposomes is reported in literature [41,42].

When in aqueous medium, lipid monomers are known to transfer from one lipid phase to another, in a time and temperature dependent mode [43]. Referring to PEG-DSPE, the micelles demonstrated good stability at the low-temperature condensed phase (< 12°C) due the higher activation barrier to monomer desorption from the micelle, but the micellar state is

thermodynamically unstable; increasing temperature the monomer desorption begins [44]. PEG transfer was then achieved at 37°C that corresponds to a temperature in which both micelles instability and DOTAP phase transition (T_m=5°C) occurred; incubation time was set at 1h to lead a thermodynamic process took place.

Several articles suggest that the percentage of PEG (exposed on surface) needed to stabilize the particles ranged between 2 and 5% mol if compared to the lipidic composition [45, 46]. Moreover, a brush conformation of PEG moieties exposed on particle surface is the most suitable to reduce plasma protein inducing opsonization [40]. Our post-pegyalated complexes, P-PEG- Lipoplexes, with a diameter in the range 300-350 nm and only a minimal amount of PEG (we used 2% molar of DSPE-PEG_{2000} respect to DOTAP) efficiently formed and protect siRNAs (complexation efficiency about 80%). Even if we did not figure-out the active conformation of PEG, the physiochemical characterization by using advanced microscopical and spectroscopical analysis demonstrated the presence of the PEG moieties prevalently arranged on the outer layer of complexes forming a dense coverage. As largely described, the presence of PEG on lipidic complex surface reduce the amount of serum protein adsorbed [48]. P-PEG-Lipoplexes formed using 2% of DSPE-PEG (respect to cationic lipid) resulted stable after incubation with different percentage (2.5%v/v and 50% v/v) of FBS, as suggested by the maintenance of the original formulative size (see supplementary, Figure S3,b). PEG coating did not interfere with the interaction and the release of siRNAs from lipoplexes into cells. In fact, P-PEG-Lipoplexes well mediated the siRNAs transfection, independently from the presence of FBS, causing BLIMP-1 inhibition and cell apoptosis (Figure 6 and supplementary Figure S2).

5. Conclusion

The post-insertion of PEG on preformed lipoplexes demonstrated great potential for masking siRNAs and minimizing the interaction with serum proteins. Then, P-PEG-Lipoplexes will be tested in *in vivo* studies evaluating both the ability to stabilize and to efficiently deliver siRNAs in PEL animal model. The safety of the carriers and the cytotoxic effect exerted by siRNAs should be evaluated to define the clinical applicability of this approach.

Acknowledgement

The authors gratefully acknowledge the professional technical assistance of Dr. Massimo Tonelli and of Centro Grandi Strumenti, University of Modena and Reggio Emilia for his assistance in microscopical analysis.

References:

[1] M. Masiero, G. Nardo, S. Indraccolo, E. Favaro, RNA interference: implications for cancer treatment, Mol. Aspects Med. 28 (2207) 143–166.

[2] V. P. Torchilin, Drug targeting, Eur. J. Pharm. Sci. 11 (2000) S81 –S91.

[3] L. Haddad, H. El Hajj, R. Abou-Merhi, Y. Kfoury, R. Mahieux, M. El-Sabban, A. Bazarbachi, KSHV-transformed primary effusion lymphoma cells induce a VEGF-dependent angiogenesis and establish functional gap junctions with endothelial cells, Leukemia. 22 (2008) 826–834.

[4] A. de Fougerolles, H.P. Vornlocher, J. Maraganore, J. Lieberman, Interfering with disease: a progress report on siRNA-based therapeutics, Nat. Rev. Drug Discov. 6, (2007) 443–453.

[5] J. Tabernero, G.I. Shapiro, P.M. LoRusso, A. Cervantes, G.K. Schwartz, G.J. Weiss, L. Paz-Ares, D.C. Cho, J.R. Infante, M. Alsina, M.M. Gounder, R. Falzone, J. Harrop, A.C. White, I. Toudjarska, D. Bumcrot, R.E. Meyers, G. Hinkle, N. Svrzikapa, R.M. Hutabarat, V.A. Clausen, J. Cehelsky, S.V. Nochur, C. Gamba-Vitalo, A.K. Vaishnaw, D.W. Sah, J.A. Gollob, H.A. Burris, First-in-Humans Trial of an RNA Interference therapeutic targeting VEGF and KSP in cancer patients with liver, lnvolvement, Cancer Discovery. 3 (2013) 40-46.

