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**Citation**
High Throughput Methods for Screening Liposome-Macrophage Cell Interaction

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Abstract:

Carriers are often an essential element of drug delivery, bestowing attributes to their cargo such as biocompatibility, enhanced delivery, extended half-life and efficacy as well as mediating specific targeting at a tissue, cell or intracellular level. Liposomes and lipid-based carriers have been investigated for decades for this purpose, many achieving clinical approval including products such as Doxil™ and Myocet™. However, reports also indicate issues with the use of cationic lipids, toxicity in particular. It is important to consider that carrier or vector systems engineered to efficiently deliver a drug may not be inert. Large-scale compound screens are routinely carried out in the field of drug discovery; however less work has been done on harnessing high throughput methods for carrier material screening. Screening the interaction of drug carriers and materials with cells is particularly critical for the development of emerging therapies, including biomedicines, in order to facilitate the development of safe and efficient drug products. Herein, a range of liposomes of neutral, anionic and cationic charge and others that are surface-modified with mannose residues were screened for cell interaction, toxicity and immune reactivity in THP-1-derived macrophages using a high throughput format.

Key words: Liposomes, nanoparticles, nanotoxicology, high content analysis, high content screening.
Introduction

Over the last decade high throughput screening of compound libraries has become a significant phase in the drug discovery and development process. With a growing range of materials now being developed for use as drug carriers, a need for high throughput screening methods for drug delivery systems is equally critical. This is of particular importance for cellular delivery where an understanding of cellular interaction, uptake and cytotoxicity is critical when comparing and optimising delivery systems (Forbes et al., 2014).

Liposomes have been evaluated for decades as drug carriers due in part to their versatility. They boast a great number of benefits over other drug carrier systems such as the capability of entrapping a wide range of drugs, biocompatibility and low toxicity (Malmsten, 2002) and are the most extensively explored delivery system for phagocyte-targeted therapies. Drug delivery to phagocytic cells such as monocytes, dendritic cells and macrophages represents an important therapeutic approach to treat inflammatory diseases and pathogenic infections through manipulation of the immune response.

Liposome drug delivery systems can be formulated to exploit the physiological role of cells to provide specific targeting and enhance drug efficacy. Currently, there are several FDA approved liposome-based products available for the delivery of drugs such as amphotericin B (AmBisome®, ABELCET® and Amphocil) and doxorubicin (Doxil®) (Puri et al., 2009). Although there are a number of amphotericin B liposome therapeutics available, the configuration of each differs considerably in terms of lipid composition, shape, size, stability, pharmacokinetics and toxicity. For example AmBiosome® liposomes are spherical in structure and <100 nm in diameter whereas ABELCET® liposomes are ribbon-like structures, 1.6 µm-11µm in diameter and associated with mild nephrotoxicity (Adler-Moore and Proffitt, 2008).

Cationic liposomes are associated with efficient cellular delivery of drug cargoes and are routinely applied for in vitro gene delivery (Zuhorn et al., 2007). Electrostatic interactions between positively charged liposomes and negatively charged cell membranes and cell surface proteoglycans (Wiethoff et al., 2001) facilitate cell uptake. Unfortunately, cationic liposomes can cause cytotoxicity, thereby
limiting their safety for clinical use (Lv et al., 2006). Cationic liposomes containing stearylamine (SA) have previously been shown to induce apoptosis through mitochondrial pathways in RAW264.7 macrophages by generating reactive oxygen species (ROS), releasing cytochrome c, caspase-3 and -8 and activating protein kinase C delta (PKCδ) possibly through cell surface proteoglycan interaction (Iwaoka et al., 2006, Aramaki et al., 2001, Arisaka et al., 2010, Takano et al., 2003). Consequently, interest has turned to neutral and anionic liposomes for drug delivery applications.

Several studies have shown enhanced uptake of anionic liposomes by macrophages. In eukaryotic cells, apoptosis results in phosphatidylserine (PS), an anionic lipid, being exposed on the outer cell surface and the stimulation of monocytic phagocytosis, most likely via scavenger receptors (SRs) (Moghimi and Hunter, 2001) and cell-surface glycoproteins (Kobayashi et al., 2007). Therefore PS composed liposomes could target these receptors. Another strategy for liposomal macrophage targeting involves coating liposomes with ligands such as antibodies, peptides and lectins (Kelly et al., 2011). For instance, mannosylated liposomes target the mannose receptor (MR) of macrophages and facilitate improved cellular uptake both in vitro and in vivo over non-ligand coated liposomes (Chono et al., 2009, Chono et al., 2007, Engel et al., 2003, Espuelas et al., 2008, Wijagkanalan et al., 2008b, Kawakami et al., 2000a, Kawakami et al., 2000b). The MR is involved in a range of processes including the recognition and internalisation of both foreign and self-materials, antigen presentation and intracellular signalling (Gazi and Martinez-Pomares, 2009). Moreover, mannose receptor activation has been linked to the initiation of an anti-inflammatory immunosuppressive programme in cells (Chieppa et al., 2003) and impaired NFκB activation (Xu et al., 2010), thus arguing for the utility of targeting strategies aimed at binding the MR, such as mannosylated liposomes.

