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Evaluation of Lipid-Stabilised Tripropionin Nanodroplets as a Delivery Route for Combretastatin A4

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Abstract

Lipid-based nanoemulsions are a cheap and elegant route for improving the delivery of hydrophobic drugs. Easy and quick to prepare, nanoemulsions have promise for the delivery of different therapeutic agents. Although multiple studies have investigated the effects of the oil and preparation conditions on the size of the nanoemulsion nanodroplets for food applications, analogous studies for nanoemulsions for therapeutic applications are limited. Here we present a study on the production of lipid-stabilised oil nanodroplets (LONDs) towards medical applications. A number of biocompatible oils were used to form LONDs with phospholipid coatings, and among these, squalane and tripropionin were chosen as model oils for subsequent studies. LONDs were formed by high pressure homogenisation, and their size was found to decrease with increasing production pressure. When produced at 175 MPa, all LONDs samples exhibited sizes between $100-300$ nm, with polydispersity index PI between $0.1-0.3$. The LONDs were stable for over six weeks, at 4 °C, and also under physiological conditions, showing modest changes in size ($< 10\%$). The hydrophobic drug combretastatin A4 (CA4) was encapsulated

in tripropionin LONDs with an efficiency of approximately 76%, achieving drug concentration of approximately 1.3 mg/ml. SVR mouse endothelial cells treated with CA4 tripropionin LONDs showed the microtubule disruption, characteristic of drug uptake for all tested doses, which suggests successful release of the CA4 from the LONDs.

Keywords: Drug Delivery, Nanoemulsion, Combretastatin

1. Introduction

The pharmaceutical industry produces many new potential therapeutic agents that have poor water solubility. Many of these hydrophobic agents show promising results during *in vitro* studies, when administered dissolved in solvents like DMSO. However, when undergoing pre-clinical testing, the hydrophobicity of drugs often makes them difficult to deliver, resulting in reduced bioavailability and efficacy.[1]. A multitude of different routes for hydrophobic drug encapsulation have been proposed,[2] from micelles[3, 4, 5] to porous nanoparticles.[2, 6] Among these methods, sub-micron emulsions, or *nanoemulsions*,[7] are a versatile option for the encapsulation and delivery of poor water soluble compounds.[8] Nanoemulsions consist of nanometer-sized oil droplets dispersed in water; the oil is generally assumed to be insoluble in the water phase. The adsorption of a surfactant in the oil-water interface allows for the existence of the nanodroplets, and helps preventing nanodroplet coalescence.[9] The use of nanoemulsions for hydrophobic drug delivery relies on the encapsulation of the therapeutic agent within the oil core of the nanodroplets. High-energy methods for the production of nanoemulsions include ultrasonication [10] and high pressure homogenisation[11, 12, 13, 14], resulting in nanodroplets below 300 nm in diameter. Such nanodroplets are particularly relevant for drug delivery as they are able to achieve good penetration though small capillaries as well as uptake across tissue.[15] There are several factors that influence the size of the nanodroplets within an emulsion. For the specific case of high-pressure homogenisation, the production pressure plays a crucial role as it determines the efficiency in dispersing the oil into the water phase. The literature about this topic is extensive, but it has mostly focused in nanoemulsions aimed at the food industry.[16, 17] The nature of the surfactant used to stabilise the nanoemulsions is also known to affect the size of the nanodroplets.[18] For example, increasing concentrations of PEG in the surface of lipid-stabilised nanoemulsions has been reported to reduce

the size of the droplets.[19] Therapeutic nanoemulsions have been successfully prepared using a range of different biocompatible surfactants, including Tween,[20] polyethylene glycol[21] and lipids.[22, 23, 24, 14, 25] Nevertheless, lipid shells are of particular relevance for drug delivery systems, as phospholipids are cheap, easy to handle and are versatile, i.e. to include different ligands for nanodroplet functionalisation to enhance biocompatibility and permit attachment of targeting agents such as antibodies.[26, 27, 19] In addition to the production pressure and the chosen surfactant, the viscosity of the oil has been observed to influence the size of the nanoemulsion nanodroplets prepared using different methods.[28, 29] Lower viscosity oils have been observed to produce smaller nanodroplets than higher viscosity oils such as long chain triglycerides. For example, Sanguansri and co-workers observed a size difference of around 33% when comparing nanodroplets prepared with peanut oil ($\sim$ 57 mPa·s at 25 °C) and hexadecane (2.66 mPa·s at 25 °C).[30] This effect is attributed to the enhancement of the droplet deformability when using lower viscosity oils, which results in a more efficient dispersion of the oil in the water phase.

To date, nanoemulsions have been used in wide range of treatments, such as antiviral,[31] and antimalarial[22, 32]. Furthermore, a number of anticancer drugs have been delivered to tumours using nanoemulsions.[33, 21, 14] For example, Dacarbazine encapsulated in soybean oil nanodroplets was found to have an increased efficacy when administered *in vivo*.[34] Combretastatin A4 (CA4) belongs to a group of vascular disrupting agents which selectively cause rapid shut down of established tumour vasculature.[35, 36]. CA4 has a high binding affinity to tubulin, which leads to morphological changes of the endothelial cells cytoskeleton. *In vivo* treatments with CA4 are impaired by the toxicity and poor water solubility of the drug. Efforts to improve treatments with CA4 have led to the development of the more soluble produg combretastatin A4 phosphate (CA4P). CA4P has been evaluated as a monotherapy in a number of Phase I clinical trials, with a reported number of adverse side effects including nausea, vomiting, tumour pain and more severe cases of acute coronary syndrome.[37, 38] There are ongoing Phase II/III clinical trials assessing CA4P in combination with other drugs such as Bevacizumab.[39]. Although converting CA4 to the water soluble pro drug CA4P permits delivery its toxicity still leads to a number of undesired side effects. Therefore there is an unmet clinical need for the development of a vehicle that is able to deliver compounds like CA4 in more targeted strategies.

