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Research paper

Development and *in vivo* validation of phospholipid-based depots for the sustained release of bupivacaine

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ABSTRACT

By direct deposition of the drug at the local site of action, injectable depot formulations – intended for treatment of a local disease or for local intervention – are designed to limit the immediate exposure of the active principle at a systemic level and to reduce the frequency of administration. To overcome known drawbacks in the production of some marketed phospholipid-based depots, here we propose to manufacture drug-loaded negatively charged liposomes through conventional technologies and to control their aggregation mixing a solution of divalent cations prior to administration. We identified phosphatidylglycerol (PG) as the most suitable phospholipid for controlled aggregation of the liposomes and to modulate the release of the anesthetic bupivacaine (BUP) from liposomal depots. *In vivo* imaging of the fluorescently-labelled liposomes showed a significantly higher retention of the PG liposomes at the injection site with respect to zwitterionic ones. *In situ* mixing of PG liposomes with calcium salts significantly extended the area under the curve of BUP in plasma compared to the non-depot system. Overall, controlling the aggregation of negatively charged liposomes with divalent cations not only modulated the particle clearance from the injection site but also the release *in vivo* of a small amphipathic drug such as BUP.

1. Introduction

Long-acting depot systems entered successfully the market in the early 2000s thanks to their tunable drug release kinetics ranging from days to months. Notable examples are the *in situ* forming implant system technology Atrigel® [1], the liquid crystalline phase technology FluidCrystal® [2], and the multivesicular liposomes (MVL) technology DepoFoam™, which includes Exparel® (bupivacaine, BUP), DepoDur® (morphine) and DepoCyte® (cytarabine) [3–9]. While DepoDur and DepoCyt were discontinued from the market in US or Europe, new therapeutic indications are explored for Exparel [7–9], one of the most prominent examples among the lipid based-drug delivery systems.

When designing a drug carrier for any route of administration, liposomes most certainly represent a carrier of choice thanks to their very low toxicity profile indeed [12]. Their high biocompatibility, their ability to encapsulate both hydrophilic and lipophilic drugs and the high variety of possible chemical modifications make them particularly suitable for depot formulations [13]. The DepoFoam multivesicular liposomes in Exparel, containing numerous internal aqueous compartments divided by non-concentric lipid bilayer, features high encapsulation efficiency and extended-release profile of the local anesthetic BUP, with a consequent pain reduction lasting up to 3 days [12–15]. Regrettably, the production of DepoFoam vesicles (a two-step, water-in-oil-in-water double-emulsification process) requires organic solvent [16,17] - a serious limitation of this system - and, due to the large size of the MVL and their temperature sensitivity, the complete production needs to be performed under aseptic conditions, resulting in a cumbersome and cost-ineffective process.

To overcome these drawbacks, our group proposed to generate a liposomal depot injectable via bedside mixing of large unilamellar

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vesicles (LUV) formulated with negatively charged phospholipids (NCP) and divalent cations (as calcium or magnesium) to prompt a controlled aggregation of the vesicles just before injection, due to the electrostatic interaction between the NCPs and cations. We identified, among several phospholipids screened, three depot-forming NCP candidates (1,2-dipalmitoyl-*sn-glycero*-3-phosphate (DPPA), 1,2-dioleoyl-*sn-glycero*-3-phosphate (DOPA) and 1,2-distearoyl-*sn-glycero*-3-phospho-(1′-*rac*-glycerol) (DSPG)) and their physicochemical characteristics evaluated *in vitro* [18]. In the current study, we explore the use of DSPG, DPPA and DOPA as building block for depot formulations to deliver BUP locally and we investigated the influence of the anesthetic drug on the aggregation process. The *in vitro* studies showed that phosphatidylglycerol (PG) is the most suitable phospholipid to modulate the BUP release from liposomal depot injectables upon addition of divalent cations. *In vivo* imaging of fluorescently labelled liposomes administered subcutaneously and pharmacokinetics studies in rats of the BUP-loaded liposomal depots confirmed the results obtained *in vitro* and *ex vivo*: the aggregation of negatively charged liposomal formulations upon addition of divalent cations can control not only the clearance of the particles but also, and more interestingly, the release of a small amphipathic drug such as BUP. Overall, we could show that by generating liposomal aggregates, drug diffusion can be slowed down and extended. Depot injectables can be formed easily prior to injection, controlling the aggregation of NCP-liposomes through the interaction with divalent cations added at concentrations enabling a rapid fusion-free aggregation. A further advantage is that sterile injectable NCP-depots can be produced with existing technologies by using sterile filtered liposomes prior to the aggregation step, which may take place in a double chamber syringe.

2. Materials and methods

2.1. Materials

1,2-dipalmitoyl-*sn-glycero*-3-phosphate (DPPA), 1,2-dioleoyl-*sn-glycero*-3-phosphate (DOPA) 1,2-dipalmitoyl-*sn-glycero*-3-phosphocholine (DPPC); 1,2-distearoyl-*sn-glycero*-3-phospho-(1′-*rac*-glycerol) (DSPG) (Lipoid, Ludwigshafen, Germany); cholesterol (Chol), bromphenolblue and sodium chloride (NaCl) (Sigma Aldrich, Schnelldorf, Germany); Bupivacaine (BUP) hydrochloride monohydrate was obtained from Fagron (Glinde, Germany); Bupivacaine-d9 was obtained from Cayman Chemical Company, USA. LC/MS grade acetonitrile, water, and formic acid 98 % – 100 % were purchased from Merck (Darmstadt, Germany); 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt (DiD) (Invitrogen, Carlsbad, USA); calcium chloride hexahydrate ($CaCl_2$), magnesium chloride ($MgCl_2$) hexahydrate and ammonium sulfate $(NH_4)_2SO_4$ (Carl Roth, Karlsruhe, Germany); isoflurane (Attane™, Piramal Pharma, India); pentobarbital (Esconarkon®; Streuli Tiergesundheit SA, Switzerland). Gibco™ water for injection (WFI) Fischer scientific (Switzerland). Acetonitrile (ACN), methanol (MeOH), chloroform, tetrahydrofuran (THF) and trifluoroacetic acid (TFA) were obtained from Carl Roth (Karlsruhe, Germany); Purified and deionized water (H_2O) was prepared with Smart2Pure3 (Thermo Scientific, Niederelbert, Germany).

2.2. Liposome preparation

Liposomes were prepared by film hydration method and subjected to freeze–thaw-cycles [19]. Appropriate amounts of lipid stock solutions in chloroform were transferred into amber glass vials and in the case of DiD labeled liposomes, the needed amount of dye stock solution (10 mg/mL in EtOH) was added together with the lipids so that the final DiD concentration being 0.02 % mol of DiD/mol total lipid. The organic solvent was removed by applying a nitrogen stream and keeping the film overnight under vacuum. After hydration with 150 mM $(NH_4)_2SO_4$ buffer and 6 freeze–thaw-cycles, liposome formulation was extruded 10 times through 2x200 nm polycarbonate membrane. To form the gradient, a spin protocol was used. Sephadex G50 columns (swollen in 150 mM NaCl overnight) were dried by centrifugation. Liposomes were loaded on the columns and elution was carried out by centrifugation (3 min at 1000 g). The eluted liposomes were collected and further characterized for lipid content by HPLC method [20]. In the case of drug-loaded formulations, the samples were incubated for 30 min at 70 °C with a BUP solution. The unencapsulated BUP was removed by SEC following a spin protocol (as described above) and the amount of encapsulated drug was quantified via HPLC (see Supplementary Methods). BUP-liposomes were concentrated by centrifugation using Amicon® Ultra-15 (regenerated cellulose, RC, Cut-off 10′000 MW; Merck Millipore, Massachusetts, USA). The osmolarity of liposomes was measured by a Knauer K-7400S semi-micro osmometer (Germany) and the obtained values were in the acceptable range for subcutaneous injection (<600 mOsm/Kg). In case of formulation produced for *in vivo* studies, water for injection (Fischer Scientific, Switzerland) was employed for preparation of buffers.

2.3. Dynamic laser light scattering measurements

The mean hydrodynamic diameter, the polydispersity index (PDI) and the zeta potential of liposomes were measured using a Litesizer500 (Anton Paar; Graz, Austria) with a 173° backscatter angle and a 633 nm helium–neon-laser. For size measurement, 100 μL of samples (10 mM) were diluted in 1 mL 150 mM NaCl (refractive index of 1.33 and a viscosity of 0.89 mPa/s) to minimize multiple scattering events. After equilibrating the sample at 25 °C for 5 min, the measurement (30 runs × 10 s) was performed. The intensity size distribution of the liposome was unimodal; therefore, the autocorrelation function was analyzed according to the cumulant method by the Kalliope™-software. In the case of zeta potential measurements (100 runs with a voltage set at 200 V) the Smoluchowski approximation with a Debye factor of 1.5 was used to calculate the mean zeta potential value.

