J Pharm Sci. Author manuscript; available in PMC 2013 November 06.

Published in final edited form as:
J Pharm Sci. 2011 July ; 100(7): . doi:10.1002/jps.22500.

Preparation, characterisation and entrapment of a non-glycosidic threitol ceramide into liposomes for presentation to invariant natural killer T cells

Randip Kaur[1], **Jili Chen**[2], **Amina Dawoodji**[2], **Vincenzo Cerundolo**[2], **Yoel R. Garcia-Diaz**[3,4], **Justyna Wojno**[3,4], **Liam R. Cox**[4], **Gurdyal S Besra**[3], **Behfar Moghaddam**[1], and **Yvonne Perrie**[1,*]

[1]School of Life and Health Sciences, Aston University, Birmingham, B4 7ET, UK

[2]Weatherall Institute of Molecular Medicine, University of Oxford, Oxford, OX3 9DS, UK

[3]School of Biosciences, University of Birmingham, Edgbaston, Birmingham, B15 2TT, UK

[4]School of Chemistry, University of Birmingham, Edgbaston, Birmingham, B15 2TT, UK

Abstract

Dendritic cells (DCs) are able to present glycolipids to invariant natural killer T (*i*NKT) cells *in vivo*. Very few compounds have been found that stimulate *i*NKT cells and of these the best-characterised is the glycolipid α-galactosylceramide (α-GalCer 1), which stimulates the production of large quantities of IFNγ and IL-4. However, αGalCer leads to overstimulation of *i*NKT cells. It has been demonstrated that the αGalCer analogue, threitol ceramide (ThrCer 2), successfully activates *i*NKT cells and overcomes the problematic *i*NKT cell activation-induced anergy. In this study, ThrCer 2 has been inserted into the bilayers of liposomes composed of a neutral lipid, 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) or dimethyldioctadecylammonium bromide (DDA), a cationic lipid. Incorporation efficiencies of ThrCer within the liposomes was 96 % for DSPC liposomes and 80 % for DDA liposomes with the vesicle size (large multilamellar vs small unilamellar vesicles) making no significant difference. Langmuir-Blodgett studies suggest both DSPC and DDA stack within the monolayer co-operatively with the ThrCer molecules with no condensing effect. In terms of cellular responses IFNγ secretion was higher for cells treated with small DDA liposomes compared to the other liposome formulations, suggesting that ThrCer encapsulation in this liposome formulation resulted in a higher uptake by DCs.

Keywords

liposomes; stability; particle size; cationic lipids; threitol ceramide; invariant natural killer T cells; dendritic cells; monolayer studies

1. Introduction

Invariant natural killer T (*i*NKT) cells are unique lymphocytes defined by their co-expression of surface markers associated with NK cells along with a T-cell antigen receptor.[1] They recognise amphipathic ligands such as glycolipids or phospholipids presented in the context of the non-polymorphic, MHC class I-like molecule CD1d.[2] To date, relatively few compounds have been found that stimulate *i*NKT cells. Of these, α-

[*]**Corresponding author:**y.perrie@aston.ac.uk.

Europe PMC Funders Author Manuscripts

galactosylceramide 1 (α-GalCer 1) (Figure 1) is one of the most potent agonists and has the ability to induce CD1d-restricted NKT cells specifically to produce high levels of both IL-4 (Th_2) and IFNγ (Th_1) *in vitro* and *in vivo*.[3-5] *i*NKT cells in different situations display both tolerogenic and immunostimulatory functions *in vivo* following αGalCer administration. Unfortunately for therapeutic applications, αGalCer 1 leads to overstimulation of *i*NKT cells; more specifically, it has been shown that a single injection of mice with 1-2 μg of αGalCer induces long-term (> 30 days) anergy of *i*NKT cells.[6,7] During this hyporesponsive state, further activation of *i*NKT cells with αGalCer is unsuccessful. The production of both Th_1 and Th_2 cytokines and the "unresponsive state" of *i*NKT cells after activation with αGalCer renders this glycolipid of limited therapeutic use as a direct activator of *i*NKT cells and has encouraged the development of αGalCer analogues that circumvent some of these problems. To this end, we recently demonstrated that the αGalCer analogue, threitol ceramide (ThrCer 2) (Figure 1), successfully activates *i*NKT cells and overcomes the problematic *i*NKT cell activation-induced anergy associated with αGalCer 1.[7] ThrCer 2 is a promising agonist for *i*NKT cells and may have therapeutic application. It has been previously demonstrated that this glycolipid, whilst preventing αGalCer-dependent *i*NKT cell over-stimulation, ensures effective dendritic cell (DC) maturation, minimises *i*NKT cell-dependent DC lysis, and promotes optimal expansion of antigen-specific T cell responses.[7] Thus by minimising *i*NKT cell over-stimulation and *i*NKT cell-dependent DC lysis, ThrCer rectifies some of the deficiencies of αGalCer.

ThrCer **2** is lipid-based with a molecular weight of 800 g/mol; it displays poor water solubility. In an effort to improve this important physical characteristic and hence the ability to deliver this immunostimulatory agent, we postulated that its incorporation into liposomes would be useful. Liposomes are phospholipid vesicles consisting of one or several concentric lipid bilayers, enclosing many aqueous compartments.[8] Liposomes based on dimethyldioctadecylammonium bromide (DDA) have previously been evaluated as carriers for drugs[9], as antimicrobial agents[10] and as adjuvants[11] for a range of vaccines for both parenteral and mucosal delivery. In recent years, they have been used as part of more complex adjuvant systems for experimental subunit vaccines.[12,13] DDA was discovered as an adjuvant by Gall in the mid 1960s[14] and has been tested in combination with a number of different viral and bacterial antigens in different animal species.[15] It is a synthetic amphiphilic lipid compound comprising a hydrophilic positively charged dimethylammonium head-group attached to two hydrophobic 18-carbon alkyl chains. In an aqueous environment, DDA assembles into closed vesicular bilayers similar to liposomes made from natural phospholipids. The adjuvant activity of DDA has been thoroughly reviewed by Hilgers and Snippe (1992), who assessed DDA to be a moderate or strong Th_2 inducer and a strong Th_1 inducer, and the mechanism of action behind the adjuvant effect of DDA has been attributed to its positive surface charge and its ability to associate antigens.[16] Recent studies provide support for this notion by showing that if DDA is replaced with a neutral lipid such as 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), the neutralisation of the surface charge of the liposomes diminishes their immunogenicity.[17] Whilst the effect of cationic liposomes on dendritic cells (DCs) is not well characterised in the literature, it is, however, well known that cationic amphiphiles can be used as liposome carriers for nucleic acids and thus enhance the transfection efficiency of cells in general.[18]

In this study, ThrCer 2 has been incorporated into the bilayers of liposomes composed of the cationic lipid DDA or the zwitterionic lipid DSPC. These systems were prepared both as multilamellar vesicles (MLV) and small unilamellar vesicles (SUV). The physiochemical characteristics (vesicle size and entrapment efficiency) and biological activity of the resulting liposomes incorporating ThrCer have been determined and these formulations were then tested in terms of uptake and delivery of ThrCer to DCs and the ability of the DCs to present ThrCer on CD1d molecules to *i*NKT cells leading to IFNγ release.

2. Materials and Methods

2.1 Materials

Dimethyl dioctadecylammonium bromide (DDA) and 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) were obtained from Avanti Polar Lipids (Alabaster, AL). The purity of the compounds was > 99% by HPLC. Sephadex® G-75 was purchased from Sigma-Aldrich Co. Ltd. (Dorset, UK). Fetal calf serum (FCS) was obtained from Biosera, UK, IODO-GEN® pre-coated iodination tubes from Pierce Biotechnology (Rockford, IL), and Ultima Gold scintillation fluid was purchased from Perkin Elmer (Waltham, MA). Methanol (extra pure), chloroform (extra pure), ethanol (extra pure) and 1 M hydrochloric acid, used to adjust pH in the Tris-buffer, were purchased from Fisher, UK. Tris (ultra pure) was obtained from ICN Biomedicals (Aurora, OH). Threitol ceramide (ThrCer, **2**) and radiolabelled ThrCer, ^{14}C-ThrCer, ^{14}C-**2**, was prepared as previously described.[19]

2.2 Methods

2.2.1 Preparation of liposomes—DDA and DSPC liposomes with and without the addition of ThrCer were prepared by the lipid hydration method.[8] The lipids and ThrCer used in these experiments were dissolved in chloroform:methanol (2:1 v/v). DDA or DSPC (30 μL of a 10 mg ml^{-1} solution) and ThrCer (60 μL of a 1 mg ml^{-1} solution) were placed in a 50 ml round-bottom Quick-fit flask and the organic solvent was removed by rotary evaporation at about 37 °C to yield a thin lipid film on the walls of the flask, which was flushed with oxygen-free nitrogen (N_2) in order to ensure complete removal of solvent. The vesicles were formed by hydrating the lipid film in Tris-buffer at pH 7.4 (600 μL of a 10 mM solution). The lipid film was hydrated for 20 min at a temperature of 10 °C above the main phase transition of either DDA (T_m ~47 °C) or DSPC (T_m ~55 °C) respectively to ensure complete hydration.

