International Journal of Pharmaceutics 426 (2012) 231–238

Contents lists available at SciVerse ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Serum-stable, long-circulating paclitaxel-loaded colloidal carriers decorated with a new amphiphilic PEG derivative

Livia Basile[a,b], Catherine Passirani[c,d,*], Ngoc-Trinh Huynh[c,d], Jérôme Béjaud[c,d], Jean-Pierre Benoit[c,d], Giovanni Puglisi[a], Rosario Pignatello[a,**]

[a] Department of Drug Sciences, University of Catania, viale A. Doria, 6, I-95125 Catania, Italy
[b] Etnalead s.r.l., c/o Etna Building -Scuola Superiore di Catania, via S. Nullo, 5/i, I-95124 Catania, Italy
[c] Inserm U 1066 MINT "Micro et nanomedicines Biomimétiques", IBS-CHU, 4 rue Larrey, F-49933 Angers cedex 9, France
[d] LUNAM Université, Université d'Angers, France

ARTICLE INFO

Article history:
Received 23 September 2011
Received in revised form 8 January 2012
Accepted 14 January 2012
Available online 24 January 2012

Keywords:
Liposomes
Lipid nanocapsules (LNC)
Lipoamino acids
Stealth® carriers
CH50 assay
Lipid PEG derivatives

ABSTRACT

The paper describes sterically stabilized lipid nanocapsules (LNC) and multilamellar liposomes (MLV) coated using a new amphiphilic conjugate of PEG_{2000} with a 2-alkyl-lipoamino acid (LAA). A complement activation assay (CH50) and uptake experiments by THP-1 macrophage cells were used to assess in vitro the effectiveness of the PEG-LAA derivative of modifying the surface behavior of nanocarriers. Administered to rats or Swiss mice, respectively, the PEG_{2000}-LAA—modified LNC and MLV showed plasma half-lives longer than the corresponding naked carriers.

To assess the ability of nanocarriers to specifically reach tumor sites, paclitaxel (PTX)—loaded LNC and MLV were administered subcutaneously to rats implanted with a 9L glioma. Animals treated with saline or naked LNC and MLV underwent a quick expansion of tumor mass, up to a volume of 2000 mm^3 25 days after the injection of tumor cells. On the contrary, treatment with a PEG-LAA modified LNC carrier reduced the growth of the tumor volume, which did not exceed 1000 mm^3 by day 25. Analogous positive results were obtained with the liposomal systems. The experimental findings confirmed that these new PEG-LAA conjugates allow to obtain sterically stable nanocarriers that behave effectively and in a comparable or even better way than the (phospho)lipid PEG derivatives commercially available.

1. Introduction

Nanocarriers are widely investigated as modified release and targeting systems for active compounds. The first generation of these carriers has been represented by liposomes, formulated mainly to ensure improved drug parenteral release (Maruyama, 2000).

Lipid nanocapsules (LNC) also belong to the first generation of colloidal carriers. They represent an alternative to liposomes, being able to embody lipophilic or amphiphilic compounds inside their oily core, which is separated from the aqueous environment by a membrane of lecithin and pegylated hydroxystearate (HS-PEG). LNC are physico-chemically stable and are prepared without employing organic solvents. Depending on the percentage of components used, LNC can be obtained with various sizes, typically 20, 50 and 100 nm (Heurtault et al., 2002). Among the applications of LNC, the administration of anticancer drugs is particularly relevant, taking advantage of the small size of nanoparticles and the unique properties of tumor vasculature (EPR effect) (Passirani and Benoit, 2005).

Upon intravenous administration these nanocarriers can be rapidly cleared from blood by the mononuclear phagocyte system (MPS) cells (Moghimi and Szebeni, 2003). To ensure a suitable longevity in the systemic circulation, the modification of nanoparticle surface was largely attempted (Woodle and Lasic, 1992). The most successful approach appears to use hydrophilic polymers, which shield the particles to complement recognition mechanisms. These modified, sterically stabilized carriers are also referred to as 'stealth®' systems. Polyethylene glycol (PEG) is the most widely used material, since PEG molecules can form a protective hydrophilic layer on the surface of the nanoparticles that hinders the interaction with blood components (Owens and Peppas, 2006). There are also many examples of lipid derivatives of PEG used to obtain long-circulating carriers (Allen et al., 1991; Woodle et al., 1994; Li et al., 2002). The 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-PEG (DSPE-mPEG) and 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-PEG (DMPE-mPEG) have been largely used to stabilize liposomal systems applied in clinical therapy, like Doxil® (Northfelt et al., 1996; Swenson et al., 2001).

* Corresponding author. Tel.: +33 02 4468 8534; fax: +33 02 4184 6555.
** Corresponding author. Tel.: +39 095 738 4005; fax: +39 095 222 239.
E-mail addresses: catherine.passirani@univ-angers.fr (C. Passirani), r.pignatello@unict.it (R. Pignatello).

0378-5173/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2012.01.038

Phospholipid-PEGs represent an optimal solution to modify the surface of liposomes: the close structural properties of vesicle phospholipids and these PEG derivatives in fact allow an ordered distribution and a stable anchorage of the modifier within the liposomal bilayers. However, when other colloidal carriers are considered, such as lipid-based ones or, even more, polymeric nanoparticles, the sterical hindrance offered by the phospholipid moiety of DSPE-mPEG or analogous compounds may not allow to achieve stable pegylated carriers. Therefore, with the aim of producing alternative amphiphilic materials than phospholipid-PEGs to realize stealth nanosized drug carriers, we have previously synthesized some new series of amphiphilic PEG derivatives, obtained by conjugating either carboxy- or amine-PEG to lipoamino acid residues (LAA) (Pignatello et al., 2008, 2010a).

LAA are α-amino acids containing a side alkyl chain in 2-position. The length and structure of the side chain and the number of LAA residues can ultimately modulate the lipophilicity, stability and solubility of their conjugates with drugs and biomolecules. LAA combine the physico-chemical properties of both lipids and amino acids, due to their amphipatic structure (Toth, 1994; Wong and Toth, 2001; Pignatello et al., 2006). PEG-LAA conjugates have shown in vitro to be able to coat and modify the surface characteristics of liposomes and nanoparticles (Pignatello et al., 2008, 2010a,b).

In this study, the protective effect of PEG-LAA conjugates in inhibiting the process of opsonization of multilamellar liposomes (MLV) or LNC encapsulating paclitaxel (PTX) has been investigated both in vitro (by the CH50 assay and macrophage uptake studies) and in vivo (by biodistribution and tumor growth studies). We focused on one of the previously described conjugates, obtained by reacting the α-methoxy-ω-carboxyPEG$_{2000}$ with a LAA residue bearing a 15 carbon atom side chain (mPEGC$_{2000}$-LAA18) (Pignatello et al., 2010a). The behavior of the PEG-LAA conjugate was also compared to commercial DSPE-mPEG, conventionally used to produce sterically stabilized vesicles.

