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PII: S0378-5173(24)01087-1
DOI: https://doi.org/10.1016/j.ijpharm.2024.124853
Reference: IJP 124853

To appear in: *International Journal of Pharmaceutics*

Received Date: 19 September 2024
Revised Date: 16 October 2024
Accepted Date: 18 October 2024

Please cite this article as: C.S. Schlosser, W. Rozek, R.D. Mellor, S.W. Manka, C.J. Morris, S. Brocchini, G.R. Williams, A lipid-based delivery platform for thermo-responsive delivery of teriparatide, *International Journal of Pharmaceutics* (2024), doi: https://doi.org/10.1016/j.ijpharm.2024.124853

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A lipid-based delivery platform for thermo-responsive delivery of teriparatide

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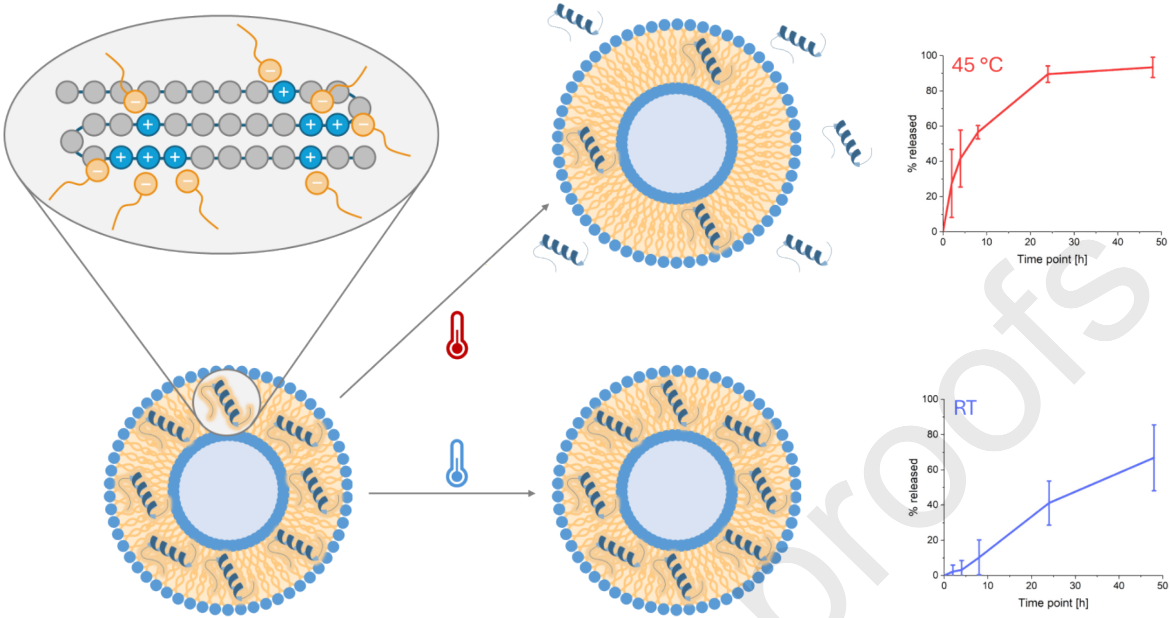
Abstract

Teriparatide (and analogue peptides) are the only FDA approved anabolic treatments for osteoporosis. Current therapies are administered as a daily subcutaneous injection, which limits patient adherence and clinical efficacy. To achieve the desired anabolic effect, a controlled delivery system must ensure a pulsatile release profile over a prolonged period. Thermo-responsive formulations (e.g. liposomes) can undergo a temperature-related phase-transition which can allow active control of drug release. Herein, thermo-responsive liposomes were developed to permit precise control over the teriparatide release rate through modulation of temperature. Entrapment of hydrophilic molecules, including peptides within liposomes remains challenging due to the large volume of hydration. In this work, hydrophobic ion pairing was employed for the first time to enhance peptide entrapment within liposomes. The method resulted in a hydrophobic complex that achieved high teriparatide entrapment (>75%) in sub-200 nm, monodispersed liposomes. Hydrophobic ion pairing outperformed other entrapment approaches. Several liposomal formulations with transition temperatures between 38 – 50 °C were obtained by modulation of the phospholipid composition. *In vitro* release assays demonstrated temperature-dependent release kinetics with faster rates of release observed at/above the transition temperature. The maintenance of biological activity of released teriparatide was demonstrated in a cell-based assay utilising the PTH1 receptor. Overall, this provides the first proof-of-concept of the suitability of thermo-responsive systems for pulsatile delivery of teriparatide and similar peptides.

Keywords: thermo-responsive liposomes, teriparatide, PTH1R, controlled release, pulsatile, hydrophobic-ion-pairin

Graphical abstract



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1. Introduction

Osteoporosis is characterised by a decrease in bone mass and deterioration of the microarchitecture of the bone tissue, and is generally associated with an increased risk of fragility fractures [1–3]. The treatment of osteoporosis aims to prevent the occurrence of fractures, which essentially is achieved by either inhibiting bone resorption or through anabolic treatments [4]. In 1987, teriparatide (Forteo®) – the biologically active fragment of human parathyroid hormone (hPTH) was the first FDA approved anabolic therapy for advanced osteoporosis, improving bone micro-architecture, mineral density, and strength [5–8]. Since 2017, abaloparatide (a PTH related peptide) is also available for the same indication. However, to achieve the bone forming effect, the dosing pattern of teriparatide is crucial [9]. Continuous exposure to teriparatide causes deleterious bone resorption, thus intermittent administration (once daily) is required for the anabolic effect [5]. Frolik et al. investigated different administration patterns of hPTH and identified that the duration of exposure to hPTH is the main determinator of an anabolic or catabolic response [10]. The exact mechanism by which this occurs is currently unknown, however different theories exist including the desensitisation of the PTH1R receptor during continuous exposure [9]. Since teriparatide is a peptide it is administered parenterally as a once daily subcutaneous injection [5,11]. The need for repeated and frequent injections leads to poor patient adherence, thus limiting its clinical efficacy [5,6].

Controlled release systems have been developed to improve the efficacy of and patient compliance to many medicines. Many of these systems seek to control drug concentrations by providing a continuous and prolonged release of a therapeutic while reducing the dosing frequency, fluctuations in plasma levels and adverse side-effects [12]. Successful sustained delivery systems have been developed for a range of different peptides (e.g. Lupron Depot and Bydureon BCise) by encapsulation into polymeric microparticles. To date, most conventional controlled release systems release therapeutics in a continuous, passive manner as a function of water imbibition and the extent of polymer degradation [12,13].

To achieve a prolonged therapeutic effect with teriparatide, a controlled release system must achieve a pulsatile release profile. Stimuli-responsive materials are of interest in drug delivery [14]. These materials can respond to endogenous or exogenous triggers leading to a property change in the material, e.g. glass transition temperature, which may be exploited to actively control drug release [13].

Liposomes are composed of phospholipids, which in an aqueous environment can non-covalently associate into bi-layer forming uni- or multilamellar vesicles [15]. Both the interior and exterior of the liposomes are in contact with an aqueous environment [16]. Liposomes have been extensively explored, mainly in the field of oncology [13,16]. Since the first authorisation of a liposomal therapeutic in 1995, several other liposomal systems have transitioned into the clinic [17].

Loading of liposomes with hydrophobic compounds often achieves high entrapment efficiencies (>90%) in a simple thin-film hydration process [18]. Indeed, hydrophobic compounds preferentially position themselves within the hydrophobic environment present in the liposomal bilayer. In contrast, hydrophilic molecules are easily dissolved in the aqueous phase (aqueous core of the liposomes and surrounding aqueous

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media). The large volume of hydration used to fabricate liposomes generally leads to low entrapment efficiencies, resulting in large discrepancies (2.1 – 72%) being reported for hydrophilic peptides and proteins [19–24].

Liposomes are inherently thermo-responsive and their transition temperature (T_c) is composition dependent, with the length and degree of saturation of the phospholipid alkyl chain influencing the transition temperature [25,26]. Upon heating above the T_c, a liposome will undergo a gel-to-liquid transition [13]. In the ordered gel phase, the phospholipids are aligned and well-packed, forming a relatively impermeable barrier. Once the temperature exceeds the T_c the bilayer becomes a disordered, liquid crystalline phase that is more permeable and permits release of the entrapped drug [13].