For the generation of small unilamellar vesicles (SUV), the multilamellar vesicles (MLV) produced were disrupted by sonication using a probe sonicator (Soniprep 150), to fracture the large liposomes into smaller structures. The DSPC liposome batches were sonicated for 1 min, whereas the DDA liposomes were sonicated for 30 s to produce SUV.

2.2.2 Measuring entrapment of ThrCer in DSPC and DDA liposomes—The degree of ThrCer incorporated in the liposomes was determined by tracking ^{14}C-labelled ThrCer. ^{14}C-Radiolabelled ThrCer (10 μl of a 1 mg ml^{-1} solution) was incorporated into liposomes prepared as described above. The DSPC or DDA ^{14}C-ThrCer liposomes (400 μl) were placed into dialysis tubing (molecular weight cut off (MWCO) of 12,000 or greater), and transferred into Tris buffer (500 ml of a 10 mM solution, pH 7.4). At various time-points, ThrCer incorporation and release from the liposome formulations was determined by removing 1 ml sample from the receiver solution, which was subsequently replaced with 1 ml Tris buffer, in order to maintain sink conditions. Incorporation of ThrCer was determined on the basis of ^{14}C-labelled ThrCer remaining in the liposome suspension after dialysis.

2.2.3 ThrCer retention in simulated *in vivo* conditions—ThrCer release from liposomes stored in simulated *in vivo* conditions was determined using liposomes entrapping ^{14}C-radiolabelled ThrCer prepared as described in Section 2.2.1. Aliquots of each formulation were diluted (1:5) using 50% FCS in Tris buffer and incubated in a shaking water bath at 37 °C for 28 days. At time intervals, samples were centrifuged (125000×*g*, 4 °C, 1 h), resuspended in Tris buffer and centrifuged again to ensure removal of all non-incorporated ThrCer. The ^{14}C-ThrCer recovery in the pooled supernatant after two washes (non-incorporated ThrCer) and the pellet (incorporated ThrCer) was measured.

The percentage of ThrCer entrapped was calculated as a percentage of the total radioactivity recovered from both supernatant and pellet.

2.2.4 Determination of liposome size—The vesicle size of DDA and DSPC liposomes with and without the inclusion of ThrCer was determined using the photon correlation spectroscopy (PCS) technique. The measurements were performed at 25 °C using a ZetaPlus (Brookhaven Instrument Corporation, USA). Polystyrene size standards 220 ± 6 nm (Duke scientific corp, Duke, NC) was used as a control. For viscosity and refractive index, the values of pure water were used (0.89 cp and 1.0, respectively). The samples were diluted with 10 mM Tris-buffer at pH 7.4 to achieve the optimal vesicle concentration.

2.2.5 Langmuir-Blodgett isotherms—An automated controlled film balance apparatus (KSV Langmuir Mini-trough, KSV Instruments Ltd, Helsinki, Finland) equipped with a platinum Wilhemy plate and placed on a vibration-free table was used to collect the surface pressure-area isotherms as previously reported (Christensen et al 2008). The size of the trough was 24,225.0 mm^2 enclosing a total volume of approximately 220 ml; the subphase was composed of filtered double-distilled water. The compounds (at fixed total concentration of 1 mg ml^{-1}) were dissolved in chloroform and 20 μl of each solution was spread onto the air/water interface with a Hamilton microsyringe, precise to ± 0.2 μl. After spreading, the monolayers were left for 10 min to allow the chloroform to evaporate. Thereafter, the molecules underwent constant compression (10 mm s^{-1}) until the required surface pressure of less than 0.2 mN/m was attained. The spread monolayer was then compressed or expanded symmetrically with the two barriers until the desired surface pressure was reached with accuracy within 0.1 mN/m. The temperature of the subphase was kept constant at 20 ± 1 °C by means of an external water bath circulation system. The temperature of 20 °C is close to the chain-melting transition temperature of DSPC and is thus suitable to clearly see the domain formation in a monolayer. At the same time, evaporation from the subphase at this temperature is limited and does not hinder the experiment. Each experiment was only compressed once and was performed three times with monolayers prepared from different solutions. KSV software (KSV Instruments Ltd, Helsinki, Finland) was used for data analysis.

2.2.6 Liposome presentation to invariant natural killer T cells—Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors' buffy coats by density gradient centrifugation over Lymphoprep (Nycomed). Monocytes were positively selected using anti-CD14 mAb-coated magnetic beads (MACS; Miltenyi Biotec) and were then cultured in 6-well plates in either X-Vivo 15 + 2% Human AB serum with 800 U ml^{-1} granulocyte monocyte colony stimulating factor (GM-CSF) and 500 U ml^{-1} IL-4 for 4 days to produce immature DCs. On day 4, liposomes (1 μg ml^{-1} or 100 ng ml^{-1}), lab grade ThrCer (1 μg ml^{-1}) and α-GalCer (100 ng ml^{-1}) were added to the immature DCs, followed by the addition of maturation cocktail (IL-1β, IL-6, TNF-α and PGE2) after 3 h. DCs were harvested and assayed with *i*NKT cells overnight, the supernatants were taken and an IFNγ ELISA performed. DCs were also analysed by FACS after addition of liposomes for expression of CD83, CD80 and CD86 to see whether there is any contamination of liposomes.

2.2.7 IFNγ ELISA—ELISA plates were coated with 1D1K antibody (Mabtech) and left overnight at 4 °C. Plates were washed with 0.05% tween 20 in PBS (v/v). To eliminate any non-specific antigen binding, the plates were coated (i.e. blocked) with 200 μL of 10% FCS in PBS (v/v) and incubated for 2 h at 37 °C. 100 μL of reaction supernatant was transferred to the ELISA plate and the standard was added starting at 50 ng ml^{-1}. The plate was then incubated at 4 °C overnight. The following day, the plates were washed with 0.05% tween

20 in PBS and the biotinylated anti-cytokine detecting mAb was added at 50 μL/well and incubated at room temperature for 2 h. After incubation, the plates were washed eight times with 0.05% tween 20 in PBS. 100 μL of working dilution of avidin-peroxidase was added per well after which the plates were incubated at room temperature for 2 h. Plates were once again washed eight times with 0.05% tween 20 in PBS. 100 μl of tetramethylbenzidine (TMB) agent was added per well. Reaction was stopped with 50 μl solution of 0.5 M H_2SO_4 and the optical density of each well was measured immediately using a microplate reader (Bio-Rad, model 680) set to 450 nm.

2.2.8 Statistical analysis—Data was analysed using analysis of variance (ANOVA) followed by Tukey test was performed to compare the mean values of different groups. Differences were considered significant when $p < 0.05$. Each value was obtained from triplicate samples from each case and expressed as mean ±SD.

3. Results and discussion

3.1 Characterisation of ThrCer liposomes

As discussed, cationic liposomes based on DDA have been evaluated and have shown potential as an adjuvant system for vaccines against a wide range of diseases.[15] In this study, two liposomal delivery systems composed of DDA and DSPC (a neutral alternative) were tested for their ability to enhance ThrCer delivery to DCs. Table 1 provides size, polydispersity and ThrCer loading of the liposomal systems with and without the inclusion of ThrCer when formulated in both multilamellar vesicles (MLV) and small unilamellar vesicles (SUV).

The addition of ThrCer to DSPC liposomes was found to significantly ($p < 0.05$) reduce the size of both MLV and SUV liposomes (Table 1). However in contrast, for the DDA-based liposomes a significant increase ($p < 0.05$) in size was found for MLV liposomes but no significant change in size was noted for SUV liposomes (Table 1). The changes in size seen in both sets of formulations suggest that ThrCer promotes a re-organisation of surfactants within the bilayers, and in the case of the DSPC liposomes promoting smaller sized vesicles. A similar effect with small-molecule drugs was found by Lopes et al[20], where sodium diclofenac, an amphiphilic molecule, interacted with the bilayers of liposomes composed of soya phosphatidylcholine, causing a decrease in liposome size. Several other studies have also suggested molecular association between amphiphilic drugs and phospholipids including anti-inflammatory compounds, results in a decrease in the size of liposome structures.[21-23] Interestingly, the effect of ThrCer on DDA liposomes was in contrast, with its addition promoting slightly larger vesicle sizes suggesting possibly the charge of the lipid headgroup was playing an influencing role.