2. Materials and methods

2.1. Materials

mPEG$_{2000}$-propionic was purchased by Iris Biotech GmbH (Marktredwitz, Germany). Ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide hydrochloride (EDAC HCl) and hydroxybenzotriazole (HOBT) were Aldrich products, while triethylamine (TEA) and dry dichloromethane (DCM) were supplied by Fluka. All reagents and solvents were commercial products of analytical grade or higher, purchased from Sigma–Aldrich Chimica Srl (Milan, Italy). The 1,2-dipalmitoyl-*sn*-glycerol-3-phosphocholine (DPPC) was purchased from Genzyme Pharmaceuticals (Liestal, Switzerland). Cholesterol (CHOL) was obtained from Sigma–Aldrich, GmbH (Steinheim, Switzerland). DSPE-mPEG$_{2000}$ was kindly provided by Avanti Polar Lipids (Alabaster, USA). 1,1′-Dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate (DiI; λ_{em} = 544 nm, λ_{exc} = 590 nm) was an Invitrogen product (Cergy-Pontoise, France). Labrafac WL 1349 (caprylic-capric acid triglycerides; Ph. Eur., 4th ed., 2002) was kindly provided by Gatefossé S.A., Saint-Priest, France; Lipoïd S75-3 (soybean lecithin at 69% of phosphatidylcholine and 10% of phosphatidylethanolamine) was supplied by Lipoïd GmbH (Ludwigshafen, Germany); Solutol HS-15 (70% of PEG 660 hydroxystearate HS-PEG, and 30% of free PEG 660, Ph. Eur., 4th ed., 2002) was provided by BASF (Ludwigshafen, Germany). NaCl was gifted from Prolabo (Fontenay-sous-Bois, France). Water was obtained from a Milli-Q plus system Millipore (Paris, France). PTX was provided by Bioxel Pharma (Saint-Foy, Quebec, Canada).

2.2. Synthesis of carboxy-PEG-LAA derivatives

The already published procedure was followed (Pignatello et al., 2010a). Briefly, mPEG$_{2000}$-propionic acid was activated with a slight molar excess of EDAC and HOBt, in dry DCM at 0 °C for 2 h. A solution of one equivalent of the LAA methyl ester hydrochloride (Gibbons et al., 1990) in dry DCM and in the presence of an equimolar amount of TEA was added, and the mixture was stirred at 0 °C for further 2 h and then at room temperature for 4 days. The solvent was evaporated off in vacuo and the residue was dissolved in water and purified by dialysis against 3× 1 l of distilled water (CelluSep H1, MWCO 2000; M-Medical, Cornaredo, Italy).

2.3. Preparation of LNC

The formulation of LNC was based on the phase inversion process described by Heurtault et al. (2002); in this study, 50-nm LNC were prepared (Heurtault et al., 2002). LNC were composed of an oily core of lipophilic chains (Labrafac WL 1349) and an outer ring of lecithin (Lipoid S75-3) and PEG (Solutol HS-15) in NaCl and water. Briefly, all the components were mixed together under magnetic stirring. Three temperature cycles were carried out to reach the phase inversion from an oil-in-water to a water-in-oil emulsion. After, the mixture underwent a fast cooling-dilution process by adding Milli-Q water, that led to the formation of LNC (1A).

The corresponding batches of fluorescent LNC were obtained by using a lipophilic marker (DiI), which was previously dissolved in acetone (0.6%, w/w) and incorporated in the Labrafac phase (1:10, w/w).

For the formulation of PTX-loaded LNC (1C and 1E; Table 1), the drug (6.5 mg/g, 2%, w/w) was first solubilized in Labrafac. Solutol HS15, Lipoid, NaCl and water were then added to this solution, and the LNC were produced as described above. In the last step, the nanoparticle suspensions were filtered by 0.20-μm Ministar high-flow filters (Sartorius) to remove the aliquot of drug that remained unencapsulated and that precipitated as large crystals in the aqueous medium.

2.4. Preparation of coated nanocapsules by post-insertion

Stealth® LNC (1B) were prepared by post-insertion of the polymer conjugate to obtain a final concentration of 15 mM of PEGC$_{2000}$LAA18. Pre-formed LNC 1A and PEGC$_{2000}$LAA18 were co-incubated in cold water at 4 °C for 2 h at 60 °C. The mixture was vortexed every 15 min and then put in an ice bath for 1 min.

Table 1
Mean size, polydispersity index (PDI), zeta potential and drug encapsulation efficiency of naked and PEGylated LNC.

Formulation	Modifier	PTX[a]	Mean size (nm)	PDI	Zeta potential (mV)	EE%
1A	None	–	54.07 ± 1.22	0.04 ± 0.01	−5.60 ± 0.61	–
1B	PEGC$_{2000}$-LAA18	–	64.37 ± 4.98	0.09 ± 0.04	−3.71 ± 2.70	–
1C	None	+	55.07 ± 1.33	0.05 ± 0.01	−5.80 ± 0.70	31.50 ± 0.02
1D	PEGC$_{2000}$-LAA18	+	61.35 ± 2.22	0.05 ± 0.08	−1.36 ± 1.45	29.45 ± 1.45
1E	DSPE-mPEG	+	60.85 ± 3.25	0.05 ± 0.22	−1.46 ± 0.45	29.95 ± 2.00

[a] The initial drug concentration was 6.5 mg/g.

PTX-loaded stealth LNC (1D, 1E) were obtained similarly by the post-insertion of $PEGC_{2000}LAA18$ or $DSPE\text{-}mPEG_{2000}$ on LNC 1C.

2.5. Preparation of MLV liposomes

Both conventional liposomes made of DPPC and CHOL (9:1, mol/mol) (2A) and pegylated liposomes modified by $PEGC_{2000}LAA18$ (2B) or DSPE-mPEG (2C) (both at 10% in mole) were prepared by the thin-film hydratation method. Briefly, the lipids and CHOL were dissolved in chloroform and transferred into a suitable conical flask. The solvent was removed under a nitrogen flow using a rotary evaporator (Heidolph®, Bioblock Scientific) at 30 °C, obtaining a thin film on the wall of the flask. Evaporation was continued by maintaining the film for 3 h under vacuum to remove any solvent trace. The film was subsequently hydrated by adding 500 μl of a phosphate buffered saline solution (PBS, pH 7.4) and the resulting suspension was subjected to three cycles of vortex-mixing for 3 min, while heating at about 60 °C. Fluorescent liposomes were obtained by dissolving DiI in chloroform (0.4% in mol) together with the lipid components.