Various developments have been made towards enhancing the delivery of CA4 through its incorporation in liposomes. Liposomal formulations rely on the incorporation of CA4 in the lipid bilayer and therefore they are greatly restricted in the amount of CA4 that can be loaded.[40, 41] Bibby and co-workers studied the effect of CA4 delivered in a 10% DMSO/oil emulsion *in vivo*. They found severe vascular shut-down and tumour growth delay as a result of the treatment with 150 mg/kg of CA4 administered in this way.[42] However DMSO is not an ideal solvent for the injection of poor water soluble drugs due to its adverse side effects.

Here we report the encapsulation and *in vitro* delivery of CA4 in lipid-stabilised oil nanodroplets (LONDs) (Figure 1). LONDs are formed in a two-step homogenisation process that progressively refines the size distribution of the colloidal solution. This process includes a blending step, and homogenisation under high pressure. To optimise the formation of LONDs, and to determine the best oil to deliver CA4, a number of biocompatible oils were screened. LONDs were prepared using seven different oils, and their size, stability and concentration determined. Of these, squalane and tripropionin were found to successfully form LONDs and were chosen as model oils for further studies. Size differences were observed in LOND samples prepared under different pressures, and also between the different oils. Squalane and tripropionin LONDs were stable for at least six weeks when kept at 4 °C, and for $>$ 2 h at 37 °C. CA4 was successfully encapsulated in tripropionin LONDs. *In vitro* evaluation of CA4 in SVR mouse endothelial cells at escalating doses revealed collapsed microtubule structures characteristic of the activity of the CA4. This suggests successful drug release and intracellular uptake of drug from LONDs, which is a promising step towards effective and safe treatments with CA4 *in vivo* .

2. Methods

2.1. Materials

All lipids were purchased from Avanti Lipids (USA), including 1palmitoyl-2-oleoyl-sn- glycero- 3-phosphocholine (POPC), 1,2- distearoyl-sn- glycero- 3-phosphocholine (DSPC), 1,2-distearoyl-sn-glycero- 3-phosphoethanolamine-N-[methoxy (polyethylene glycol)- 2000] (PEG2000-DSPE) and 1,2-distearoyl-sn-glycero- 3-phosphoethanolamine-N-[biotinyl (polyethylene glycol)- 2000] (biotin-PEG2000-DSPE). Fluorescently tagged 1,2-dioleoyl-sn- glycero- 3-phosphoethanolamine (DOPE), Atto590 DOPE and Atto488 DOPE, were

obtained from Atto-TEC (Germany). All oils were purchased from Sigma-Aldrich (UK), including squalane, squalene, triacetin, olive oil, isoamyl acetate (IA), tripropionin and eicosapentaenoic acid (EPA). Combretastatin A4 (CA4) was obtained from Sigma (UK). The primary mouse monoclonal anti-β-tubulin antibody was also obtained from Sigma (UK). Secondary antibodies biotin-labelled polyclonal rabbit anti-mouse was obtained from DAKO (UK) and fluorescein-isothiocyanate-labelled Avidin D was obtained from Vector Laboratories (UK). Antibody diluent ready to use reagent and Prolong Gold with DAPI were obtained from Thermo Fisher Scientific (UK).

2.2. LONDs formation

LONDs are emulsion droplets stabilised with phospholipids. LONDs were formed in a two-step homogenisation process of oil-in-water mixtures containing lipid. All lipid mixtures were initially dissolved in a chloroform-methanol mixture (1 : 1) and then dried under nitrogen for at least 30 min. The lipid shell was tuned for the different oils, informed by preliminary optimisation on the production (data not shown). Hence, and unless otherwise stated, POPC + 5% biotin-PEG2000-DSPE was used for stabilising squalane LONDs, whereas DSPC + 20% Cholesterol + 5% biotin-PEG2000-DSPE was used for the preparation of tripropionin LONDs; these lipid shell combinations enhanced the stability of the LONDs and reduced premature coalescence. When required, fluorescence tracking of LONDs was performed by incorporating $0.1\,mol\%$ Atto590-DOPE or Atto488-DOPE to the lipid mix. The lipids were re-suspended in 0.7 ml ($\phi = 0.07$) of oil by vortexing. The oil fraction was kept constant during the experiments. Occasionally the dried lipid took longer to disperse in the oil, and an additional bath-sonication step was required. For samples encapsulating drugs or drug mimics, the compound was solubilised in the oil to the desired concentration before adding it to the lipids. The homogenisation process began with a blending step using the rotor-stator system Polytron PT1300 D (Kinematica AG, Switzerland). Polytron blending of the sample was carried out at 12500 rpm for 10 min, at 40 ° C and atmospheric pressure. LONDs formation was completed in a high-pressure homogeniser EmulsiFlex-C5 (Avestin Europe GmbH, Germany) for 20 min. The pressure was varied between $35 - 175$ MPa to study the dependence of LONDs size with the production pressure. Other than the LOND samples used to study the effect of the production pressure, LONDs were prepared under 175 MPa unless otherwise stated. Excess lipid and non-encapsulated oil were removed from the LONDs solution by filtration using

a KrosFlow Research IIi Tangential Flow Filtration System (SpectrumLabs Europe, The Netherlands) and 60 kDa Mini-Discover12 pore size column (WaterSep, USA) for at least 2 h.

2.3. LONDs size and concentration

Filtered LONDs were sized by Dynamic Light Scattering (DLS) using a Zetasizer Nano ZSP (Malvern Instruments, UK). For the measurements, 10 μl of the LOND sample were dissolved in 1 ml of PBS in an ordinary disposable cuvette. Three measurements of the sample distribution were performed, each of which consisted of $10 - 17$ runs as determined by the Malvern ZetaSizer Software. DLS was also used to study the stability of LONDs size over time, with storage at 4 °C between measurements. The stability of LONDs at 37 °C was also investigated using DLS. In this case, the sample was kept at 37 °C for 2 h and measurements of its size distribution were taken every 15 min. The sample was gently shaken between measurements to avoid creaming (or sedimentation). The size of LOND samples was alternatively determined by nanoparticle tracking analysis (NTA) using a NanoSight instrument (Malvern Instruments, UK). This device was also used to determine the concentration of LOND samples. NTA relies on the detection of light scattered by sub-micron sized particles, which is used to calculate individual diffusion coefficients and estimate their size.[43] Samples containing around 10^6 LONDs/ml were used for the measurements with the NanoSight instrument (normally involving a $1 : 10^6$ dilution of the original sample). All data regarding LOND size and concentration was analysed using Origin 9 (OriginLab Corporation, USA).