Hence, it is clear from the outset that targeted drug carriers can elicit responses related to not only toxicity and immune reactivity in macrophages but also more subtle cell signalling pathway effects. When tasked with developing a macrophage-targeted delivery system it is therefore critical that comprehensive and rapid tools are available for screening of potential carriers to determine lead formulations for progress to pre-clinical in vivo testing. In this study, a range of different liposomes were prepared representing cationic, anionic, uncharged (neutral) and coated (mannosylated) classes and high throughput in vitro screening techniques were explored to assess targeted uptake,
cytotoxicity and ability to activate macrophages in a human macrophage cell model. Liposomes were seen to be efficacious in a concentration-dependent and mannosylated cholesterol linker length-dependent manner.
Methods

Liposome Preparation
Cholesten-5-yloxy-N-(4-((1-imino-2-α-thioglycosylethyl)amino)butyl)formamide (Mann-C4-Chol) and its C2 and C6 derivatives were synthesized using a method described previously by Kawakami et al. (Kawakami et al., 2000a). Mannosylated cholesterol derivatives differ by their carbon linker lengths (C2, C4 and C6). Phospholipids 1,2-Dioleoyl-3-trimethylammonium-propane (DOTAP), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (PE-rhodamine) were purchased from Avanti Polar Lipids Inc. Liposomes containing phospholipids, mannosylated cholesterol and cholesterol were formed by dehydration-rehydration. Briefly, the liposome components were dissolved in a minimal volume of Folch (Chloroform: Methanol; 2:1 (v/v)) and mixed in a round bottomed flask at the molar ratios described in Table 1. PE-Rhodamine (0.1% of mole ratio) was added to fluorescently label liposomes. Solvents were evaporated using a rotary evaporator (Büchi Rotavapor R200) and the lipid film was rehydrated in pH 7.4 Phosphate Buffered Saline (PBS; GIBCO). Size reduction of rehydrated lipids was achieved by extrusion using a LIPEX™ Thermobarrel Extruder or Avanti® Mini-Extruder 10 times through a polycarbonate membrane filter with 100, 200, 400 or 1000 nm pores (Whatman).

Liposome Characterization
Liposome concentration was determined using cholesterol and phospholipid assays (WAKO). Size and zeta potential were determined using a zetasizer (nanoseries, Malvern). For transmission electron microscopy (TEM) studies copper thin bar 200-mesh grids were coated with pioloform. Liposomes (2µl) were placed on the grid for 1 minute and blotted. The grid was negatively stained with a drop of (2% w/v) phosphotungstic acid (PTA) for 1 minute and again blotted followed by washing with deionized water. The grids were viewed the same day using a Hitachi H-7650 TEM.

Cell Culture
Human monocyte THP-1 cells were maintained in RPMI 1640 (BioSera) supplemented with 10% heat inactivated fetal bovine serum (FBS, BioSera) in a humidified atmosphere at 37°C and 5% CO₂.
THP1-XBlue™ cells are stably transfected THP-1 cells expressing secreted embryonic alkaline phosphatase (SEAP) gene which is induced by the transcription factors NF-κB and activator protein-1 (AP-1). THP1-XBlue™ cells were maintained in RPMI 1640 supplemented with 10% heat inactivated FBS and 200µg/ml of selection marker Zeocin™. Cells were differentiated by the addition of 100nM phorbol myristate acetate (PMA) and incubated in a humidified incubator at 37°C and 5% CO₂ for 72 hours.

**Immunofluorescence Microscopy to Determine Mannose Receptor Expression**

THP-1 cells were seeded at 1x10⁵ cells/ml in 96-well plates, differentiated and fixed with 4% paraformaldehyde (PFA) for 20 minutes. Cells were blocked with 1% bovine serum albumin (BSA) and incubated with 0 or 1 µg/ml rabbit polyclonal mannose receptor antibody (Abcam) followed by goat anti-rabbit secondary antibody Alexa Fluor® 488 and counterstained with Hoechst 33342 and phalloidin-tetramethyl rhodamine isothiocyanate (TRITC). Images were acquired using an INCELL 1000 cell analyser and 10x objective.

**Liposome-Cell Interaction Study: High Content Cell Analysis**

A High Content Cell Analysis (HCA) method was developed for analysis of liposome-cell interaction to enable high throughput studies. THP-1 cells were differentiated at a density of 1x10⁵ cells/ml in 96-well plates. Rhodamine-labelled anionic, neutral and mannosylated liposomes were incubated with differentiated THP-1 cells in triplicate according to Table 2. Cells were washed, fixed with 4% PFA and stained with phalloidin-fluorescein isothiocyanate (FITC) and Hoechst 33342. HCA was carried out using INCELL 1000 analyser. Images were acquired in three channels; excitation/emission wavelengths of 360/460nm (Hoechst 33342), 480/535nm (phalloidin-FITC) and 535/600nm (rhodamine tagged liposomes), 5 fields per well and analysed using INCELL1000 analyser software. Multi-target analysis module was used to segment nuclei, cells and liposomes (Figure 2) and count the number of liposomes associated with the identified cell regions.

