Photoactive liposomal formulation of PVP-conjugated chlorin e6 for photo-dynamic reduction of atherosclerotic plaque

Wojciech Kalas¹*, Edyta Wysokińska¹, Magdalena Przybyło², Marek Langner², Agnieszka Ulatowska-Jarża³, Dariusz Biały⁴, Magdalena Wawrzyńska⁴, Ewa Ziolo⁴, Wojciech Gil³, Anna Trzeciak³, Halina Podbielska², and Marta Kopaczynska⁴

¹Ludwik Hirschfeld Institute of Immunology and Experimental Therapy, PAS, R. Weigla 12, 53-114 Wroclaw, Poland; secretarl@hirschfeld.pl
²Department of Biomedical Engineering, Wroclaw University of Science and Technology, Wybrzeze Wyspianskiego 27, 50-370 Wroclaw, Poland; martakopaczynska@pwr.edu.pl
³Faculty of Chemistry, University of Wroclaw, 14 F. Joliot-Curie St., 50-383 Wroclaw, Poland; annatrzeciak@chem.uni.wroc.pl
⁴Department of Emergency Medical Service, Wroclaw Medical University, Parkowa 34, 51-616 Wroclaw, Poland; ratownictwo@umed.wroc.pl
⁵Clinic of Cardiology, Wroclaw Medical University, Parkowa 34, 51-616 Wroclaw, Poland; kardiologia@umed.wroc.pl

* Correspondence: wojciech.kalas@hirschfeld.pl

Received: date; Accepted: date; Published: date

** Abstract:** Background: Liposomes are delivery systems for biologically active compounds. Current technologies inefficiently encapsulate large hydrophilic macromolecules such as PVP-conjugated chlorin e6 (Photolon). This photo-active drug is widely tested for therapeutic applications, including photo-dynamic reduction of atherosclerotic plaque. Methods: Novel formulation of Photolon was produced using “gel hydration technology”. Its pharmacokinetics was tested in Sus scrofa f. domestica. Its cellular uptake, cytotoxicity and ability to induce phototoxic reaction was demonstrated in J774.A1, RAW264.7 macrophages and vascular smooth muscle (TG-HA-VSMC) and vascular endothelial (HUVEC) cells. Results: Developed liposomes were (heavily) loaded with Photolon (encapsulation efficiency > 80%) and average diameter of 124.7±0.6 nm (PDI = 0.055). Its half-life in Sus scrofa was 20 minutes with AUC equals to 14.7. The formulation was non-cytotoxic. It was rapidly (10min) and efficiently accumulated by macrophages, but not TG-HA-VSMC or HUVEC. Accumulated quantity of photo-sensitizer was sufficient for induction photo-toxicity in J774.1, but not TG-HA-VSMC. Conclusions: Due to the excellent physical and pharmacokinetics properties and selectivity for macrophages our novel ultra-dense liposomal formulation of...
Photolon is a promising therapeutic candidate for use in arteriosclerosis treatment, where targeting macrophages, but not accompanying vascular tissue, is critical for effective and safe therapy.

**Keywords:** liposomes, Photolon, photo-dynamic therapy, arteriosclerosis, macrophages, pharmacokinetics, vascular smooth muscle cells, vascular endothelial cells, arteriosclerosis