2.4. Transmission electron microscopy

Transmission electron microscopy (TEM) images of LONDs were taken using a JEM1400, 120 kV instrument (JEOL, USA). Samples for TEM imaging were prepared by depositing 15 μl of the LONDs samples (10^{11} LONDs/ml, in PBS) on a carbon coated grid. The solution was left on the grid for 30 s, and the excess liquid dried out using blotting paper. Samples were negatively stained using uranyl acetate by dropping 15 μl of uranyl acetate (1%) on the grid and incubating for 15 s, after which the excess liquid was removed with blotting paper. Images were analysed using ImageJ.

2.5. Encapsulation efficiency of CA4 in LONDs

The concentration of CA4 in LOND preparations was determined by comparing their UV-VIS absorption to a calibration curve for CA4 dissolved in tripropionin. A Lambda 35 UV-VIS Spectrometer (Perkin Elmer, USA) was used to record absorption spectra of the samples. All absorption spectra were recorded between $200-700$ nm. The peak absorption for CA4 diluted in the oil (not encapsulated) lies around 309 nm. To measure the amount of CA4 encapsulated in the LONDs, 10 μl of LONDs solution was dispersed in 990 μl of DMSO. In this way, the LONDs are broken and their contents released, eliminating light scattering from intact LONDs in the solution, which can cause detection issues.

2.6. In vitro *cell culture*

SVR mouse pancreatic islet endothelial cells transformed with *Ras* oncogene, were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% (v/v) foetal calf serum (FCS) at 37 °C and 5% CO_2. SVR cells were grown to $80-90$% confluence, trypsinised and seeded in μ-slide VI (ibidi, Germany) at a density of 3×10^5 overnight after which they were treated with CA4 tripropionin LONDs . All treatments and sequential staining were carried out directly in the μ-slide. Escalating doses of CA4 tripropionin LONDs (4 mM stock concentration) were added to SVR cells in fresh media for 2 h at 37 °C. Following the 2 h treatment the cells were washed with PBS and fixed with 4%(v/v) paraformaldehyde in PBS for 10 min and permeabilised with 0.1% Triton X-100 in PBS for 5 min. To reduce non specific binding the cells were blocked with antibody diluent for 5 min at room temperature. Immunofluorescence staining was performed for β-tubulin 1:500 in antibody diluent for 2 h at room temperature. The cells were then washed with PBS and incubated with biotin-labelled polyclonal rabbit anti-mouse 1:200 in antibody diluent for 1h at room temperature followed by fluorescein isothiocyanate-labelled Avidin D 1:250 in antibody diluent for 1h at room temperature. The μ-slide was mounted with Prolong Gold and DAPI (4,6-diamidino-2-phenylindole) to visualise nuclei. Fluorescent images were acquired using a ZEISS Axioimager Z1 microscope (Carl Zeiss Microscopy, USA) with AxioVision software at x40.

2.7. Quantification of CA4 effect

To quantitate the extent of disruption to microtubules caused by CA4 tripropionin LONDs, fluorescence images of control untreated cells and cells

treated with CA4 tripropionin LONDs were taken ($n = 5$). The length of 10 microtubules from five randomly selected cells were measured in each of the five images (total of 250 microtubules). Image J and GraphPAD were used to measure the microtubules and data plotting, respectively. Statistical analysis was carried using a Mann-Whitney test.

3. Results and discussion

3.1. Preparation of LONDs with candidate oils

LONDs were prepared in a two-step homogenisation process of an oil-in-water and phospholipids mixture. The crude emulsions was initially blent in a rotor-stator system for around 10 min to create a rough dispersion of the oil into the water phase, and to dissolve big lipid agglomerates. This mixing step was followed by high pressure homogenisation for 20 min in an Emulsiflex. In this device, the crude emulsion was forced though a *homogenising nozzle*, where it experienced great mechanical stress for a short time. Figure 2 shows the size distribution of squalane LONDs after processing under high pressure, as measured with DLS, as an example. The high pressure homogenisation step was performed at 175 MPa, and reduced the size distribution of the sample giving a polydispersity index (PI) lower than 0.3 (figure 2b). The average size of the LONDs was (165 ± 5) nm. After filtration, the average size of the sample increased slightly to (188 ± 11) nm (figure 2). This apparent size change of about 12% was accompanied by a slight increase in distribution width, as indicated by the increase of the full width at half maximum (FWHM). This perhaps indicates that the filtration process gave rise to mild sample coalescence.

A number of biocompatible oils were screened to determine their ability to form LONDs stabilised with POPC + 5% biotin-PEG2000-DSPE and at 175 MPa. Table 1 summarises the results obtained for the different oils, including the size and concentration of the LONDs, where applicable. Isoamyl acetate (IA) and eicosapentaenoic acid (EPA) did not produce stable LONDs, thus no further experiments were performed with these. The concentration of triacetin LONDs was found to be lower than that of LONDs prepared with any other oil. This was attributed to the higher solubility of triacetin in water. Among all of the oils tested, tripropionin LONDs exhibited the smaller size, and also the highest concentration. LONDs prepared with squalane, squalene or olive oil produced LONDs of similar size, PI and concentration. Pure oils were chosen over blends such as olive oil, whose composition

strongly depends on its production environment and manufacturer. The size of the LONDs prepared with different candidate oils remained stable for a period of more than six weeks, at 4 °C. Details for the case of tripropionin and squalane LONDs are provided below. Squalane and tripropionin were used as oils for the optimisation of LONDs production, in order to compare LOND production with oils of different density and viscosity.