**Liposome High Content Toxicity Screen**

Cellomics® Multiparameter Cytotoxicity 3 kit and an INCELL 1000 analyser were used to determine cytotoxicity following liposome treatment in differentiated THP-1 cells. Cells were seeded in flat-
bottomed 96-well plates and were treated with rhodamine-labelled anionic, neutral and mannosylated liposomes at concentrations of 0, 100 or 300μM in triplicate and incubated at 37°C for 23.5 hours. Valinomycin (120μM) served as a positive toxic control. Permeability and mitochondrial membrane potential (MMP) dyes were added to wells and incubated at 37°C for 30 minutes. Cells were fixed with 4% PFA, permeabilised and blocked. Cytochrome c staining was carried out using a cytochrome c primary antibody and DyLight 649 secondary antibody. Nuclei were stained using Hoechst. Images were acquired by an INCELL 1000 analyser within 24 hours in 4 fields per well using excitation/emission wavelengths of 360/460nm (Hoechst 33342 dye), 480/535nm (Permeability dye), 535/600nm (MMP dye) and 620/700nm (DyLight 649). Cell loss, nuclear area, nuclear intensity, cytochrome c release, mitochondrial membrane potential and cell permeability were determined using INCELL analysis software using multi-parameter target analysis and the settings in Table 3. Images were acquired at 10x objective magnification.

**Macrophage Activation Screen**

THP1-XBlue™ cells (NF-κB/AP-1 Reporter Monocytes) were differentiated for 72 hours at 1x10⁶ cells/ml in a 96 well plate. PMA free media was replaced daily for a further 5 days to remove residual PMA. Liposomes were added to cells in fresh media at 0, 100, 200 and 300μM final concentration in triplicate. Cells were stimulated with 100ng/ml lipopolysaccharide (LPS) as a positive control. After 24 hours 20µl of media was removed and added to 180µl of QUANTI-Blue™. After a 4 hour incubation absorbance was measured at 630nm using a multiplate reader to determine SEAP activity.

THP-1 cells were differentiated in 24 well plates at 1x10⁵ cells/ml. Cells were incubated in fresh complete media (untreated); RPMI supplemented with 100ng/ml LPS or anionic, neutral, cationic or mannosylated liposomes at 100μM or 300μM for 24 hours. Media was collected and stored at -80°C until assayed. Production of a range of cytokines secreted following macrophage activation (IFNy, IL-1β, IL-2, IL-4, IL-5, IL-8, IL-10, IL-12p40, IL-13 and TNFα) were simultaneously determined using a MesoScale Discovery (MSD) multiplex assay. MSD plates were analysed on a SECTOR Imager using the MSD DISCOVERY WORKBENCH® software.
Statistics
Results are expressed as means ± SD. One way or two way ANOVA was used to test for differences between treatments with p-values < 0.05 considered significant, < 0.01 very significant and < 0.001 highly significant.
Results

Liposome Characterization

Particle size and zeta potential were determined for each batch of liposomes prepared. Spherical liposomes (Figure 1) with representative average sizes for 3 batches following extrusion through 200nm pore filters of cationic, anionic, neutral and mannosylated liposomes shown in Table 4 with polydispersity indices (PDI) of less than 0.227. The zeta potential of liposomes composed of 50% cationic lipid DOTAP was 61.8 ± 6mV whilst anionic, neutral and mannosylated (MC6C) liposomes had mean surface charges of -68.3 ± 8mV, -4.4 ± 3mV and 45.2 ± 3mV.

THP-1 cells as a Macrophage Cell Model: Mannose Receptor Expression

THP-1 cells are a monocytic cell line that can be differentiated by phorbol esters into macrophage like cells (Tsuchiya et al., 1982, Daigneault et al., 2010, Lawlor et al., 2012, O'Sullivan et al., 2007) which express both SRs and MRs upon differentiation (Kohro et al., 2004, Liao et al., 1999). The visible presence of Alexa Fluor 488 (green fluorescence) in Figure 3B demonstrates MR expression in differentiated THP-1 cells, confirming this cell line as an appropriate in vitro model for mannosylated liposome targeting.

High Content Cell Analysis (HCA) of Liposome Interaction with THP-1 cells

A HCA protocol was established to count liposomes per cell. Several conditions were screened including a range of liposome sizes (100nm to >1μm in diameter), concentrations (0 to 1mM), incubation times (0.5 to 24 hours) and temperatures (4 and 37°C). Overall, HCA data (Figure 4) indicates that DOPS and mannosylated liposomes had significantly better cellular association compared to untargeted DOPC controls.