1. Introduction

Liposomes, lipid nano-aggregates, are promising delivery systems for biologically active compounds including biological macromolecules and polymers [1,2]. Despite numerous studies on various delivery systems like micelles, dendrimers, carbon nanotubes, and poly(lactide-co-glycolide) [3] or polyphosphoester nanoparticles [4], only liposomes are considered safe and some acquire FDA approval [5]. Physical encapsulation can increase macro-molecule stability, modify blood circulation time or enhance specificity. All these factors play important role in photo-sensitizer application in photo-dynamic therapy. Most, clinically approved, photo-sensitizers are administered intravenously. Low stability in blood, poor specificity for target tissue and substantial dark or non-specific toxicity are still the great challenges of photo-dynamic therapy (PDT). Liposomes can aid some of these problems by enhancing stability following administration, improving pharmacokinetics or tissue specificity [6]. But still, current technologies such as "dry lipid film", reverse phase evaporation or "ethanol injection" methods allow for the encapsulation of a hydrophilic active compound, but typically with limited efficiency. The major limitation regarding the type of encapsulated compound is its size and these methods are not practical when encapsulated compound is a large macromolecule [7]. Additionally, it is very difficult to generate uniform population of liposomes loaded with large macromolecules [8]. For this reason, liposomes encapsulating macromolecules have rarely been studied or frequently used before. One of such molecules is Photolon. This PVP-derivative of chlorin e6, is widely tested as a photo-sensitizer in photo-dynamic therapy of various types of cancer [4,9] oral leukoplakia [10] and resistant microbial infections [11,12]. Notably, one of the emerging implementations of PDT and chlorin e6 derivatives is photo-dynamic reduction of cardiovascular plaque [13]. In recent studies Temoporfin (m-Tetra(hydroxyphenyl)chlorin, mTHCP) was encapsulated into polymeric micelles to reduce side effects. Despite relatively low encapsulation efficiency (17%) inside of these micelles, the formulation successfully targeted and repolarized THP-1 macrophages in *in vitro* system. Similarly, some liposomes, especially these with negative surface charges, are efficiently internalized by macrophages [14–17]. Targeting this particular cell subset, which are believed to transform into
arteriosclerotic foam cells and crucial for development of cardiovascular plaque can enhance effectivity of the PDT modalities tested for treatment of arteriosclerosis [18].

In this paper we show novel liposomal Photolon formulation obtained via our unique "gel hydration technology". The resulting lipo-aggregates are stable, biocompatible and are uniform with respect to their sizes. Next, this new Photolon formulation was thoroughly characterized and its effectiveness was demonstrated on cellular and animal models relevant for application as anti-macrophage and anti-arteriosclerotic agent.

2. Results

2.1. Liposomal formulation of photo-active drug and encapsulation efficiency

In order to reduce unspecific toxicity of Photolon by encapsulation and its possible selective delivery we developed the liposomal carriers in the form of the homogeneous aqueous suspension. To overcome the limitations of known methods of liposomes generation, especially the low encapsulation efficiency of large macromolecules, we applied our own novel gel hydration technology (patent no. PL232799). For formation of liposomes the phosphatidylcholine from soya beans as the well-defined as biocompatible lipid was selected. The resulting aggregate are considered stable, biocompatible and immunologically inert thanks to the outer antigen free lipid surface [1]. The liposomes were visualised by the transmission electron microscopy (TEM) (Fig. 1a-b). Images illustrates presence of large number of nano-vesicles loaded with PVF-conjugated chlorin e6 that are uniformly distributed inside of liposome. The external layer of liposome consisted of a single spherical lipid bilayer about 3 nm thick, as is expected for a single lipid bilayer. The encapsulation efficiency of Photolon was determined by ultrafiltration technique using membranes with 30kDa cut-off pores. The resulting encapsulation efficiency from of 3 independently prepared samples equals to 93% +/- 6%.

Additionally, each of the prepared batches of liposomes have been characterized by the dynamic light scattering method (DLS). The suspensions of Photolon-loaded liposomes were uniform with respect to their shape and size. The average size equals to 124.7 nm ±0.6 nm, with polydispersity index PDI = 0.055 and zeta potential = -5 mV (Table 1).

<table>
<thead>
<tr>
<th>Table 1. Summary of physical characterisation of liposomal formulation of Photolon.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hydrodynamic Size</strong></td>
</tr>
<tr>
<td>[nm]</td>
</tr>
<tr>
<td>Liposomal</td>
</tr>
</tbody>
</table>
Figure 1. Transmission electron micrographs of liposomal formulation of Photolon of a) single liposome with the spherical bilayer of 3 nm in thickness and b) separate, uniform, liposomes packed with photosensitizer. c) Fluorescence 30 kDa filtered liposomes, along with fluorescence of supernatant indicating high encapsulation efficiency.
Figure 2. The homogeneity of vesicles population encapsulating Photolon. (a) Vesicle size distribution obtained from DLS experiment; (b) quality of the single population model fitting to experimentally derived autocorrelation function.