Thermo-responsive liposomes are currently deployed in oncology with ThermoDox being the most prominent system. It has been investigated in several clinical trials in combination with radiofrequency ablation [17,27]. [27]. The possibility to trigger drug release in an “on/off” manner may be particularly promising as an approach in the development of pulsatile release systems. We conceptualised that teriparatide loaded thermo-responsive liposomes would release the peptide upon a brief and localised temperature increase. Hyperthermia may be achieved through incorporation of near infrared (NIR) responsive materials (e.g. gold nanoparticles or polydopamine). These materials absorb energy from light and convert it into heat. NIR in particular, has high tissue penetration due to low absorption of NIR from biological materials. By adapting the intensity and duration of irradiation, the dose released is controlled [28].

Here, thermo-responsive liposomes were investigated for their potential to actively control the release of teriparatide after subcutaneous administration. At first, different entrapment methods were explored to identify the most suitable approach to maximise peptide loading within the liposomes. Initially, a once weekly formulation was desired, providing 7 individual doses of teriparatide (20 – 40 µg/dose) [5]. To achieve this, the liposomal formulation must contain a minimum of 140 – 280 µg of teriparatide. The liposome composition was optimised to achieve a target transition temperature of 43 – 45 °C and the temperature-dependent release of pharmacologically active teriparatide confirmed using a cell-based PTH1R receptor binding assay.

2. Materials and Methods

2.1. Materials

Teriparatide was supplied by Insight Biotechnolgoy (Wembley, UK). Dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), and distearoylphosphoethanolamine-polyethyleneglycol-2000 (DSPE-PEG) were provided by Lipoid (Ludwigshafen, Germany). Acetonitrile (HPLC grade), sucrose, trifluoroacetic acid and water (HPLC grade) were obtained from Fisher (Loughborough, UK). 3-isobutyl-1-methylxanthine, 4-(3-butoxy-4-methoxybenzyl) imidazolidinone, formic acid, fetal bovine serum, and isopropanol were obtained from Merck Life Sciences (Darmstadt, Germany). Ethanol, phosphate buffered saline, sodium dodecyl sulfate, sodium phosphate monobasic, sodium phosphate dibasic, and Trizma were purchased from Sigma Aldrich (Steinheim, Germany). PTH1R encoding plasmid (OHu15045D) was from GenScript (Oxford, UK). Hydrochloric acid

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was obtained from Honeywell (Seelze, Germany). A cAMP assay was provided by Promega. Oleic acid was purchased from Scientific Laboratory Suppliers Ltd. (Nottingham, UK). Dulbecco's modified essential media, trypsin, and geneticin (G418) were obtained from ThermoFisher (Rockford, IL, USA).

2.2. Liposome preparation

Liposomes were prepared by the thin-film method, where DPPC:DSPC were dissolved in ethanol at a concentration of 10 mg/mL. A series of different ratios of the two phospholipids were prepared, to which 5% *w/w* DSPE-PEG with respect to the total lipid content was added for enhanced colloidal stability. A thin, lipidic film was consequently obtained by removing ethanol using a rotary evaporator. The lipidic film was rehydrated with phosphate buffer (10 mM, pH 7.4) to a concentration of 10 mg/mL of lipids, yielding a cloudy suspension. Small, monodispersed liposomes were obtained by sequential extrusion through polycarbonate membranes of decreasing size (800 µm, 400 µm, 200 µm, and 100 µm) using a mini-extruder (Avanti Polar Lipids Inc, US). Extrusion was conducted at 41 ± 3 °C with a minimum of 10 extrusion cycles per membrane.

2.3. Teriparatide entrapment methods

2.3.1. Direct mixing

Liposomes were prepared by controlled mixing of the lipidic solution and aqueous solution using a V-junction device which was equipped with 2 inlet tubes for the feed solution and one outlet tube collecting the liposomal formulation. The inlet tubes were equipped with one-way valves and connected to a syringe pump controlling the flow rate (Figure 1). DPPC and DSPC (8:2 molar ratio) were prepared in ethanol at 5 mg/mL, to which 5% (*w/w* to lipids) DSPE-PEG was added. The liposomes were prepared by mixing of the ethanol-lipid solution with an aqueous teriparatide solution (0.25 mg/mL) in 20 mM phosphate buffer (pH 7.2) at a flow rate of 450 mL/h using a water: ethanol ratio of 4:1 *v/v*. The resulting liposomes were purified by gel filtration (see Section 2.4.1).

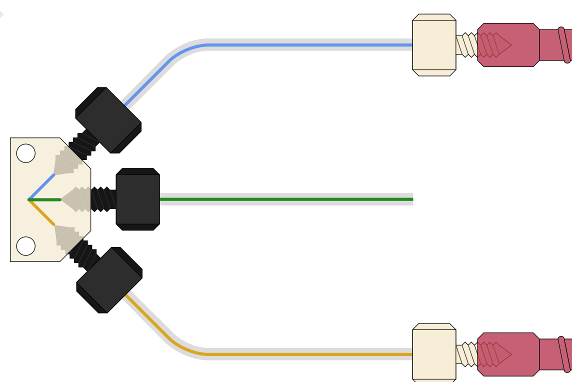

Figure 1: Schematic of the controlled mixing set up showing the two inlet lines (orange = lipids in ethanol, blue = aqueous phase) as well as the outlet (green = liposome formulation) connected to the V-shaped mixing piece (JR-9000-0670). All tubing was PEEK 1/16" x 0.75 mm ID (JR-T-6011-M3) connected via PEEK connectors (JR-55021-5). All items were supplied by VICI Jour, Schenkon, Switzerland.

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2.3.2. Dehydration-rehydration vesicles

Dehydration-rehydration vesicles (DRVs) were prepared using a modified protocol from Kirby and Gregoriadis as described by Mugabe et al. [20,29]. Empty liposomes were prepared as described under Section 2.2 with slight modifications. Liposomes were prepared by the thin-film method, where DPPC: DSPC (8:2 molar ratio) with 5% of DSPE-PEG *(w/w)* were dissolved in ethanol. A thin film was created by removal of ethanol. The lipidic film was rehydrated to a concentration of 6.25 mg/mL with 20 mM Tris buffer (pH 7.0). Small, monodispersed, liposomes were obtained by sequential extrusion through polycarbonate membranes as described under Section 2.2.

A physical mixture of the empty liposomes (800 µL), teriparatide (20 µL, 10 mg/mL) and sucrose (10 µL, 100 mg/mL) was prepared and freeze-dried (dehydration). Peptide loaded liposomes were then obtained by rehydration of the freeze-dried cake. The rehydration procedure was adapted from [29]. Briefly, 20 mM Tris-HCl buffer (pH 7.0) was pre-heated to 55 °C. Initially, the cake was rehydrated with 50 µL of the buffer followed by 30 min incubation at 55 °C. After 30 min, a further 150 µL and then 850 µL of the buffer was added, allowing for a 30 min incubation between each addition. The resulting liposomes were purified by gel filtration (see Section 2.4.1).

2.3.3. Charged liposomes

Charged liposomes were prepared as described under Section 2.3.1 but by adding oleic acid to the lipidic mixture (1:1 molar ratio with respect to the phospholipids) prior to preparation of the thin film. The film was rehydrated to a concentration of 6.25 mg/mL of phospholipids. Following preparation of the thin film, teriparatide loaded charged liposomes were obtained by dehydration rehydration. A physical mixture of the empty liposomes (800 µL), teriparatide (20 µL, 10 mg/mL) and sucrose (10 µL, 100 mg/mL) was prepared and freeze-dried (dehydration), then rehydrated as described under Section 2.3.1.