3.2. Dependence of LONDs size and concentration on production pressure

Control over the size of the LONDs is important for reproducible pharmacokinetic/pharmacodynamic studies. Furthermore, the size of nanocarriers greatly influences their biodistribution and bioavailability.[15] The production of LONDs was monitored as a function of the homogenisation pressure using two different oil/shell combinations: i) squalane LONDs stabilised with POPC, and ii) tripropionin LONDs stabilised with DSPC + 20mol% Cholesterol + 5mol% biotin-PEG2000-DSPE. Size measurements of both LOND samples prepared under 35, 70, 105, 140 and 175 MPa were performed using DLS and NanoSight. Figure 3 shows the changes in the size distribution of the two LOND samples prepared under different pressures, as determined by DLS. In the case of squalane, the average size of the droplets d was fitted to the relationship $d = C \cdot P^{-b}$, where P is the pressure applied at the homogenising nozzle in the Emulsiflex. The parameter b is determined by the turbulent regime existent in the homogenising device, which depends mostly on the size of the appliance,[44] whereas C depends on efficiency of the droplet disruption.[45] Through the fitting of the sizing data for squalane LONDs, b was found to be $b = (0.70 \pm 0.06)$. This value is in agreement with previous observations, which reported values of b between $0.6 - 0.75$ for regimes dominated by inertial or shear forces, respectively.[46, 45] In the case of tripropionin LONDs, their size was found to reduce with increasing production pressure. It is also interesting to note that the size change undergone by tripropionin LONDs between 35 and 175 MPa was of around 25%, whereas the size change for squalane LONDs was of about 70%. This is thought to relate primarily to the differences in viscosity between tripropionin and squalane, as viscosity is known to influence the size of the nanodroplets in a nanoemulsion.[30]

Upon preparation of a nanoemulsion, the number of nanodroplets formed is generally assumed to be determined by the oil volume fraction ϕ. Although this assumption is true in general, non-negligible solubility of the oil in water

could result in a reduced number of nanodroplets, since the disperse phase could partially dissolve in the continuous prior to surfactant stabilisation. The concentration and size of LONDs prepared under different pressures (35, 70, 105, 140 and 175 MPa) were determined with the NanoSight instrument. Figure 4 shows concentration change in LOND samples as a function of the production pressure, for squalane (a) and tripropionin LONDs (b). Squalane LONDs show a rapid decrease in their size distribution with increasing pressure. The size reduction coincides with an increase in the number of LONDs in the solution. The concentration reached a steady value at around $(2.2 \pm 0.4) \times 10^{13}$ ml^{-1}. This is in agreement with the predicted increase in the efficiency of the oil breakage upon increase of the shear forces at the homogenising nozzle (i.e. increasing production pressure). These values are, within experimental error, consistent with the theoretical value for the concentration of LONDs calculated though size considerations for $\phi = 0.07$ ($\sim 2 \times 10^{13}$ LONDs/ml). In the case of tripropionin LONDs (figure 4b), increasing production pressures ($35 - 145$ MPa) resulted in distribution sizes changes of around 4%. Sample processing under 175 MPa for 10 min resulted in a significant size reduction ($\sim 16\%$), accompanied by an increase of the LOND concentration ($\sim 53\%$). As expected, the concentration of the the tripropionin LOND sample prepared under 175 MPa ($(1.4 \pm 0.1) \times 10^{14}$ LONDs/ml) is in agreement with the theoretical value for the concentration of LONDs, based on LONDs dimension and oil volume fraction ($\phi = 0.07$), 1.3×10^{14} LONDs/ml.

3.3. Stability of LONDs

The stability of LONDs as a function of time was followed by monitoring the size distribution of LOND samples using DLS. Sample agglomeration or degradation was expected to translate into changes in the size distribution, such as broadening of the original size distribution and increases in the PI. Two different studies were performed: i) to determine the stability of LONDs during six weeks, and ii) to determine the stability of LONDs at 37 °C in *in vitro* conditions for 2 h. There was an interest in monitoring the stability of LONDs for a time period that would resemble the time required for the LONDs to initially reach the target site and accumulate over time in an *in vivo* setting. Hence the choice of a 2 h period for this experiment. The temperature conditions in this experiment aimed to better resemble those to which the LONDs would be exposed to *in vivo*.

As reflected in table 1, LONDs prepared from all the candidate oils showed good stability over a six weeks period. During this time, the PIs were < 0.3 in all cases. As an example of the type of data recorded, figure 5a shows the size variation of squalane and tripropionin LONDs over six weeks. Each experimental point was produced averaging three DLS runs. This graph shows a maximum size variation of around 16% for squalane LONDs, and around 15% for tripropionin LONDs. This small variation of the LONDs diameter over time suggests good stability of the LONDs against agglomeration and coalescence. Figure 5b shows the size variation for squalane and tripropionin LONDs during 2 h, and incubated 37 $^{\circ}C$ more consistent with conditions *in vitro*. The sizes of both LOND types did not change, showing good stability under these conditions. This suggests that LONDs are suitable vehicles for the delivery of hydrophobic compounds *in vivo* and have an excellent potential to enhance the delivery of poor water soluble drugs.

3.4. TEM imaging of LONDs

TEM was used to image LOND samples. Figure 6 shows the TEM images of squalane and tripropionin LONDs. Squalane LONDs appeared as globular structures ranging $100 - 300$ nm, with an average size of 111 nm ($n = 187$, with a standard deviation of the sample $\sigma = 85$ nm). In contrast, tripropionin LONDs appeared to lose their structure under vacuum conditions in TEM, and exhibited a shell appearance. These LONDs were found to have an average size 89 nm ($n = 71$, with a standard deviation of the sample $\sigma = 23$ nm). A small discrepancy was observed between the TEM and DLS sizing of both squalane and tripropionin LONDs. This was expected and it is attributed to the different conditions under which the samples are studied in one and another techniques (i.e. the sample is dried for TEM imaging, whereas the hydrodynamic radius of the colloids are measured by DLS).