2.4. Aggregation study

To determine the extent of aggregation, 50 μL of BUP-loaded or unloaded liposomes formulations (total phospholipid concentration between 5 mM and 50 mM) were mixed in clear 96-well microtiter plates with 150 μL of calcium chloride or magnesium chloride solutions so to achieve a final cation concentration from 0 mM to 10/20 mM. After 2 min incubation, the optical density at $\lambda = 400$ nm (OD_{400}) was measured with a multimode microplate reader (Tecan Spark 20 M or Tecan Infinite M Pro 200F-PlexNano, Tecan, Männedorf, Switzerland). The ΔOD_{400} was calculated by subtracting the absorbance of liposomes without cation addition from every absorbance value of the aggregated samples.

2.5. Transmission electron microscopy

Blank liposomes and BUP-liposomes (total phospholipid concentration 10 mM) in the aggregated or unaggregated state were morphologically characterized via transmission electron cryomicroscopy (cryo-TEM at the Electron Microscopy Center, University Hospital Jena, Friedrich-Schiller-University Jena, Germany). Liposomes were mixed in a 1:4 ratio with water, calcium chloride or magnesium chloride solutions (final cation concentration 10 mM) and 5 μL of each sample were applied to a copper grid covered by holey carbon film (R1/2, 300 mesh, Quantifoil Micro Tools GmbH, Jena, Germany) and excess of liquid was blotted automatically between two strips of filter paper. The grids were mounted on a plunger, blotted and vitrified in liquid ethane at –180 °C. A Gatan 626 cryo-holder (Gatan, Pleasanton, California) was used to transfer the frozen specimen to the Philips CM120 transmission electron cryomicroscope (Philips, Eindhoven, The Netherlands) equipped with a LaB6 cathode source and operated at 120 kV accelerating voltage. Images were recorded with a 2 k CMOS camera TemCam-F216 (TVIPS-

GmbH, Gauting, Germany). Depot liposomes aggregated in presence of calcium were imaged at the Microscopy Imaging Center (MIC), University of Bern, Switzerland. Quantifoil 200 mesh 2/1 grids were glow discharged for 20 min at 10 mA using a Balzers Union CTA010. Samples (3 μL) were applied to the grids and vitrified by plunging in liquid ethane using a FEI Vitrobot Mach4 at 100 % humidity at 21 °C. Samples were loaded onto a pre-cooled Gatan 626 cryoholder and transfered into a FEI Tecnai F20 microscope operated at 200 kV. Images were recorded with a 4 k DED Falcon 3 as aligned frame averages at different magnifications using EPU.

2.6. Differential scanning calorimetry

The thermal behavior of unloaded and BUP-loaded liposomes and their aggregates was investigated using a DSC 250 (TA Instruments, New Castle, Delaware) with TRIOS software (TA Instruments). Multilamellar liposomes (MLVs) were produced by film hydration method in a concentration of 50 mM. BUP was encapsulated passively using a 5 mg/mL BUP solution. Additionally, LUVs were prepared, BUP actively encapsulated, centrifuged (20,000 × g, 1 h) and the pellet resuspended in a low volume to increase the concentration. The liposome samples with or without BUP (15 μL) were transferred in a TZero® aluminum pan (TA Instruments) and hermetically sealed. The aggregate formation was done in the pan by adding different amounts of calcium or magnesium chloride solutions. The liposomes were heated once above their phase transition temperature and afterwards three cycles with a heating rate of 2 °C/min performed. The last cycle was used for the evaluation of the thermal profile and the calculation of the temperature and enthalpy of the phase transition.

2.7. Release studies via "ex vivo dialysis"

Porcine skin with subcutaneous tissue was used to mimic the physiological conditions after *in vivo* subcutaneous injection of the formulations. BUP-loaded liposomes were prepared and centrifuged, and the pellet resuspended to achieve higher concentrations. Liposomes were mixed with calcium or magnesium cations (cation concentration 10 mM) and 80 μL of the aggregated or unaggregated BUP-liposomes or 80 μL of a BUP solution (7.5 mg/mL) injected in the middle of the subcutaneous tissue of porcine skin discs (20 mm diameter, porcine excised skin with subcutaneous layer was obtained from the Institut für Versuchstierkunde und Tierschutz, Jena, Germany). The skin disks containing the samples were placed in dialysis inserts (Slide-A-Lyzer Mini Dialysis Devices, 2 mL, 3.5 kDa MWCO, Thermo Fisher Scientific) and incubated at 37 °C for 10 days under the conditions described above using TRIS buffer (50 mM with 200 mM NaCl) pH 7.4 with physiological concentrations of calcium chloride (2.5 mM) and magnesium chloride (1.5 mM) containing 0.02 % (w/v) of sodium azide. At several timepoints aliquots of the release medium were taken, lyophilized and after reconstitution the BUP amount quantified via HPLC (for details on the method: see Supplementary Material).

2.8. In vivo experiments

All animal experiments were performed on 12 weeks old Lewis rats (LEW/OrlRj, Janvier Laboratories, France) in accordance with institutional and federal regulations governing animal care and use, according to the ARRIVE guidelines and were approved by the Cantonal Veterinary Office of Bern (Switzerland) (BE47/2021). Rats were housed in specific pathogen-free (SPF) conditions in compliance with the and Federation of European Laboratory Animal Science Associations (FELASA) guidelines. Up to 3 rats can be accommodated in autoclaved IVCs cages (Blue line, Tecniplast, Italy) with aspen wood bedding (J. Rettenmeier & Söhne GmbH, Germany) and cotton nestlets as nesting material; a black PVC tunnel (Plexx, Netherlands) and aspen wood stick (LAB & VET Service GmbH, Austria) are present in the home cages as enrichment. The housing conditions are controlled with a 12:12 light: dark cycle, room temperature in the range of 22+/-2°C° and relative humidity in the range of 45 %-65 %. Autoclaved tap water and irradiated rodent chow (Mouse and Rat Maintenance 3432, Granovit, Switzerland) are provided ad libitumin. The rats had an acclimatization period of one week and were regularly handled by the personnel for gentling and habituating to the procedures. On the day of experiment, animals were randomly allocated in different treatment-groups. Each group, consisting of 3 males and 3 females, received a different treatment and the sex was then imposed as blocking factor for the statistical analysis. The results are presented as mean ± σ (n = 6). Multiple comparison between the groups were performed by a non-parametric Kruskal-Wallis test and a *post hoc* correction for multiple comparison was done by using Dunn' s test. The adjusted p-values are reported as: * 0.1; ** 0.05, *** 0.001 and **** 0.0001.

2.8.1. Clearance kinetics of liposome formulations from the injection site

The clearance kinetics of the fluorescently labelled formulations (**Depot-Liposomes**: negatively charged liposomes aggregated with 10 mM $CaCl_2$; **Non-Depot-Liposomes**: negatively charged liposomes without $CaCl_2$ and **Neutral-Liposomes** (DPPC/Chol 70/30 mol/mol) were determined by *in vivo* imaging. *In vivo* near-infrared fluorescence imaging was performed with an IVIS Spectrum scanner (IVIS Spectrum CT; PerkinElmer). Rats (6 animals/group) were anesthetized with 5 % isoflurane in 100 % O_2 at 1 L/min for the induction and anesthesia was maintained with 1.2 minimum alveolar concentration via face mask, and injected subcutaneously in the plantar side of the right hind paw (using 1 mL insulin syringes with a 29G needle) with the DiD labelled formulations (150 μL; 40 mM lipid contrition and 0.02 % mol of DiD) and images of the injection site were acquired (under anesthesia) at different time points immediately before and after injection, at 0.5 and 6 h, 1, 2, 3, 4, 7, 8 9, 10, 11 and 14 days). Animals were euthanized with a pentobarbital injection (150 mg/Kg intraperitoneal) under anesthesia after the last time point and organs were harvested to assess the formulations biodistribution. The IVIS Spectrum scanner was set to fluorescence imaging mode, time and an emission filter positioned at 650 nm. The focus was kept stable using subject high of 1.5 cm whereases the temperature into the chamber was set at 37 °C. Analysis of images (set with a fixed counts scale from 1′000 to 40′000 and acquired with a 0.2 s exposure time) were computed by first define the regions of interest (ROI; a representative image with the ROIs are shown in SI) which was kept consistent across images and then the sum of all counts for all pixels inside the ROI (Total Fluorescence Counts- TFC; photons/second) recorded. The clearance kinetics profile was obtained by plotting the normalized percentage of TFC (as defined in Equation1) *vs* time (day).

$$\%TFC = (TFC_t - TFC_{blank})/TFC_{t0} \quad (1)$$

Where TFC_t, TFC_{blank} and TFC_{t0} are the Total Fluorescence Counts inside the ROI recorded at different time point, before injections and immediately after the injection, respectively.