2.3.4. Hydrophobic ion-pairing

A hydrophobic complex between teriparatide and sodium dodecyl sulfate (SDS) was prepared. Teriparatide was dissolved in 1 mM HCl at a concentration of 1.7 mg/mL, to which an equal volume of SDS (1.008 mg/mL) was added dropwise. A white precipitate formed which was collected by centrifugation at 10,000 rpm for 15 min. The supernatant was removed, and the white pellet was dissolved in ethanol (to a theoretical concentration of 1.7 mg/mL). Teriparatide loaded liposomes were obtained by addition of ion-pair dispersion (0.1 mL) to the phospholipid mixture in ethanol prior to the thin-film rehydration procedure as described under Section 2.2. Liposomes were rehydrated to a concentration of 5 mg/mL total lipid and unentrapped peptide was initially removed by centrifugation using 50 kDa Amicon filters. For release experiments, more concentrated liposomes were prepared by adding 0.85 mL of the ethanolic ion pair dispersion to the phospholipids. Liposomes were prepared at 10 mg/mL in 100 mM phosphate buffer and further purified by dialysis using 100 kDa Float-A-Lysers (Repligen, US).

2.4. Liposome purification methods

2.4.1. Gel filtration

Free peptide was removed from the liposome formulation by gel filtration using a Sephadex G50 filled column. 750 µL of the liposome suspension was transferred to the column. Once fully permeated into the column 200 µL of 20 mM Tris buffer (pH 7.4) was added. Next, fractions were collected by sequential addition of 750 µL of buffer. The liposomes eluted in fractions 4-6. After collection of the liposomes, 8 mL of buffer was flown through the column to remove free peptide (Supporting Information, Section S1).

2.4.2. Dialysis

Alternatively, liposome formulations were purified from free peptide by dialysis with 100 kDa Float-A-Lysers against 800 mL of 100 mM phosphate buffer (pH 7.4). Dialysis was typically performed overnight (Supporting Information, Section S2).

2.5. Liposome characterisation

2.5.1. Determination of peptide loading

Teriparatide concentration was measured with an Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA, USA) coupled with an UV-detector. A Supleco Biowide C18 column (4.6 mm × 150 mm × 5µm) was employed to determine peptide concentrations from 20 µL injections. The mobile phase consisted of 0.1% trifluoroacetic acid (TFA) in water (mobile phase A) and 0.1% TFA in acetonitrile (mobile phase B). Gradient elution started with 20% B at a flow rate of 1.0 mL/min and was held for 2 min prior to increasing to 60% over the course of 4 min. The % B was then increased to 100% over 0.2 min where it was held for 1 min prior to decreasing back to 20% B. The column temperature was set at 25 °C, and UV absorbance monitored at 210 nm. Sample teriparatide concentrations were determined against a calibration curve (range 5 – 100 µg/mL).

2.5.2. Determination of particle size and zeta potential

Particle size and polydispersity index (PDI) were determined by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern, UK). Liposomes were diluted (to 1 mg/mL) in relevant buffer prior to measurements. For each sample three consecutive measurements were performed at a scattering angle of 173° and at a temperature of 25 °C. For zeta potential measurements the liposomal sample was loaded into folded capillary cells and three consecutive measurements were conducted at a temperature of 25 °C.

2.5.3. Imaging of the liposomes

Frozen-hydrated liposome samples for cryo-TEM with varied HIP content (0-600 µg/ml) were prepared at a concentration of 10 mg/ml in 100 mM phosphate buffer (pH 7.4) and 4 µl of each sample were deposited on glow-discharged C-Flat™ Holey Carbon CF-224C 400 Mesh TEM grids (Electron Microscopy Sciences) in the chamber

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of the Leica GP2 plunger. The chamber was set to 20 °C and 40% humidity. After 10 s incubation, the grids were blotted for 2-3 s (with 2 mm push and without additional drain time) and plunge-frozen in liquid ethane maintained at −183 °C by liquid nitrogen.

Cryo-micrographs were acquired on a Talos L120C G2 microscope (FEI/Thermo Fisher) operated at 120 kV, using a 4k × 4k BM-Ceta camera, an objective aperture of 100 µm and nominal magnification of 4200-92000. Grids were inserted into the microscope using a side-entry Dual Grid Cryo Transfer Holder Model 205 from Simple Origin, Pittsburgh, USA. Three independent rounds of liposome preparation were screened with at least 4 grids per sample at each round.

2.5.4. Characterisation of thermal properties

The transition temperature of the liposome formulations was determined using a TA X3 differential scanning calorimeter (TA instruments). A 10 µL aliquot of each liposome formulation was loaded into a hermetic aluminium pan. A buffer aliquot was prepared as reference. Each sample was subjected to two heating cycles from 25 °C to 65 °C at a rate of 1 °C/min with the gas flow (nitrogen) maintained between 300 – 400 mL/min. The obtained data were analysed using the TRIOS Software.

2.6. Evaluation of in vitro drug release

After purification, the loaded liposomes were diluted to obtain a target peptide concentration between 50 – 175 µg/mL. The diluted liposome formulation (1.0 – 1.5 mL) was filled into 100 kDa Float-A-Lysers and were dialysed at different temperatures (room temperature, 34 °C, and 45 °C). At pre-defined time points 50 µL of each liposome formulation was withdrawn and quantified for remaining peptide concentration. The percentage of peptide released at every time point was calculated as described in Equation 1:

Equation 1: Determination of percentage release of teriparatide

$$Released\ peptide\ (\%) = 100 - \left(\frac{Peptide\ concentration\ at\ t_x}{Peptide\ concentration\ at\ t_0} \times 100\right)$$

2.7. Functionality assessment of released teriparatide

2.7.1. HEK293 cell culture

A cell line overexpressing PTH1R was created by transfecting HEK293 cells with a pcDNA3.1+/C-(K)DYK plasmid vector including the PTH1R gene (GenScript, United Kingdom). After lipofection (Lipofectamine 3000, Invitrogen) cells were grown in DMEM containing 10% fetal bovine serum and 600 µg/mL geneticin for two weeks to create a stably over-expressing line, HEK293-PTH1R (see Supporting Information, Section S3 for details). All cells were cultured in humidified incubators at 37 °C/5% CO_2 and were passaged at confluency.

2.7.2. Determination of peptide functionality with cellular cAMP assay

The functionality of the formulated teriparatide was determined using the HEK293-PTH1R cell line, by measuring and comparing intracellular cAMP levels after cellular binding of native and processed teriparatide. Release of the formulated peptide was obtained by incubating 1mL of the liposomal formulations overnight at 45 °C. The liposomal formulation (1 mL) was then dialysed against 5 mL phosphate saline buffer (PBS) with 100 kDa Float-A-Lysers for approximately 24 h. Teriparatide concentration in the dialysate was quantified by HPLC. A teriparatide stock was prepared in induction buffer (PBS containing 500 µM isobutyl-1-methylxanthine and 100 µM 4-(3-butoxy-4-methoxybenzyl) imidazolidinone). A total of five samples at concentrations in the range of 0.2 pM - 1.8 nM were prepared in induction buffer for all released teriparatide samples, as well as for the native peptide.

HEK293-PTH1R were plated in white clear bottom 96-well plates at a density of 10,000 cells/well and cultured overnight. At 70-80% confluency, the media was removed and 20 µL of all teriparatide samples added to separate wells before the plate was incubated at 37 °C/5% CO_2 for 30 minutes. cAMP levels were then measured with the Promega cAMP Glo Assay according to the manufacturer's instructions. A cAMP calibration curve between 1.9 nM – 62.5 µM was prepared separately.

2.8. Statistical analysis

Unless otherwise specified, experiments were conducted in triplicates (n=3) and results are presented as mean ± standard deviation. Statistical analysis was performed using ANOVA for comparing > two methods or Students t-test for the comparison of two methods. When ANOVA showed significant differences, a Tukey post-test was employed. Statistical significance was set at $p < 0.05$.

3. Results and discussion

3.1. Comparison of peptide entrapment methods

Liposomal entrapment of hydrophilic molecules, especially peptides and proteins, remains a challenge. Thus, a wide range of entrapment methods such as dehydration-rehydration, solvent injection, reverse phase evaporation, freeze-thawing have been developed to maximise entrapment [19,20,30] Drug loading may also be achieved through adsorption onto the surface of liposomes through electrostatic interactions [26].