3.5. Encapsulation of CA4 in LONDs

Drug delivery using nanoemulsions, and LONDs in particular, rely on drug encapsulation within their structure. It is generally accepted that the drug is encapsulated within the oil core of the nanoemulsions, and that its lipophilicity directly affects its release from the nanodroplets and therefore its biodistribution.[47] It is also known that the polarity of a therapeutic compounds defines its solubility in oils.[32, 48] CA4 is a largely polar molecule with poor water solubility. However, its polarity made it difficult to dissolve in squalane, as it is a saturated, non-polar oil. Triacetin and tripropionin

were profiled as more suitable oils for the preparation of LONDs encapsulating CA4, as the polarity of the oil enhanced the solubility of CA4. Tripropionin was chosen over triacetin, in light of its reduced water solubility. The concentration of the drug encapsulated in LONDs was determined by measuring its absorption in the UV-VIS. The calibration curve produced for the CA4 in tripropionin is presented in the supplementary. The encapsulation efficiency of CA4 in tripropionin LONDs was approximately 76%, with 1270 μg/ml in the final LOND sample.

3.6. CA4 tripropionin LONDs cause microtubule disruption

To qualitatively and quantitatively assess LOND-based drug delivery, CA4 tripropionin LONDs were used to treat SVR endothelial cells. The cells were treated with escalating concentrations of CA4 tripropionin LONDs from $2-100$ nM for 2 h at 37 °C. Figure 7 is a representative panel of images showing the effect caused by CA4 tripropionin LOND exposure. CA4 tripropionin LONDs caused complete microtubule disruption evident in all SVR cells treated with $40-100$ nM, with microtubules appearing as a uniform fluorescence surrounding the cells nuclei. At concentrations lower than $2-20$ nM, some short microtubules could be seen. Control untreated SVR cells showed normal microtubule structures.

To quantitate the effect on SVR cells and to evaluate the effect caused to microtubules by CA4 tripropionin LOND treatment, the lengths of the cellular microtubules were measured. Figure 8 shows the average microtubule length for the different treatment concentrations. Compared to control, cells treated with doses of CA4 tripropionin LONDs between $4-100$ nM are significantly different $^{****}P < 0.001$. A significant difference $^{*}P< 0.024$ was observed in the smallest concentration of 2 nM. It should be noted that the exact mechanism of drug uptake is unclear however the fact that CA4 tripropionin LONDs at 37°C are stable suggests that the lipid membrane of the LONDs fuses with cell membranes leading to drug release or the LONDs are endocytosed leading to intracellular release of CA4.

4. Conclusion

We have carried out a systematic study on the formation of lipid-stabilised oil nanodroplets (LONDs) with a range of biocompatible oils, towards medical applications. LONDs are formed in a two-step process that includes

blending using a rotor-stator system and high pressure homogenisation. Production pressure has been found to be a factor affecting the size of the LONDs, an it also affects LONDs concentration in the final sample. LOND samples exhibit excellent stability over time and under different conditions. The hydrophobic therapeutic drug Combretastatin A4 (CA4) was encapsulated in tripropionin LONDs with an efficiency of around 76%. Preliminary studies on LOND-based delivery of this compound to SVR cells have shown successful drug release from the LONDs, attending at the effect on cellular microtubules by the encapsulated CA4. Tripropionin LONDs show promising results for the delivery of CA4 *in vitro*, and they hold exciting potential for facilitating the delivery of the compound *in vivo*. We anticipate that studies concerned with the use of CA4 in anticancer combination therapies will benefit from the enhancement of the delivery routes for CA4.

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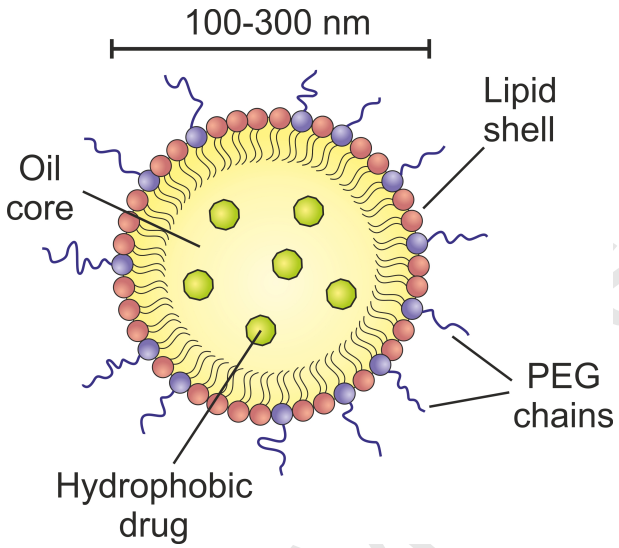

Figure 1: Schematic of a lipid-stabilised oil nanodroplet (LONDs). Hydrophobic drugs, such as CA4, are contained in the oil core of the LONDs, which is stabilised by a lipid monolayer.

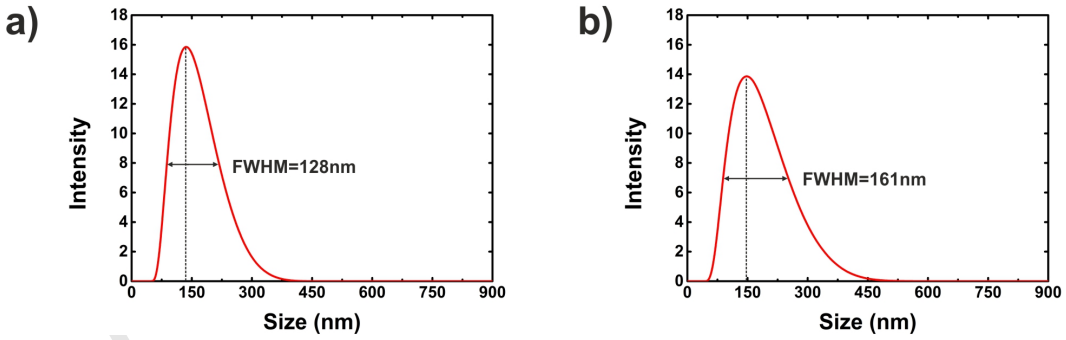

Figure 2: Changes in the size distribution of a squalane LONDs sample: a) after high pressure homogenisation (at 175 MPa), with average size (165 ± 5) nm and b) after sample cross-filtration, mode size (188 ± 11) nm. The width of the distribution (full width at half maximum, FWHM) is indicated in the figures.