For the in vivo tumor growth inhibition studies, PTX-loaded MLV (2D) and PTX-loaded stealth liposomes made using either $PEGC_{2000}LAA18$ (2E) or DSPE-mPEG (2F) were prepared. PTX (1 mg/g) was dissolved in the organic solvent together with the lipid phase. After the MLV production, the unentrapped aliquot of drug was removed by centrifugation at 20,000 rpm for 30 min at 10 °C. The supernatant was discarded and the liposomal pellet was washed with PBS (pH 7.4) and centrifuged again. The washing step was repeated and the final amount of phospholipids in the pellet was measured by the Stewart assay (Stewart, 1980).

2.6. Determination of mean particle size and zeta potential

The size and surface charge distribution of the prepared nanocarriers were analyzed using a Malvern Zetasizer Nano Serie DTS 1060 (Malvern Instruments S.A., Worcestershire, UK). Sample suspensions were diluted in deionized water to ensure a convenient scattered intensity on the detector.

2.7. Determination of drug loading

An HPLC method was used to calculate PTX concentration in the produced samples; each measurement was made in triplicate. A determined amount of LNC or MLV dispersions was dissolved in a 96:4 (v/v) methanol/tetrahydrofuran mixture. A Waters Alliance 2690 HPLC system was used (Waters S.A., Saint-Quentin en Yvelines, France). A 20 μl aliquot of the filtrate was injected in triplicate into an HPLC XTerra RP18 column (5 μm, 4.6 mm × 150 mm) (Waters). Analysis was performed with water and acetonitrile as the mobile phase, with a gradient elution program of 50–80% acetonitrile, at a flow rate of 1 ml/min. Eluting fractions were revealed with a Waters 2487 Dual λ absorbance Detector, at a wavelength of 227 nm. Data were analyzed by the Empower Pro® software, version 5.00. PTX gave a retention peak at 2.37 min.

2.8. Measurement of complement activation: CH50 assay

Complement consumption was tested in the presence of normal human serum (NHS) (Mayer, 1991) by measuring the residual hemolytic capacity of the complement after contact with the test plain nanocarriers (samples LNC 1A and 1B, and MLV 2A, 2B and 2C) (Figs. 1 and 2). This test determines the amount of serum able to lyse 50% of a fixed number of sensitized sheep erythrocytes (CH50) (Passirani et al., 1998). This analysis was conducted by using VBS (Veronal buffered saline) and VBS^{2+} (VBS containing 0.15 mM Ca^{2+} and 0.5 mM Mg^{2+}) prepared as described elsewhere (Passirani et al., 1998). The data can be expressed in terms of CH50 units, which are calculated by a linear fit to a log–log version of the Von Krogh equation (Mayer, 1991):

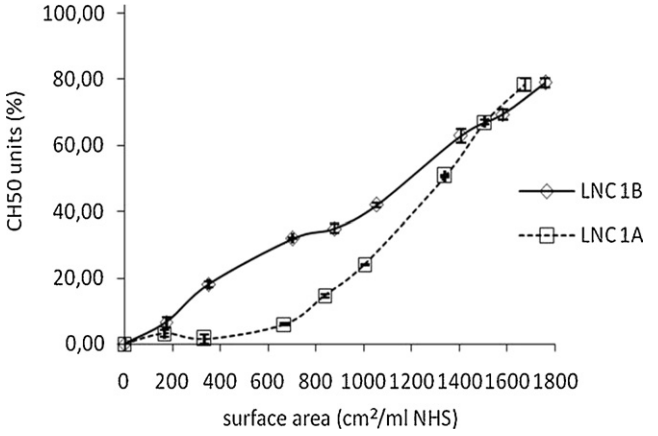

Fig. 1. Influence of $PEGC_{2000}LAA18$ on complement consumption variation induced by LNC.

$$\text{Consumption } (\%) = \frac{CH50_{sample} - CH50_{control}}{CH50_{control}} \times 100 \quad (1)$$

To compare particles with different mean diameters, the complement consumption was plotted as a function of the surface area. Surface areas of nanoparticles were calculated, as described in detail elsewhere (Mayer, 1991; Kazatchkine et al., 1986), by using Eq. (2):

$$S = n4\pi r^2 \quad \text{and} \quad V = n\left(\frac{4}{3}\right)(\pi r^3) \quad (2)$$

leading to:

$$S = \frac{3V}{r} = \frac{3m}{r\rho} \quad (3)$$

where S is the surface area (cm^2) and V the volume (cm^3) of n spherical beads of average radius r (cm), weight m (μg), and voluminal mass ρ (μg/cm^3). All experiments were performed in triplicate and the data were expressed with standard deviation.

2.9. Macrophage uptake studies

THP-1 cells (human monocyte/macrophage cell line obtained by ATCC, Manassas, VA, USA) were grown in suspension at 5% CO_2 and 37 °C in ATCC medium. The experiment was performed on differentiated cells and the differentiation was induced in the

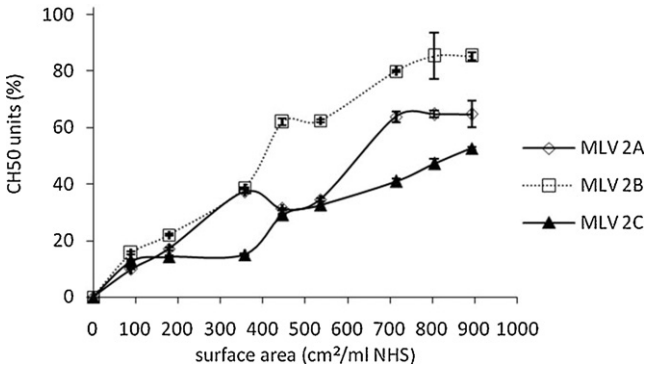

Fig. 2. Influence of $PEGC_{2000}LAA18$ and $DSPE\text{-}mPEG_{2000}$ on complement consumption variation induced by MLV.

same medium by adding 200 mM of Phorbol 12-myristate 13-acetate (PMA, Sigma, Saint-Quentin Fallavier, France) for 24 h to allow their adherence (Tsuchiya et al., 1982). After aspiration of the medium to eliminate non-adhered cells, the cells were incubated in a fresh medium for other 24 h before adding the nanoparticles. Cells (0.6×10^6/ml) were plated on 24-well cell culture and left to grow for 24 h at 37 and 4 °C. After 24 h, the suspensions of DiI-loaded (fluorescent) LNC (1A and 1B) or MLV (2A, 2B and 2C) were deposited on the cell monolayer and incubated for 90 min at either 37 °C or at 4 °C. The amount of phagocytized nanoparticles was determined quantitatively by FACS.

2.10. In vivo studies

2.10.1. Pharmacokinetic and biodistribution study

Animal studies on Swiss mice and Wistar rats were carried out in accordance with the French regulation and EC Directive 86/609/EEC for animal experiments. Fluorescent LNC (1A and 1B) and liposomes (2A, 2B, and 2C) were injected intravenously in the vein tail of mice (150 μl) and in the penile vein of the rat (1 ml) under gaseous isoflurane anesthesia. Blood samples were withdrawn on three animals at 1, 10 and 30 min and 1, 3 and 24 h after injection and then introduced into BD Vacutainer tubes, centrifuged at 4000 rpm for 10 min. The optical density was measured using a fluoroskan (Fluoroskan Ascent, FL, USA).