2.8.2. Pharmacokinetics (PK)

Rats (6 animals/group) were injected subcutaneously in the plantar part of the right hind paw (using 1 mL insulin syringes with a 29G needle) with 150 μL of Depot, Non-Depot and Neutral liposomes, or free BUP. All the formulations had the same final BUP concentration (6.5 mg/mL). For each animal, 100 μL of blood (in compliance with the guidelines provided by National Centre for the Replacement, Refinement and Reduction of Animals in Research [21] was collected from the lateral tail vein at −2 days, 0.25, 2 and 8 h, 1, 2, 3, 4, 7, 9, 11, and 14 days after injection, with the conscious animals placed in a restrainer. Animals were euthanized after the last sampling point with CO_2 (slow fill rate and organ harvesting performed after confirmation of death). Blood was collected and stored in K2-EDTA BD-Microtainer™ (Fisher Scientific AG, Switzerland), centrifuged at 4 °C, at 3000 g for 5 min and

the obtained plasma (25 μL) stored at −20 °C. BUP was extracted from plasma by adding 125 μL of methanol (containing the deuterated bupivacaine; IS) and 100 μL of acetonitrile. After 1 h centrifugation at 20′000 g (4 °C) BUP concentration was determined in the obtained clear supernatant using LC-MS/MS analysis. $AUC_{0\text{-}14d}$ were calculated according to the trapezoid method. The data were furthermore analyzed by population PK modelling with the saemix package (v 3.0) in R (v 4.2.0). One- and two-compartment models with intravascular or extravascular administration as well as a model with a combination of two one-compartment models, one with intravascular and one with extravascular administration to simulate two fractions of differently behaving BUP, were applied. The formulation and sex were tested as potential covariates. However, these analyses failed for the Depot and Non-Depot liposomes as not all parameters were sufficiently defined by the data.

3. Results and discussion

3.1. BUP encapsulation and liposomes characterization

Our group recently showed how liposomes formulated with L-α-phosphatidylcholine from egg (EPC) comprising 25 mol% of various NCP form large aggregates not undergoing fusion upon addition of calcium and magnesium ions and we identified DSPG, DOPA and DPPA as the most suitable phospholipids to generate aggregates using physiological concentration of divalent cations [18].

To encapsulate efficiently a small amphiphilic drug as BUP and to ensure a reduced drug leakage from the liposomes upon storage, our original EPC/NPC formulation was enriched with Chol to modulate the bilayer fluidity. Besides EPC, also the synthetic 1,2-dipalmitoyl-*sn-glycero*-3-phosphocholine (DPPC) was tested as alternative zwitterionic (overall neutral) component. The use of DPPC instead of EPC did not influence the hydrodynamic diameter or zeta potential (*vide infra*). As the EPC-based formulations turned out to be less stable in shelf-life studies and more prone to fusion than DPPC-based liposomes (**Figure S1 and S2**), here we report exclusively data on formulations using DPPC as main zwitterionic phospholipid.

Following the established protocols to generate a transmembrane ammonium sulfate gradient [10,11,22–25], BUP was loaded into liposomes composed by 25 mol% NCPs (DSPG, DOPA and DPPA), 30 mol% Chol and 45 mol% DPPC starting from various drug to lipid ratios (D/L = 1, 2, or 3) with high efficiency (Fig. 1**a**). A D/L ratio of 1 resulted in the highest amount of encapsulated BUP (up to 2.7 mg/mL) whereas an initial D/L ratio of 3 led to the highest final D/L ratio of 0.57 for DSPG based-liposomes, higher than other values reported in literature where only neutral liposomes were used [24,26]. We hypothesize that the electrostatic interactions between the negative surface charge of the liposomes and the positively charged drug remotely loaded in the core as sulfate salt might have enhanced BUP encapsulation. All the formulations exhibit a mean hydrodynamic diameter of around 160 nm (Fig. 1**b**) with a low PDI (<0.15) indicating a homogenous size distribution independently of the NCP used.

We observed an increase of the hydrodynamic diameter and PDI of the formulations after BUP encapsulation and a decrease of the liposomes' zeta potential (Fig. 1**c**), likely caused by the reported interaction of the amphiphilic BUP with the NCPs [28] that we further investigated via DSC (*vide infra*). Notably, all formulations were stable for at least one month either at 4 or 25 °C, meeting the requirements for a drug formulation with a long shelf-life (**Figure S1 and S2**).

3.2. Aggregation study

Although it has been already demonstrated that either the drugs or the cations are able to induce aggregation of NCP-containing liposomes [29–31], the influence of an encapsulated drug on the aggregation behavior of negatively charged liposomes with divalent cations has not been investigated so far. Thus, the aggregation profiles of liposomes encapsulating BUP were followed by turbidimetry, mixing the liposome dispersions with calcium or magnesium chloride and measuring their OD_{400} in high throughput fashion via a microplate reader. BUP-loaded and unloaded DSPG liposomes (+BUP *vs* -BUP, respectively) exhibited the highest threshold cation concentration for the start of the aggregation either with calcium or magnesium (Fig. 2**a and 2d**). The presence of BUP, likely associated with the bilayer as our DSC results suggested (*vide infra*), affected the DSPG liposomes aggregation profile with a higher amount of cation needed for the formation of the depot system with respect to unloaded liposomes.

The two different cations and the presence of the drug did not affect the aggregation profile of DOPA liposome (Fig. 2**b and 2e**), whereas DPPA liposomes aggregates with slightly higher magnesium concentration in the presence of BUP (Fig. 2**c and 2f**). The same results were obtained when BUP was loaded in highly concentrated liposomes (**Figures S3**).

Despite the chain length match in the DPPA/DPPC formulation, only the combination of DPPC with DSPG did not induce any fusion at the explored experimental conditions (**Figure S4**). We decided to carry out all the subsequent *in vitro* and *in vivo* with the DSPG/DPPC formulation, since the existence of isolated, drug-loaded liposomes (without cochleate structures) is essential for a predictable drug release profile to take place from the depot.

A parallel morphological investigation, also aimed at excluding possible fusion processes derived from the cation-mediated aggregation, was carried out via cryo-TEM. Representative micrographs of the Depot BUP-liposomes are displayed in Fig. 3.

All three formulations containing BUP showed LUV in the unaggregated state within a size range of around 150 nm. After the addition of cations (10 mM, NCP: cation ratio 1:16) large, overlaid aggregates could be detected. The individual liposomes in the DSPG and DPPA formulations retained their initial size and shape. Thanks to the inclusion of Chol in the formulations and to the presence of the fully saturated DPPC, the flexibility of the membrane was reduced and we did not observe any deformed contact areas with a flat bilayer region [32]. The same results were obtained when DPPC Depot liposomes were

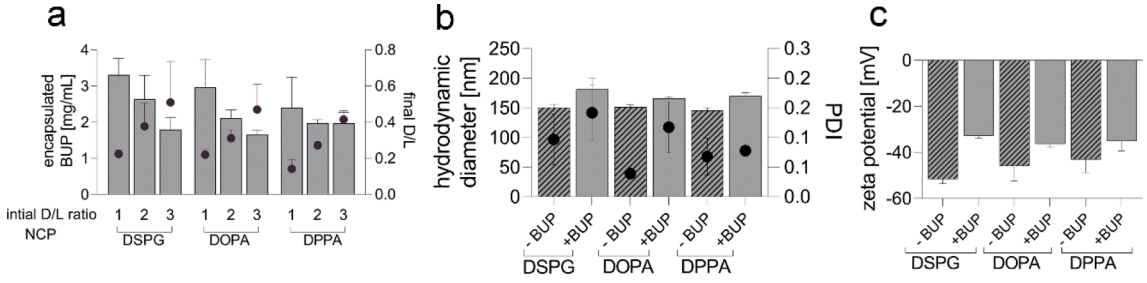

Fig. 1. (**a**) Encapsulation of BUP obtained using different initial D/L ratios (represented as bars plot; left axis) and the final D/L ratio (represented as scatter plot; right axis). (**b**) Mean hydrodynamic diameter (represented as bars plot; left axis) and PDI (represented as scatter plot; right axis) and (**c**) zeta potential of the liposomes. Data are presented as mean ± STDV (n = 3).