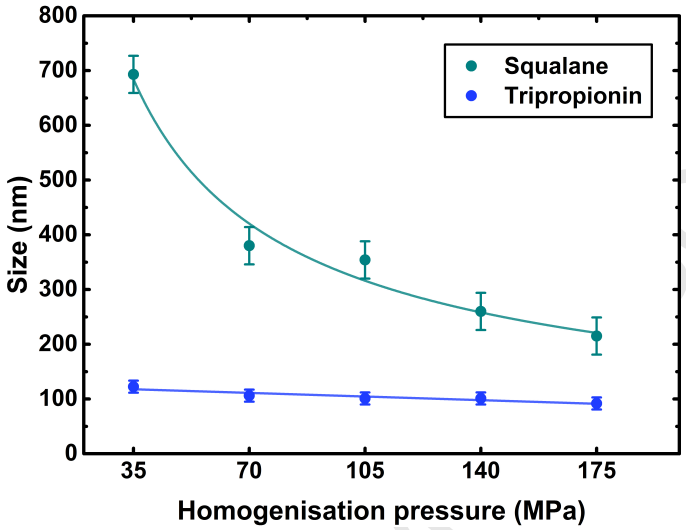

Figure 3: DLS sizing of squalane and tripropionin LOND samples prepared at different pressures. Squalane LONDs were stabilised with POPC, and the shell of the tripropionin LONDs was DSPC + 20% Cholesterol + 5% biotin-PEG2000-DSPE. The lines show fits to the data.

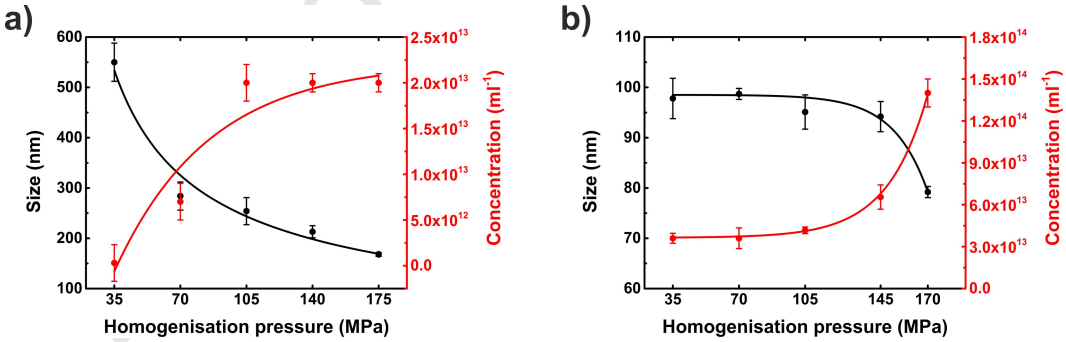

Figure 4: Nanoparticle Tracking Analysis (NTA) measurements on a) squalane LONDs stabilised with POPC and b) tripropionin LONDs stabilised with DSPC + 20% cholesterol + 5% biotin-PEG2000-DSPE. The graph shows the changes in size and concentration of the LONDs in the solution, and the data points were fitted as a guide to the eye.

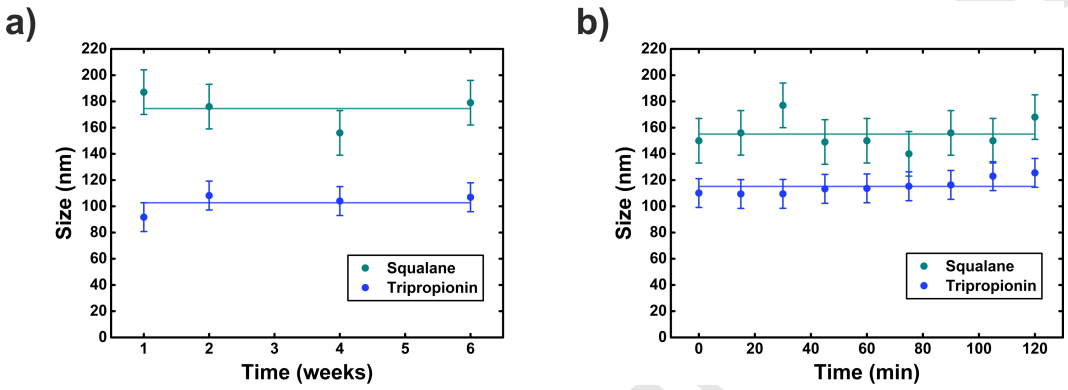

Figure 5: a) Size change of squalane and triacetin LONDs over a six week period, stored at 4 °C, and measured using DLS. b) Size change of squalane and triacetin LONDs at 37 ° over 2 h, as measured by DLS. Experimental points in both graphs were produced averaging three size measurements on the same sample.

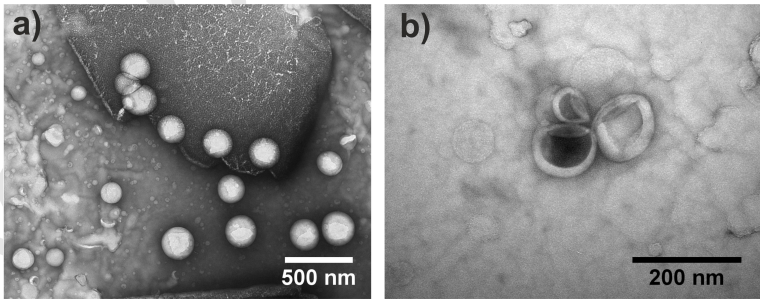

Figure 6: TEM images of LONDs. a) Squalane LONDs stabilised with POPC + 5% biotin-PEG2000-DSPE. b) Tripropionin LONDs stabilised with DSPC + 20% Cholesterol + 5% biotin-PEG2000-DSPE. Both samples were prepared under at 175 kPa.