2.2. Pharmacokinetics of photo-active liposomes formulation

The prepared formulation was made to be suitable for intravenous administration and intra-coronary PDT. Regarding such implementation the *Sus scrofa f. Domesticica* was selected as a model for pharmaceutical studies. The pharmacokinetics of Photolon liposome formulation was measured using the multi-labelling protocol. The fluorescent properties of Photolon are suitable for monitoring presence and concentration of its liposomal formulation in serum or cells using $\lambda_{ex}=405$ nm and $\lambda_{em}=670$ nm Figure 3a. Using that set concentration can be determined in large range of concentrations (Figure 3b). The lipid vesicles were marked with fluorescently labelled lipids, NBD-PE ($\lambda_{ex}=460$ nm, $\lambda_{em}=535$ nm) and/or Rhodamine-PE ($\lambda_{ex}=560$ nm, $\lambda_{em}=583$ nm). The time of decay of lipid derived fluorescence and Photolon fluorescence are correlated as presented on Figure 4a. The 10 mL of the multi-labelled formulation (0.45 mg/g Photolon active liposomal formulation was injected intravenously to the jugular vein of *Sus scrofa f. Domesticica*. Then, following the administration, fluorescent signals of labelled lipids along with the intrinsic fluorescence of Photolon ($\lambda_{ex}=405$ nm, $\lambda_{em}=670$ nm) was measured (Figure 4b). The calculated half-time of Photolon equals $t_{1/2}=20$ minutes, whereas AUC equals $14.7$ $\mu$g/mL*minute. The linear dependence of Photolon and labelled lipid fluorescence indicates that formulation is stable in animal serum and Photolon remains inside liposomes. Additionally, animals were observed for 3 days after administration of Photolon formulation and no signs of delayed toxicity were observed.

Not clear if there is interference in the measurement of void liposomes and C6 containing due to fluorescent or NBD-PE and C6...
Figure 3. (a) The spectra of Photolon liposomes. (b) The fluorescence signal 405 nm/670 nm is concentration dependent. Representative graphs are shown.

Figure 4. The pharmacokinetics characteristics of liposomal Photolon injected in jugular vein of Sus scrofa f. domestica. Panel (a) shows the correlation between fluorescence of Photolon and NBD-PE or Rhodamine-PE measured in blood. The experimental data can be fitted with straight lines what indicates that Photolon remains in liposomes throughout the duration of the experiment. Panel (b) shows pharmacokinetics curve for fluorescently labelled lipids and Photolon.

2.3. Macrophage selective biological activity

To assess biological activity of liposomal Photolon formulation we use four cell lines relevant to planned application for the atherosclerotic plaque treatment: vascular smooth muscle cells (TG-HA-VSMCs), umbilical vein endothelial cells (HUVECs) and two activated macrophage cell lines RAW264.7 and J774.1. The dark cytotoxicity was studied using MTS assay in high range of concentration up to 1 mg/mL. After 48h, we found that liposome formulations did not exert any cytotoxic effect regardless the cell type (Figure 5).
Figure 5. The dark toxicity of liposomal formulation of Photolon in cell lines relevant for atherosclerotic plaque formation. The viability was measured by MTS assay and results shown as a percentage of untreated control. Averages and SD are shown.

To monitor the uptake of Photolon to the cells, again, we took advantage of the fluorescence properties of studied compound (Figure 3). We found that all cell lines can accumulate the Photolon from liposomal formulation over the time. As would be expected, uptake to macrophage cell lines was more rapid and efficient than to the smooth muscle or endothelial cells. Notably, the 10' incubation was sufficient for maximum uptake in J774.1, while in VSMCs the time required for building-up maximum uptake was 45'. Moreover, the value of the maximum uptake differed greatly between the macrophages and other cell lines. The macrophages could accumulate the Photolon corresponding to over the 1000 RLU, while the vascular smooth muscle cells or umbilical vein endothelial cells only corresponding up to 500 RLU (Figure 6a). The availability of drug will also depend on kinetic of its release. In this regard the cell lines differ greatly. The RAW264.7 or TG-HA VSMCs sustain the level of Photolon, while the J774.1 or HUVECs released the drug. Nevertheless, the content of photo-sensitizer was higher in macrophage cell lines (Figure 6b). Presented data indicates the preferential accumulation of photo-active drug in macrophages, the cells engaged in aetiology of atherosclerotic plaque formation.
Figure 6. a) The accumulation of Photolon inside of the indicated over the time of incubation with liposomal formulation. Average RLU±SD are shown. b) Release of Photolon formulation. Amount of Photolon in the cells treated with liposomal for 15 minutes and its decrease over the time. Average RLU±SD are shown.