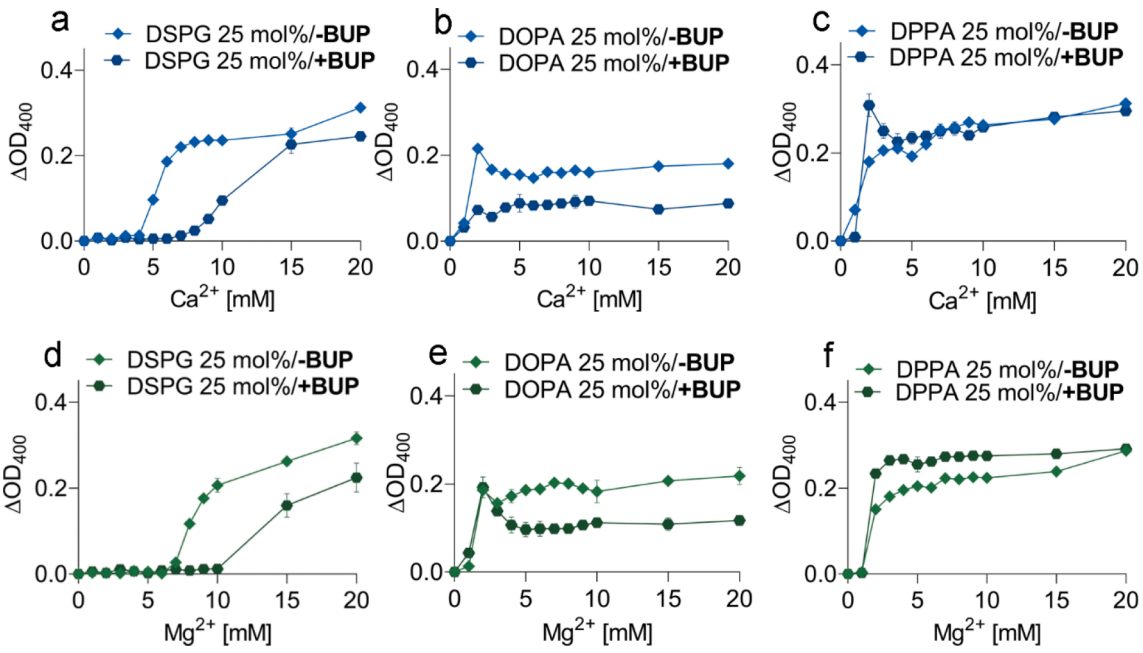

Fig. 2. Aggregation profiles of DPPC liposome formulations containing 25 mol% NCP (DSPG, DOPA or DPPA), 30 mol% Chol and 45 mol% DPPC in presence (+BUP) or absence (-BUP) of BUP in direct comparison (PL concentration 5 mM): DSPG (**a, d**), DOPA (**b, e**) and DPPA (**c, f**). The aggregation was induced by addition of calcium chloride (panels **a, b** and **c**) or magnesium chloride (panels **d, e** and **f**) solutions (NCP:cation ratio 1:32) and measured after 2 min incubation. Data are presented as mean ± STDV (n = 3).

aggregated with a lower NCP: cation ratio of 1:0.4 (**Figure S5**).

Among other techniques, thermal analysis is a powerful tool to understand the interaction between drugs and excipients and, in this specific case, to elucidate why a higher cation concentration was needed to aggregate BUP-loaded formulations. BUP seems to perturb the membrane at a higher extent than the divalent cations do and it has a fluidizing effect on the membrane, consistently with what reported in the literature for local anesthetics [28,33–38]. All BUP-loaded formulations showed indeed a downshift of the T_m either in the case of liposomes composed only by NCPs or in the case of mixed liposomes (**Tables S1, S2 and S3**; representative thermograms are reported **Figure S6**). It is indeed reported that the amphiphilic BUP interacts with the hydrophobic parts of the phospholipid bilayer [28], inducing a perturbation of the lipid bilayer order, weakening the molecular interactions and fluidizing the membrane, with a resulting reduced T_m [36,39,40]. On the contrary, the addition of high concentrations of cations reversed this phenomenon and the T_m values almost raised at the same temperature of drug free liposomes.

BUP-loaded DSPG (and DPPA) liposomes aggregated with the lowest NCP:cation ratio exhibits the same T_m as the respective formulation in absence of cations, confirming a concentration-dependent effect of the cations. The reduction of the T_m is particularly evident in the case of DPPA liposomes, where BUP interacts with the small negatively headgroup of the phospholipids promoting a better penetration of the drug into the hydrophobic region of the bilayer resulting in a modulation of the thermal behavior. On the contrary, this interaction seems to be sterically challenged with DSPG-based liposomes [26,41–43].

3.3. *In vitro release study*

Conventional drug release analysis methods [44,45], using dialysis [46] or a flow-through cell [47] are not applicable to appreciate the long-sustained drug release offered by depot formulations intended for subcutaneous administration. A scientific and pharmacopoeial consensus on standard *in vitro* release tests for this administration route is still missing [48].

To mimic closely the *in vivo* conditions following subcutaneous injection, we decided to inject different liposome formulations encapsulating BUP in presence or absence of cations (10 mM, NCP:cation ratio 1:0.4) into a modified dialysis setup, that we will name "*ex vivo dialysis*" (Fig. 4**a**).

In this setup, a 20 mm disk of porcine skin rich of subcutaneous tissue is first injected with the sample, then placed in a dialysis insert and incubated at 37 °C for 10 days using TRIS buffer pH 7.4 with sodium azide and physiological concentrations of divalent cations.

While all neutral liposomes (Fig. 4**b**) show an identical release profile with 80 % of the drug released after 10 days, the NCP liposomal formulations show different release behaviors. DSPG-containing liposomes was the only NCP-formulation showing a difference in the release profile of BUP between the unaggregated and aggregated liposomes (Fig. 4**c**). The non-aggregated system released about 60 % of BUP over 10 days whereas the aggregated formulations released a maximum of 43 %. In case of DOPA and DPPA formulations, no difference between the aggregated and unaggregated formulations can be detected (cumulative release of < 40 % over 10 days) (Fig. 4**d**).

We hypothesize that the initial non-aggregated DOPA and DPPA liposomes form aggregates *in situ* with the polyvalent cations intrinsically present in the subcutaneous tissue thus leading to similar release profiles compared to the depot formulations. These findings are consistent with the obtained aggregation results, where DSPG liposomes were less pronounced at physiological cation concentrations in comparison to the DOPA and DPPA formulations.

All the NCP-based depot liposomes released only about 40 % of the initially loaded BUP, suggesting a strong compaction of the vesicles containing NCPs after aggregation. This might imply a delayed and hindered diffusion of BUP through the aggregated dispersions until reaching the release milieu.

To confirm this hypothesis, a hydrophilic dye (bromphenolblue) was encapsulated into the liposomes to visualize its distribution in the skin discs during the *in vitro* release (**Figure S7**). In correspondence of the injection site of DSPG liposomes into the skin disc, a dark blue spot from the encapsulated bromphenolblue, still retained at the injection site after 10 days incubation either in the presence or in absence of cations, could be detected, whereas for DOPA and DPPA liposomes the colored