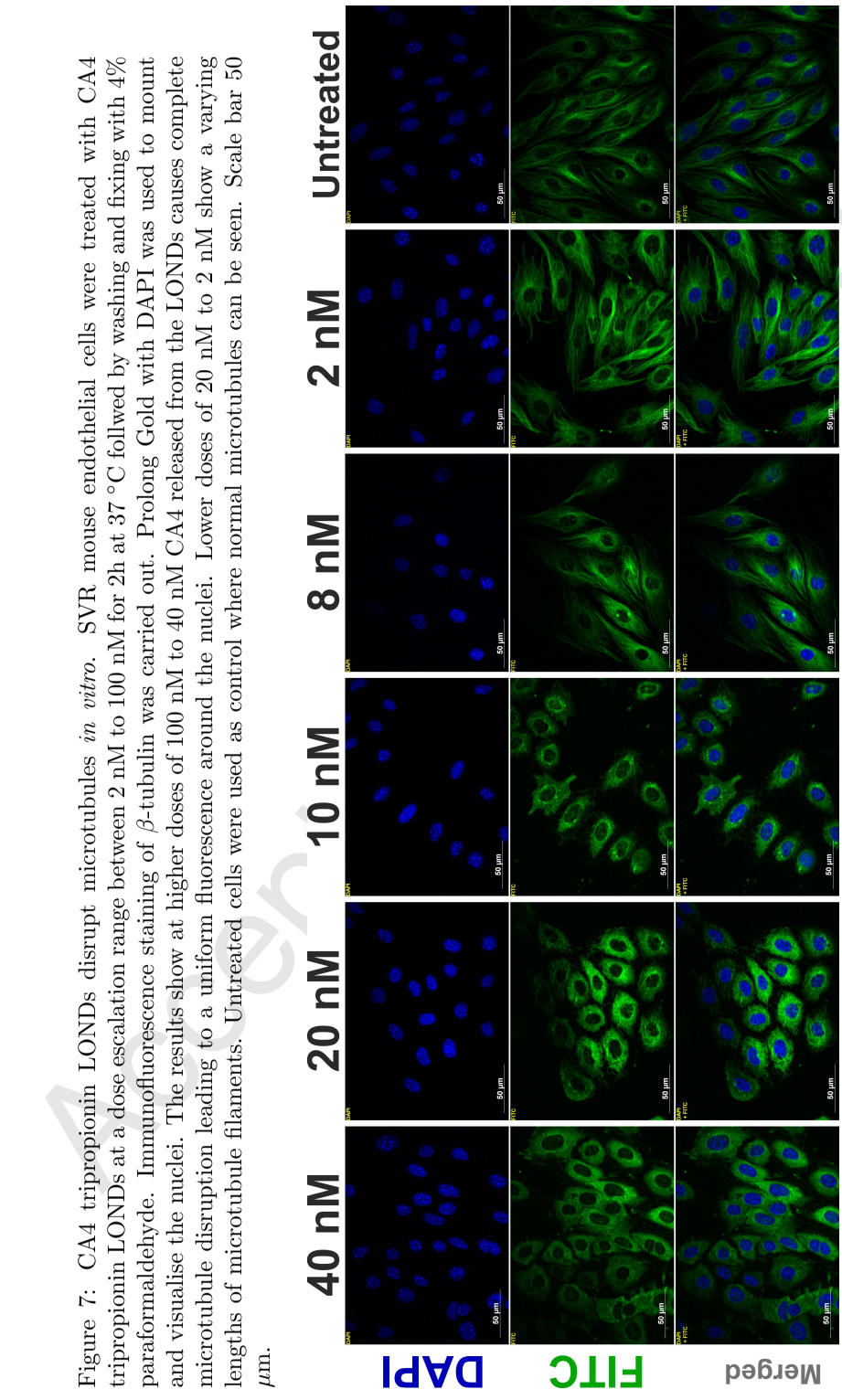

Figure 7: CA4 tripropionin LONDs disrupt microtubules *in vitro*. SVR mouse endothelial cells were treated with CA4 tripropionin LONDs at a dose escalation range between 2 nM to 100 nM for 2h at 37 °C follwed by washing and fixing with 4% paraformaldehyde. Immunofluorescence staining of β-tubulin was carried out. Prolong Gold with DAPI was used to mount and visualise the nuclei. The results show at higher doses of 100 nM to 40 nM CA4 released from the LONDs causes complete microtubule disruption leading to a uniform fluorescence around the nuclei. Lower doses of 20 nM to 2 nM show a varying lengths of microtubule filaments. Untreated cells were used as control where normal microtubules can be seen. Scale bar 50 μm.