Size can contribute significantly to cellular uptake efficiency of liposomes. Cell association of anionic DOPS liposomes increased with increasing particle size (Figure 4G) while neutral DOPC and MC2C and MC4C mannosylated liposomes showed an increase up to 200nm however the cell association decreased with particles ≥ 400nm in diameter. The most significant increases in liposome cell association when compared to unextruded counterparts were of MC4C 200 and 400nm (p < 0.001)
and MC6C 200nm (p < 0.001) (Figure 4G). DOPS and MC6C liposomes showed the greatest level of cell association with DOPS liposome association size dependent and MC6C liposome size independent in the 100 - 1000nm range. In general, increasing liposome concentration lead to an increase in THP-1 cell association. Significant increase in targeted liposome cell association particularly DOPS and MC6C liposomes was observed compared to concentration matching non-target DOPC liposomes at all concentrations (Figure 4H). Cells were treated with 200nm liposomes at 200µM and fixed after 0.5, 1, 2, 4 and 24 hours. Cell association of DOPC, MC2C and MC4C liposomes did not significantly change over 24 hours. MC6C liposome cell association was significantly higher at all time points in comparison to neutral DOPC counterparts. However, DOPS liposome cell association was significantly enhanced following 0.5 and 1 hour treatments but decreased after 24 hours. Cell association of DOPS liposomes at 24 hours was almost 4-fold less than association of MC6C liposomes at the same time point. Overall, optimal concentrations were above 100µM (Figure 4H) and incubation times greater than 2 hours (Figure 4I) for the leading DOPS and MC6C liposomes.

Additionally liposome cell association at 4°C and 37°C was compared. Cells were incubated either at 4 or 37°C with 100µM of 200nm-sized rhodamine labelled anionic (DOPS), neutral non-mannosylated (DOPC) and mannosylated (MC2C, MC4C and MC6C) liposomes for 2 hours. THP-1 cell association of DOPS, MC4C and MC6C liposomes was highly significantly impeded at 4°C (p < 0.001) suggesting an active uptake process (Figure 5).

**Liposome Toxicity: Multiparameter Cytotoxicity Testing**

Differentiated THP-1 cells were used to assess the effects of liposome treatment on cell viability. Cationic (DOTAP), anionic (DOPS), neutral (DOPC) and mannosylated (MC2C, MC4C and MC6C) liposomes at 100µM and 300µM were incubated with differentiated THP-1 cells for 24 hours, fixed and stained. Negative controls were incubated under normal conditions and served as the healthy cell comparison for other treatments. HCA was used to acquire and analyze images based on staining for nuclei, cell permeability, MMP and cytochrome c. Chromatin condensation is associated with apoptosis which can be monitored by changes in nuclear size and intensity. The positive control valinomycin induced significant cell loss (p < 0.001) (Figures 6B&6I). Significant cell loss was also
found following 300μM treatments of mannosylated liposomes (Figure 6I). Valinomycin treatment decreased the mean nuclear area (Figure 6J) and increased the total nuclear intensity albeit not significantly. Increased cell permeability occurs during necrosis, however, no significant elevation in permeability was determined following any liposome treatment (Figure 6L). Valinomycin, and 300μM DOTAP, DOPS and MC2C liposome treatments induced a fall in MMP (Figure 6M), which is associated with apoptosis and cytochrome c release.

**Macrophage Activation: NFκB Activation and Cytokine Screen**

When dealing with targeting of any immune cell, including macrophages, it is of particular importance that the ability to activate the cells in question is compared for different carriers. In order to assess this response in our model system, NFκB activation and induction of a range of cytokines were determined, including cytokines associated with inflammation and a TH1 response such as TNFα, IL-1β, IL-2, IL-12p70, IFNγ and IL-8 and a TH2 response such as IL-2, IL-4, IL-5, IL-10 and IL-13 (Table 5). NFκB can induce transcription of many pro-inflammatory genes such as TNFα, IL-1β, IL-6 and IL-8 (Tak and Firestein, 2001). Here activation was assessed using a NFκB reporter cell line, THP1-XBlue™ cells, following incubation with liposomes. LPS stimulation of differentiated THP1-XBlue™ cells was used as a positive control and to validate the macrophage activation screen. A significant increase in NFκB activation compared to untreated cells was found following 24 hour treatment with the positive control LPS (p < 0.001) (Figure 7). As expected, TNFα was significantly induced by LPS (p < 0.05) whilst elevations in mean concentrations of IL-1β, IFNγ and IL-8 compared to untreated cells were also observed, further validating this screening assay. THP1-XBlue™ cell cytotoxic and immune reactivity to cationic, neutral, anionic and mannosylated liposomes are discussed in detail below.