Herein, four different strategies were explored covering the different mechanisms of drug loading. The strategies comprised simple mixing via a fluidic, controlled ethanol injection process, dehydration-rehydration, and charged liposomal adsorption. Finally, hydrophobic ion pairing (HIP) a well-known approach to enhance peptide loading within lipid-based delivery systems such as self-emulsifying drug delivery systems (SEDDS) and solid lipid nanoparticles, was explored for the first time as a strategy to enhance entrapment of peptides within liposomes [31,32]. All methods were compared for their efficiency of entrapping teriparatide into liposomes.

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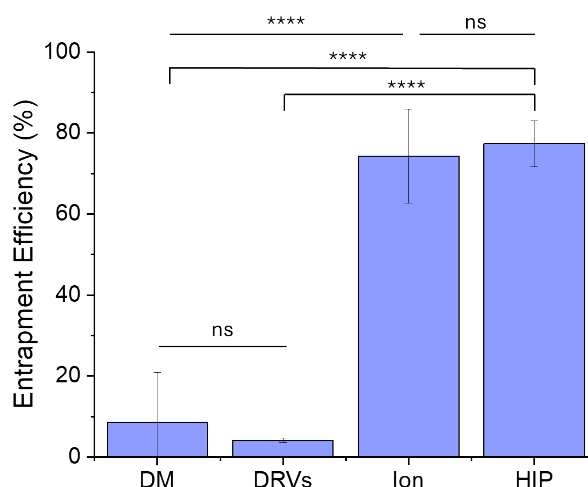

Figure 2: Comparison of the different methods explored to incorporate teriparatide into DPPC:DSPC (8:2) liposomes containing 5% w/w DSPE-PEG with respect to the total lipid content. Entrapment efficiencies obtained by direct mixing (DM), dehydration-rehydration (DRVs), charged liposomes (Ion) and hydrophobic ion pairing (HIP) are shown. Statistically significant difference in encapsulation efficiency is indicated by (****) P < 0.0001. Results are presented as mean ± s.d. (n=3).

The efficiencies of different entrapment methods are shown Figure 2. It can be observed that when direct mixing or DRV was used to obtain peptide loading into preformed liposomes, the encapsulation efficiencies were poor, with less than 10% of the peptide entrapped (8.6 ± 12.3 % and 4.1 ± 0.6 % respectively). The liposomes were composed of two zwitterionic lipids (DPPC and DSPC) presenting a net neutral charge. The incorporation of oleic acid into these liposomes (Ion) prior to dehydration-rehydration of the physical mix of liposomes and teriparatide resulted in a significant increase in teriparatide loading (74.3 ± 11.6%). The incorporation of oleic acid into the lipid composition conferred the (unloaded) liposomes a negative charge with oleic acid liposomes presenting a zeta potential of -24.0 ± 2.4 mV compared to 0.6 ± 3.5 mV without oleic acid. The dramatic increase in loading could be explained by electrostatic interactions of the overall positively charged teriparatide (pI = 8.3) with the negative charges of oleic acid. Further, this is consistent with the literature, where adsorption of various polypeptides resulted in high loading efficiencies. Indeed, van Slooten et el. achieved high association with >90% of human IFNγ through association with negatively charged phospholipids [33].

The use of hydrophobic ion pairing (HIP) of teriparatide resulted in an entrapment efficiency of 77.3 ± 5.7 % (n=3). The positively charged teriparatide was first complexed with sodium dodecyl sulfate (SDS) at a 1:8 molar ratio (1:1 charge ratio). At low pH, all of teriparatide's basic amino acids (8) were assumed to be protonated and forming electrostatic interaction with the negatively charged sulfate group (1) of SDS. The electrostatic interactions result in an overall neutral complex covered by a hydrophobic C12 carbon chain, thereby increasing the hydrophobicity of the peptide and leading to precipitation from aqueous solution. The hydrophobic complex is dispersible and has been observed to be stable in ethanol (Supporting Information Section S4), thus allowing incorporation of the peptide directly into the thin film. Upon rehydration of the thin film the hydrophobic complex would be expected to

preferentially locate within the lipid bilayer (hydrophobic environment), resulting in high entrapment efficiency within the liposomes.

Although high entrapment efficiencies (>70%) were obtained for both charged and hydrophobic ion paired liposomes, the size distribution and polydispersity between the two formulations differed. Upon rehydration of the charged liposomes their initial size distribution was not recovered, but a disperse formulation (PDI = 0.439 ± 0.056) with larger (potentially aggregated) liposomes was obtained (Figure 3a). Conversely, hydrophobic ion pairing permits direct incorporation of the peptide within the lipidic thin-film and extrusion of the final formulation, resulting in small and monodispersed liposomes (PDI = 0.236 ± 0.006, Figure 3b).

Furthermore, with the ionised liposomes – as described above - it is likely that teriparatide loading is predominantly achieved by association via electrostatic interactions with oleic acid (Supporting Information, Section S5), thus further explaining the increase in zeta potential from -24.0 ± 2.4 mV to -16.1 ± 2.6 mV [26]. It is uncertain whether a temperature-dependent release of the peptide could be achieved from an adsorbed therapeutic. Therefore, physical entrapment of the peptide within the liposomal bilayer may be preferable, presenting higher chances of demonstrating temperature-dependent release upon gel-sol transition of the phospholipids.

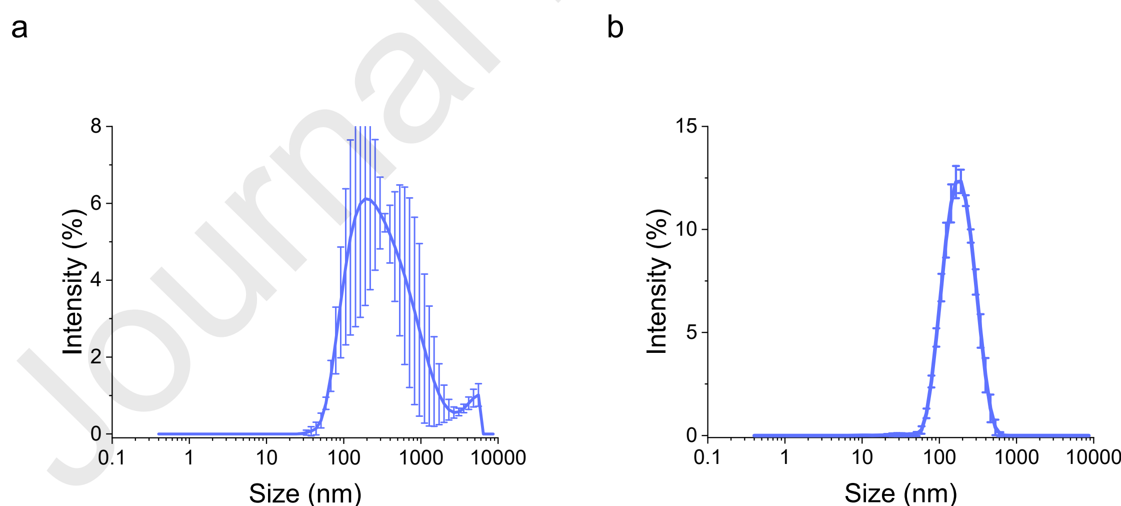

Figure 3: Comparison of size distribution of teriparatide loaded liposomes prepared using a) charged oleic acid containing liposomes and b) peptide ion pair containing liposomes. Results are presented as mean ± s.d. (n=3).

The use of hydrophobic ion pairing is a versatile approach, which has been successfully employed to generate hydrophobic complexes of many peptides and proteins [34]. Through the choice of an appropriate counter-ion, the approach is applicable to both positively and negatively charged peptides and proteins and could

further be extended to biomolecules (e.g. RNA). Indeed, current mRNA delivery systems employ a similar strategy where ionisable/cationic lipids allow complexation with the negatively charged mRNA and consequently encapsulation within lipid nanoparticles [35].

Herein, we demonstrated for the first time that hydrophobic ion pairing is a promising strategy to enhance peptide entrapment within liposomes. Indeed, pre-forming hydrophobic peptide-surfactant ion pairs prior to entrapment into the liposomes was deemed to be the most promising approach to producing peptide loaded monodispersed liposomes. The teriparatide loading ranged between 10 – 30 µg of teriparatide per mg of lipid. A range of different teriparatide loading levels up to 70 µg per mg of liposome was achieved by adapting the amount of peptide ion pair dispersion (0.1 mL – 0.85 mL per mL) that is added to the phospholipid mixture prior to formation of the thin film (Figure 4).