Next, we asked if more efficient accumulation of the Photolon in J774.1 cell will correspond to its higher sensitivity in PDT. The illumination of J774.1 cells diminished its viability, in light dose dependent manner, by 60% (Figure 7a, grey bars) and the reduction of number of cells can be also easily observed in light inverted microscope. (Figure 7c). At the same time, the illumination of TG-HA-VSMC resulted in only up to 10% reduction of viability of the cultures. (Figure 7a, dark bars). The general mechanism of induction of cell death by the photoactivated Photolon is induction of reactive oxygen species (ROS). The amount of ROS in the illuminated cells was assessed using fluorescent probe of ROS H2DCFDA. We observed light dose dependent induction of ROS in J774.1 (Figure 7b), which was consistent with viability loss.
Figure 7. Assessment of *in vitro* PDT. a) The cells were pre-incubated for 30 minutes with liposomal formulation of Photolon and illuminated with X nm diode laser for indicated time. The viability of the cell cultures was assayed using MTS assay. Results are expressed as percentage of untreated control. Average±SD are shown. b) Irradiation induced ROS assayed with fluorescent H2DCFDA probe. Average RLU±SD are shown. c) Morphology and density of illuminated J774.1 cell cultures subjected to photo-sensitization.

3. Discussion

In this paper, we present and characterise our novel liposomal formulation of Photolon, PVP-modified chlorin e6, suited for intra-vascular delivery and PDT of cardiovascular plaque. To our best knowledge, analogue liposomal formulation of chlorin e6 suited for corresponding applications has not been reported, yet.

Encapsulation of Photolon in liposomes was performed using our patented technology, LipoShell. Although, there are the reports of obtaining heavy-loaded liposomes, but it are using smaller drugs with favourable physical properties [19,20], the chlorin e6 derivate Temoporfin, similar to Photolon, was encapsulated into polymeric micelles with only about 10 % [21] or 17% loading capacity [13]. It could be contrasted with 93 +/- 6% loading in case of our formulation. Notably, despite relatively low loading the Temoporfin preparations remained biologically active, showing potential of a chlorin e6 derivate in PDT. Obtained liposomes have relatively low average diameter equals 124.7 nm ± 0.6 nm, which is similar to Temoporfin formulation [21] or to liposomes loaded with unmodified chlorin e6 [12]. Our method allows preparation of uniform formulation, which is reflected by excellent PDI=0.055, superior to registered photo-active formulations Foslip or Fospeg characterised with PDI over 0.1 [22] or experimental formulations of chlorin e6, which varied greatly in size [12].

Thus, our technology provides novel and unique opportunity for encapsulation of hydrophilic substances in liposomes, such as polymers, small hydrophilic molecules, biological macromolecules such as peptides, enzymes,
DNA with efficiency that is not available in the literature data, so far. Again, to our best knowledge, Photolon, which is PVP polymer of 11kDa size, could not be encapsulated with such high efficiency by any other known method of liposome manufacturing [1]. High packing density of hydrophilic substances inside vesicles with homogeneous distribution of liposomes allows for relative easy production upscaling and possible, future application of the studied formulation as drug.

To ensure good biocompatibility of resulting liposomes pharmacy grade pure phosphatidylcholine stabilized with 0.1% ascorbyl palmitate (Phospholipon 90G), was used for liposomes preparation. Phosphatidylcholine for numerous occasions were shown to be well-suited for in vivo application [23,24]. In our case, as expected, intravenous administration of liposomal formulation does not result in any immediate toxic effect. Although, the incidence of immunological side effects are still possible or liver or kidney toxicity need to be establish. Nevertheless, the incidence of such reactions should be attributed to impurities of phospatydiocholine preparations or Photolon itself. In studied formulation the majority of Photolon is enclosed within the liposome membrane and lipid bilayer forms continuous barrier around photo-sensitizer. It greatly decreases exposure of Photolon to physiological fluids after injection and is standard procedure to increase stability of drug and to lower the unspecific toxicity [6,15,23,25].

Having in mind the proposed application, the large animal, Sus scrofa f. domestica, was selected for pharmacokinetic measurements. We have shown that the liposomes remain intact in serum following the injection. They remain in circulation for relatively short time (t₁/₂ =20 minutes).