2.10.2. Tumor implantation and animal treatment

Syngeneic Fischer F344 female rats weighing 150–180 g were bought from Charles River Laboratories France (L'Arbresle, France). All experiments were performed on 10–11-week old female Fisher rats. The animals were manipulated under isoflurane/oxygen anaesthesia. Animal manipulation was conducted under the French Ministry of Agriculture regulations.

Rat 9L gliosarcoma cells were given by the European Collection of Cell Culture (Salisbury, UK, No. 94110705). Purified newborn rat primary astrocytes were obtained as reported elsewhere (McCarthy and Vellis, 1980). Cells were grown at 37 °C/5% CO_2 in Dulbecco modified Eagle medium (DMEM) enriched by glucose and L-glutamine (BioWhittaker, Verviers, Belgium) containing 10% fetal calf serum (FCS) (BioWhittaker) and 1% antibiotic and antimycotic solution (Sigma, Saint-Quentin Fallavier, France).

On the day of implantation, cells were detached with trypsin-ethylene diamine tetraacetic acid, washed twice with EMEM without FCS or antibiotics, counted, and resuspended to the final concentration desired. 1.5×10^6 9L cells were injected subcutaneously on rat right thigh allowing the formation of tumor. On day 6 after cell injection, a single injection (400 μl) of different treatments of PTX was administered to rats treated with 9L cells. Group 1 was treated with physiological saline (control; $n = 9$ animals), Group 2 was injected with LNC 1A ($n = 9$ animals), Group 3 received a dose of LNC 1C (6.5 mg/g; 16.2 mg/kg; $n = 10$ animals), Group 4 received LNC 1D (6.5 mg/g; 16.2 mg/kg; $n = 10$ animals), Group 5 received LNC 1E. Group 6 was treated with liposomes 2D (1 mg/g; 2.5 mg/kg; $n = 10$ animals), Group 7 received liposomes 2E (1 mg/g; 2.5 mg/kg; $n = 10$ animals), and Group 8 received liposomes 2F (1 mg/g; 2.5 mg/kg; $n = 9$ animals). The length and width of each tumor were regularly measured using a digital caliper, and tumor volume was estimated with the mathematical ellipsoid formula given in Eq. (4). At the end of the study (day 25), the rats were sacrificed and the weight of each tumor was evaluated.

$$\text{Volume } (V) = \left(\frac{\pi}{6}\right) \times \text{width}^2 \ (l) \times \text{length } (L) \quad (4)$$

3. Results and discussion

3.1. Nanocarrier characterization

LNC were prepared according to an organic solvent-free process based on phase inversion of emulsions (Heurtault et al., 2002). PCS measurement showed that all the batches, either plain or loaded with PTX, were formed by weakly negative nanoparticles with a mean size between 54 and 64 nm, in good accordance with the used production method (Heurtault et al., 2002) (Table 1). Post-insertion of $PEGC_{2000}$LAA18 caused only a 10 nm increase in the particle size. The obtained LNC were stable over time, their mean size and surface charge remaining constant up to one month after production (data not shown).

The prepared liposomes were multilamellar vesicles with a particle size around 200 nm (by volume). In the presence of either the PEG-LAA or DSPE-PEG modifier, the surface charge (zeta potential) of the nanocarriers decreased: this behavior can be explained by considering that the modifier masked the slightly positive charges due to LNC or liposomal components. Moreover, in the pegylated MLV the measured negative charge can be referred to the formation of dipoles in the PEG chains, as already demonstrated in previous studies (Vonarbourg et al., 2005).

PTX encapsulation efficiency (EE%) in LNC and MLV is reported in Tables 1 and 2, respectively. This parameter remained almost constant between naked and pegylated LNC (Table 1); conversely, in the corresponding liposomes the presence of any PEG modifier dramatically increased the EE% (Table 2); a possible explanation is that the drug was in part encapsulated within the PEG chains.

3.2. In vitro and in vivo studies

The protective effect of the tested $PEGC_{2000}$-LAA18 conjugate was investigated by the CH50 assay, using unloaded batches (LNC 1A and 1B, Table 1). In this test, the classical complement pathway of NHS is activated after the contact with sensitized sheep erythrocytes, leading to the lysis of erythrocytes and hemoglobin release. When the human serum is in presence of activating nanoparticles, less complement proteins are available in the serum to lyse the sheep erythrocytes, causing a reduction of CH50 units.

The consumption of CH50 units was measured at a fixed level of human serum in the presence of an increasing surface area of particles (Figs. 1 and 2). Fig. 1 shows the behavior of LNC 1A and LNC 1B, revealing that the latter induced a high response of the complement system in the range of studied surfaces (0–1700 cm^2). Both unmodified and pegylated nanoparticles caused the activation of

Table 2
Mean size, polydispersity index (PDI), zeta potential and drug encapsulation efficiency of naked and PEGylated MLV.

Formulation	Modifier	PTX[a]	Mean size (nm)	PDI	Zeta potential (mV)	EE%
2A	None	–	210.34 ± 5.15	0.78 ± 0.20	+4.97 ± 0.25	–
2B	$PEGC_{2000}$-LAA18	–	183.72 ± 15.98	0.17 ± 0.07	−3.12 ± 0.87	–
2C	DSPE-mPEG	–	230.24 ± 4.14	0.48 ± 0.18	−20.33 ± 5.13	–
2D	None	+	223.50 ± 7.16	0.68 ± 0.17	+1.15 ± 2.53	57.31 ± 3.73
2E	$PEGC_{2000}$-LAA18	+	204.56 ± 8.96	0.73 ± 0.12	−11.66 ± 7.82	71.89 ± 1.14
2F	DSPE-mPEG	+	255.52 ± 5.67	0.72 ± 0.36	−37.60 ± 9.88	73.49 ± 1.31

[a] The initial drug concentration was 1 mg/g.