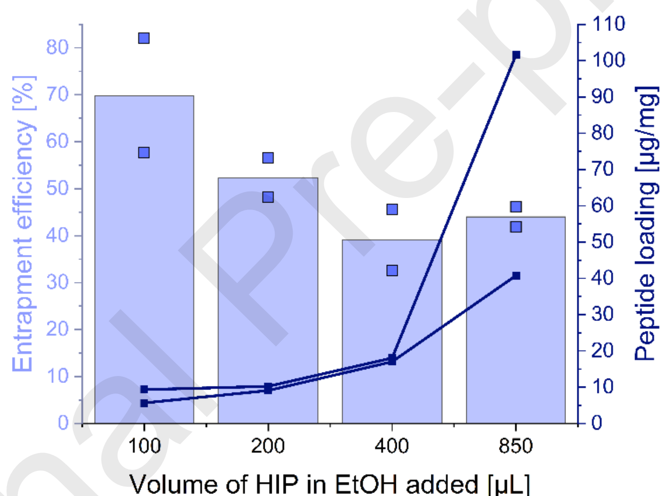

Figure 4: Effect of increased ion-pair addition on entrapment efficiency and peptide loading for liposomal formulations presented as individual result from two independent replicates. Entrapment efficiency is represented as average with individual replicates indicated (□).

From Figure 4 it can be observed that the increase in hydrophobic ion pair loading is inversely correlated with entrapment efficiency. Where the volume has been increased from 100 µL of ion-pair dispersion to 850 µL a decrease in entrapment from 70% to 40% was observed. The decrease in entrapment appears to stabilise around 30-40% within the tested range. While the increase in ion-pairs incorporated within the thin-film resulted in a decrease in entrapment, an increase in peptide loading was observed from 7.5 µg/mg of liposomes up to 70 µg/mg within the range explored. The difference in loading levels between the two repeats containing 850 µL HIPs may be attributed to differences in the HIP dispersion. The decrease in entrapment efficiency is due to the increase in drug:lipid ratio. As lipid concentrations are relatively low (<15 mM), this could be improved by increasing the lipid concentration [19].

3.2. Liposome characterisation

Teriparatide loaded liposomes were produced by pre-forming hydrophobic peptide-surfactant ion pairs and then incorporating these into the lipidic thin-film to produce liposomes. A range of liposomes with different phospholipid composition was prepared and characterised for peptide loading, size distribution, and transition temperature. All further experiments were conducted at the highest HIP loading (1.4 mg of HIPs per 10 mg of phospholipids). The liposomes were comprised of DPPC (T_c = 41°C) and DSPC (T_c = 54°C) mixtures, ranging from a DPPC:DSPC molar ratio of 8:2 to pure DSPC (0:10) liposomes, thereby increasing the transition temperature [36]. Table 1 presents an overview of the different liposomal formulations and their physico-chemical properties.

Table 1: Physico-chemical properties of liposomal formulations of different phospholipid composition. Liposomes were loaded with teriparatide-SDS ion pairs and prepared by thin-film rehydration prior to extrusion through 0.2 µm polycarbonate membranes. All formulations contained 5% w/w DSPE-PEG with respect to the lipid content. Results are presented as mean ± s.d. (n=3).

	Molar ratio DPPC:DSPC	**z-average [nm]**	**PDI**	**T_c onset [°C]**	**T_c peak [°C]**
DSPC-2	8:2	85 ± 12	0.266 ± 0.021	38.7 ± 0.4	41.3 ± 0.4
DSPC-5	5:5	125 ± 17	0.242 ± 0.034	41.5 ± 0.7	45.7 ± 1.0
DSPC-8	2:8	191 ± 41	0.312 ± 0.094	46.7 ± 0.3	49.7 ± 0.5
DSPC-10	0:10	165 ± 20	0.206 ± 0.102	49.7 ± 0.1	52.3 ± 0.3

The two phospholipids (DSPC and DPPC) differ in the number of carbons in their fatty acid chain. Indeed, the increased carbon chain length of DSPC increases the transition temperature to 52 °C (pure DSPC), from 41 °C (pure DPPC) [36]. Liposomes composed of different phospholipids combine into one phase with a single phase transition [37]. Therefore, by preparing composite DPPC:DSPC liposomes the transition temperature of the resulting formulation may be tailored within the range of 41 – 52 °C. Four different liposomal formulations were prepared with increasing DSPC content ranging from DPPC:DSPC ratios of 8:2 to pure DSPC. As expected, an increase in onset and peak transition temperature is observed with increasing DSPC content (Table 1). Further, the incorporation of HIP into the liposomes resulted in a

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slight reduction of the transition temperature. Empty DSPC-2 liposomes presented a transition temperature onset of 40.9 ± 0.4 °C, whereas when incorporating HIPs a slight reduction in transition temperature was observed. A slight reduction of transition temperature upon incorporation of drug into the lipid bilayer has been well described in the literature, thus suggesting that the HIPs are located in the lipid bilayer of the liposomes [37,38]. The desired transition temperature of the system is 43 – 45 °C, slightly above physiological temperature. This will avoid premature release, while keeping the required temperature increase minimal, thus ensuring tolerability and avoiding tissue damage. Apart from the DSPC-2 formulation, all formulations present transition temperatures above physiological temperatures (37 - 40 °C) while also accounting for disease (e.g. fever) and within or slightly above the target range of 43 - 45 °C.

The teriparatide entrapment efficiency within the different liposomal formulations can be found in Figure 5. For all formulations entrapment efficiencies between 30-53 % were obtained representing peptide loadings between 25 – 100 µg per mg of phospholipid.

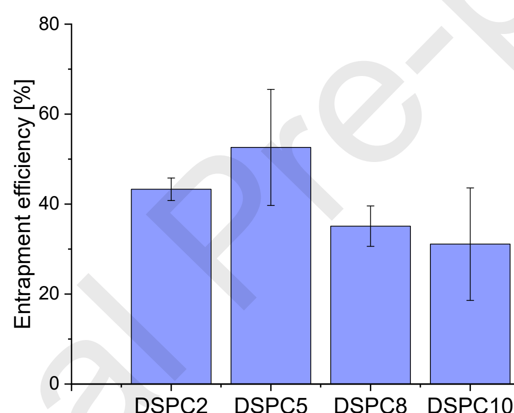

Figure 5: Entrapment efficiency of teriparatide through hydrophobic ion pairing within liposomal formulations comprised of DPPC and DSPC at various molar ratios. All formulations contained 5% w/w DSPE-PEG with respect to the lipid content. Results are presented as mean ± s.d. (n=3).

Compared to previous entrapment achieved by hydrophobic ion pairing (>70%, see section 3.1), the entrapment of teriparatide is significantly lower ($p < 0.05$, One-way ANOVA). This effect is mainly attributed to the increase in peptide loading (see Figure 4). In addition, in the current experiment liposomes were prepared in 100 mM phosphate buffer (pH = 7.4) in order to improve physiological relevance and cellular biocompatibility. The subsequent increase in ionic strength (and pH) may further contribute to the decrease in entrapment efficiency by favouring de-complexation of the ion-pairs during the rehydration process. Indeed, de-complexation is driven by counter-ion competition (e.g. through salts) or through pH-dependent charge negation [34]. Nevertheless, entrapment efficiencies are still comparable to those observed when loading hydrophilic compounds by other methods such as reverse phase evaporation [18]. Overall, peptide loadings (250 – 1000 µg/mL corresponding to 12-50 doses / mL) were deemed satisfactory and encapsulation efficiencies comparable

to the most successful techniques for passive entrapment of hydrophilic molecules (30-50%) [18].

The entrapment efficiency of teriparatide ion pairs within the DPPC:DSPC liposomes appears to be higher with lower DSPC content, however this observation was not significant (p>0.05, One-way ANOVA). Incorporation of hydrophobic molecules within the lipid bilayer generally depends on physicochemical properties of the cargo molecule as well as bilayer composition and volume [39].