**Cationic DOTAP Liposomes Induce High Levels of Toxicity and a Proinflammatory Response from Differentiated THP-1 cells**

DOTAP liposomes induced significant cell loss while increasing cell permeability, decreasing MMP and promoting cytochrome c release at 100 and 300μM indicative of both apoptosis and necrosis. Cytochrome c intensity was significantly reduced (p < 0.05) compared to healthy control cells.
following 300μM DOTAP liposome treatment suggestive of cytochrome c release (Figure 6). DOTAP liposomes also induced a proinflammatory response in the macrophage-like cells with increases in IL-1β and IFN-γ and decreases in IL-4 being observed, although these were not statistically significant (Table 5).

Neutral DOPC Liposomes Induce Minimal Toxicity and Inflammatory Cytokine Production in Differentiated THP1 cells

Cell loss resulted only from 300μM treatments with DOPC liposomes in THP-1 cells however, other signs of apoptosis or necrosis were not observed (Figure 6). NFκB was significantly activated (p < 0.05) following 24 hour DOPC liposome treatment at 100μM (Figure 7) and IL-12p70 was (non-significantly) induced by a 300μM dose of DOPC liposomes (Table 5). In general however, DOPC liposome treatment in THP-1 cells resulted in reduction of immune mediators compared to untreated control cells.

Anionic DOPS Liposomes at High Concentrations Induce Toxicity and Immune Reactivity in Differentiated THP1 cells

Anionic liposomes were composed of DOPS which targets macrophage scavenger receptors by mimicking apoptotic cells. At the higher concentration of 300μM DOPS, liposomes were found to reduce cell number and MMP, a sign of induced apoptosis (Figure 6). Furthermore, NFκB activity was significantly induced (p < 0.001) at 300μM and was concentration dependent (Figure 7). IL-8 is an important chemokine for neutrophil recruitment and therefore is upregulated during inflammation. Significant induction of IL-8 (p < 0.05) was observed after 24 hour treatment (6615.5±1400.6pg/ml) with 300μM DOPS liposomes (Table 5). DOPS liposomes were the most potent inducer of cytokines and chemokines in differentiated THP-1 cells with all measured immune mediators induced except IL-12p70.
Mannosylated Liposomes show Immunosuppressive Effects but at High Concentrations Induce Toxicity in Differentiated THP1 cells

Significant cell loss was found following cell treatment of cells with mannosylated liposomes at 300μM (Figure 6I), of which MC2C liposomes caused significant (p < 0.01) increase in nuclear intensity (Figure 6K). Of the targeted liposomes, MC2C liposomes at 300μM caused the most significant effect, leading to apoptosis in THP-1 cells after 24 hours with a highly significant cell loss (p < 0.001), a significant increase in nuclear intensity (p < 0.01) and a decrease in MMP. MC6C liposomes composed of mannosylated cholesters with longer linker length at 300μM induced significant cell loss but all other measures of cell health were comparable with untreated control cells. However, mannosylated liposome treatment showed no significant difference in NFκB activation compared to untreated cells but in comparison to non-mannosylated DOPC liposome treatment at equal concentrations, NFκB activation was significantly lower following 200μM MC4C (p < 0.01), 300μM MC4C (p < 0.05), 200μM MC6C (p < 0.05) and 300μM MC6C (p < 0.05) liposome treatment (Figure 7). However, although MC2C liposomes did not significantly alter NFκB activation, they induced increased synthesis of IL-12p70 (to a lesser extent than DOPC liposomes), IFNγ (at 100μM, similar to MC4C), IL-13, IL-5 and IL-10, indicating that they elicited a cytokine response. MC6C liposomes also led to IFNγ and IL-8 suppression compared to untreated control levels. Previously, TNFα, IL-1β and cytokine-induced neutrophil chemoattractant-1 (CINC-1) levels have been monitored in vitro in alveolar macrophages and in vivo in rat lungs following mannosylated liposome delivery of dexamethasone (Wijagkanalan et al., 2008a). However, the ability of mannosylated liposomes to induce immune reactivity alone has not been investigated.
Discussion

An essential step in targeted cellular delivery is internalization of the carrier system by the target cells. Cell interaction of liposomes has been studied using a range of techniques such as flow cytometry, confocal microscopy and spectrofluorimetry (Cryan et al., 2006, Wijagkanalan et al., 2008b, Lawlor et al. 2011, Gilleron et al., 2013). We have developed a more powerful method for studying particle-cell interaction using High Content Analysis (HCA) that combines imaging and drug/carrier quantification, from single cells to cell populations, in a high throughput format (Hibbitts et al., 2011, Lawlor et al., 2011). HCA also enables multiple, parallel experiments to be carried out to optimize the liposome formulation for macrophage targeting.

HCA assessment of liposome association with differentiated THP-1 cells showed overwhelming confirmation of DOPS and MC6C liposomes as lead delivery platforms (Figure 4). Moreover, the level of cell interaction of mannosylated liposomes also appeared to be linker dependent with levels for liposomes composed of MC6C greater than those composed with MC4C which both facilitated higher uptake than MC2C liposomes. Engel et al. investigated the influence of spacer length between alkyl mannosides and liposome surface, on liposome interaction with phagocytic cells (Engel et al., 2003). Spacers were 0 to 8 ethyleneoxy units long (Man0 - Man8) with longer spacers mannosylated liposome uptake by mannose receptor expressing cells was more enhanced. Furthermore, Gal-C6-Chol composed liposomes were previously shown to mediate a higher DNA transfection efficiency than Gal-2-Chol and Gal-C4-Chol liposomes (Kawakami et al., 1998).