As we observed very fast accumulation of Photolon inside the macrophage cells in vitro, such short half-time should be sufficient for photo-sensitisation of macrophages and should lower the probability of systemic toxic reaction. There are only a few studies of chlorin-related photo-sensitizers that were performed on large animals. None of them included intravenous administration [26–28]. Very comprehensive pharmacokinetics studies of registered Temoporfin formulations, Foslip and Foscan, were performed on rodent model. The half-times of both compounds varied from 8 to 11 hours [29], which is several times longer than times observed for our formulation in pig model. In our opinion the difference could be primary attributed to species differences [30]. On the other hand the Temoporfin, has half life time in human serum as long as 65 hours [31], suggesting that other properties also affect pharmacokinetics.

The good photo-sensitizer should not be toxic prior the exposure to light. The Photolon alone exhibit low dark toxicity [9,32]. Nevertheless, using cell lines relevant to planned application vascular smooth muscle, vascular endothelial and activated macrophage cell line we have shown that our liposomal formulation did not exert any significant dark cytotoxicity, which is consistent with data obtained with other chlorin related compound [13,17,21].

Molecular mechanism of the vascular disease have been extensively studied and provide guidance for the development of medical strategies based on
nanomaterials targeted selectively to diseased cells and/or tissues [33]. Macrophages are believed to be one of the most important factors in atherosclerotic progression and are known to release inflammatory factors and chemokines that affect healthy blood vessels and thus contribute to neointimal hyperplasia or restenosis following interventional therapies [15,18]. Therefore, targeting macrophage proliferation via specially design drug delivery systems called nanocarriers is a promising strategy to treat atherosclerotic lesions. There are several reports suggesting the preferential uptake of liposomes by macrophages, which could make the liposomes excellent tool for targeting this particular cell subset. It could ensure some specificity of drug delivery in macrophage related pathologies.

The cause of that selectivity has not been studied. It could be related with high phagocytic activity of macrophages, accumulating great variety of nanomaterial. It results in greater sensitivity to treatments with particulate matter [34]. On the other hand the cause of selectivity could be related to expression of scavenger receptors on macrophages [35,36]. If this is the case it would additionally enhance selectivity towards the macrophages residing in vascular plaque [37].

In this regard we have shown that Photolon liposomal formulation is preferentially accumulated in macrophages, but not smooth muscle or endothelial cell lines. Consequently, exposure to light resulted in much greater cytotoxicity towards macrophages then the other cells. This is very important, especially in the context of arteriosclerosis treatment, as the sparing the endothelial cells during PDT would be beneficial for preserving the vascular wall haemostasis.

4. Materials and Methods

4.1. Liposomal formulations

Highly purified phosphatidylcholine from soya beans (Phospholipon 90G) was purchased from Lipoid (Switzerland) and its purity was checked by HPLC - ELSD method, as recommended by the manufacturer and validated in our laboratory, according to OECD and ICH requirements. Fluorescent dyes; Rhodamine PE and NBD - PE, as chloroform solutions, were purchased from Avanti Polar Lipids (USA), and were tested for fluorophore - lipid stability with HPLC-ELSD - FLUO. Buffered aqueous solutions were prepared from PBS tablets purchased from Sigma-Aldrich and were always filtered through 200 m (Whatman, USA) after preparation. Conductivity and pH of solutions were checked before each experiment. Photolon was purchased, as a pharmaceutical ingredient in the powder form, from Belmedpreparaty (Minsk, Belarus). Liposomes were prepared by the "the gel hydration method" (EUP 17 162 568.4). Specifically, lipids at high concentrations (> 20% w/w) with or without fluorescent dye, were dissolved in propylene glycol and hydrated with aqueous phase containing Photolon. Next, the final liposomal gel was extruded 5 times through a 100 nm pore size polycarbonate
Whatman® filter. The final liposome suspension consists of 20% w/w Phospholipon 90G, 25% w/w of propylene glycol, 49.55% w/w water and 0.45% w/w of Photolon. For pharmacokinetics studies lipid fraction was doped with small quantity (0.05 % w/w) of fluorescently labelled lipids; NBD-PE and Rhodamine-PE.