Incorporation of ThrCer was also influenced by the charge of the lipids employed with cationic liposomes prepared from DDA having a reduced loading compared to their zwitterionic counterpart; both MLV and SUV liposomes prepared using DSPC incorporated more ThrCer than their cationic DDA counterparts (Table 1). This suggests that the zwitterionic lipid DSPC is better able to accommodate the ThrCer within the bilayer structure compared to DDA.

3.2 Determination of vesicle stability over time

Stability was investigated in terms changes of vesicle size over time, the 8 formulations were prepared and stored at room temperature (25 °C) for 28 days (Figure 2a and b). Whilst after the initial preparation, the DSPC MLV were 1346.6 ± 267.0 nm, the size of these vesicles increased rapidly on storage to 1944.2 ± 102.2 nm after 14 days and after 28 days

storage visible aggregation became apparent (Figure 2a). In contrast, addition of ThrCer to these DSPC liposomes promoted enhanced stability with liposomes showing no significant change (p <0.05) in particle size over the 28 day period with sizes remaining around 296.1± 39.2 nm, indicating that the presence of ThrCer in the DSPC liposome bilayers leads to improved liposome stability potentially through promoting the reduced vesicle sizes which were less likely to sediment and aggregate (Figure 2a).

The MLV liposomes produced with DDA alone again showed instabilities in terms of particle size when stored at room temperature with a twofold increase in size being noted over the 28 day study (Figure 2a), however unlike the DSPC liposomes the addition of ThrCer did not improve the stability of DDA liposomes with vesicle size increasing by 59 % over the 28 days. These results could again suggest that the smaller vesicle size of the DSPC-ThrCer liposomes (~175 nm; Table 1) compared to the larger MLV DDA liposomes (~409 nm) was supporting their improved stability. However this is not supported by stability studies of SUV formulations (Figure 2b). Of the four SUV formulations, again only the DSPC:ThrCer liposomes showed no significant change (p >0.05) in size over the 28 day period with sizes remaining around 177 nm, whilst DSPC only SUV increased by 57 % from their initial size (Figure 2b). The DDA SUV with and without ThrCer also showed size instability over time with particle sizes increasing from around 100 nm to 300 nm by day 14 with no further significant changes in size thereafter (Figure 2b). Therefore whilst both liposome size and surface charge in general are known to have an influencing effect on the rate of sedimentation and potential aggregation, the change in lipid packaging with the addition of ThrCer to liposomes formulated as both MLV and SUV improved DSPC liposome stability but not those based on DDA.

3.3 ThrCer is retained in liposomes under simulated *in vivo* conditions

For therapeutic application, it is important that drugs are retained within liposomes for an appropriate time, and release kinetics may vary depending on the drug delivered, the site of action and the therapeutic application. Among the various factors that dictate the liposomal release of a drug, the bilayer composition is a key factor.[24] ThrCer retention was measured in simulated *in vivo* conditions i.e. in 50% FCS in Tris buffer and compared to control with formulations only in Tris buffer. The percentage of ThrCer incorporation was calculated as a percentage of the total radioactivity recovered from both supernatant and pellet in both conditions. In all four of the formulations tested high levels of ThrCer retention was measured in the presence of 50 % FCS (Figure 3). In the case of the MLV formulations (Figure 3a), the DSPC:ThrCer and DDA:ThrCer MLV retained over 88 % and 80 % of the initial ThrCer loading over the period of the study (Figure 3a) with 7 % being lost over the initial 24 hour period.

For the SUV-based formulations (Figure 3B), both the DSPC and DDA based vesicles gave significantly higher (p <0.05) ThrCer release in serum over time compared to the MLV, with DSPC vesicles showing ThrCer retention of 63 % and DDA vesicles 69 %. This increased instability of the SUV compared to the MLV maybe an outcome of the larger total surface area of these SUV suspensions which results in higher interactions of the lipids with the serum moieties. In general releases in serum were higher than those measured for Tris buffer (Figure 3).

3.4 Langmuir-Blodgett isotherms

To investigate the packaging of ThrCer with the DDA or DSPC surface pressure/area isotherms of monolayers of the lipids on their own or in combination were investigated. Such monolayer studies can be used to thermodynamically analyse interactions between components in mixed monolayers, allowing interactions between lipid components and their

molecular arrangement at the air/water interface.[25] It is clear that the structural differences of lipids influence the interactions among them and these interactions dominate the organisation of membranes.[26] Many studies have been conducted on the behaviour of different lipids in mixed monolayers[27,28] and these can be used to interpret interaction is in liposome bilayers.[29,30] Surface properties of mixed monolayers are generally studied on the basis of surface pressure (π)/mean molecular area (A) measurements in which bidimensional phases can be detected, each separated by a phase transition. The 2D phases in increasing pressure order are (1) bidimensional gas, (2) expanded liquid, (3) liquid, (4) condensed liquid, and (5) solid. Once the solid phase has been reached and the pressure is further increased, collapse occurs, i.e., the monolayer breaks and aggregates or multilayers are formed. Importantly, the shape and location of the isotherms are indicative of the interactions both between molecules in the monolayer and of the subphase. To investigate the geometrical interactions between DSPC or DDA with ThrCer at the air/water interface surface pressure studies of the various surfactants and mixtures were investigated (Figure 4a and b, with calculations presented in Table 2).

The isotherm of pure DSPC (one component system) shows the molecular area as 48.2 ± 2.7 A^2 per molecule and the condensed phase collapsing at 58.5 ± 0.6 mN m^{-1} (Figure 4a; Table 2), which are in agreement with results collated by Cardenas et al[31]. For DDA, monolayer collapse occurred at 44.8 ± 1.9 mN m^{-1}, similar to literature reports of DDA on pure water, which was found to be 45 mN m^{-1} [32,33] (Figure 4b; Table 2). Of the three pure molecules, ThrCer has a smallest area/molecule of 27.2 ± 1.3 A^2 per molecule and a collapse pressure similar to DSPC of 60.3 ± 2.4 mN m^{-1}, which would be expected from this type of surfactant structure (a small headgroup and saturated tail groups; Figure 1).

In both cases, the isotherms of the lipid mixtures were found to lie between the ranges of those of the pure components (Figure 4a and b) and for both the DSPC:ThrCer and DDA:ThrCer mixtures the measured extrapolated area per molecule was significantly higher than the predicted A^2 per molecule (5 % deviation for the DSPC:ThrCer mix and 14 % for the DDA:ThrCer mix; Table 2). It has been suggested[34] that positive deviation (which is when the experimental area is more than the ideal area) occurs with saturated lipids which stack co-operatively and that there is no condensing effect as is noted when lipids such as DSPC are mixed with cholesterol.[30]

In terms of monolayer collapse pressure, the addition of ThrCer to DSPC made no significant difference to the collapse pressure of the monolayer. In contrast, ThrCer was shown to significantly increase the surface pressure at which the monolayer collapsed compared to DDA alone (increasing the collapse pressure by 8.7 mN/m; Table 2). Recent surface pressure studies of DDA in combination with trehalose 6,6′dibehenate (TDB)[29] have shown that the addition of TDB to DDA monolayers increased the collapse pressure compared to DDA alone indicating that the attractive forces between the trehalose headgroup of TDB and water are greater than those between the quaternary ammonium head group of DDA and water. From the data in Figure 4b, it would suggest that ThrCer is having a similar effect and improving the interaction of the monolayer mixture with water. However this did not translate into improved stability as seen in Figure 2, whilst the addition of ThrCer to DSPC liposomes was seen to improve their stability, this was not the case with DDA liposomes (Figure 2) and it may be that higher concentrations of ThrCer than those employed in the liposomes would be required to stabilise the cationic formulations.

In general, these collapse pressures at high surface pressure and small molecular area can be attributed to the lipids (DDA, DSPC and ThrCer) used for the study which are saturated lipids. Previous studies noted that when a lipid is unsaturated with one or more double bonds, which in most naturally occurring lipids are in the *cis* configuration, this makes the

chain bend and accordingly, the more double bonds the chain has in the *cis* configuration, and the more curved the chain.[34] This results in a decreased packaging efficiency and an increased area per molecule. In contrast, saturated lipids are known to form straight chains resembling rods. The saturated lipids therefore occupy a smaller molecular area than unsaturated lipids and their collapse pressure is higher.[25]

3.5 Uptake of ThrCer by DCs

The uptake of the immunostimulatory agent, ThrCer by DCs, and presentation on CD1d molecules to *i*NKT cells were determined by measuring IFNγ release (Figure 5). Previous studies have shown that ThrCer efficiently activates *i*NKT cells, resulting in DC maturation and activated *i*NKT cells rapidly produce IFNγ.[7] To examine whether both the cationic and neutral ThrCer-containing liposomes were able to enhance this action, we measured secretion of IFNγ by *i*NKT cells stimulated with different concentrations (1 μg ml^{-1} and 100 ng ml^{-1}) of liposomes, which were loaded on to DCs. The results (Figure 5) show that both types of ThrCer-containing liposomes could sufficiently activate *i*NKT cells. When comparing concentration of ThrCer, 1 μg ml^{-1} and 100 ng ml^{-1}, a higher IFNγ secretion was found at higher concentrations for all formulations. In particular the DDA SUV based liposome formulation was found to produce a higher IFNγ secretion at both concentrations 1 μg ml^{-1} and 100 ng ml^{-1}compared to the neutral DSPC based liposomes. In all cases the negative control liposomes (not containing ThrCer) induced minimal levels of IFNγ secretion.