In general, nanoparticle-induced toxicity is connected to particle size and surface chemistry (Ai et al., 2011). Smaller liposomes can be more toxic than larger liposomes (Mayhew et al., 1987). Additionally a study previously compared the toxicity of liposomes composed of cationic (SA and cardiolipin), anionic (phosphatidylglycerol and phosphatidylserine) or neutral (phosphatidylcholine or dipalmitoylphosphatidylcholine) lipids (Mayhew et al., 1987). Toxicity was established as inhibition of cell growth and determined following exposure to 200μM, 130-3000μM and 3000-4000μM cationic, anionic and neutral liposome formulations in a range of human cell lines (Mayhew et al., 1987). It was observed that SA containing liposomes were toxic to cells and negatively charged liposomes had higher toxic effects on cells than similar uncharged formulations. However, inhibition of cell growth
alone was used as a marker of toxicity. Here, HCA allowed the screening of multiple parameters including cell number to determine cell health in differentiated THP-1 cells following liposome treatment for 24 hours. For these assays the ionophor valinomycin served as a positive control and validated the assay in terms of detection of changes in cell number, nuclear morphology, cell permeability, mitochondrial membrane potential (MMP) and cytochrome c release. Typically, assays such as MTT are used to assess cell viability but this method enables more subtle changes in cell health to be detected with more detail. Drugs and compounds affect cell health at various levels and cellular changes can vary depending on concentration and exposure time to these materials. Measuring a single parameter or end point may not provide the information necessary to determine an accurate cytotoxicity profile and under certain conditions may lead to false positive or false negative results (McKim Jr, 2010).

Aside from toxicity, drug delivery systems can also induce immune responses, the extremes of which have been witnessed in clinical trials and in some cases have been fatal (Cotrim and Baum, 2008). To assess the effects of the liposome carriers on immune reactivity in differentiated THP-1 cells we looked at NFκB activation and at cytokine/chemokine induction following 24 hours treatment with LPS (positive control) and liposomes. NFκB is a transcription factor that can be activated through various receptors including TLRs and TNF receptors and has a central role in inflammation (Tak and Firestein, 2001).

DOTAP liposomes significantly reduced cell numbers at 100μM and 300μM (p < 0.05), increased cell permeability, reduced MMP and lead to significant cytochrome c release indicating the induction of both necrosis and apoptosis in these cells. DOTAP is a cationic lipid that has been shown to cause toxicity in macrophages (Filion and Phillips, 1997, Filion and Phillips, 1998). DOTAP liposomes also induced an inflammatory response in the macrophage-like cells with increases in IL-1β and IFN-γ and decreases in IL-4 (Table 5). DOTAP containing liposomes at 100μM have been shown to down-regulate TNFα synthesis in activated macrophages harvested from mice (Filion and Phillips, 1998) and therefore the lack of TNFα induction following DOTAP liposome treatment was expected.
Activation of the mannose receptor has previously been shown to have an anti-inflammatory effect with impaired NFκB activation (Chieppa et al., 2003, Xu et al., 2010). In this study, mannosylated liposomes reduced NFκB activation in differentiated THP-1 cells compared to non-mannosylated DOPC liposomes in a concentration- and mannosylated cholesterol linker length-dependent manner. In general this lack of immune reactivity of mannosylated liposomes was corroborated in cytokine screens.
Conclusions

Overall, a range of liposomes were screened for their targeting ability and biocompatibility with macrophage cells. HCA was applied to study the interaction of liposomes with macrophage cells, including representative formulations of neutral, charged and targeted liposomes. Anionic and mannose (MC6C) coated liposomes showed the most significant cellular interaction, specifically DOPS and mannosylated MC6C liposomes emerged as the leading formulations for intracellular delivery. At the lower concentration range tested liposomes were non-toxic, however, DOPS liposomes caused an inflammatory response in differentiated THP-1 cells. MC6C liposomes showed immunosuppressive characteristics such as a hampering of NFκB activation and reduced IL-8 and IFNγ production. HCA allowed liposome formulation interaction with macrophages to be assessed in a high throughput format for the first time.
Acknowledgements


Declaration of Interest

No potential conflicts of interest.
References


Tables:

Table 1: Prepared liposome compositions: DOTAP (liposome positive control), DOPS (anionic), DOPC (neutral), mannosylated (MC2C, MC4C, MC6C) liposomes.