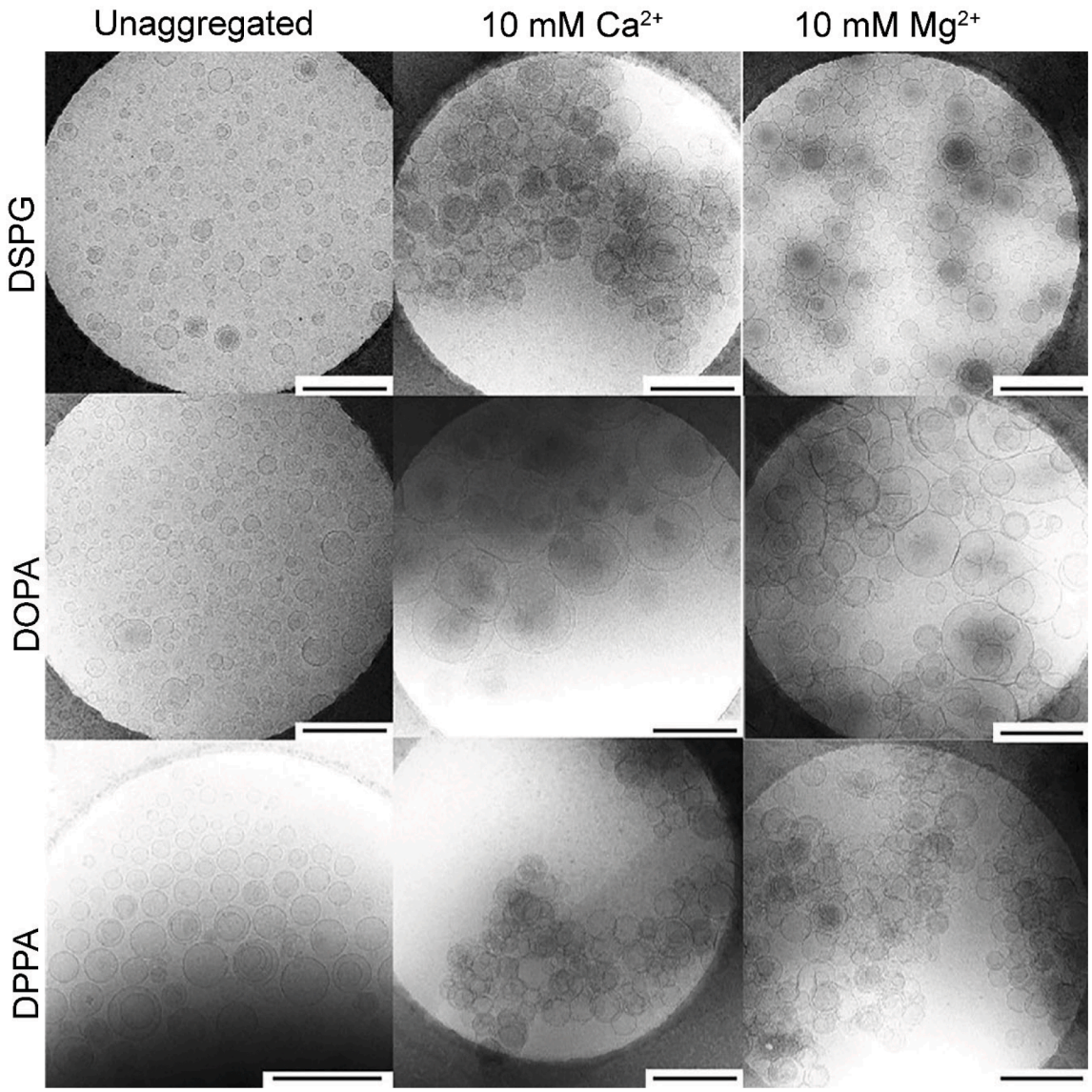

Fig. 3. Representative cryo-TEM micrographs of depot BUP-loaded liposomes (total phospholipid concentration 10 mM) containing 25 mol% NCP (DSPG top, DOPA middle, DPPA bottom row), 30 mol% Chol and 45 mol% DPPC. The first column shows the unaggregated drug-loaded liposomes, the following columns the aggregates with 10 mM calcium cations or 10 mM magnesium cations respectively (NCP:cation ratio 1:16). Scale bar represents 500 nm.

spot was only visible when the vesicles were aggregated prior to injection.

Taken together, our *in vitro* investigations revealed the superiority of liposomes formulated with DSPG as NCP and DPPC as phospholipid to form depot upon addiction of cations and in absence of fusion and, most importantly, to release slowly the encapsulated BUP. The *ex vivo dialysis* assay showed that the presence of cations was decisive to modulate the BUP release only with the 25 mol% DSPG, 30 mol% Chol and 45 mol% DPPC formulation, and we thus decided to continue with this NCP-liposomes in our *in vivo* investigation.

3.4. In vivo studies

To produce suitable formulations for the *in vivo* studies, a scale-up process was performed to increase the BUP concentration in Depot, Non-Depot and Neutral liposomes (Supplementary Material). The formulations prepared for the *in vivo* studies, for the preparation of which a D/L ratio of 5 was employed, were analyzed before administration and the concentration of either the lipids or the BUP was assessed, guaranteeing that the same drug dose (6.5 mg/mL) was used across the groups in the PK and the same lipid concentration (40 mM) was administer in the imaging study. Moreover, either the viscosity or the injection force required to administer the formulations were evaluated. Both values were close to that one obtained for NaCl solution thus the administration (through a 28G needle) guarantees minimal pain or distress for the animal during the injection (Supplementary Material, viscosity and injectability studies and **Figures S8 and S9**).

Firstly, the clearance kinetics of the fluorescently labelled formulations (Depot-Liposomes: 25 mol% DSPG, 30 mol% Chol and 45 mol% DPPC aggregated with $CaCl_2$; Non-Depot-Liposomes: 25 mol% DSPG, 30 mol% Chol and 45 mol% DPPC without $CaCl_2$ and Neutral-Liposomes 30 mol% Chol and 70 mol% DPPC) were determined by noninvasive *in vivo* fluorescence imaging (IVIS®). Clinically, no local or systemic side or adverse effect occurred. The study (Fig. 5) demonstrated that depot formulation had a significantly slower clearance compared to neutral formulation at all timepoints tested and these differences are qualitatively visible in the imaging of the site of injection over time (Fig. 5**A**). On the other hand, no significant differences were observed between Depot and Non-Depot formulation. It is indeed worth mentioning that Non-Depot and Neutral formulations, as well as Depot and Neutral

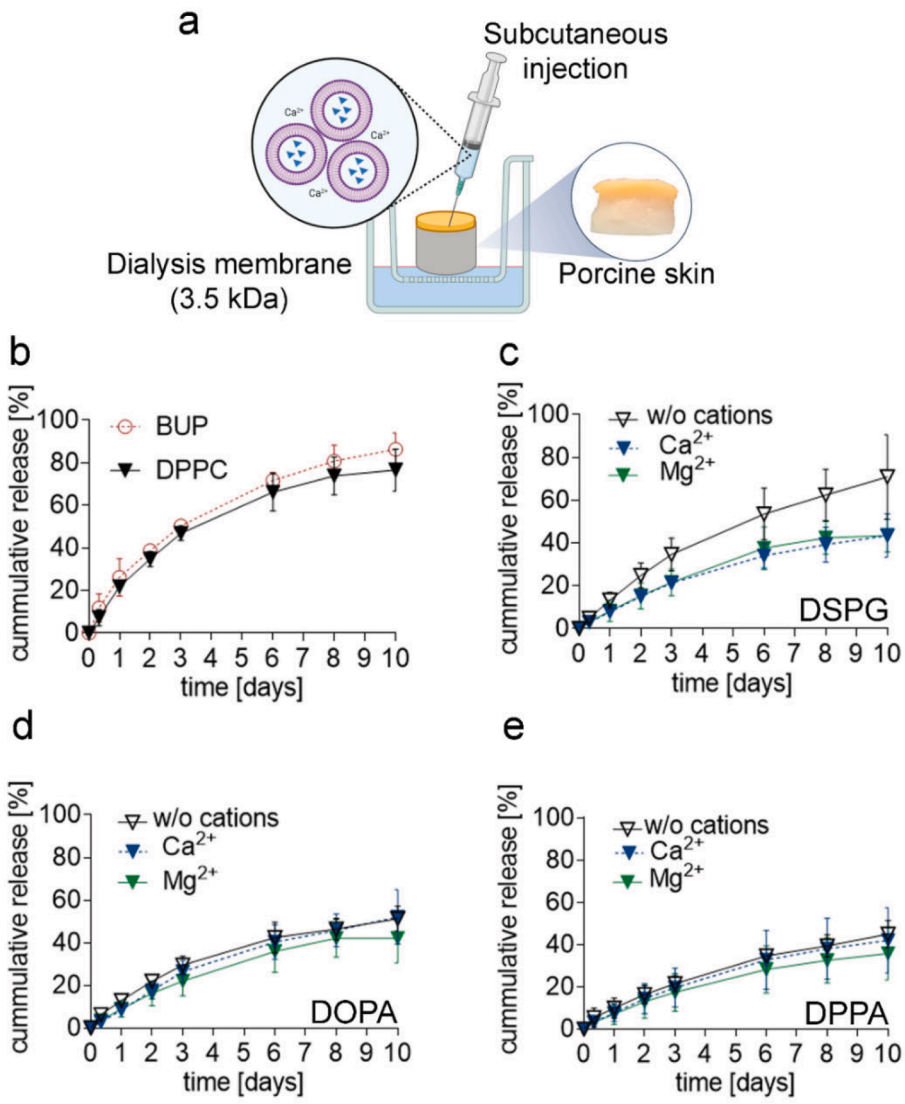

Fig. 4. *Ex vivo dialysis* setup (**a**) and cumulative release profiles of BUP after s.c. injection of liposomes (100 mM) composed of 25 mol% NCP, 30 mol% CHOL and 45 mol% DPPC into porcine skin: Neutral liposomes deprived of aggregation behavior (30 mol% CHOL and 70 mol% DPPC) and a BUP solution (**b**), DSPG (**c**), DOPA (**d**), DPPA (**e**). The aggregation was induced by addition of calcium chloride (blue triangles) or magnesium chloride (red triangles) solutions (NCP: cation ratio 1:0.4). Data are presented as mean ± STDV (n = 3). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

formulations, showed a significantly different clearance profile and a different area under the curve (AUC, obtained from %TFC *vs* time profile; Fig. 5**C**) highlighting the essential role of negatively charged liposomes in the particle-clearance kinetics [49]. As we expected, the fluorescence of the internal organs was indeed negligible after 14 days (**Figure S14**).