4.2. Encapsulation efficiency determination

The encapsulation efficiency was determined using ultrafiltration method. Samples of liposomal gels were 5x diluted before ultrafiltration, which was performed using ultrafiltration PS Microkros filter modules (C02-E050-05-N from Spectrum Labs) composed of 20 cm polysulfone tubes with 30 kDa cutting size pores. Three successive ultrafiltrations were performed on each sample to make sure the extravascular polymer was removed. A small fraction of permeate (about 1 mL) as well as initial solutions and retentate were collected for subsequent evaluation. The evaluation of Photolon quantity inside liposomes was based on fluorescence measurements (λ_ex=405 nm, λ_em=670 nm) following the destabilization of lipid bilayer by 100 μL of 20% Triton X-100. The encapsulation efficiency, η was calculated according to the following formula:

$$\eta = \frac{C}{C_{tot}}$$

where C_in and C_tot are Photolon concentration inside liposomes and total Photolon concentration, respectively.

4.3. Characterization of liposomal formulations using TEM

Photolon containing liposomes were imaged with a FEI Tecnai G2 20 X-TWIN transmission electron microscope operated at 200 kV. The samples were placed onto a grid covered with a collodium film. Measurements were then performed without or with staining solutions containing 1% of uranyl acetate or phosphotungstate which was blotted off after 60 sec. following sample deposition.

4.4. Characterization of liposomal formulations dynamic light scattering (DLS)

Liposome’s size and Zeta-potential were determined using the dynamic light scattering (DLS) technique (Malvern Zetasizer Nano ZS, Malvern Instruments, UK). The instrument was equipped with a He/Ne laser emitting at 633 nm, a measurement cell, a photomultiplier and a correlator. The samples were diluted around 100 times with the buffer, so the sample osmotic balance was maintained, and placed in 1 cm polystyrene cells. The refractive index of the material and viscosity were fixed to 1.33 and 0.9025 cP, respectively. The scattering intensity was measured at 25°C at a scattering angle of 173° relative to the laser source. The liposome sizes were derived from the correlation function using software provided with the instrument. Each measurement was performed three times with 12 repetitions.
4.5. Cell culture

J774A.1 (mouse monocyte/macrophage cells) cell line was obtained from IITD collection. Cells were cultured in high-glucose Dulbecco Modified Eagle’s Medium (DMEM; IITD) supplemented with 10% fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific), glutamate, HEPES, sodium pyruvate and with Antibiotic and Antimycotic Solution (Sigma-Aldrich). Culture plates and flasks were purchased from Corning Incorporated (Tewksbury, MA, USA). Cells were grown at 37°C, in 5% CO₂, and 95% humidity (NuAire, Plymouth, MN, USA). The cells were seeded on 96 well plate at densities of 8×10³ cells/0.1 mL (cell viability assay and microscopy images) and 30×10³ cells/0.1 mL (kinetic of liposomal Photolon formulation uptake and release) and rested for 24 h for culture stabilisation.

4.6. Cytotoxicity studies

Dark and phototoxicity of liposomal Photolon (3%) formulation was determined using the CellTiter 96 AQueousOne Solution Cell Proliferation Assay MTT (Promega, Madison, WI, USA) and microscopic observations. Cells were treated with liposomal Photolon formulation suspended in 100 µl of DMEM, the final concentration of Photolon was 2 µg/mL. After 45 minutes of incubation, cells were washed twice with 2.5% FCS in PBS (IITD) and fresh cultured media was added. Then, the cells were illuminated (λ=655 nm, E=500 mW/cm², H=15 J/cm²) for 30, 60 or 90s. After 48 h, the changes in J774A.1 cell morphology were visualized using bright field microscopy (Olympus IX81). Next cells were incubated in the presence of the MTS solution. Subsequently the values of 490nm absorbance corresponding to the number of metabolically active cells were measured on a WallacVictor2 plate reader (Perkin Elmer, Waltham, MA, USA). The cell viability was calculated as the % of control sample (100%). The experiments were performed in triplicate.

4.7. Intracellular reactive oxygen species level

Reactive oxygen species (ROS) concentration was assessed by measuring the 485nm/535nm fluorescence of H₂DCFDA (6-carboxy-2',7'-dichlorodihydrofluorescein diacetate, di(acetoxymethyl ester); Molecular Probes). Briefly, cells were treated 45 minutes with liposomal Photolon formulation suspended in 100 µl of DMEM, the final concentration of Photolon was 2 µg/mL. Then, the cells were washed and stained with 10 µM of H2DCFDA for 30 minutes in PBS with 10% FCS. Next cells were washed twice with warm PBS with 2.5% FCS and 100 µl of fresh DMEM without phenol red was added. After basal fluorescence measurement cells were illuminated (λ=655 nm, E=500 mW/cm², H=15 J/cm²) for 30, 60 or 90s and the fluorescence of ROS was measured immediately on Microplate Reader EON (BioTek). The experiments were performed in triplicate.