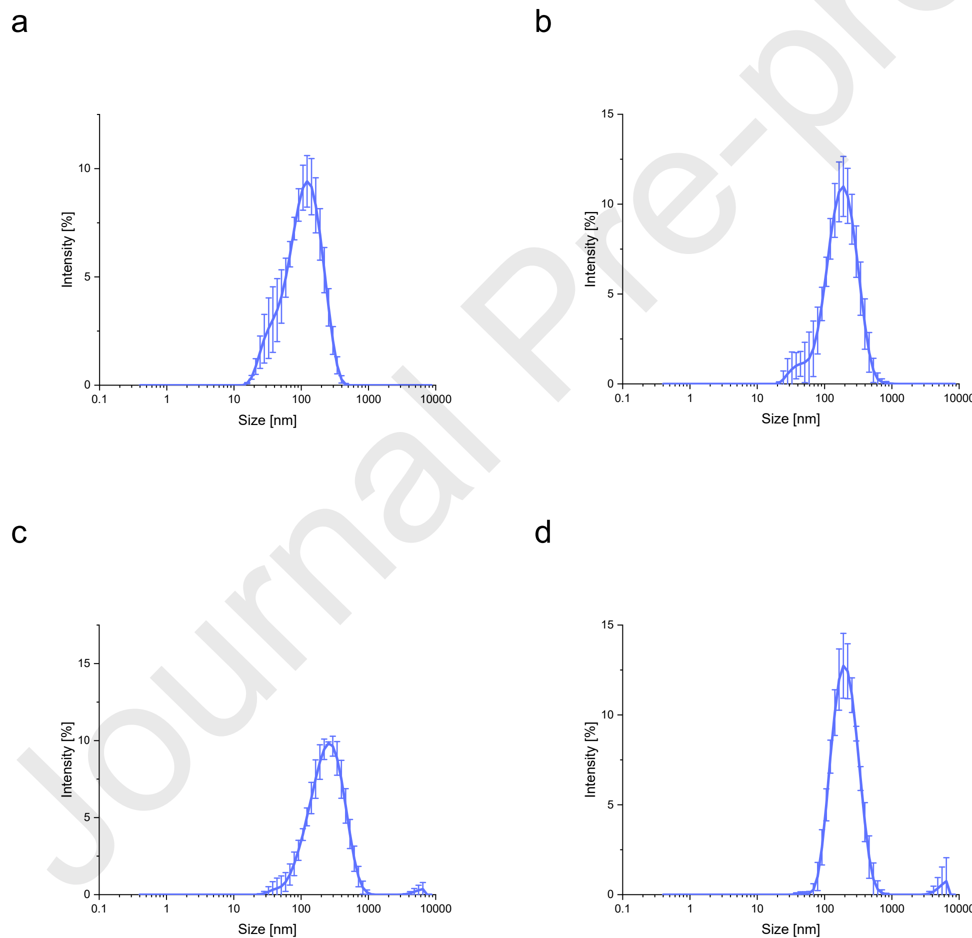

Figure 6: Size distribution of the teriparatide loaded liposome formulations composed of different DPPC:DSPC ratios. Teriparatide entrapment into (a) DSPC2, (b) DSPC5, (c) DSPC8, and (d) DSPC10 liposomes was achieved through hydrophobic ion pairing. All formulations contained 5% w/w DSPE-PEG with respect to the lipid content. Results are presented as mean ± s.d. (n=3).

The average size of the individual formulations ranged between 100 – 200 nm, with the exception of DSPC-2, which presented a size of 85 ± 12 nm (see Figure 6). The difference is likely to be related to the extrusion process. Initially, formulations (DSPC-2) were extruded through 100 nm membranes. It was observed that increasing T_c led to more resistance during the extrusion process, thus more manual strain. As extrusion through 100 nm membranes was not required to achieve small (<200 nm) liposomes, the final extrusion of all other formulation was achieved with 200 nm membranes.

All formulations were largely monodispersed (Figure 6), which is reflected in their polydispersity indexes (PDI, see Table 1).

3.3. Evaluation of in-vitro drug release

The temperature-dependent peptide release from the different liposomal formulations was compared, to assess suitability of thermo-responsive liposomes as a pulsatile delivery system. Brief and localised hyperthermia may be achieved through incorporation of near infrared (NIR) responsive materials within the liposomes [28]. Each formulation was continuously stored at three different temperatures (room temperature (22 ± 2 °C), 34 °C, and 45 °C), where 34 °C represents the temperature in the subcutaneous environment and 45 °C is the target temperature to trigger the release of the peptide [40]. Figure 7 presents the release of teriparatide over a 48-hour period for the three liposome formulations.

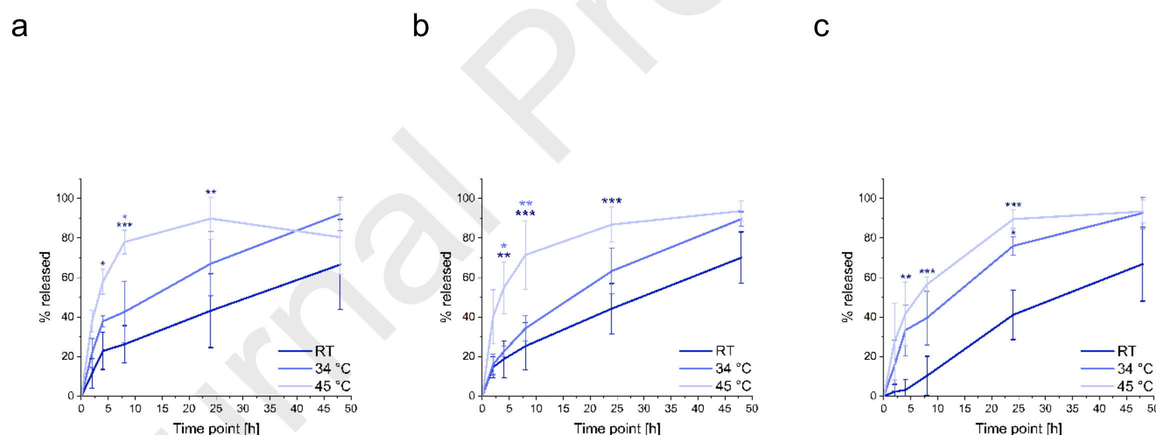

Figure 7: Teriparatide release from the three liposome formulations investigated over 48h at room temperature, 34 °C, and 45 °C comparing three liposomal formulation a) DSPC5, b) DSPC8, and c) DSPC10. These formulations differ in their transition temperature and range from an on-set of transition of 41.5 ± 0.7 °C, 46.7 ± 0.3 °C, and 49.7 ± 0.1 °C respectively (n=3, mean ± s.d.). All formulations contained 5% w/w DSPE-PEG with respect to the lipid content. Where the amount of teriparatide release was statistically significant higher to either room temperature (dark blue asterisk) or 34 °C (light blue asterisk) at a given time point, this has been indicated by (*) p < 0.05, (**) p < 0.01, and (***) p < 0.001.

Figure 7 shows a temperature dependency of teriparatide release for all three formulations. For each formulation the amount of peptide release is significantly higher (Two-way ANOVA, $p < 0.05$) between 4 and 24 h when incubated at 45 °C compared to room temperature. However, no difference (Two-way ANOVA, $p > 0.05$) is observed at 48 h. This is because when incubated at 45 °C, most (>80%) of the peptide is released in the first 24h. Further release between 24 and 48 h is minimal. Conversely, at RT and 34 °C only 30 - 60% is released after 24 h, allowing for continued release

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of the peptide between 24 and 48 h. Moreover, significantly higher amounts of the peptide (Two-way ANOVA, $p < 0.05$) were released at 45 °C compared to 34 °C for some formulations at 4h and 8h (see Figure 7), thus further demonstrating the temperature dependence and possibility for pulsatile release systems.