Although the preclinical and clinical properties of BUP are well documented [27,50], a pharmacokinetic (PK) study was conducted to determine the retention of the drug at the injection site after subcutaneous administration of free BUP, and compare it with the retention of BUP loaded in Neutral, Non-Depot and Depot liposomes. Similarly to the *in vivo* imaging, also the PK study was carried out for 14 days to follow either the drug systemic absorption or its complete clearance from the body.

As hypothesized, the plasma concentration–time profiles of BUP solution and BUP loaded-formulations were different. Loading BUP in Depot and Non-Depot liposomes resulted in a significantly higher plasma concentrations, in particular at time points from 24 h to 14 d, where no BUP was detectable anymore after administration of BUP solution or BUP in neutral liposomes. The $AUC_{0\text{-}14d}$ of BUP from Non-Depot and Depot liposomes were significantly higher than from solution or neutral liposomes. Comparing the Depot and Non-Depot formulations, the AUC of the Depot formulation was higher by trend with $p = 0.056$. The PK analysis suggested that the Depot formulation, retained at the injection site for a prolonged time compared to neutral formulation, hold also more efficiently the embedded drug. As it is evident from the PK parameters (Fig. 6), the Depot system resulted in a significantly higher AUC than free BUP, than the Neutral formulation, and remarkably, even than the Non-Depot.

The similarities of the clearance kinetics of Depot and Non-Depot liposomes observed in the imaging studies correlated only partially with the PK parameters, which interestingly showed a difference between the two DSPG-based formulations, revealing a distinctive role of the divalent cations in modulating BUP release profile *in vivo*, as the *ex vivo dialysis* release already suggested. We can conclude that the presence of Ca^{2+} in the Depot formulation is not redundant as the bromphenolblue diffusion assay and the *in vivo* imaging of the injection site could have initially indicated: the controlled aggregation of negatively charged liposomal formulations upon addition of divalent cations can control not only the clearance of the particles but also, and more interestingly, the release of a small amphipathic drug such as BUP *in vivo*.

Population PK analysis failed as the terminal phases of the Depot and Non-Depot liposomal formulations were not sufficiently defined by the data. The concentrations of the terminal phases did not significantly decrease over time. This would be in favor of the model assuming two states of BUP, one with fast liberation (burst effect) from the liposomes and injection site as observed for the neutral liposomes, and one fraction

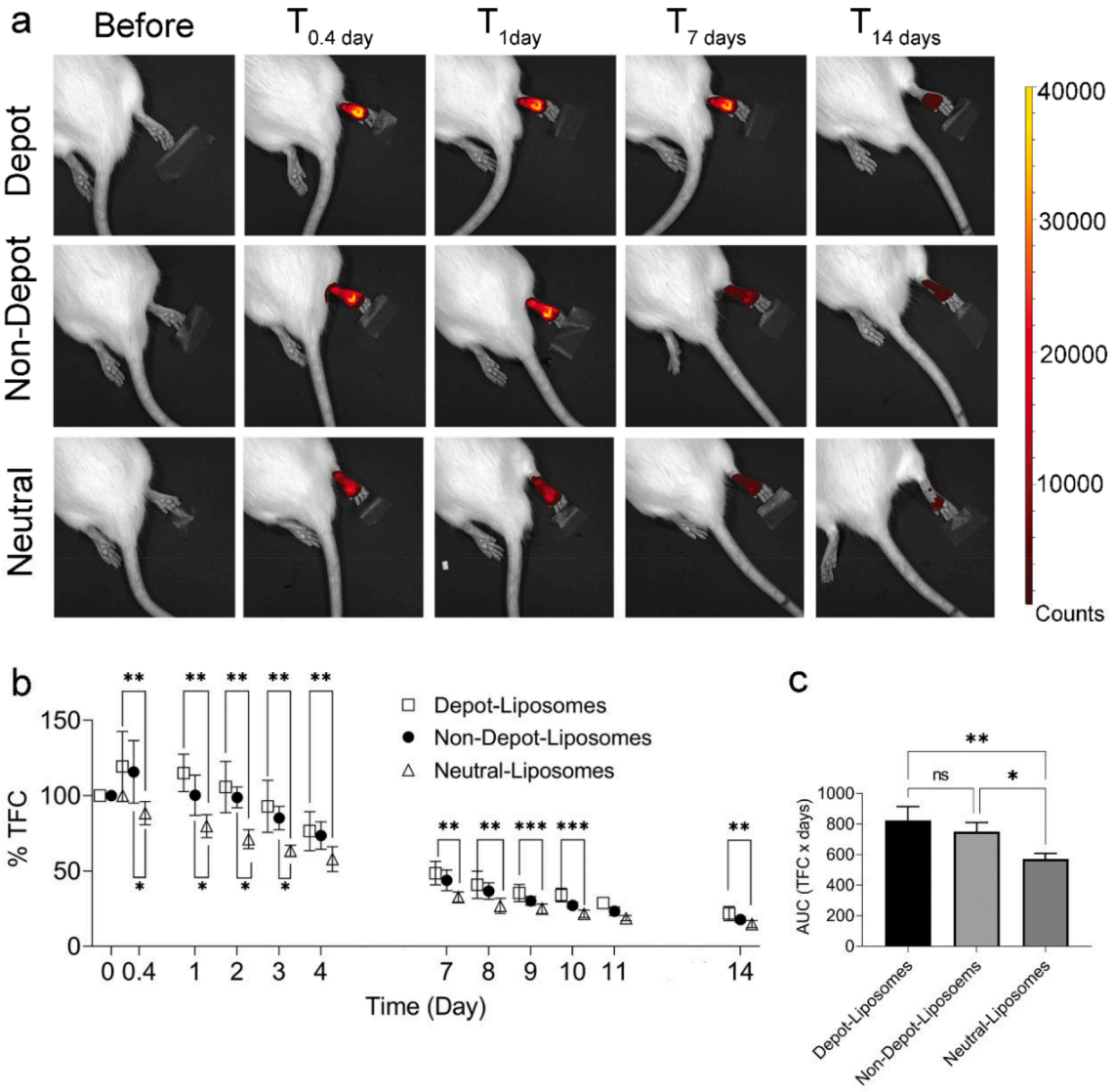

Fig. 5. Representative *in vivo* images of the site of injection after subcutaneous administration of DiD-labeled formulations injected in the ventral part of the hind paw of rats (**a**), clearance kinetics reported as % of initial injected dose accumulated at site of injection is presented as % total fluorescence counts TFC (**b**) and the calculated area under the curve,AUC (**c**).

of BUP with slow liberation from the liposomal membrane, to which the drug is strongly associated via electrostatic interactions.

4. Conclusion

We demonstrated that drug-loaded liposomal formulations containing NCPs are able to form injectable depots as a result of the aggregation with calcium cations. The cation-mediated aggregation of liposomes was investigated with different methods. It was evident that low concentrations of cations do not alter the fluidity of the bilayer but prolong the drug release in comparison to a non-depot liposome. The excellent injectability of the aggregated depot liposomes was confirmed by rheological characterisation *in vivo* studies during which the NCP-depots were administered subcutaneously. Independently from the pre-mixing with calcium, NCP containing-liposomes showed a significantly slower clearance from the injection site with respect to the control neutral formulation. The PK study revealed that Ca^{2+}-aggregated BUP-loaded liposomes did not induce any burst release of the drug after the administration, as often observed with biodegradable polymers such as PLGA. Our depot injectable was instead characterized by an extended release of BUP over 14 days, as indicated by its AUC. A correlation between BUP plasma concentrations and its pharmacological efficacy has not been established yet, but the obtained results suggest the potential of BUP-loaded depot liposomes as a possible new platform for a prolonged drug release. It has to be underlined that our LUV-based approach ensures a streamlined manufacturing process that can be first traditionally optimized for large-scale production in function of the drug to be delivered, and then followed by a final sterile filtration step prior to bedside mixing with cations and administration. This latter aspect represents a crucial and cost-effective difference with other existing technologies, such as DepoFoam, which (i) relies on a double-emulsification process requiring organic solvents and (ii) imposes to manufacture the large size MVV under aseptic conditions.