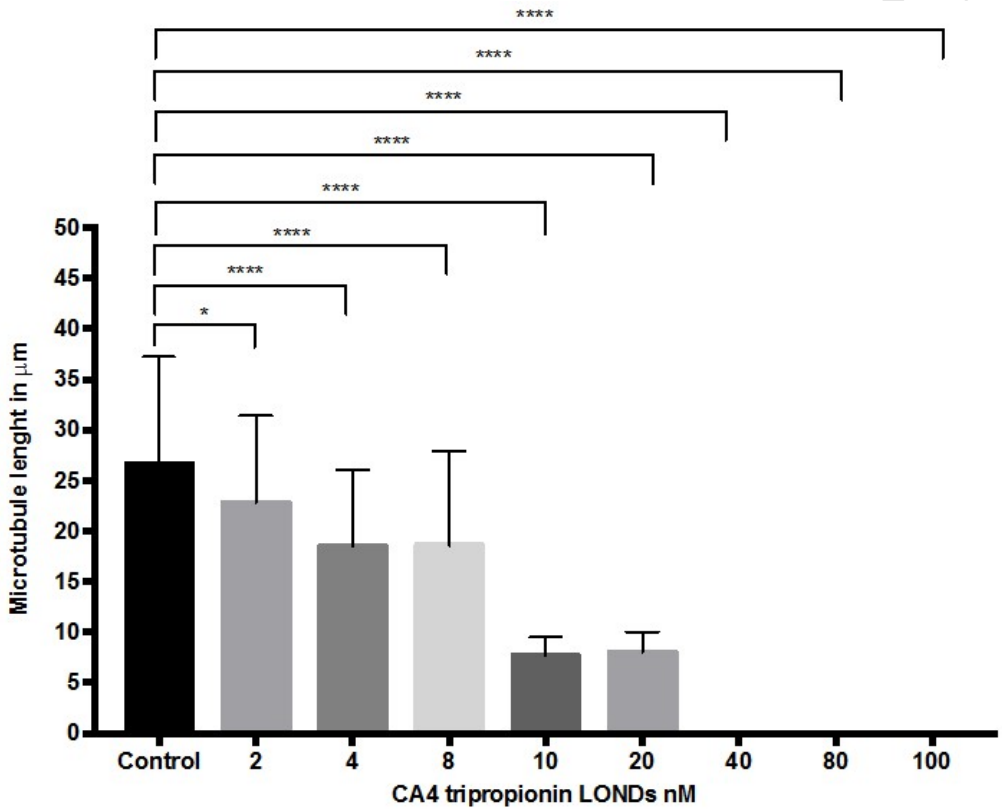

Figure 8: Quantification of microtubule lengths following CA4 tripropionin LOND exposure of 2 h at 37 °C. A total of 250 microtubule filaments were measured with *Image J* from five images taken from different positions on the ibidi μ-Slide. Error bars were calculated by Mann-Whitney test. $^{*}P< 0.0240$, $^{****}P< 0.0001$. Results shown as media with interquartile range.

Table 1: LOND preparation and stability for different oil types and a POPC + 5% biotin-PEG200-DSPE* shell.

Oil	Solubility in H_2O (g/L)	Viscosity at 20 $°C$ (cP)	Density (g/cm^3)	Lipid stabilised	Diameter [a] (nm)	Concentration (ml^{-1})	Stability (weeks)
Squalane	-	12	0.805	Yes	165	10^{13}	> 6
Squalene	0.124×10^{-3}	11	0.858	Yes	133	10^{13}	-
Olive oil	-	85	0.918	Yes	191	-	> 6
Triacetin	61	23	1.160	Yes	250	10^{11}	> 6
Tripropionin	0.003	7	1.082	Yes	100	10^{14}	> 6
IA	2	7.2	0.876	No	-	-	-
EPA	0.284×10^{-3}	35	0.923	No	-	-	-

* Tripropionin LONDs shell was $75mol\%$ DSPC + $5mol\%$ Cholesterol + $5mol\%$ PEG2000-DSPE. Olive oil, IA and EPA had POPC shells.
[a] Two-step preparation process and cross filtration. Emulsiflex pressure was 175 kPa in all cases. Sizing data on this table was measured with DLS. Concentration data for squalane and squalene LONDs was obtained with NTA, and Qnano was used to obtain the concentration of triacetin LONDs.

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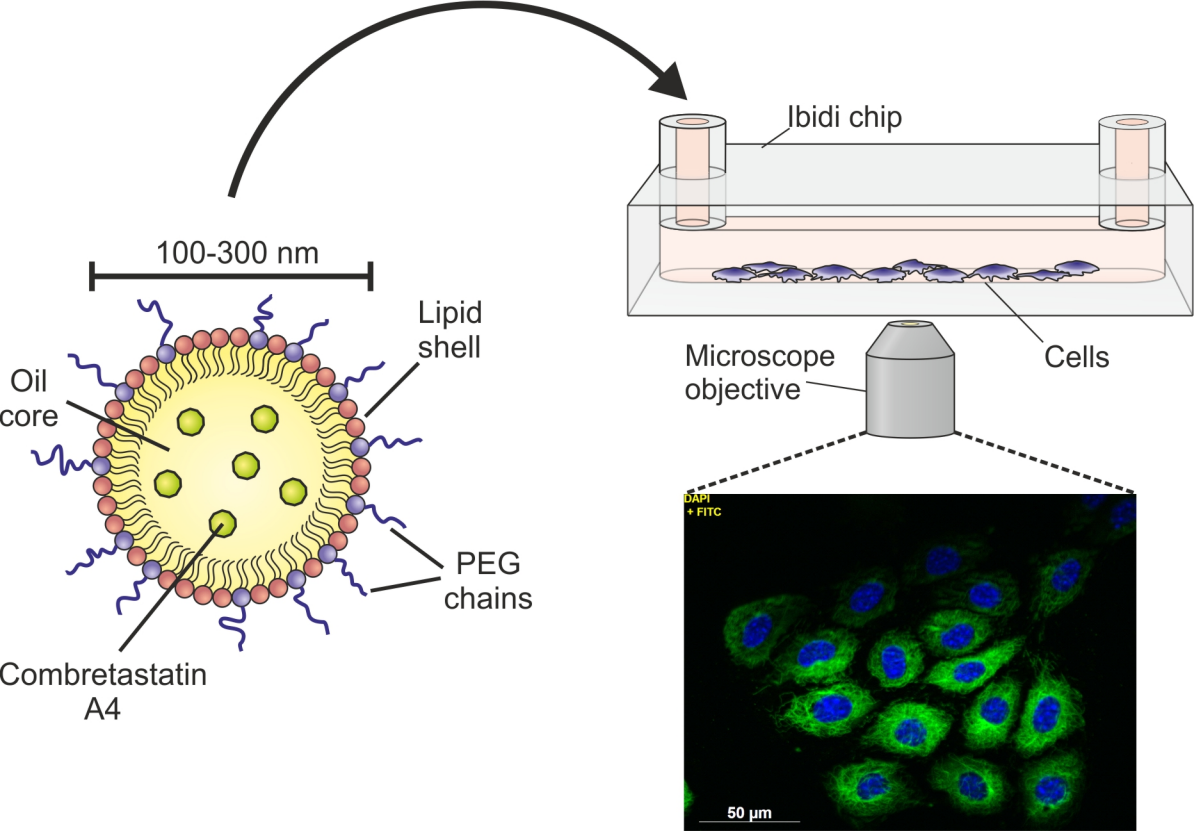
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Lipid shell
Oil core
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Combretastatin A4
Ibidi chip
Cells
Microscope objective
DAPI + FITC
50 µm