[6] J.C. Burnett, J.J. Rossi, K. Tiemann, Current progress of siRNA/shRNA therapeutics in clinical trials, Biotechnol. J. 6 (2011) 1130-1146.

[7] G. Ozcan, B. Ozpolat, R.L. Coleman, A.K. Sood, G. Lopez-Berestein, Preclinical and clinical development of siRNA-based therapeutics, Adv. Drug Deliv. Rev. (2015), doi: 10.1016/j.addr.2015.01.007. [Epub ahead of print]

[8] B. Ozpolat, A.K. Sood, G. Lopez-Berestein, Nanomedicine based approaches for the delivery of siRNA in cancer, J. Intern. Med. 267 (2010) 44-53.

[9] Y.C. Tseng, S. Mozumdar, L. Huang, Lipid-based systemic delivery of siRNA, Adv. Drug Deliv. Rev. 61 (2009) 721–731.

[10] S.Y. Wu, N.A. McMillan, Lipidic Systems for In Vivo siRNA Delivery, AAPS J.11 (2009) 639-652.

[11] T. Tagami, T. Suzuki, M. Matsunaga, K. Nakamura, N. Moriyoshi, T. Ishida, H. Kiwada, Anti-angiogenic therapy via cationic liposome-mediated systemic siRNA delivery, Int. J. Pharm. 422 (2012) 280-289.

[12] G. Riva, I. Lagreca, A. Mattiolo, D. Belletti, L. Lignitto, P. Barozzi, B. Ruozi, D. Vallerini, C. Quadrelli, G. Corradini, F. Forghieri, R. Marasca, F. Narni, G. Tosi, F. Forni, M. A. Vandelli, A. Amadori, L. Chieco-Bianchi, L. Potenza, M. L. Calabrò, M. Luppi, Antineoplastic effects of liposomal siRNA treatment targeting BLIMP1/PRDM1 in primary effusion lymphoma. Haematologica. Accepted. (2015).

[13] A. Carbone, A. Gloghini, HHV-8-associated lymphoma: state-of-the-art review, Acta Haematol. 117 (2007) 129-131.

[14] W. Li, F.C. Jr Szoka, Lipid-based nanoparticles for nucleic acid delivery, Pharm. Res. 24 (2007) 438–449.

[15] H. Eliyahu, N. Servel, A.J. Domb, Y. Barenholz, Lipoplex-induced hemagglutination: potential involvement in intravenous gene delivery, Gene Ther. 9 (2002) 850–858.

[16] F. Sakurai, T. Nishioka, F. Yamashita, Y. Takakura, M. Hashida, Effects of erythrocytes and serum proteins on lung accumulation of lipoplexes containing cholesterol or DOPE as a helper lipid in the single-pass rat lung perfusion system, Eur. J. Pharm. Biopharm. 52 (2001) 165–172.

[17] J.S. Zhang, F. Liu, L. Huang, Implications of pharmacokinetic behavior of lipoplex for its inflammatory toxicity, Adv Drug Deliv Rev. 57 (2005) 689-698.

[18] J. D. Tousignant, A. L. Gates, L. A. Ingram, C. L. Johnson, J.B. Nietupski, S.H. Cheng, S.J. Eastman, R.K. Scheule, Comprehensive analysis of the acute toxicities induced by systemic administration of cationic lipid: plasmid DNA complexes in mice, Hum Gene Ther. 11 (2000) 2493–2513.

[19] P. Resnier, P. LeQuinio, N. Lautram, C. Gaillard, G. Bastiat, J.P. Benoit, C. Passirani, Efficient in vitro gene therapy with PEG siRNA lipid nanocapsules for passive targeting strategy in melanoma, Biotechnol. J. 9 (2014) 1389-13401.