<table>
<thead>
<tr>
<th>Liposome Nomenclature</th>
<th>Molar Ratios of Constituent Lipids</th>
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<tr>
<td></td>
<td>DOTAP</td>
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<tr>
<td>DOTAP</td>
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<tr>
<td>DOPS</td>
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<td>DOPC</td>
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</tr>
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<td>MC2C</td>
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<td>MC4C</td>
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<td>MC6C</td>
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Table 2: Liposome treatments for differentiated THP-1 cell–interaction assessment by high content screening

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<th>HCA Assay Variable</th>
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<th>Concentration (μM)</th>
<th>Time (hours)</th>
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<td>Liposome Incubation Time</td>
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<td>200</td>
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Table 3: Representative parameters for high content screening of liposome toxicity in THP-1 cells

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<th>Parameters</th>
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<td>Cell</td>
<td>-</td>
<td>Collar; 11μm</td>
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<td>Permeability</td>
<td>480/535</td>
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<tr>
<td>Mitochondrial Membrane Potential</td>
<td>535/600</td>
<td>Reference; In Cell</td>
</tr>
<tr>
<td>Cytochrome c</td>
<td>620/700</td>
<td>Reference; In Cell</td>
</tr>
</tbody>
</table>

Table 4: Sizing and zeta potentials of liposomes extruded to 200nm (n = 3)

<table>
<thead>
<tr>
<th>Liposome</th>
<th>Average Size (d&lt;sub&gt;50&lt;/sub&gt; nm)</th>
<th>Polydispersity Index (PDI)</th>
<th>Zeta Potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOTAP</td>
<td>198.9 ± 39</td>
<td>0.214</td>
<td>61.8 ± 6</td>
</tr>
<tr>
<td>DOPS</td>
<td>168.6 ± 28</td>
<td>0.227</td>
<td>-68.3 ± 8</td>
</tr>
<tr>
<td>DOPC</td>
<td>190.3 ± 22</td>
<td>0.164</td>
<td>-4.4 ± 3</td>
</tr>
<tr>
<td>MC2C</td>
<td>206.9 ± 35</td>
<td>0.145</td>
<td>36.9 ± 14</td>
</tr>
<tr>
<td>MC4C</td>
<td>204.7 ± 15</td>
<td>0.207</td>
<td>44.9 ± 5</td>
</tr>
<tr>
<td>MC6C</td>
<td>178.2 ± 11</td>
<td>0.108</td>
<td>45.2 ± 3</td>
</tr>
</tbody>
</table>
Table 5 Cytokine production in differentiated THP-1 cells following 24 hour liposome treatment.

Differentiated THP-1 cells were incubated for 24 hours with complete media (negative control), 100ng/ml LPS (positive control) or DOTAP (liposome positive control), DOPS (anionic), DOPC (neutral), mannosylated (MC2C, MC4C, MC6C) liposomes at 100μM or 300μM. Supernatants were analyzed using a MSD 10-plex cytokine assay.

Data represented as means ± SD. (n = 3) Statistical significance was determined by two-way ANOVA with Bonferroni’s Post-hoc test (* p < 0.05, ** p < 0.01, *** p < 0.001)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration</th>
<th>TH1</th>
<th>TH2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TNFα</td>
<td>IL-1β</td>
</tr>
<tr>
<td>Untreated</td>
<td>-</td>
<td>272.5 ± 104.9</td>
<td>4.6 ± 2.2</td>
</tr>
<tr>
<td>LPS</td>
<td>100ng/ml</td>
<td>2316.4 ± 2860.4 *</td>
<td>85.3 ± 114.2</td>
</tr>
<tr>
<td>DOTAP</td>
<td>100μM</td>
<td>290.3 ± 88.1</td>
<td>69.9 ± 87.5</td>
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<td>300μM</td>
<td>380.3 ± 63</td>
<td>43.1 ± 24.5</td>
</tr>
<tr>
<td>DOPS</td>
<td>100μM</td>
<td>379 ± 60.6</td>
<td>6.9 ± 3.7</td>
</tr>
<tr>
<td></td>
<td>300μM</td>
<td>631.9 ± 238.3</td>
<td>36.6 ± 16.3</td>
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<tr>
<td>DOPC</td>
<td>100μM</td>
<td>286.7 ± 33.9</td>
<td>6.3 ± 4.0</td>
</tr>
<tr>
<td></td>
<td>300μM</td>
<td>418 ± 116.8</td>
<td>11.0 ± 8.6</td>
</tr>
<tr>
<td>MC2C</td>
<td>100μM</td>
<td>294.9 ± 45.4</td>
<td>6.3 ± 4.3</td>
</tr>
<tr>
<td></td>
<td>300μM</td>
<td>325.8 ± 26.7</td>
<td>9.2 ± 8.0</td>
</tr>
<tr>
<td>MC4C</td>
<td>100μM</td>
<td>215.8 ± 93.9</td>
<td>4.0 ± 2.1</td>
</tr>
<tr>
<td></td>
<td>300μM</td>
<td>305 ± 89.5</td>
<td>10.9 ± 7.3</td>
</tr>
<tr>
<td>MC6C</td>
<td>100μM</td>
<td>259.5 ± 51.4</td>
<td>4.7 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>300μM</td>
<td>320.3 ± 32.6</td>
<td>15.3 ± 12.5</td>
</tr>
</tbody>
</table>
Figure 1: Transmission electron microscopy images of DOPS:Chol (7:3) liposomes at 100 kV high voltage and 50,000x magnification negatively stained with 2% phosphotungstic acid.