Only a few compounds stimulate *i*NKT cells, the best characterised is the glycolipid α-GalCer, which stimulates the production of large quantities of IFNγ and IL-4 by these cells. However, αGalCer 1 leads to overstimulation of *i*NKT cells. ThrCer 2 successfully activates *i*NKT cells and overcomes the problematic *i*NKT cell activation-induced anergy associated with αGalCer 1[19]. From this data it has been demonstrated that the IFNγ secretion was higher for the DDA SUV based formulation, suggesting that ThrCer encapsulation in this liposome formulation resulted in a higher uptake by dendritic cells.

Previous studies have shown that DDA is an effective type of adjuvant promoting a cell-mediated immune response against intracellular pathogen *M. tuberculosis*.[12] A number of studies have looked at DDA and other immunomodulating agents.[35,36] Administration of Arquad 2HT, which comprises DDA, in humans was promising and did not induce apparent side effects.[35] An experimental vaccine based on culture filtrate proteins from *M. tuberculosis* and DDA generated a protective immune response against TB in mice.[36] Moreover, DDA has been used as an adjuvant for a DNA vaccine against pseudorabies virus leading to enhanced T-cell responses and antiviral immunity.[37]

4. Conclusion

ThrCer 2, a new class of immunostimulatory agent, has been entrapped in DSPC and DDA liposomes, MLV and SUV, respectively. The entrapment efficiency of ThrCer within the lipid bilayers was high for both zwitterionic (DSPC) and cationic (DDA) liposomes. The release of ThrCer from liposomes was measured in Tris and 50% FCS (to simulate *in vivo* conditions) and it was found that both the MLV based formulations had a higher ThrCer retention compared to the SUV counterparts. In terms of ThrCer packaging it was found that the addition of ThrCer improved the stability of the DSPC liposomes, however this was not the case for the DDA-based liposomes and it may be that higher concentration of ThrCer than those employed in the liposomes would be required to stabilise the cationic formulations. For the *in vitro* work, it was demonstrated that the IFNγ secretion was higher for DDA SUV liposome formulation ($p < 0.05$), suggesting that whilst these systems had

reduced stability compared to their DSPC counterparts, ThrCer encapsulation in this liposome formulation resulted in a higher uptake by dendritic cells.

Acknowledgments

The NMR spectrometers used in this research were funded in part through Birmingham Science City: Innovative Uses for Advanced Materials in the Modern World (West Midlands Centre for Advanced Materials Project 2), with support from Advantage West Midlands (AWM) and part-funded by the European Regional Development Fund (ERDF). GSB acknowledges support in the form of a Personal Research Chair from Mr. James Badrick, Royal Society Wolfson Research Merit Award, as a former Lister Institute-Jenner Research Fellow, the Medical Council and The Wellcome Trust (084923/B/08/7).

References

1. Schmieg J, Yang G, Franck R, Van Rooijen N, Tsuji M. Glycolipid presentation to natural killer T cells differs in an organ-dependent fashion. P Natl Acad Sci USA. 2005; 102:1127–1132.
2. Godfrey D, Hammond K, Poulton L, Smyth M, Baxter A. NKT cells: facts, functions and fallacies. Immunol Today. 2000; 21:573–583. [PubMed: 11094262]
3. Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, Motoki K, Ueno H, Nakagawa R, Sato H, Kondo Eea. CD1d-Restricted and TCR-Mediated Activation of V9 (alpha) 14 NKT Cells by Glycosylceramides. Science. 1997; 278:1626–1629. [PubMed: 9374463]
4. Spada F, Borriello F, Sugita M, Watts G, Koezuka Y, Porcelli S. Low expression level but potent antigen presenting function of CD1d on monocyte lineage cells. Eur J Immunol. 2000; 30:3468–3477. [PubMed: 11093166]
5. Matsuda J, Naidenko O, Gapin L, Nakayama T, Taniguchi M, Wang C, Koezuka Y, Kronenberg M. Tracking the response of natural killer T cells to a glycolipid antigen using CD1d tetramers. J Exp Med. 2000; 192:741–753. [PubMed: 10974039]
6. Parekh V, Lalani S, Kim S, Halder R, Azuma M, Yagita H, Kumar V, Wu L, Van Kaer L. PD-1/PD-L Blockade Prevents Anergy Induction and Enhances the Anti-Tumor Activities of Glycolipid-Activated Invariant NKT Cells. J Immunol. 2009; 182:2816–2826. [PubMed: 19234176]
7. Silk J, Salio M, Reddy B, Shepherd D, Gileadi U, Brown J, Masri S, Polzella P, Ritter G, Besra Gea. Nonglycosidic CD1d lipid ligands activate human and murine invariant NKT cells. J Immunol. 2008; 180:6452–6456. [PubMed: 18453560]
8. Bangham A, Standish M, Watkins J. Diffusion of univalent ions across the lamellae of swollen phospholipids. J Mol Biol. 1965; 13:238–252. [PubMed: 5859039]
9. Carmona-Ribeiro A, Ortis F, Schumacher R, Armelin M. Interactions between cationic vesicles and cultured mammalian cells. Langmuir. 1997; 13:2215–2218.
10. N L, Mamizuka E, Carmona-Ribeiro A. In vivo activity of a novel amphotericin B formulation with synthetic cationic bilayer fragments. J Antimicrob Chemoth. 2003; 52:412–418.
11. Dzata G, Wyckoff J, Confer A. Immunopotentiation of cattle vaccinated with a soluble Brucella abortus antigen with low LPS content: an analysis of cellular and humoral immune responses. Vet Microbiol. 1991; 29:15–26. [PubMed: 1835212]
12. Hilgers L, Snippe H. DDA as an immunological adjuvant. Res Immunol. 1992; 143:494–503. [PubMed: 1439129]
13. Kirby D, Rosenkrands I, Agger E, Andersen P, Coombes A, Y P. Liposomes act as stronger subunit vaccine adjuvants when compared to microspheres. J Drug Target. 2008; 16:543–554. [PubMed: 18686124]
14. Gall D. Adjuvant activity of aliphatic nitrogenous bases. Immunol Today. 1966; 11:369.
15. Davidsen J, Rosenkrands I, Christensen D, Vangala A, Kirby D, Perrie Y, Agger E, Andersen P. Characterization of cationic liposomes based on dimethyldioctadecylammonium and synthetic cord factor from M. tuberculosis (trehalose 6,6 dibehenate) a novel adjuvant inducing both strong CMI and antibody responses. Biochim Biophys Acta. 2005; 1718:22–31. [PubMed: 16321607]
16. Hilgers L, Snippe H, Jansze M, Willers J. Combinations of two synthetic adjuvants: synergistic effects of a surfactant and a polyanion on the humoral immune response. Cell Immunol. 1985; 92:203–209. [PubMed: 2581702]