These results concur with the literature, demonstrating a more efficient retention of compounds when the liposomes are in their solid gel phase (below T_c) than in the liquid phase (above T_c) [37]. Chen et al. measured calcein release from liposomal formulations of different phosphatidylcholine composition at a temperature below the T_c of all formulations. The authors demonstrated that calcein release correlated to the T_c with as little as 7% released after 11 h for HSPC containing liposomes (T_c = 53.7 °C) compared to 43% for pure DPPC liposomes (T_c = 42.4 °C) [37]. While DSPC-10 (T_c = 49.7 °C) liposomes present a slower peptide release (<5% at RT) over the first 4 h compared to the other two formulations (Figure 7a-c), this was not statistically significant (Two-way ANOVA, $p > 0.05$) and otherwise no marked difference between formulations of different T_c.

Surprisingly, retention of the peptide within the formulation was not achieved as well as in other published reports of thermo-responsive liposomal formulations, with up to 25% released in the first 8h incubated at RT. It was hypothesised that improvements may be achieved by replacing the counter-ion used in the hydrophobic ion pairing (currently SDS). The impact of counter-ion employed for HIP formation was shown to determine the release in similar delivery systems. Indeed, Bonengel *et al.* observed counter-ion dependent release profiles for octreotide from self-emulsifying drug delivery systems [41]. Ristroph *et al.* discovered that the nature of the counter-ion strongly influenced the resulting internal ordering of polymyxin B ion-pair loaded nanocarriers [42]. The authors further demonstrated that the internal ordering strongly impacted the release of polymyxin B from the nanocarriers, with more ordered phases resulting in a slower release

Additionally, we hypothesised that the presence of free SDS (from unentrapped and dissociated ion-pairs) may result in solubilisation of the lipid bilayers, resulting in the presence of mixed micelles and further contributing to premature release of teriparatide [43]. Sudbrack *et al*. demonstrated that the formation of mixed micelles is concentration dependent, where at low concentrations SDS will be incorporated into the bilayer up until a saturation point. Following saturation, the SDS rich bilayer will co-exist with mixed micelles. Finally, above the solubility limit, complete disappearance of the bilayer is obtained and only mixed micelles will be present [44]. This hypothesis was investigated by preparing liposomes of different ion-pair loadings and observing the formulations by cryo-transmission electron microscopy (cryo-TEM).

Cryo-TEM images (Figure 8) were obtained for empty liposomal formulations as well as HIP loaded liposomes. Loaded liposomes were prepared at three different levels of HIP loading ranging from 8 – 60 µg teriparatide per mg of liposomes (80 µg/mL – 600 µg/mL teriparatide), corresponding to entrapment efficiencies between 18 – 40%.

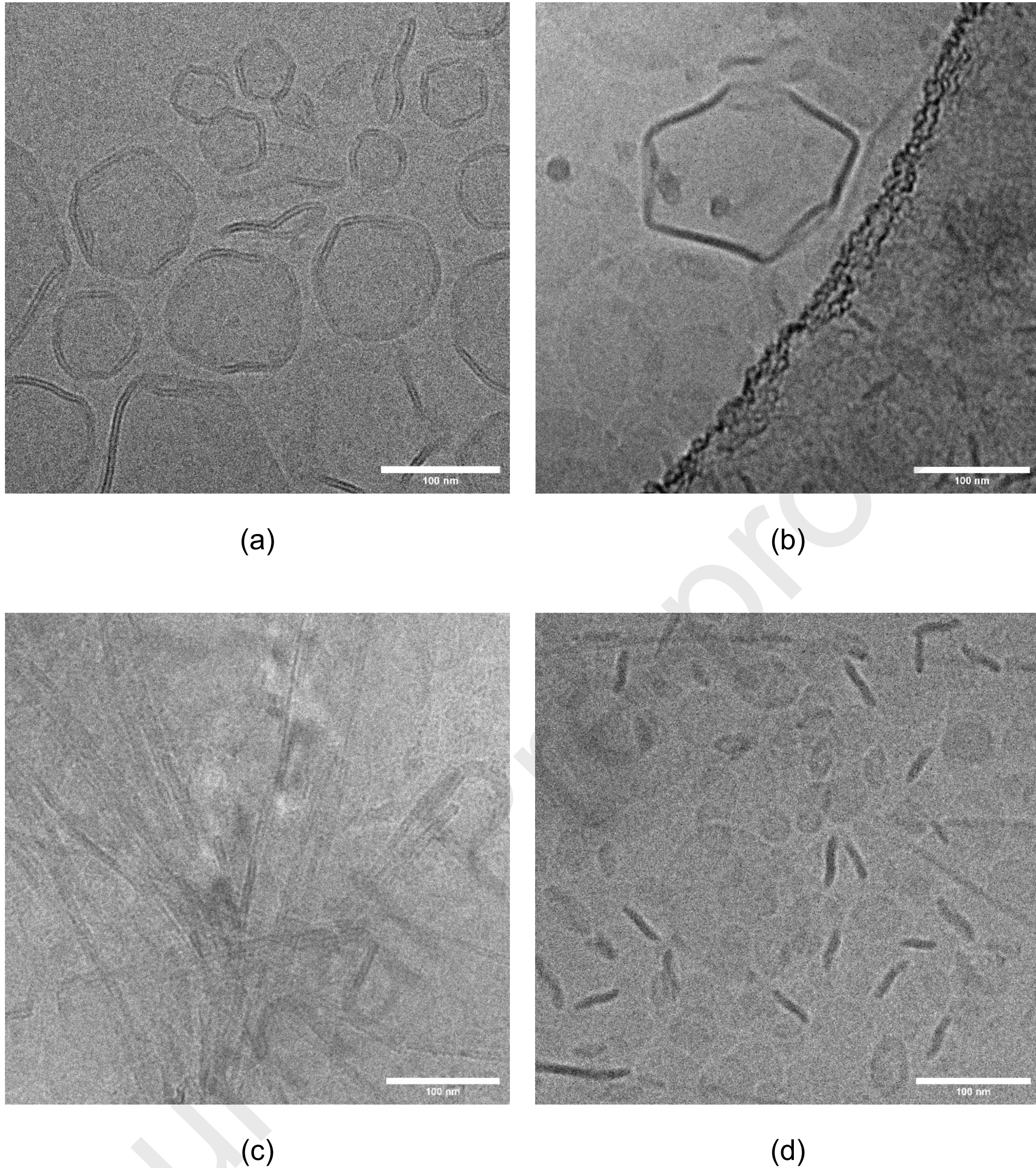

Figure 8: Cryo transmission electron micrographs of a) empty liposomes, b) 80 µg/mL HIP (low), c) 140 µg/mL HIP (medium), d) 600 µg/mL HIP (high) in the liposomal formulation.

From Figure 8 it may be observed that with increasing teriparatide content, the formulation increases in heterogeneity. The empty formulations (Figure 8a) show the typical ring-shaped structure where high contrast regions represent the lipid bilayer [45]. These formulations do not present a round but rather an angular shape, which is due to the phase transition temperature of the liposomes being above room temperature. Additionally, disk-like shapes are observed, which have previously been described to originate from the incorporation of PEGylated phospholipids forming additional colloidal structures (e.g. mixed micelles) [38].

For the liposomal formulations at low teriparatide content (Figure 8b), liposomal vesicles may still be present, and the appearance of globular shaped systems as well as small rods of high contrast were found. Similar observations were made with high concentration of PEGylated phospholipids [45]. The appearance of mixed micelles was attributed to an increasing presence of free SDS originating from unentrapped HIPs. The appearance of thread-like micelles as present in Figure 8c is typically observed in the solubilisation of lipid membranes by surfactants, prior to formation of shorter threads with increasing concentrations (Figure 8d) [43]. The zeta potential of all four liposomal formulations was measured and the decrease from +0.4 mV (empty liposomes) to up to -11.7 ± 2.1 mV (high HIP liposomes) attributed to the presence of SDS, further concurring with the observations.

While increasing peptide concentration through addition of larger volumes of HIP dispersion results in a more heterogenous lipid-based formulation presenting not only liposomes but also mixed micelles, all formulations present a thermo-responsive character with comparable transition temperatures. Overall, teriparatide release form the lipid-based formulations was shown to be temperature-dependent thus providing a first proof-of-concept of the suitability of thermo-responsive liposomes for pulsatile delivery. Similarly, Che *et al*. recently described a biomimetic bone structure coated with polydopamine and loaded with thermo-sensitive (DPPC) liposomes containing calcein and teriparatide for local bone regeneration. The authors demonstrate a pulsatile release of teriparatide over 14 days in an *in vitro* assay and *in vivo* bone regeneration. Nevertheless, the teriparatide loading within the thermo sensitive liposomes was not reported and thus it remains unknown if the system would be suitable to produced therapeutically relevant teriparatide levels [46].