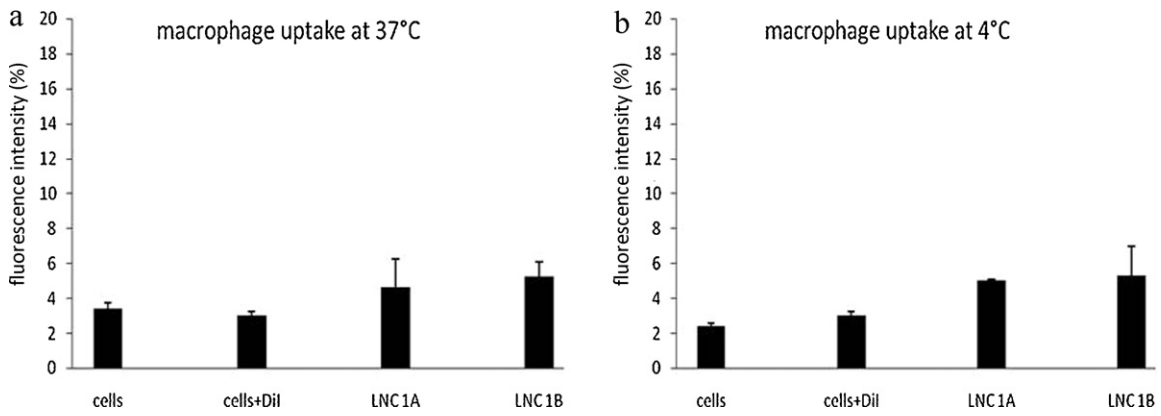

Fig. 3. Evaluation of macrophage uptake of LNC 1A and 1B after 90 min incubation with THP-1 cells at 37 °C (a) and at 4 °C (b).

complement proteins, up to a value of 70% at 1700 cm^2/ml, despite the LNC had small sizes (50 nm) and a low zeta potential. In a previous study of Vonarbourg et al., the 50-nm LNC determined a low activation of complement proteins at low surface values (until 1000 cm^2) (Vonarbourg et al., 2006), demonstrating that the activation of complement proteins by LNC is size-dependent and the presence of a PEG layer on LNC surface does not change the behavior of nanoparticles. Also naked LNC actually still possess small PEG chains (PEG 660) that protect them from complement activation. However, when using the high molecular weight PEG modifier, bigger LNC were produced (around 10 nm more) and this was sufficient to promote the activation of proteins, due to the higher facility they have to adsorb on larger surfaces.

Fig. 2 shows the CH50 data relative to the MLV samples. As expected, the DPPC-CHOL-based MLV caused a high activation of the immune system proteins, CHOL having been demonstrated to be a highly immunogenic molecule (Alving and Swartz, 1991). It is known from literature that adding amphiphilic derivatives of PEG to liposome or nanoparticle surface increase their permanence time in the blood (Gref et al., 2000; Li et al., 2010). Contrarily to expectations, addition of the $PEGC_{2000}$LAA18 conjugate did not modify the response in the CH50 test. Neither the decoration with DSPE-mPEG stabilized the liposomes, even if the value of consumption of complement reached a value of 50% at 800 cm^2/ml against a response of 80% for the MLV modified with $PEGC_{2000}$LAA18. Therefore, the prepared MLV seemed to be stronger complement activators than LNC.

To confirm the CH50 test data, parallel in vitro studies on THP-1 cells were performed, using DiI-loaded (fluorescent) nanocarriers. The results are reported in Figs. 3 and 4 and were expressed as the percentage of fluorescence after FACS analysis. With respect to a control, represented by cell alone incubated with DiI, naked and PEGylated LNC did not show any difference in terms of macrophage uptake (Fig. 3a). These data are in agreement with those obtained in the CH50 test: the fluorescence recorded was only 5%, meaning that naked and PEG-LAA—modified LNC were not internalized after contact with THP-1 cells. Experiments were also conducted at 4 °C and the results are reported in Fig. 3b. In this case, the internalization of all LNC was minimal, with only small differences between the various nanoparticles. Even the differences in the fluorescence associated with cells were very low among the various test samples. The uptake of LNC was therefore an ATP-dependent mechanism. The slight increase of fluorescence recorded was due only to interactions of the systems with the cell membrane.

The situation changed when THP-1 cells were incubated with the liposomes (Fig. 4a). Particularly, the fluorescence related to DPPC-CHOL MLV was slightly higher than that one registered for the MLV modified with the PEG-LAA conjugate. These results are encouraging if compared to those ones measured with the MLV modified with commercial DSPE-mPEG, which were internalized more quickly. In analogous experiments carried out at 4 °C (Fig. 4b), the internalization of the MLV was reduced to a minimum, with only small differences between the two samples. Even the differences in the fluorescence associated with cells were minimal. Thereby, also the uptake of MLV can be considered an ATP-dependent mechanism, and the slight increase in fluorescence might only due to some interaction of the vesicles with the cell membrane.

To evaluate the ability of the novel PEG-LAA conjugate to stabilize the nanoparticles in the blood, we studied their behavior after intravenous administration to Swiss mice and rats of DiI-loaded naked or PEG-LAA decorated LNC (batches 1A and 1B, Table 1).

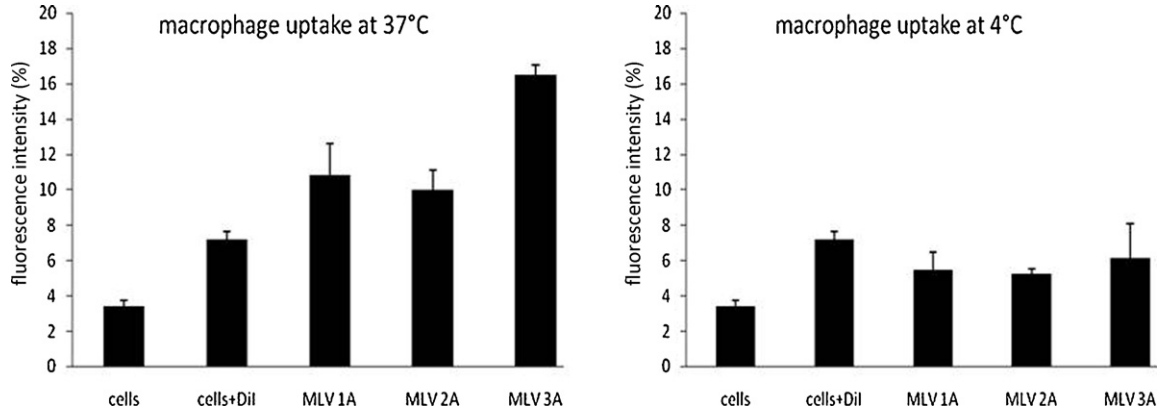

Fig. 4. Evaluation of macrophage uptake of MLV 2A, 2B and 2C after 90 min incubation with THP-1 cells at 37 °C (a) and at 4 °C (b).

Table 3
Pharmacokinetic profile of LNC 1A and 1B following systemic administration in Swiss mice.

Formulation	$t_{1/2}\alpha$[0–3](h)	$t_{1/2}\beta$[0–24](h)	$AUC_{[0-3\,h]}$ (%injected dose/h)	$AUC_{[0-24\,h]}$ (%injected dose/h)	$t_{50\%}$(h)
LNC 1A	11.31	6.28	516.23	569.53	1.61
LNC 1B	6.35	7.80	564.00	480.43	1.51

Table 4
Pharmacokinetic profile of LNC 1A and 1B following systemic administration in Wistar rats.