Overall, our approach has the potential to overcome the production drawbacks linked to other vesicle-based technologies (such as Exparel) while enabling a prolonged retention of the depot at the site of injection and a sustained release of small hydrophilic molecules *in vivo*.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

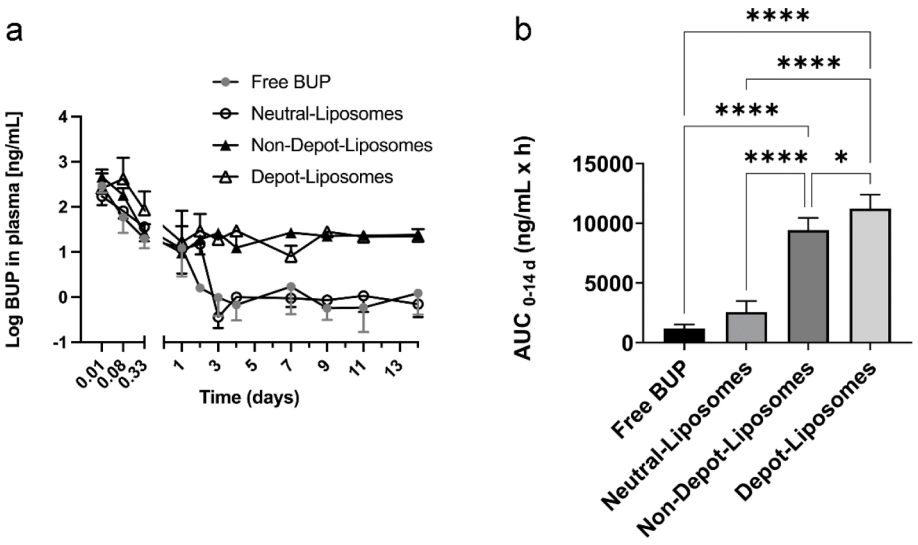

Fig. 6. Concentration time profile (**a**) and $AUC_{0\text{-}14\ d.}$ (**b**) of BUP in plasma. The results are presented as mean ± STDV; n = 6. Multiple comparison between the groups were performed by a non-parametric Kruskal-Wallis test and a *post hoc* correction for multiple comparison was done by using Dunn' s test. The adjusted p-values are reported as: * 0.1; ** 0.05, *** 0.001 and **** 0.0001.

the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejpb.2022.11.019.

References

[1] J. Siepmann, R.A. Siegel, M.J. Rathbone, eds., Fundamentals and applications of controlled release drug delivery, Springer US, Boston, MA, 2012. https://doi.org/10.1007/978-1-4614-0881-9.

[2] M. Albayaty, M. Linden, H. Olsson, M. Johnsson, K. Strandgården, F. Tiberg, Pharmacokinetic evaluation of once-weekly and once-monthly buprenorphine subcutaneous injection depots (CAM2038) versus intravenous and sublingual buprenorphine in healthy volunteers under naltrexone blockade: an open-label Phase 1 study, Adv. Ther. 34 (2017) 560–575, https://doi.org/10.1007/s12325-016-0472-9.

[3] U. Bulbake, S. Doppalapudi, N. Kommineni, W. Khan, Liposomal formulations in clinical use: An updated review, Pharmaceutics. 9 (2017) 12, https://doi.org/10.3390/pharmaceutics9020012.

[4] M.S. Angst, D.R. Drover, Pharmacology of drugs formulated with DepoFoam™, Clin. Pharmacokinet. 45 (2006) 1153–1176, https://doi.org/10.2165/00003088-200645120-00002.

[5] D.J.A. Crommelin, P. van Hoogevest, G. Storm, The role of liposomes in clinical nanomedicine development. What now? Now what? J. Control. Release. 318 (2020) 256–263, https://doi.org/10.1016/j.jconrel.2019.12.023.

[6] Public statement EMA Available online https://www.ema.europa.eu/en/medicines/human/withdrawn-applications/depocyte (access date: 27/09/2022).

[7] A. Burnett, B. Faley, T. Nyirenda, Z.M. Bamboat, Liposomal bupivacaine reduces narcotic use and time to flatus in a retrospective cohort of patients who underwent laparotomy, Int. J. Surg. 59 (2018) 55–60, https://doi.org/10.1016/j.ijsu.2018.09.018.

[8] C.Y. Lee, D.A. Robinson, C.A. Johnson, Y. Zhang, J. Wong, D.J. Joshi, T.-T. Wu, P. A. Knight, A randomized controlled trial of liposomal bupivacaine parasternal intercostal block for sternotomy, Ann. Thorac. Surg. 107 (1) (2019) 128–134.

[9] H.M. Yalmanchili, S.N. Buchanan, L.W. Chambers, J.D. Thorns, N.A. McKenzie, A. D. Reiss, M.P. Page, V.V. Dizon, S.E. Brooks, L.E. Shaffer, S.T. Lovald, T. H. Hartranft, P.D. Price, Postlaparotomy pain management: Comparison of patient-controlled analgesia pump alone, with subcutaneous bupivacaine infusion, or with injection of liposomal bupivacaine suspension, J. Opioid Manag. 15 (2019) 169, https://doi.org/10.5055/jom.2019.0498.

[10] E.M. Davidson, Y. Barenholz, R. Cohen, S. Haroutiunian, L. Kagan, Y. Ginosar, High-dose bupivacaine remotely loaded into multivesicular liposomes demonstrates slow drug release without systemic toxic plasma concentrations after subcutaneous administration in humans, Anesth. Analg. 110 (2010) 1018–1023, https://doi.org/10.1213/ANE.0b013e3181d26d2a.

[11] G.J. Grant, Y. Barenholz, E.M. Bolotin, M. Bansinath, H. Turndorf, B. Piskoun, E. M. Davidson, M. Bansinath, G.J. Grant, E.M. Davidson, E.M. Bolotin, Y. Barenholz, H. Turndorf, A novel liposomal bupivacaine formulation to produce ultralong-acting analgesia, Anesthesiology 101 (2004) 133–137, https://doi.org/10.1097/01.sa.0000151238.77039.f4.

[12] A.H. Ali, X. Zou, S.M. Abed, S.A. Korma, Q. Jin, X. Wang, Natural phospholipids: Occurrence, biosynthesis, separation, identification, and beneficial health aspects, Crit. Rev. Food Sci. Nutr. 59 (2019) 253–275, https://doi.org/10.1080/10408398.2017.1363714.

[13] L. Rahnfeld, P. Luciani, Injectable lipid-based depot formulations: Where do we stand? Pharmaceutics. 12 (2020) 567, https://doi.org/10.3390/pharmaceutics12060567.

[14] M.J. Ang, R.Z. Silkiss, The use of long-acting liposomal bupivacaine (Exparel) for postoperative pain control following enucleation or evisceration, Ophthal. Plast. Reconstr. Surg. 34 (2018) 599, https://doi.org/10.1097/IOP.0000000000001218.

[15] A.L. Balocco, P.G.E. Van Zundert, S.S. Gan, T.J. Gan, A. Hadzic, Extended release bupivacaine formulations for postoperative analgesia, Curr. Opin. Anaesthesiol. 31 (2018) 636–642, https://doi.org/10.1097/ACO.0000000000000648.

[16] N.V. Katre, Liposome-based depot injection technologies: How versatile are they? Am. J. Drug Deliv. 2 (2004) 213–227, https://doi.org/10.2165/00137696-200402040-00002.

[17] S. Mantripragada, A lipid based depot (DepoFoam® technology) for sustained release drug delivery, Prog. Lipid Res. 41 (2002) 392–406, https://doi.org/10.1016/S0163-7827(02)00004-8.