To date, most stimuli-responsive drug delivery systems predominantly focus on enhancing bioavailability and selective cytotoxicity in oncology and improving the delivery of nucleic acids. Few reports exploit a stimuli-responsive system for on-demand or pulsatile delivery [47,48]. In two reports organic solvents (e.g. ethanol) were employed for preparation of the stimuli-responsive system. Ge *et al.,* for example, synthesized polypyrrole (PPy) nanoparticles by emulsion polymerisation. During synthesis two drug (fluorescein and daunorubicin) were loaded into the PPy nanoparticles. Not only were those two drugs exposed to organic solvent their hydrophobicity was key to encapsulation [48]. However, such systems would typically be unsuitable for peptides as exposure to organic solvents often results in denaturation and loss in bioactivity [49]. The formation of peptide-ion pairs and concomitant improvement of peptide stability in solvents such as ethanol (see Supporting Information, Section S4) as well as increased hydrophobicity is likely to expand the possibility for other peptides and possibly some proteins.

3.4. Pharmacological assessment of released teriparatide

The preservation of teriparatide's biological activity was assessed in a cell-based assay measuring intracellular cAMP levels. Teriparatide binds to parathyroid hormone 1 receptor (PTH1R), a receptor belonging to the family of G-protein coupled receptors (GPCR) primarily expressed in bone, kidney and cartilage. PTH1R couples to several intracellular signalling pathways transmitting stimuli in response to binding of different ligands (e.g. PTH/teriparatide). Upon teriparatide binding, the PTH1R signals primarily via $G\alpha_s$ resulting in a series of downstream signals including an increase in cAMP

levels (see Figure S6.1) [50]. In this assay, cAMP levels in a PTH1R overexpressing HEK293 cell line were measured after exposure to teriparatide and the downstream cell signalling through ERK 1/2 confirmed by Western blotting (see Supporting Information Section S3). The cells were exposed to identical concentrations of the native (unprocessed) teriparatide as well as to teriparatide released from a liposomal formulation (DSPC-10), as determined by HPLC analysis. Figure 9 shows cAMP levels measured in HEK-PTH1R cells after a 30-minute exposure to 0.14 ng/mL teriparatide samples. The native peptide and teriparatide released from the formulation showed no significant difference (Student's t-test, $p > 0.05$) in cAMP levels (0.024 µM and 0.032 µM respectively), thus demonstrating that the functionality of teriparatide is retained.

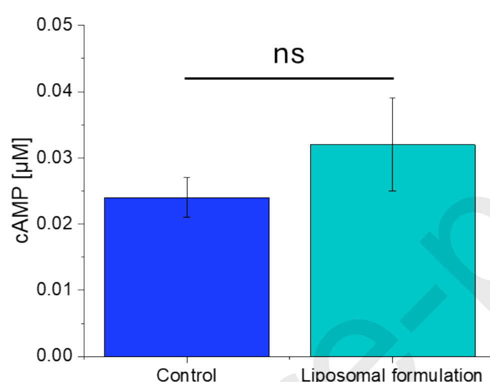

Figure 9: cAMP levels measured in a PTH1R overexpressing HEK293 cell line after exposure to identical concentrations of native (unprocessed) teriparatide (control) and teriparatide released from the liposomal formulation (mean ± s.d., n=3).

4. Conclusion

Teriparatide loaded thermo-responsive liposomes with high entrapment efficiency (> 75%) were prepared. The generation of hydrophobic ion pairs (HIPs) was explored for the first time as an approach to improve liposome entrapment of a peptide. Utilising a HIP approach improved peptide stability in ethanol and improved processing through direct incorporation of peptide ion pairs within the thin film. TEM analysis of empty liposomes showed vesicles presenting the typical liposomal bilayer. However, with higher levels of HIP entrapment a more heterogenous system of liposomes and micelles was evidenced by the presence of globular vesicles, thread like micelles and small rods. This was attributed to solubilisation of the lipid bilayer by SDS. The transition temperature of the liposomal formulations is modulated by the acyl chain length of phospholipid, and by modification of DPPC and DSPC ratios may be tailored within the range of 38 – 50 °C. *In vitro* release experiments demonstrated temperature-dependent release kinetics providing a first proof-of-concept of the suitability of thermo-responsive liposomes for pulsatile delivery. Most importantly, no loss in biological activity of liposomal teriparatide was observed in a cell-based assay. Further development of this platform will afford active control over the release of the therapeutic through incorporation of NIR responsive materials. The incorporation of liposomal formulation into a hydrogel would permit a depot system, where localised

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laser-stimulation of the construct will generate brief hyperthermia triggering the release of teriparatide.

Funding

This work was supported by the UK Engineering and Physical Sciences Research Council (EPSRC) [EP/S023054/1]. S.W.M. is supported by the Medical Research Council (MRC) [MR/X007332/1].

CRediT authorship contribution statement

Conceptualization, C.S.S., C.J.M., S.B. and G.R.W; methodology, C.S.S., R.D.M, S.W.M., formal analysis, C.S.S., investigation, C.S.S. W.R., resources, C.J.M., S.W.M., S.B., and G.R.W., writing – original draft preparation, C.S.S.; writing – review and editing C.S.S, W.R., R.D.M., S.W.M., C.J.M., S.B., G.R.W.; visualisation, C.S.S.; supervision, S.B. and G.R.W., funding acquisition, G.R.W.

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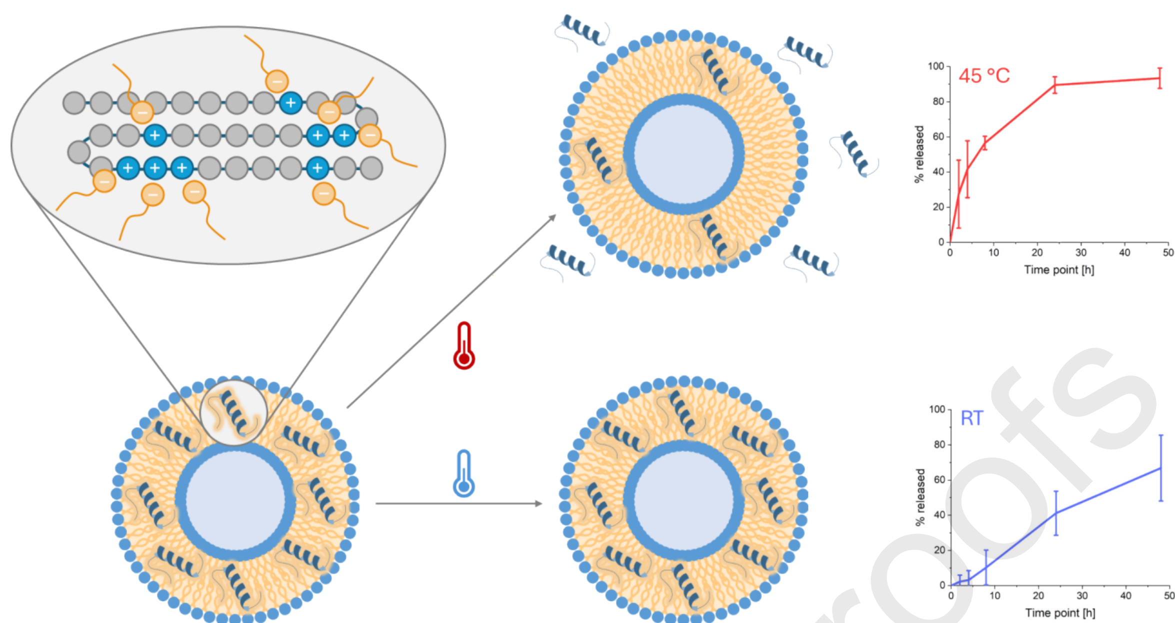

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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