Figure 2: INCELL cell analysis. Images acquired at 3 excitation/emission wavelengths 360/480 blue (nuclei; Hoechst 33342), 480/535 green (cells; phalloidin-FITC) and 535/620 (red; liposomes). Nuclei, cells and liposomes were segmented. Fused images show overlays of the segmentation. The number of liposomes in each cell was counted.

Figure 3: Immunofluorescence to determine the expression of mannose receptor by differentiated THP-1 cells. Fixed cells were blocked with 1% BSA followed by incubation with (A) 1% BSA or (B) polyclonal mannose receptor antibody (1μg/ml). Cells were then incubated with secondary Alexa Fluor 488 antibody (green) and stained with Hoechst 33342 (nuclei; blue) and phalloidin-TRITC (F-Actin; Red). Images were acquired using an INCELL 1000 analyser with a 10x objective.

Figure 4: Cell association analysis by INCELL HCA of rhodamine labelled liposomes with differentiated THP-1 cells. Cells were incubated without liposomes (untreated) or with fluorescently tagged (rhodamine; red) anionic (DOPS), non-mannosylated neutral (DOPC) and mannosylated liposomes (7.5% MC2C, MC4C and MC6C), fixed and stained for nuclei (Hoechst; blue) and F-Actin (phalloidin-FITC; green). Images were acquired by an INCELL 1000 cell analyser with a 20x objective. Representative images show (A) untreated cells and cells treated with 200nm DOPS liposomes at concentrations of (B) 50, (C) 100, (D) 200, (E) 300 and (F) 1000μM for 2 hours. Liposomes were counted per cell using INCELL 1000 analysis software following treatment with liposomes ranging (G) in size at 200μM for 2hrs, (H) in concentration at 200nm for 2hrs and (I) in incubation time at 200nm and 200μM.

Data represented as means ± SD (n = 6)

Statistical differences were determined by two-way ANOVA with Bonferroni’s post-hoc test (* p < 0.05, ** p < 0.01) vs. unextruded liposome or DOPC-treated counterparts.
Figure 5: Cell association analysis by INCELL HCA of rhodamine labelled liposomes by differentiated THP-1 cells at 4°C and 37°C. Cells were incubated without liposomes (untreated) or with 100μM 200nm fluorescently tagged (rhodamine; red) anionic (DOPS), neutral non-mannosylated (DOPC) and mannosylated (7.5% MC2C, MC4C and MC6C) liposomes at 4 and 37°C for 2 hours. THP-1 cells were fixed and stained for nuclei (Hoechst; blue) and F-actin (phalloidin-FITC; green) and images were acquired using an INCELL 1000 cell analyser. Representative images show (A) untreated cells and cells treated with 100μM of 200nm MC6C liposomes at (B) 4°C and (C) 37°C for 2 hours. Images were analysed and liposomes counted per cell (G). Data represented as means ± SD (n = 6)

Statistical differences were determined by two-way ANOVA with Bonferroni’s post-hoc test (* p < 0.05, ** p < 0.01, *** p < 0.001)

Figure 6: High content cell assessment of liposome associated cytotoxicity in differentiated THP-1 cells. Representative images show control treatments with cells treated with (A) complete media as a negative control, (B) 120μM valinomycin as a positive control and cells treated with 100μM (C) DOTAP, (D) DOPS, (E) DOPC, (F) MC2C, (G) MC4C and (H) MC6C liposomes. Images were acquired by an INCELL 1000 analyser in 4 channels and (A-H) show nuclei (Hoechst; blue), cell permeability (green) and mitochondrial membrane potential (MMP; red). Differentiated THP-1 cells were treated with 100µM or 300µM anionic (DOPS), neutral (DOPC) or mannosylated liposomes. Positive controls were treated with 120μM valinomycin or 100 or 300μM cationic (DOTAP) liposomes. Cells were treated for 24 hours, fixed and stained.

Figure 7: Liposome induced NFκB Activation in THP1-XBlue™ Cells. Differentiated THP1-XBlue™ reporter cells were incubated with anionic (DOPS), neutral (DOPC) and mannosylated (MC2C, MC4C and MC6C) liposomes at 100μM and 300μM for 24 hours. Positive controls were treated with 100ng/ml LPS. Mean absorbance determined following treatment with 100μM DOPC is shown by the dashed line. SEAP activity was determined by absorbance at 630nm. Data represented as means ± SD (n = 3)
Figures:

Figure 1.
Figure 2.
Figure 3.
Figure 4.
Figure 5.
Figure 7.