17. Henriksen-Lacey M, Bramwell V, D C, Agger E-M, Andersen P, Perrie Y. Liposomes based on dimethyldioctadecylammonium promote a depot effect and enhance immunogenicity of soluble antigen. J Control Release. 2010; 142:180–186. [PubMed: 19874860]
18. Dass C. Lipoplex-mediated delivery of nucleic acids: factors affecting in vivo transfection. J Mol Med. 2004; 82:579–591. [PubMed: 15221077]
19. Garcia-Diaz Y, Wojno J, Cox L, Besra G. Synthesis of threitol ceramide and [14C] threitol ceramide, non-glycosidic analogues of the potent CD1 antigen ♋-galactosyl ceramide. Tetrahedron: Assymetr. 2009; 20:747–753.
20. Lopes L, Scarpa M, Silva G, Rodrigues D, Santilli C, Oliveira A. Studies on the encapsulation of diclophenac in small unilamellar liposomes of soya phosphatidylcholine. Colloid Surface B. 2004; 39:151–158.
21. Schreier S, Malleiros S, de Paula E. Surface active drugs: self-association and interaction with membranes and surfactants. Physicochemical and biological aspects. Biochim Biophys Acta. 2000; 1508:210–234. [PubMed: 11090827]
22. Kriwet K, Muller-Goymann C. Mutual interactions between diclofenac diethylamine and phospholipids – investigation on the microstructure of the arisen systems. Pharmazie. 1994; 49:187–191.
23. Stoye I, Schroder K, Muller-Goymann C. Transformation of a liposomal dispersion containing ibuprofen lysinate and phospholipids into mixed micelles physicochemical characterization and influence on drug permeation through excised human stratum corneum. Eur J Pharm Biopharm. 1998; 46:191–200. [PubMed: 9795056]
24. Devaraj G, Parakh S, Devraj R, Apte S, Rao B, Rambhau D. Release studies on niosomes containing fatty alcohols as bilayer stabilisers instead of cholesterol. J Colloid Interf Sci. 2002; 251:360–365.
25. Hac-Wydro K, Wydro P, Jagoda A, Kapusta J. The study on the interaction between phytosterols and phospholipids in model membranes. Chem Phys Lipids. 2007; 150:22–34. [PubMed: 17632093]
26. Andrade C, Santos-Magalhaes N, de Melo C. Thermodynamic characterization of the prevailing molecular interactions in mixed floating monolayers of phospholipids and usnic acid. J Colloid Interf Sci. 2006; 298:145.
27. Chou T-H, Chang C-H. Thermodynamic behavior and relaxation processes of mixed DPPC:cholesterol monolayers at the air:water interface. Colloid Surface B. 2000; 17:71.
28. Gzyl B, Paluch M. Mixed monolayers of dipalmitoyl phosphatidylcholine and ethyl palmitate at the air/water interface. Appl Surf Sci. 2005; 246:357.
29. Christensen D, Kirby D, Foged C, Agger E, Andersen P, Perrie Y, Nielsen H. alpha,alpha' -trehalose 6,6′ -dibehenate in non-phospholipid-based liposomes enables direct interaction with trehalose, offering stability during freeze-drying. Biochim Biophys Acta. 2008; 1778:1365–1373. [PubMed: 18261458]
30. Ali M, Kirby D, Mohammed A, Perrie Y. Solubilisation of drugs within liposomal bilayers: alternatives to cholesterol as a membrane stabilising agent. J Pharm Pharmacol. 2010; 62:1646–1655. [PubMed: 21039548]
31. Cardenas M, Nylander T, Jonsson B, Lindman B. The interaction between DNA and cationic lipid films at the air-water interface. J Colloid Interf Sci. 2005; 286:166–175.
32. Hato M, Minamikawa H, Okamoto K, Iwahashi M. Monolayers of [omega] hydroxyalkyldimethyloctadecylammonium bromide at water-air interface. J Colloid Interf Sci. 1993; 161:155–162.
33. Taylor, D.; Dong, Y.; Jones, C. Thin Solid Films Seventh International Conference on Organized Molecular Films; 1996; p. 130-133.
34. Hac-Wydro K, Wydro P. The influence of fatty acids on model cholesterol/phospholipid membranes. Chem Phys Lipids. 2007; 150:66–81. [PubMed: 17651712]
35. Stanfield J, Gall D, Bracken P. Single-dose antenatal tetanus immunisation. Lancet. 1973; 1:215–219. [PubMed: 4119372]

36. Andersen P. Effective vaccination of mice against Mycobacterium tuberculosis infection with a soluble mixture of secreted mycobacterial proteins. Infec Immun. 1994; 62:2536–2544. [PubMed: 7910595]

37. van Rooij E, Glansbeek H, Hilgers L, Te Lintelo E, De Visser Y, Boersma W, Haagmans B, Bianchi A. Protective antiviral immune responses to pseudorabies virus induced by DNA vaccination using dimethyldioctadecylammonium bromide as an adjuvant. J Virol. 2002; 76:10540–10545. [PubMed: 12239334]

Europe PMC Funders Author Manuscripts

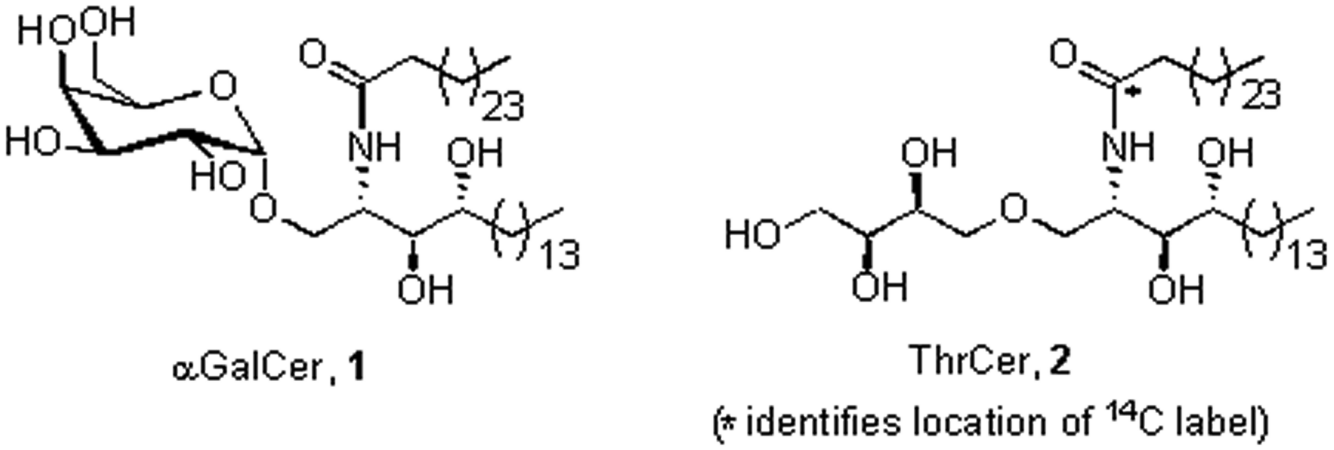
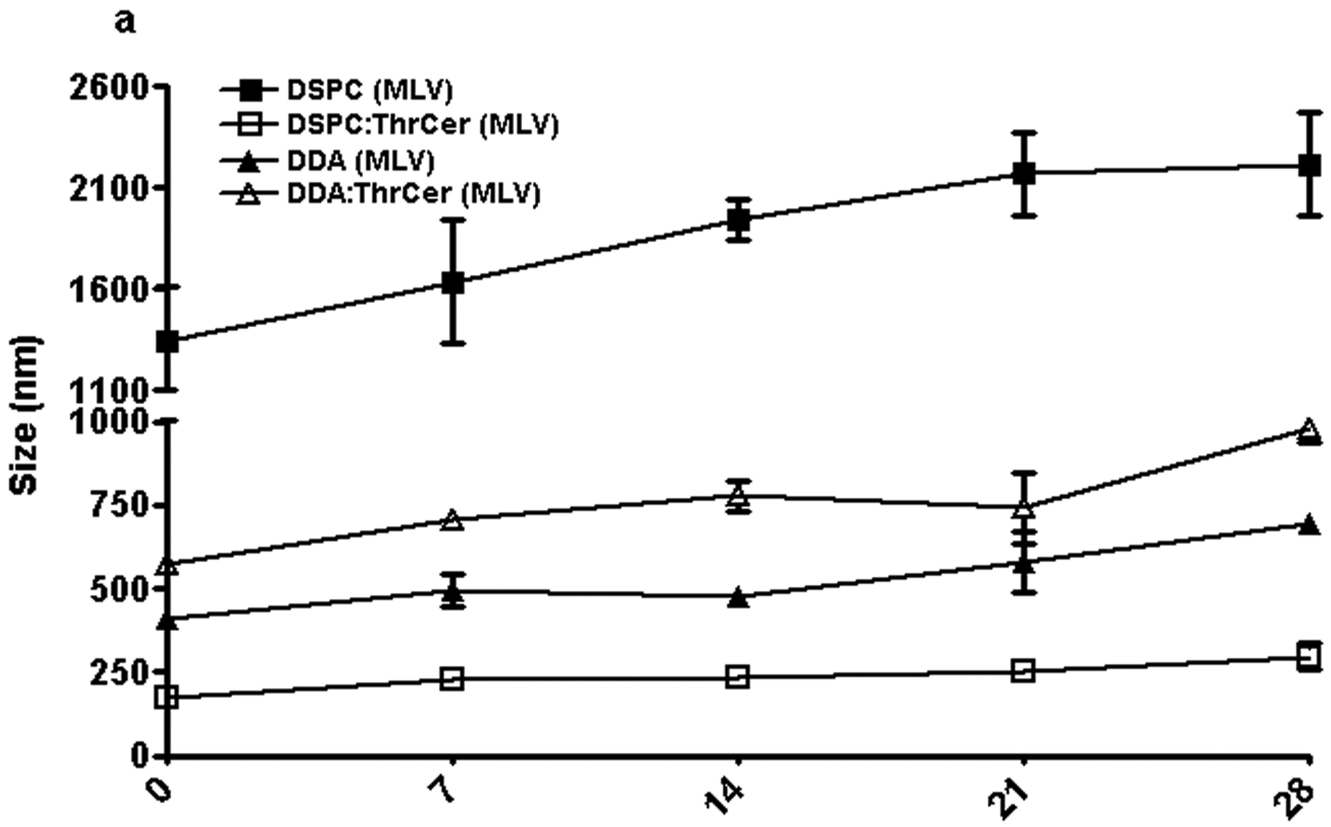