Formulation	$t_{1/2}\alpha$[0–3](h)	$t_{1/2}\beta$[0–24](h)	$AUC_{[0-3\,h]}$ (%injected dose/h)	$AUC_{[0-24\,h]}$ (%injected dose/h)	$t_{50\%}$(h)
LNC 1A	3.90	7.40	489.52	604.61	11.16
LNC 1B	10.81	10.35	1091.01	2110.01	19.30

Table 5
Pharmacokinetic profile of MLV 2A, 2B and 2C following systemic administration in Swiss mice.

Formulation	$t_{1/2}\alpha$[0–3](h)	$t_{1/2}\beta$[0–24](h)	$AUC_{[0-3\,h]}$ (%injected dose/h)	$AUC_{[0-24\,h]}$ (%injected dose/h)	$t_{50\%}$(h)
MLV 2A	6.81	28.55	411.08	1295.24	0.61
MLV 2B	3.71	10.23	849.71	1077.03	3.50
MLV 2C	9.88	9.65	1390.05	1268.52	2.52

Two animal models were chosen due to the differences in the immunity system existing between these species. The nanoparticle concentration in the bloodstream was calculated in relation to the fluorescence intensity of DiI and was reported vs. time of withdrawal (Figs. 5 and 6). The main pharmacokinetic parameters, such as $t_{50\%}$, $t_{1/2}\alpha$ (distribution), $t_{1/2}\beta$ (elimination), and AUC (as the percentage of injected dose) are gathered in Tables 3 and 4 for the LNC and in Table 5 for the liposomal batches.

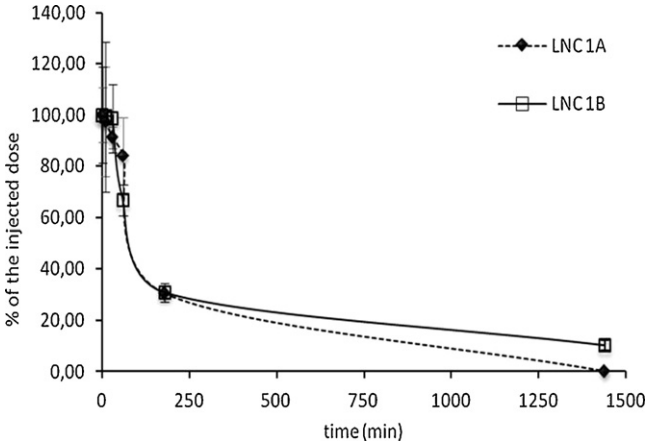

Fig. 5. Blood residence time of LNC 1A and 1B in Swiss mice.

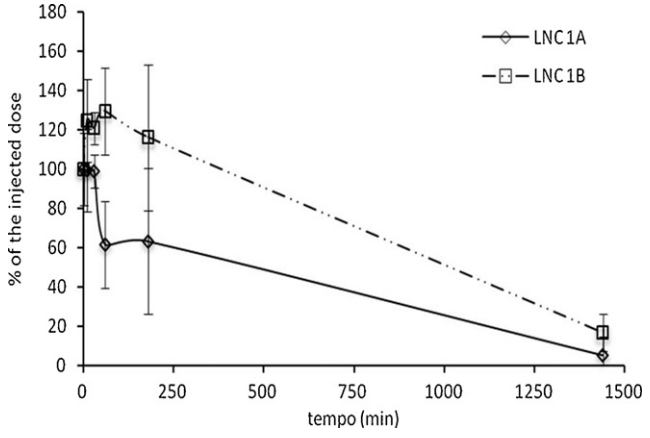

Fig. 6. Blood residence time of LNC 1A and 1B in Wistar rats.

Results showed that the LNC 1B after administration to Swiss mice did not exhibit plasma half-lives higher than LNC 1A (Fig. 5), with $t_{50\%}$values that rose from 1.6 h for the LNC 1A to 1.5 h for LNC 1B (Table 3). Findings were substantially different when the LNC were administered to rats (Fig. 6): as mice and rats do not possess the same immunity system, and mice have no or a very little complement system, the '*stealth*' effect can be considered useful only in rats. In general, the above findings confirmed that the $PEGC_{2000}$LAA18 conjugate was able to confer long-circulating properties to the LNC (Fig. 6).

With regard to the tested liposomes, the pegylated MLV showed higher plasma half-lives ($t_{50\%}$) in mice than naked ones (Fig. 7), with values rising to 3.5 h for the PEG-LAA modified MLV (2B) and to 2.5 h for the DSPE-PEG MLV (2C) (Table 5). A clear inverse correlation with the $t_{1/2}\beta$ values was also observed, ranging from 28.55 h for MLV 2A to 9.65 h for MLV 2C.

Therefore, the tested PEG-LAA derivative confirmed to be able to impart long-circulating properties to these MLV carriers when administered to mice.

Finally, the ability of the prepared nanoparticles to reach a specific tumor tissue was assessed. For this purpose, a 9L subcutaneous glioma model was used. PTX-loaded LNC and MLV were administered after the tumor has reached a volume of 100 mm^3, and the study was performed on six groups of animals, that were randomly separated to reduce weight and tumor size differences inside the groups. Rats treated with physiological saline or unmodified LNC and MLV underwent a quick expansion of tumor mass (Fig. 8), up

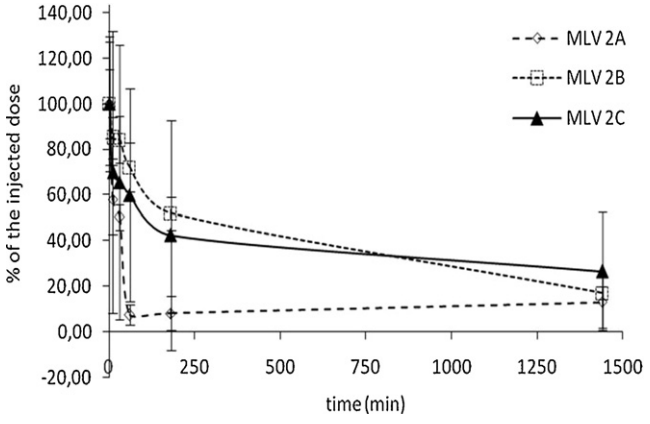

Fig. 7. Blood residence time of MLV 2A, 2B and 2C in Swiss mice.