[20] E. Alaaeldin, A.S. Abu Lila, N. Moriyoshi, H.A. Sarhan, T. Ishida, K.A. Khaled, H. Kiwada, H. The co-delivery of oxaliplatin abrogates the immunogenic response to PEGylated siRNA-lipoplex, Pharm Res. 30 (2013) 2344-2354.

[21] K. Buyens, J. Demeester, S.S. De Smedt, N.N. Sanders, Elucidating the encapsulation of short interfering RNA in PEGylated cationic liposomes, Langmuir. 25 (2009) 4886-4891.

[22] T. Tagami, T. Suzuki, M. Matsunaga, K. Nakamura, N. Moriyoshi, T. Ishida, H. Kiwada, Anti-angiogenic therapy via cationic liposome-mediated systemic siRNA delivery, Int. J. Pharm. 422 (2012) 280-289.

[23] U. Rungsardthong, M. Deshpande, L. Bailey, M. Vamvakaki, S.P. Armes, M.C. Garnett, S. Stolnik, Copolymers of amine methacrylate with poly(ethylene glycol) as vectors for gene therapy, J. Control. Release. 73 (2001) 359–380.

[24] S. Pirotton, C. Muller, N. Pantoustier, F. Botteman, S. Collinet, C. Grandfils, G. Dandrifosse, P. Degée, P. Dubois, M. Raes, Enhancement of transfection efficiency through rapid and noncovalent post-PEGylation of poly(dimethylaminoethyl methacrylate)/DNA complexes, Pharm. Res. 21 (2004) 1471–1479.

[25] D. Oupicky, M. Ogris, K.A. Howard, P.R. Dash, K. Ulbrich, L.W. Seymour, Importance of lateral and steric stabilization of polyelectrolyte gene delivery vectors for extended systemic circulation, Mol. Ther. 5 (2002) 463–472.

[26] V. Knorr, M. Ogris, E. Wagner, An acid sensitive ketal-based polyethylene glycol-oligoethylenimine copolymer mediates improved transfection efficiency at reduced toxicity, Pharm. Res. 25 (2008) 2937–2945.

[27] H. Hatakeyama, H. Akita, H. Harashima A multifunctional envelope type nano device (MEND) for gene delivery to tumours based on the EPR effect: a strategy for overcoming the PEG dilemma Adv. Drug Deliv. Rev. 63 (2011), pp. 152–160

[28] H. Xu, K. Q. Wang, Y. H. Deng, W. Chen, Effects of cleavable PEG-cholesterol derivatives on the accelerated blood clearance of PEGylated liposomes, Biomaterials. 31 (2010) 4757-4763.

[29] L. Zhang, Y. Wang, Y. Yang,Y. Liu, S. Ruan, O. Zhang, X. Tai, J. Chen, T. Xia, Y. Qiu, H. Gao, Q. He, High Tumor Penetration of Paclitaxel Loaded pH Sensitive Cleavable Liposomes by Depletion of Tumor Collagen I in Breast Cancer, ACS Appl Mater Interfaces. 13 (2015) 9691-9701.

[30] C. Lu, D. J. Stewart, J. J. Lee, L. Ji, R. Ramesh, G. Jayachandran, M. I. Nunez, I. I Wistuba, J. J. Erasmus, M. E. Hicks, E. A. Grimm, J. M. Reuben, V. Baladandayuthapani, N. S. Templeton, J. D. McMannis, J. A. Roth, Phase I clinical trial of systemically administered TUSC2(FUS1)-nanoparticles mediating functional gene transfer in humans. PLoS One. 7 (2012) e34833.

[31] I. Tranchant, B. Thompson, C. Nicolazzi, N. Mignet, D. Scherman, Physicochemical optimisation of plasmid delivery by cationic lipids, J. Gene Med. 6 (2004) S24–S35.

[32] B. Ruozi, G. Riva, D. Belletti, P. Barozzi, P. M. Luppi, F. Forni, M.A. Vandelli, Immunoliposomal systems targeting the primary effusion lymphoma (PEL): in vitro study, Nanomedicine (UK). 5 (2010) 1051-1064.