[18] L. Rahnfeld, J. Thamm, F. Steiniger, P. van Hoogevest, P. Luciani, Study on the in situ aggregation of liposomes with negatively charged phospholipids for use as injectable depot formulation, Colloids Surf B Biointerfaces. 168 (2018) 10–17.
[19] M.J.J. Hope, M.B.B. Bally, G. Webb, P.R.R. Cullis, Production of large unilamellar vesicles by a rapid extrusion procedure. Characterization of size distribution, trapped volume and ability to maintain a membrane potential, Biochim. Biophys. Acta 812 (1985) 55–65, https://doi.org/10.1016/0005-2736(85)90521-8.
[20] F. Weber, L. Rahnfeld, P. Luciani, Analytical profiling and stability evaluation of liposomal drug delivery systems: A rapid UHPLC-CAD-based approach for phospholipids in research and quality control, Talanta 220 (2020), 121320, https://doi.org/10.1016/j.talanta.2020.121320.
[21] NC3Rs, Blood sampling: Rat | NC3Rs, Natl. Cent. Replace. Refinement Reduct. Anim. Res. (2021). https://nc3rs.org.uk/3rs-resources/blood-sampling/blood-sampling-rat (accessed June 21, 2022).
[22] J.O. Eloy, M. Claro de Souza, R. Petrilli, J.P.A. Barcellos, R.J. Lee, J.M. Marchetti, Liposomes as carriers of hydrophilic small molecule drugs: Strategies to enhance encapsulation and delivery, Colloids Surf. B Biointerfaces. 123 (2014) 345–363, https://doi.org/10.1016/j.colsurfb.2014.09.029.
[23] R. Cohen, H. Kanaan, G.J. Grant, Y. Barenholz, Prolonged analgesia from Bupisome and Bupigel formulations: From design and fabrication to improved stability, J. Control. Release. 160 (2012) 346–352, https://doi.org/10.1016/j.jconrel.2011.12.030.
[24] J.J. Mowat, M.J. Mok, B.A. MacLeod, T.D. Madden, Liposomal bupivacaine extended duration nerve blockade using large unilamellar vesicles that exhibit a proton gradient, J. Am. Soc. Anesthesiol. 85 (1996) 635–643, https://doi.org/10.1097/00000542-199609000-00024.
[25] G. Haran, R. Cohen, L.K. Bar, Y. Barenholz, Transmembrane ammonium sulfate gradients in liposomes produce efficient and stable entrapment of amphipathic weak bases, BBA - Biomembr. 1151 (1993) 201–215, https://doi.org/10.1016/0005-2736(93)90105-9.
[26] R. Cohen, A. Steiner, H. Kanaan, Y. Barenholz, Chemical and physical characterization of remotely loaded bupivacaine liposomes: comparison between large multivesicular vesicles and small unilamellar vesicles, J. Mater. Chem. B. 1 (2013) 4619–4627, https://doi.org/10.1039/c3tb20609b.
[27] Exparel (Bupivacaine Liposome Injectable Suspension): Uses, Dosage, Side Effects, Interactions, Warning. Available online: https://www.accessdata.fda.gov/drugsatfda_docs/label/2018/022496s9lbl.pdf (access date 27/09/2022).
[28] D.E. Becker, K.L. Reed, Essentials of local anesthetic pharmacology, Anesth. Prog. 53 (2006) 98–110, https://doi.org/10.2344/0003-3006(2006)53[98:EOLAP]2.0.CO;2.
[29] K. Arnold, S. Okhi, M. Krumbiegel, Interaction of dextran sulfate with phospholipid surfaces and liposome aggregation and fusion, Chem. Phys. Lipids. 55 (1990) 301–307, https://doi.org/10.1016/0009-3084(90)90168-Q.
[30] M.J. Fonseca, E.C.A. van Winden, D.J.A. Crommelin, Doxorubicin induces aggregation of small negatively charged liposomes, Eur. J. Pharm. Biopharm. 43 (1997) 9–17, https://doi.org/10.1016/S0939-6411(96)00018-5.
[31] F. Van Bambeke, P.M. Tulkens, R. Brasseur, M.-P. Mingeot-Leclercq, Aminoglycoside antibiotics induce aggregation but not fusion of negatively-charged liposomes, Eur. J. Pharmacol. Mol. Pharmacol. 289 (1995) 321–333, https://doi.org/10.1016/0922-4106(95)90110-8.
[32] S.T. Yang, A.J.B. Kreutzberger, J. Lee, V. Kiessling, L.K. Tamm, The role of cholesterol in membrane fusion, Chem. Phys. Lipids. 199 (2016) 136–143, https://doi.org/10.1016/j.chemphyslip.2016.05.003.
[33] K.o. Sugahara, N. Shimokawa, M. Takagi, Thermal stability of phase-separated domains in multicomponent lipid membranes with local anesthetics, Membranes 7 (3) (2017) 33.
[34] J.G. Paiva, P. Paradiso, A.P. Serro, A. Fernandes, B. Saramago, Interaction of local and general anaesthetics with liposomal membrane models: A QCM-D and DSC study, Colloids Surf. B Biointerfaces 95 (2012) 65–74, https://doi.org/10.1016/j.colsurfb.2012.02.027.
[35] R. Pérez-Isidoro, F.J. Sierra-Valdez, J.C. Ruiz-Suárez, Anesthetic diffusion through lipid membranes depends on the protonation rate, Sci. Rep. 4 (2015) 7534, https://doi.org/10.1038/srep07534.
[36] M.M. Moreno, P. Garidel, M. Suwalsky, J. Howe, K. Brandenburg, The membrane-activity of ibuprofen, diclofenac, and naproxen: A physico-chemical study with lecithin phospholipids, Biochim. Biophys. Acta - Biomembr. 1788 (2009) 1296–1303, https://doi.org/10.1016/j.bbamem.2009.01.016.
[37] W. Cong, Q. Liu, Q. Liang, Y. Wang, G. Luo, Investigation on the interactions between pirarubicin and phospholipids, Biophys. Chem. 143 (2009) 154–160, https://doi.org/10.1016/j.bpc.2009.05.005.
[38] D. Yonar, M.M. Sunnetcioglu, Effect of cis-(Z)-flupentixol on DPPC membranes in the presence and absence of cholesterol, Chem. Phys. Lipids. 198 (2016) 61–71, https://doi.org/10.1016/j.chemphyslip.2016.06.002.
[39] T.T. Wei, H.Y. Sun, G. Deng, J.Y. Gu, H.Y. Guo, J. Xu, R.G. Wu, The interaction of paeonol with DPPC liposomes, J. Therm. Anal. Calorim. 132 (2018) 685–692, https://doi.org/10.1007/s10973-017-6894-z.
[40] R.L. Biltonen, D. Lichtenberg, The use of differential scanning calorimetry as a tool to characterize liposome preparations, Chem. Phys. Lipids. 64 (1993) 129–142, https://doi.org/10.1016/0009-3084(93)90062-8.
[41] H. Tsuchiya, T. Ueno, M. Mizogami, K. Takakura, Local anesthetics structure-dependently interact with anionic phospholipid membranes to modify the fluidity, Chem. Biol. Interact. 183 (2010) 19–24, https://doi.org/10.1016/j.cbi.2009.10.006.
[42] T. Ueno, H. Tsuchiya, M. Mizogami, K. Takakura, Local anesthetic failure associated with inflammation: verification of the acidosis mechanism and the hypothetic participation of inflammatory peroxynitrite, J. Inflamm. Res. 1 (2008) 41–48, https://doi.org/10.2147/JIR.S3982.
[43] K. Græsbøll, H. Sasse-Middelhoff, T. Heimburg, The thermodynamics of general and local anesthesia, Biophys. J. 106 (2014) 2143–2156, https://doi.org/10.1016/j.bpj.2014.04.014.
[44] M.P. Even, S. Bobbala, B. Gibson, S. Hook, G. Winter, J. Engert, Twin-screw extruded lipid implants containing TRP2 peptide for tumour therapy, Eur. J. Pharm. Biopharm. 114 (2017) 79–87, https://doi.org/10.1016/j.ejpb.2016.12.033.
[45] Y. Xing, H. Chen, S. Li, X. Guo, In vitro and in vivo investigation of a novel two-phase delivery system of 2-methoxyestradiol liposomes hydrogel, J. Liposome Res. 24 (2014) 10–16, https://doi.org/10.3109/08982104.2013.822395.
[46] Y. Zhao, J. Liu, X. Sun, Z.-R.-R. Zhang, T. Gong, Sustained release of hydroxycamptothecin after subcutaneous administration using a novel phospholipid complex-DepoFoam™ technology, Drug Dev. Ind. Pharm. 36 (2010) 823–831, https://doi.org/10.3109/03639040903520975.
[47] W. Tian, S. Schulze, M. Brandl, G. Winter, Vesicular phospholipid gel-based depot formulations for pharmaceutical proteins: development and *in vitro* evaluation, J. Control. Release. 142 (2010) 319–325, https://doi.org/10.1016/j.jconrel.2009.11.006.
[48] S. Manna, Y. Wu, Y. Wang, B. Koo, L. Chen, P. Petrochenko, Y. Dong, S. Choi, D. Kozak, B. Oktem, X. Xu, J. Zheng, Probing the mechanism of bupivacaine drug release from multivesicular liposomes, J. Control. Release. 294 (2019) 279–287, https://doi.org/10.1016/j.jconrel.2018.12.029.
[49] C. Oussoren, G. Storm, Liposomes to target the lymphatics by subcutaneous administration, Adv. Drug Deliv. Rev. 50 (2001) 143–156, https://doi.org/10.1016/S0169-409X(01)00154-5.
[50] EMA/CHPMP/528272/2020. https://www.ema.europa.eu/en/medicines/human/EPAR/exparel-liposomal (access date, 23/11/2022).