Figure 1.
CD1d agonists αGalCer **1** and ThrCer **2.**



Europe PMC Funders Author Manuscripts

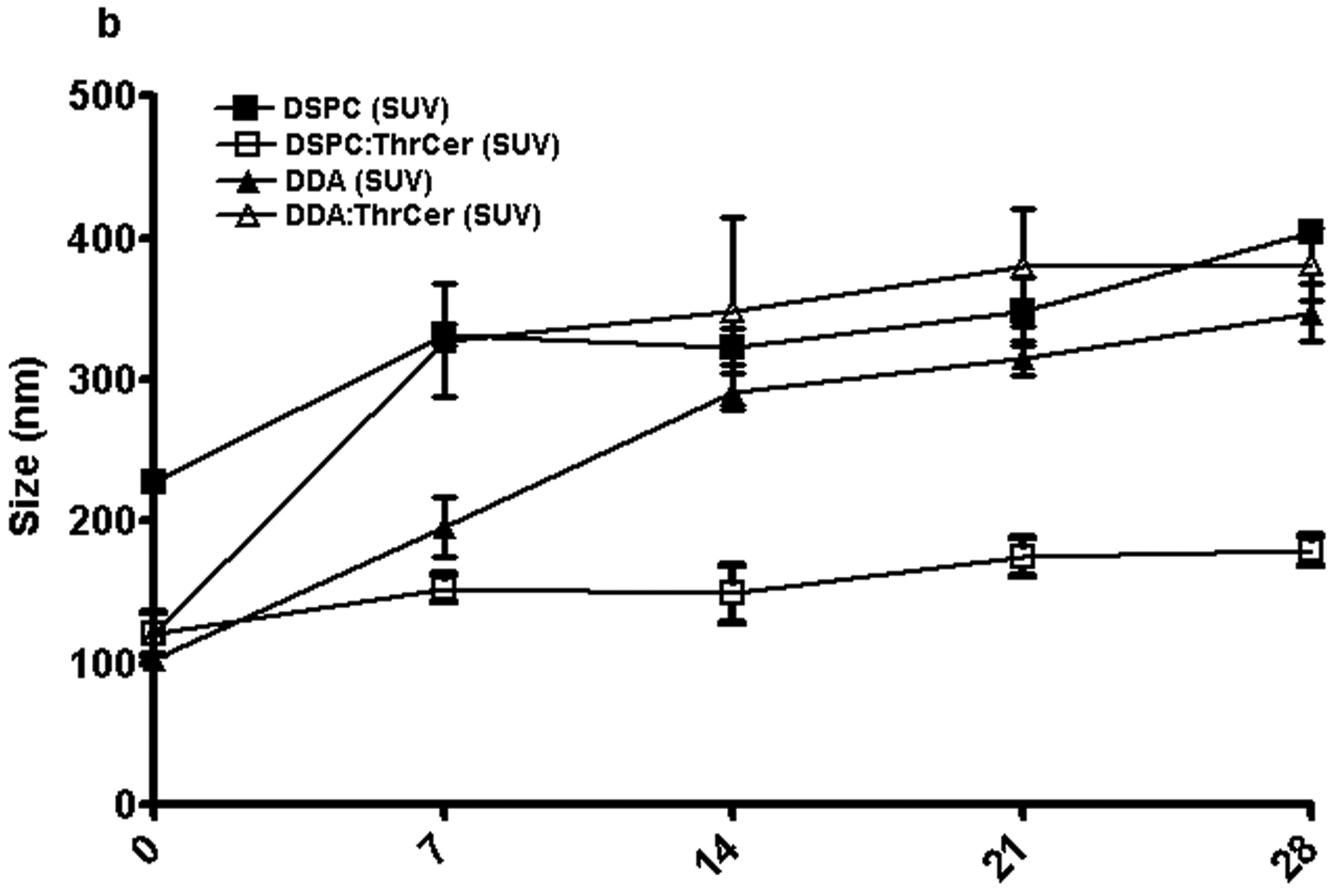
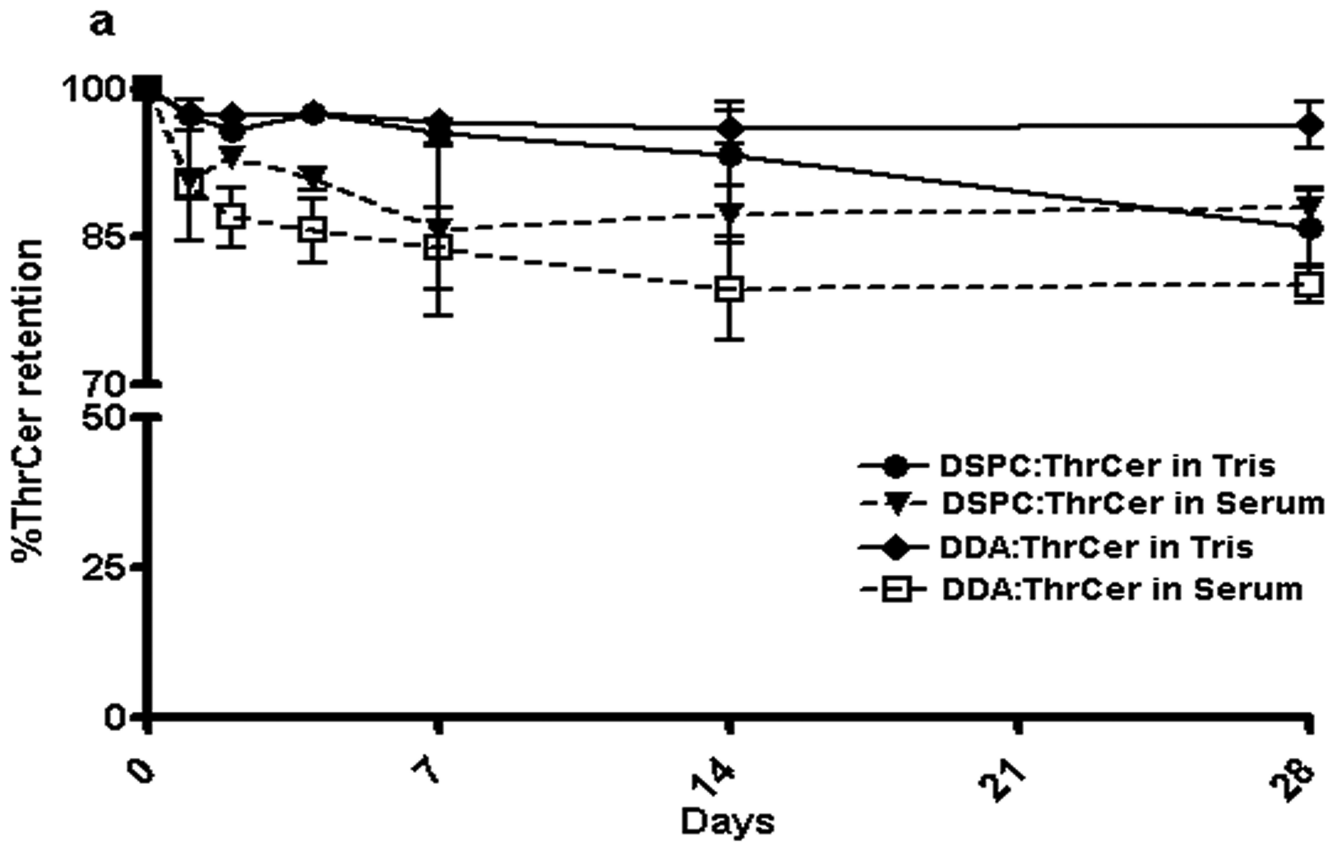


Figure 2.

Vesicle size (nm) of MLV (a) and SUV (b) of DSPC and DDA liposomes with and without the inclusion of ThrCer represented by storage at 25 °C. Size were measured in Tris buffer (1 mM) using a Brookhaven ZetaPlus instrument. Results represent mean ± SD of triplicate experiments.