[33] D. Belletti, M. Tonelli, F. Forni, G. Tosi, M.A. Vandelli, B. Ruozi, AFM and TEM characterization of siRNAs lipoplexes: A combinatory tools to predict the efficacy of complexation, Colloids Surf. A Physicochem. Eng. Asp. 436 (2013) 459-466.

[34] D. Nečas, P. Klapetekm, Gwyddion: an open-source software for SPM data analysis, Central European Journal of Physics. 10 (2012) 181-188.

[35] B. Ruozi, G. Riva, D. Belletti, G. Tosi, F. Forni, A. Mucci, P. Barozzi, M. Luppi, M.A. Vandelli, Cidofovir-loaded liposomes: an intro-study using BCBL-1 cell line as a model for primary effusion lymphoma, Europ. J. Pharm. Sci. 41 (2010) 254-264.

[36] M. Shapiro-Shelef, K.I. Lin, D. Savitsky, J. Liao, K. Calame, Blimp-1 is required for maintenance of long-lived plasma cells in the bone marrow, J. Exp. Med. 5 (2005) 1471-1476.

[37] F.R. Lin, H.K. Kuo, H.Y. Ying, F.H. Yang, K.I. Lin, Induction of apoptosis in plasma cells by B lymphocyte-induced maturation protein-1 knockdown, Cancer Res. 15 (2007) 11914-11923.

[38] M.C. Pedroso de Lima, S. Simoes, P. Pires, H. Faneca, N. Düzgüneş, Cationic lipid–DNA complexes in gene delivery: from biophysics to biological applications, Adv. Drug Del. Rev. 47 (2001) 277–294.

[39] O. Zelphati, L.S. Uyechi, L.G. Barron, F.C. Szoka, Effect of serum components on the physico-chemical properties of cationic lipid/oligonucleotide complexes and on their interactions with cells, Biochim Biophys Acta. 1390 (1998) 119-133.

[40] 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, J. Control. Release 126 (2008) 67–76.

[41] P. Vader, B.J. Crielaard, S.M. van Dommelen, R. van der Meel, G. Storm, R.M. Schiffelers, Targeted delivery of small interfering RNA to angiogenic endothelial cells with liposome-polycation-DNA particles, J. Control. Release. 160 (2012) 211-216.

[42] I. Tatsuhiro, D. Iden, T.M. Allen, A combinatorial approach to producing sterically stabilized (Stealth) immunoliposomal drugs, FEBS Letters. 460 (1999) 129–133.

[43] J.W. Nichols, Polymerizable phospholipids. In: Phospholipids Handbook. Cevc, G (Ed.), Marcel Dekker, New York, 1993; pp 663-685.

[44] M. Kastantin, B. Ananthanarayanan, P. Karmali, E. Ruoslahti, M. Tirrell, Effect of the lipid chain melting transition on the stability of DSPE-PEG(2000) micelles, Langmuir. 25 (2009) 7279–7286.

[45] L. Peeters, N.N. Sanders, A. Jones, J. Demeester, S.C. De Smedt, Post-pegylated lipoplexes are promising vehicles for gene delivery in RPE cells, J. Control. Release. 28 (2007) 208-217.

[46] R.E. Vandenbroucke, S.C: De Smedt, J. Demeester, N.N. Sanders, Cellular entry pathway and gene transfer capacity of TAT-modified lipoplexes. Biochim. Biophys. Acta. 1768 (2007) 571-579.

[47] C.L. Li, J.X. Cui, C.X. Wang, L. Zhang, Y:H: Li, L. Zhang, X. Xiu, Y.F. Li, N. Wei, Development of pegylated liposomal vinorelbine formulation using "post-insertion" technology, Int. J. Pharm. 31 (2010) 230-236.

[48] G. Caracciolo, Liposome-protein corona in a physiological environment: Challenges and opportunities for targeted delivery of nanomedicines, Nanomedicine. (2014) doi: 10.1016/j.nano.2014.11.003. [Epub ahead of print].