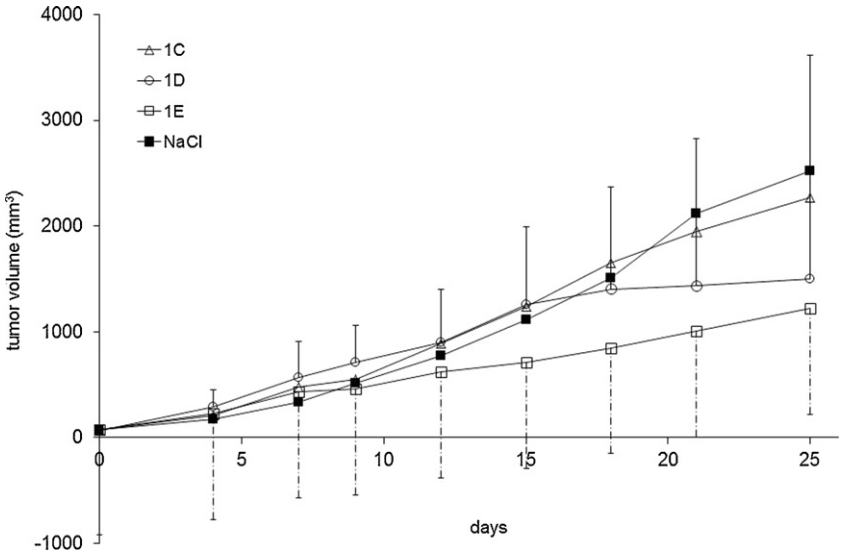

Fig. 8. Effects of in vivo treatment with PTX-loaded LNC on the growth of 9L glioma cells implanted in Fisher rats. For clarity, only the error bars were shown of the carriers modified with the $PEGC_{2000}LAA18$ (1D, full lines) or DSPE-$mPEG_{2000}$ (1E, dotted lines).

to a volume of 2000 mm^3 25 days after the injection of tumor cells. Conversely, the rats treated with pegylated LNC 1C or 1E were characterized by a slow increase of the tumor volume, which did not exceed 1000 mm^3 by day 25.

Similar positive results were also obtained with the liposomal system (Fig. 9). MLV 2D maintained the cytostatic activity of PTX in respect to control rats, that presented a very rapid progression of the tumor mass (5000 mm^3 at day 25) when treated with an equivalent dose of the anticancer drug. These results are also very interesting if compared to those obtained after the injection of MLV 2F, since they showed the same profile of therapeutic efficacy, blocking the tumor growth at 1600 mm^3 at day 25.

In agreement with many other literature evidences, these experimental data confirmed that, contrarily to conventional colloidal carriers that are rapidly recognized by macrophages, long circulating (stealth®) carriers lead to an important accumulation of their cargo in accessible pathological sites. For this reason, pegylated nanocarriers are considered to be potential carriers for drug targeting to the site of action, particularly in solid tumors due to the enhanced permeability and retention (EPR) effect (Maeda et al., 2000). Both PTX-loaded LNC (Lacoeuille et al., 2007; Hureaux et al., 2010) and liposomes (Zhao et al., 2010) have been reported to increase the antitumor activity of the drug than free PTX formulations.

In summary, this work investigated the ability of a novel amphiphilic PEG conjugate to stabilize colloidal drug carriers with respect to their permanence on the bloodstream. Through flow cytometry and blood permanence measurements it was possible to shed light on the ability of these particles to escape to macrophage uptake. Using a new PEG conjugate with an amphiphilic LAA, it has been possible to prepare sterically stable nanocarriers and it has been confirmed that this conjugate behaves effectively in a

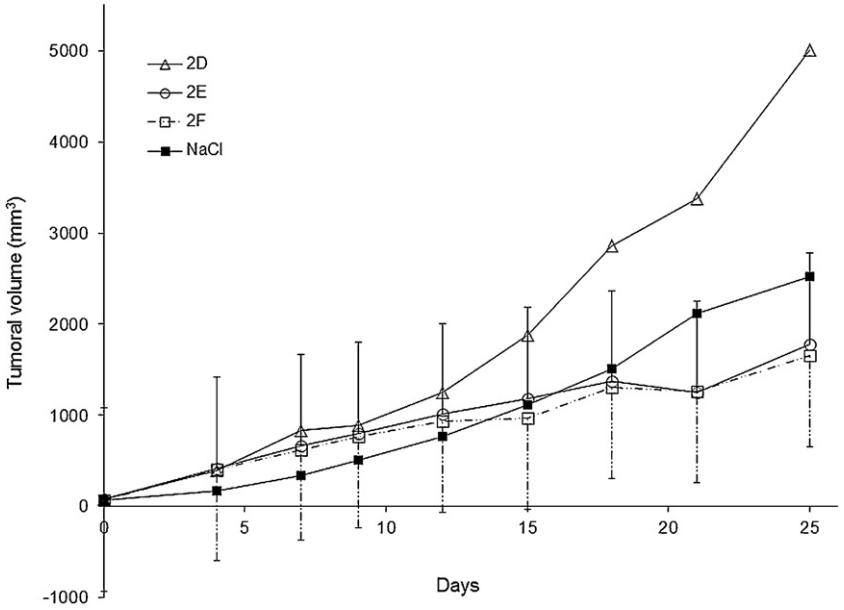

Fig. 9. Effects of in vivo treatment with PTX-loaded MLV on the growth of 9L glioma cells implanted in Fisher rats. For clarity, only the error bars were drawn of the vesicles modified with the $PEGC_{2000}LAA18$ (2E, full lines) or DSPE-$mPEG_{2000}$ (2F, dotted lines).

comparable way or in some cases better than a commercially available lipid PEGF derivative.

Future experiments will focus on loading other model drugs to assess the potentiality of these nanocarriers of active targeting to specific therapeutic targets, such as tumor tissues or inflamed areas.

Conflict of interest

None declared.

Acknowledgments

L.B. performed this study within her Research Doctorate under an EU financial support (FSE funds). A partial support of the University of Catania (Ricerca di Ateneo) is acknowledged. The authors would like to thank Pierre Legras (Service Commun d'Animalerie Hospitalo-Universitaire (SCAHU), Angers, France) for his skilful technical support.

References

Allen, T.M., Hansen, C., Martin, F., Redemann, C., Yau-Young, A., 1991. Liposomes containing synthetic lipid derivatives of poly(ethylene glycol) show prolonged circulation half-lives in vivo. Biochim. Biophys. Acta 1066, 29–36.

Alving, C.R., Swartz Jr., G.M., 1991. Antibodies to cholesterol, cholesterol conjugates and liposomes: implications for atherosclerosis and autoimmunity. Crit. Rev. Immunol. 10, 441–453.

Gibbons, W.A., Hughes, R.A., Choralambous, M., Christodoulou, M., Szeto, A., Aulobaugh, A.E., Mascagni, P., Toth, I., 1990. Synthesis, resolution and structural elucidation of lipidic amino acids and their homo- and hetero-oligomers. Liebigs Ann. Chem., 1175–1183.

Gref, R., Luck, M., Quellec, P., Marchand, M., Dellacherie, E., Harnisch, S., Blunk, T., Muller, R.H., 2000. 'Stealth' corona-core nanoparticles surface modified by polyethylene glycol (PEG): influence of the corona (PEG chain length and surface density) and of the core composition on phagocytic uptake and plasma protein adsorption. Colloids Surf. 18, 301–313.

Heurtault, B., Saulnier, P., Pech, B., Proust, J.E., Benoit, J.P., 2002. A novel phase inversion-based process for the preparation of lipid nanocarriers. Pharm. Res. 19, 875–880.