Europe PMC Funders Author Manuscripts

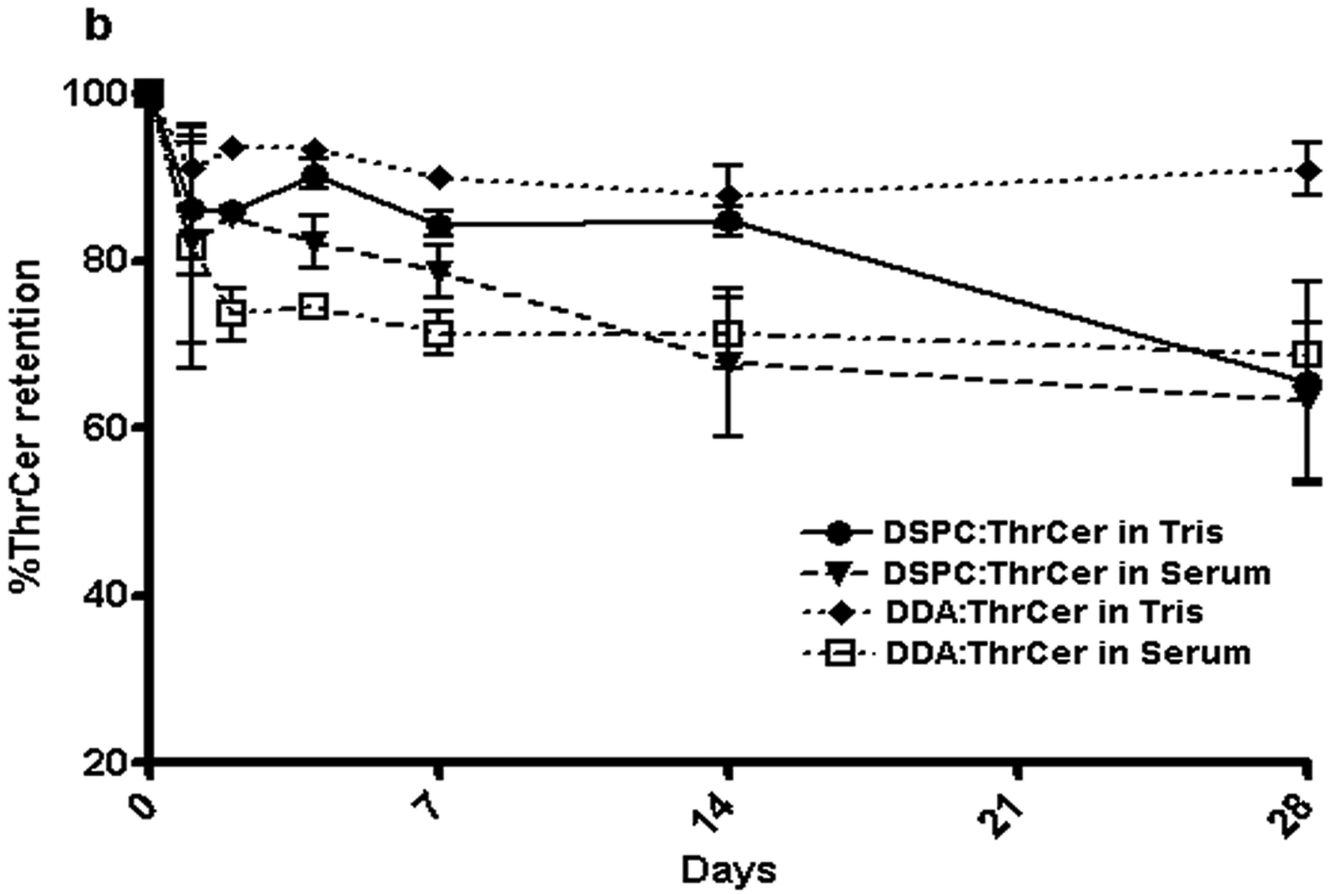
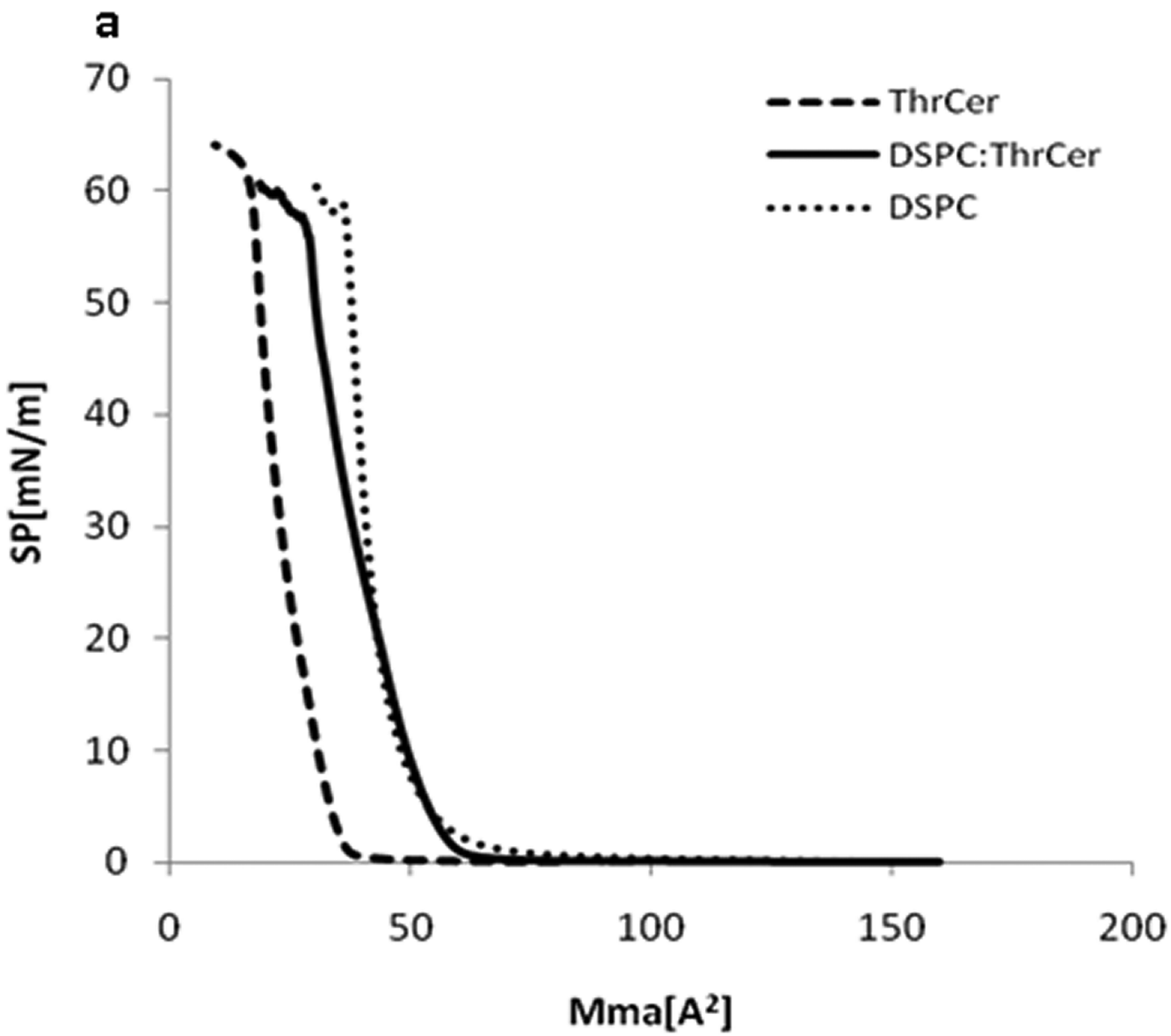


Figure 3.
Entrapped ThrCer release profile of DSPC and DDA MLV (a) and SUV (b) from liposomes when stored in Tris and under simulated *in vivo* conditions (50% FCS, 37 °C).