4.8. Evaluation of the liposomal Photolon formulation release/uptake
Release of liposomal Photolon formulation was determined by the Photolon fluorescence measurements. Briefly, J774A.1 cells were incubated 45 minutes with liposomal Photolon formulation (2 µg/mL Photolon in 0.2 mL media). Next, medium was transferred to other wells on the same plate, cells were washed with PBS. Then, PBS was transferred to other wells, the fresh medium was added to cells and fluorescence of Photolon (λ<sub>ex</sub>=405 nm, λ<sub>em</sub>=670 nm) was measured on Microplate Reader EON. This procedure was repeated at indicated time intervals after first measurement (0 minutes).

2.9 Pharmacokinetic protocol

Pigs were anesthetized with isoflurane and surgically equipped with a permanent catheter (Cook Espana, Barcelona, Spain) placed in the jugular vein. The aim of the study was to determine the pharmacokinetic profile of liposomal formulation (0.45 mg/g of lipid and Photolon) in Sus scrofa f. domestica. Animals were anesthetized with isoflurane and surgically equipped with a permanent catheter placed in the jugular vein for withdrawing blood samples following the injection of 10 mL solution of liposomal Photolon was injected into the anaesthetised animal and its blood was collected in 6 time points. Reference blood sample was drawn shortly prior the injection of liposomal formulation. Photolon concentrations in blood samples were determined by the fluorescence measurements with JobinYvon spectrofluorimeter in quartz 5 mm x 5 mm cuvettes (Hellma, Germany). The excitation wavelength was set to 405 nm with monochromator slit of 4 nm emission spectra were corrected in the range 550-700 nm with 4 nm slit. Spectra were acquired directly from the plasma (blood after erythrocyte centrifugation).

5. Conclusions

The novel Photolon liposomal formulation have been developed. The major advantage of this new formulation is its very high encapsulation efficiency reflected with over 90% loading with Photolon. Liposomes loaded with Photolon have shape of regular spheres with average diameter of 124.7±0.6nm and form uniform population with polydispersity index PDI = 0.055.

The Photolon liposomal formulation:

- is suitable for intravenous injection
- is stable in Sus scrofa f. Domestica serum, its haltime in animal serum is 20 minutes and calculated AUC equals 14.7 µg/mL*minute
- does not caused toxic reaction in animals or dark cytotoxicity in cells in vitro
is rapidly uptaken by macrophages, but not vascular smooth muscle cells or vascular endothelial cells in vitro

- can induce reactive oxygen species and viability loss in macrophages, but not vascular smooth muscle cells in vitro

All this features, including high density and selectivity for macrophages reveal a great potential of this Photolon formulation for use in atherosclerosis treatment by photo-dynamic reduction of atherosclerotic plaque.

6. Patents

Part of presented work resulted in patent no.PL232799.


Funding: This research was funded by Polish National Centre of Science, grant number UMO-2013/09/B/NZ5/02764 and statutory program no.3 of Ludwik Hirsfeld Institute of Immunology and Experimental Therapy.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

**AUC** Area under the curve

**DLS** Dynamic Light Scattering

**ELSD-FLUO** Evaporative light scattering detector

**FBS** fetal bovine serum

**FDA** U.S. Food and Drug Administration

**HPLC** High-performance liquid chromatography

**ICH** International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use

**IITD** Institute of Immunology and Experimental Therapy

**MDPI** Multidisciplinary Digital Publishing Institute

**NBD** nitrobenzoxadiazole

**OECD** Organisation for Economic Co-operation and Development

**PDI** polydispersity index

**PE** Phycocerythrin

**PVP** polyvinylpyrrolidone

**RLU** Relative light unit
ROS  reactive oxygen species
SD  Standard deviation
TEM  Transmission Electron Microscopy

References


1. Author 1, A.B. Title of Thesis. Level of Thesis, Degree-Granting University, Location of University, Date of Completion.

2. Title of Site. Available online: URL (accessed on Day Month Year).

19 by the authors. Submitted for possible open access publication under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/)