Hureaux, J., Lagarce, F., Gagnadoux, F., Rousselet, M.C., Moal, V., Urban, T., Benoit, J.P., 2010. Toxicological study and efficacy of blank and paclitaxel-loaded lipid nanocapsules after i.v. administration in mice. Pharm. Res. 27, 421–430.

Kazatchkine, M.D., Hauptmann, G., Nydegger, U.E., 1986. Techniques du Complément. In: INSERM collection technique en immunologie. Livre, Paris, pp. 22–23.

Lacoeuille, F., Hindre, F., Moal, F., Roux, J., Passirani, C., Couturier, O., Cales, P., Le Jeune, J.J., Lamprecht, A., Benoit, J.P., 2007. In vivo evaluation of lipid nanocapsules as a promising colloidal carrier for paclitaxel. Int. J. Pharm. 344, 143–149.

Li, W.M., Mayer, L.D., Bally, M.B., 2002. Prevention of antibody-mediated elimination of ligand-targeted liposomes by using poly(ethylene glycol)-modified lipids. J. Pharmacol. Exp. Ther. 300, 976–983.

Li, C.L., Cui, J.X., Wang, C.X., Zhang, L., Li, Y.H., Zhang, L., Xiu, X., Li, Y.F., Wei, N., 2010. Development of pegylated liposomal vinorelbine formulation using post-insertion technology. Int. J. Pharm. 391, 230–236.

Maeda, H., Wu, J., Sawa, T., Matsumura, Y., Hori, K., 2000. Tumor vascular permeability and the EPR effect in macromolecular therapeutics. J. Control. Release 65, 271–284.

Maruyama, K., 2000. In vivo targeting by liposomes. Biol. Pharm. Bull. 23, 91–99.

Mayer, M.M., 1991. Complement and complement fixation. In: Kabat, E.A., Mayer, M.M. (Eds.), Experimental Immunochemistry, vol. 2. Charles C. Thomas, Springfield, IL, pp. 133–240.

McCarthy, K.D., De Vellis, J., 1980. Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue. J. Cell Biol. 85, 890–902.

Moghimi, S.M., Szebeni, J., 2003. Stealth liposomes and long circulating nanoparticles: critical issues in pharmacokinetics, opsonization and protein-binding properties. Prog. Lipid Res. 42, 463–478.

Northfelt, D.W., Martin, F.J., Working, P., Volberding, P.A., Russell, J., Newman, M., et al., 1996. Doxorubicin encapsulated in liposomes containing surface-bound polyethylene glycol: pharmacokinetics tumor localization, and safety in patients with AIDS-related Kaposi's sarcoma. J. Clin. Pharmacol. 36, 55–63.

Owens III, D.E., Peppas, N.A., 2006. Opsonization biodistribution, and pharmacokinetics of polymeric nanoparticles. Int. J. Pharm. 307, 93–102.

Passirani, C., Benoit, J.P., 2005. Complement activation by injectable colloidal drug carriers. In: Mahato, R.I. (Ed.), Biomaterials for Delivery and Targeting of Proteins and Nucleic Acids. CRC Press, pp. 187–230.

Passirani, C., Barratt, G., Devissaguet, J.P., Labarre, D.D., 1998. Interactions of nanoparticles bearing heparin or dextran covalently bound to poly(methyl methacrylate) with the complement system. Life Sci. 62, 775–785.

Pignatello, R., Guccione, S., Castelli, F., Sarpietro, M.G., Giurato, L., Lombardo, M., Puglisi, G., Toth, I., 2006. Enhancement of drug affinity for cell membranes by conjugation with lipoamino acids II. Experimental and computational evidence using biomembrane models. Int. J. Pharm. 310, 53–63.

Pignatello, R., Pantò, V., Puglisi, G., Ballistreri, A., Impallomeni, G., 2008. Derivati anfifilici del poliossietilenglicole (PEG), procedimento di preparazione e loro usi nella preparazione di sistemi farmaceutici. It. Patent no. RM 2008 A 551.

Pignatello, R., Pantò, V., Basile, L., Impallomeni, G., Ballistreri, A., Pistarà, V., Craparo, E.F., Puglisi, G., 2010a. New amphiphilic conjugates of mono- and bis(carboxy)-PEG2000 polymers with lipoamino acids as surface modifiers of colloidal drug carriers. Macromol. Chem. Phys. 211, 1148–1156.

Pignatello, R., Basile, L., Musumeci, T., Cardile, V., Vicari, L., Martinetti, D., Gulisano, M., Puglisi, G., 2010b. In vitro assessment of the sterical stabilization of colloidal carriers decorated with novel amphiphilic PEG derivatives. In: 2nd Conference Innovation in Drug Delivery: From Preformulation to Development through Innovative Evaluation Process, Aix-en-Provence (France), October 3–6.

Stewart, J.C.M., 1980. Colorimetric determination of phospholipids with ammonium ferrothiocyanate. Anal. Biochem. 104, 10–14.

Swenson, C.E., Perkins, W.R., Roberts, P., Janoff, A.S., 2001. Liposome technology and the development of Myocet™ (liposomal doxorubicin citrate). Breast 10 (Suppl. 2), 1–7.

Toth, I., 1994. A novel chemical approach to drug delivery: lipidic amino acid conjugates. J. Drug Target. 2, 217–239.

Tsuchiya, S., Kobayashi, Y., Goto, Y., Okumura, H., Nakae, S., Konno, T., Tada, K., 1982. Induction of maturation in cultured human monocytic leukemia cells by a phorbol diester. Cancer Res. 42, 1530–1536.

Vonarbourg, A., Saulnier, P., Passirani, C., Benoit, J.P., 2005. Electrokinetic properties of non charged lipid nanocapsules: influence of the dipolar distribution at the interface. Electrophoresis 26, 2066–2075.

Vonarbourg, A., Passirani, C., Saulnier, P., Simard, P., Leroux, J.C., Benoit, J.P., 2006. Evaluation of pegylated lipid nanocapsules versus complement system activation and macrophage uptake. J. Biomed. Mater. Res. A 78, 620–628.

Wong, A., Toth, I., 2001. Lipid sugar and liposaccharide based delivery systems. Curr. Med. Chem. 8, 1123–1136.

Woodle, M.C., Lasic, D.D., 1992. Sterically stabilized liposomes. Biochim. Biophys. Acta 1113, 171–199.

Woodle, M.C., Newman, M.S., Cohen, J.A., 1994. Sterically stabilized liposomes: physical and biological and biological properties. J. Drug Target. 2, 397–403.

Zhao, P., Wang, H., Yu, M., Cao, S., Zhang, F., Chang, J., Niu, R., 2010. Paclitaxel-loaded, folic-acid-targeted and TAT-peptide-conjugated polymeric liposomes: in vitro and in vivo evaluation. Pharm. Res. 27, 1914–1926.