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts



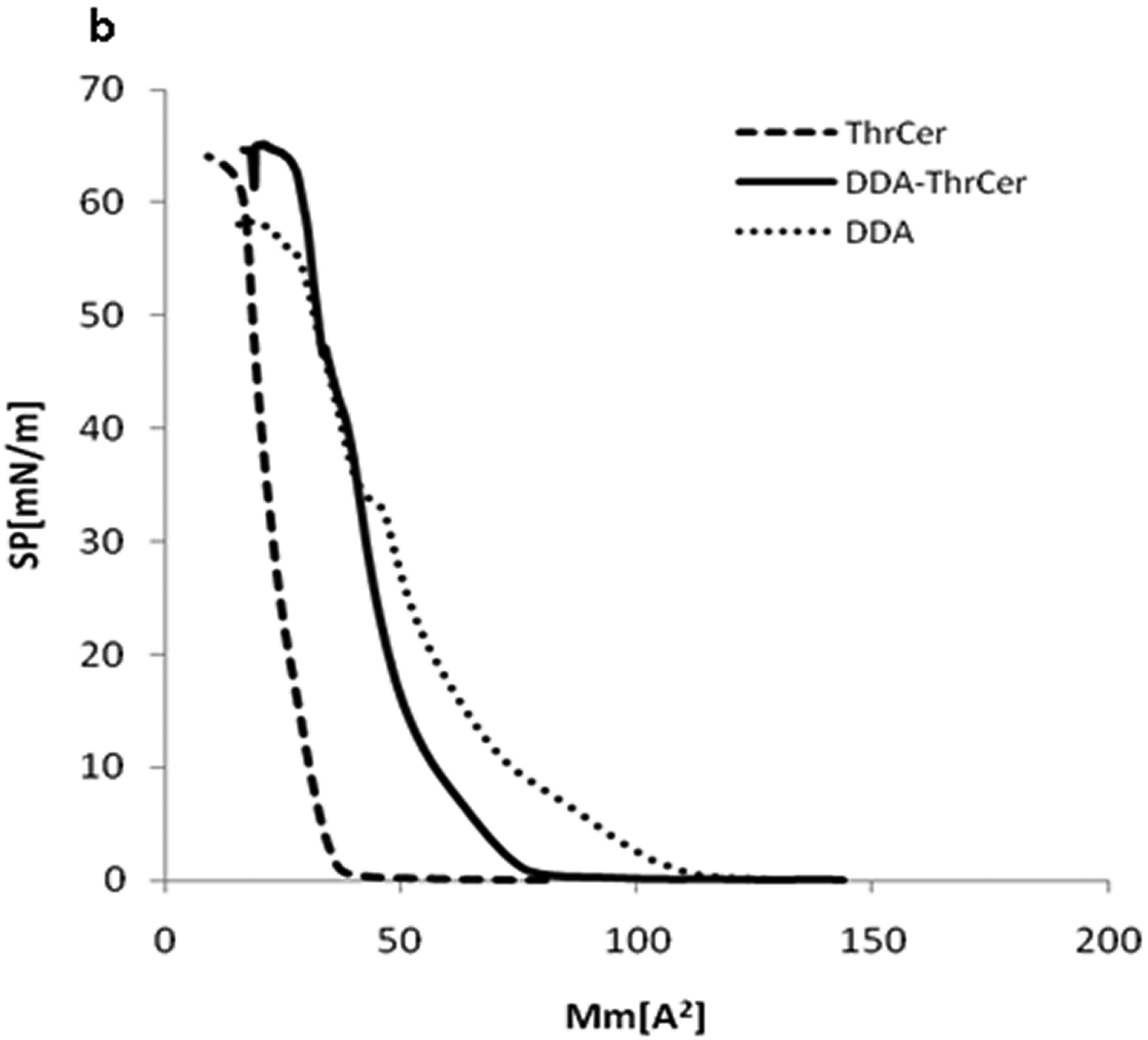


Figure 4.
The surface pressure-area isotherms of mixed and pure monolayers at the air/water interface. The mixed and pure monolayers were of (a) DSPC, (b) DDA and ThrCer. The air/water interface was at 20 °C. Results denote mean ± SD, from 3 independent batches.

Europe PMC Funders Author Manuscripts

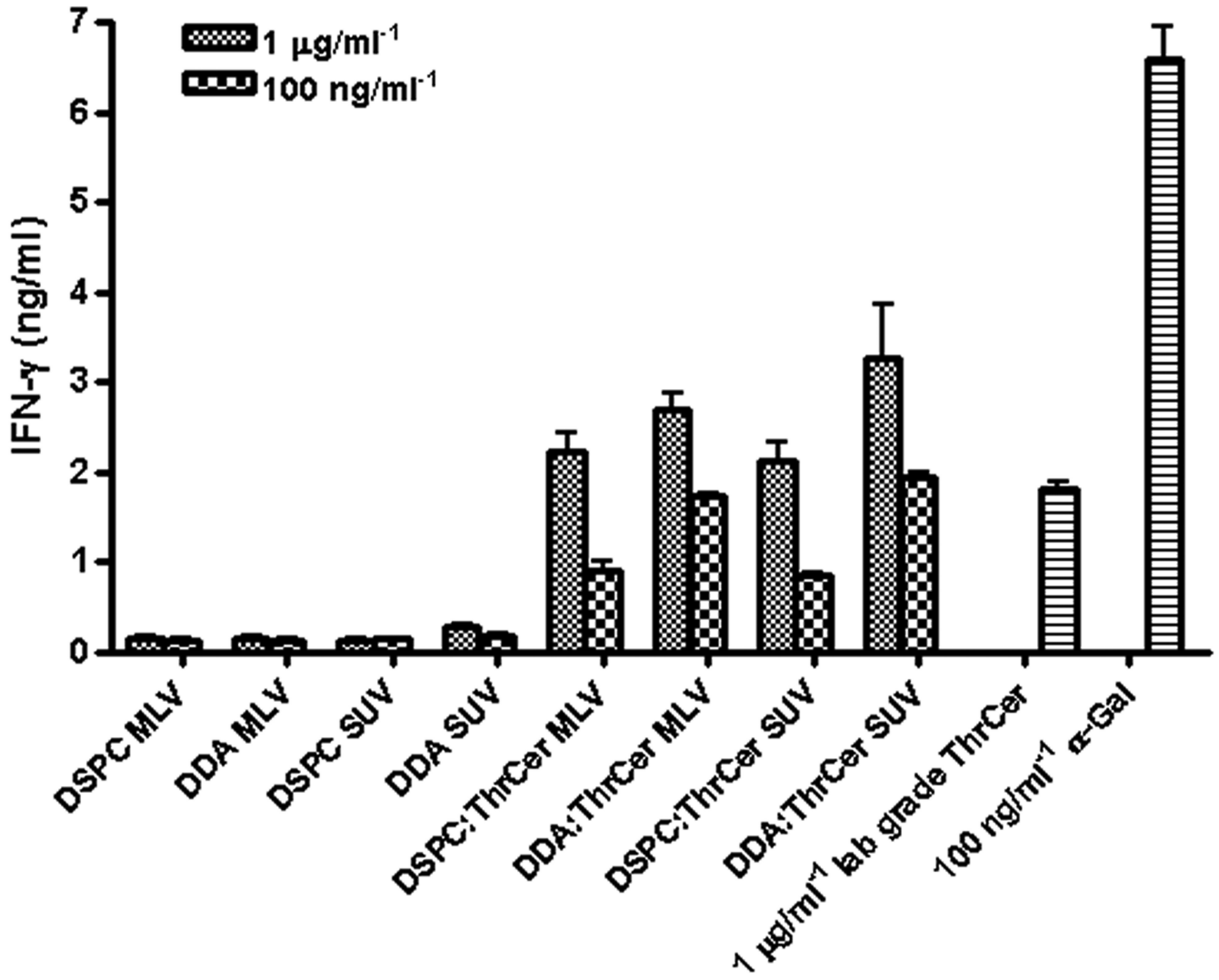


Figure 5.
IFNγ release from iNKT cells co-cultured with DCs pulsed with liposomes

Europe PMC Funders Author Manuscripts

Table 1

Characterisation of MLV or SUV liposomes prepared from DSPC or DDA with and without the inclusion of ThrCer. Size (nm) and polydispersity (PI) were measured by photon correlation spectroscopy. Results represent mean ± SD for 3 independent batches.

Formulation	MLV			SUV		
	Size (nm)	Polydispersity Index (PI)	ThrCer loading (% of amount used)	Size (nm)	Polydispersity Index (PI)	ThrCer loading (% of amount used)
DSPC	1346.6 ± 267.0	0.378 ± 0.00	-	228.4 ± 3.20	0.302 ± 0.03	-
DSPC-ThrCer	174.5 ± 3.10	0.324 ± 0.05	96 ± 2.5 %	121.1 ± 2.30	0.302 ± 0.00	94 ± 2.8%
DDA	408.7 ± 14.90	0.345 ± 0.03	-	103.1 ± 1.40	0.296 ± 0.07	-
DDA-ThrCer	572.8 ± 18.30	0.327 ± 0.03	80 ± 1.2%	121.2 ± 4.70	0.357 ± 0.00	74 ± 4.2%

Europe PMC Funders Author Manuscripts

Table 2

The experimental extrapolated area and area compressibility of mixed and pure monolayers at the air/water interface (at 20 °C) by DSPC, DDA and ThrCer in dH_2O. Results denote mean ± SD, from 3 independent batches.

Formulation	Extrapolated area at zero pressure (A^2/Molecule)	Ideal Extrapolated area at zero pressure (A^2/Molecule)	Deviation from ideality (%)	Collapse pressure (mN m^{-1})
DSPC	48.1 ± 2.7	-	-	58.5 ± 0.6
DDA	61.5 ± 2.7	-	-	44.8 ± 1.9
ThrCer	27.2 ± 1.3	-	-	60.28 ± 2.4
DSPC:ThrCer (1:1)	42.6 ± 2.6	37.65	+ 4.95	57.1 ± 1.1
DDA:ThrCer (1:1)	50.4 ± 2.6	44.35	+ 13.64	63.5 ± 1.5