Chapter 6

Chapter 6 contains the manuscript of a technical note to be submitted to Cytometry. The technical note contains the introduction, aims, experimental methods, results and discussion of the determination of the influence of lipid drug delivery systems on ROS and haemolysis assays. The technical note is prepared according to the author guidelines (http://onlinelibrary.wiley.com/journal/10.1002/%28ISSN%291552-4930/homepage/ForAuthors.html). Some formatting have been changed to help with ease of reading. Language and grammar is consistent with UK English. References style used as described by guideline for authors (Reference style as found in Refworks).

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Optimization of *in vitro* assays to study biocompatibility of lipid drug delivery systems

C. Slabbert, L.H. du Plessis*, A.F. Kotzé

Unit for Drug Research and Development, North-West University, Hoffman Street, Potchefstroom, 2531, South Africa

* Corresponding author: Dr. L.H. Du Plessis
Lissinda.DuPlessis@nwu.ac.za
Tel: +27 18 299 4246
Fax: +27 18 299 2248

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- Pheroid™ Technology
- Liposomes
- Efficacy
- Haemolysis

Standard List of Abbreviations for Cytometry

DNA	Deoxyribonucleic acid
FACS	Fluorescence-activated cell sorting
FCM	Flow cytometry measurement
FSC	Forward light scatter
PBS	Phosphate buffer saline
PI	Propidium Iodide
RNA	Ribonucleic acid
SSC	Side light scatter

List of Abbreviations

iRBC	Infected erythrocytes
FL1	Green fluorescent
FL2	Red fluorescent
RBC	Uninfected erythrocytes

1. Introduction

In vitro evaluation of new compounds is used to give an overall profile of the compound before expensive *in vivo* and clinical trials are conducted (1). When an existing compound is formulated in a drug delivery system with pharmaceutical excipients, it is necessary to determine the effect of the system in its entirety (2). Drug delivery systems involve the formulation of various excipients to overcome poor physiochemical properties (3) to deliver a compound at a specific site and to maintain a certain drug concentration (2,4). Lipids are used as excipients in drug delivery systems due to their versatility and variety of dosage forms it can be formulated in. It is important to determine the biocompatibility for the drug delivery systems without drugs due to the ability of the drug delivery system to elicit its own response (5). A promising lipid drug carrier is liposomes, a spherical structure consisting of a lipid bilayer enclosing an aqueous volume. Lipophillic and hydrophilic drugs can be entrapped in these phospholipid vesicles (6,7). Pheroid™ vesicles made from essential and natural fatty acid are similar in size and structure to liposomes (8,9). They vary in size from 25 nm to 10 µm and can be manipulated for different applications (7,8).

Biocompatibility evaluation is the determination of the influence of the drug delivery system on the biological environment. This is usually done through *in vitro* and *in vivo* assay specific for certain problems that may be expected (5). A specific limitation for biocompatibility evaluation is the lack of sufficient *in vitro* and *in vivo* analysis methods for determination of efficacy, toxicity and other response elicited by the lipid drug delivery systems (7,10). It is therefore necessary to develop methods to analyze the influence of drug delivery systems on different cell types as well as on the assay itself. Erythrocytes

infected with *Plasmodium* spp. can be used to determine the efficacy of newly formulated compounds or drug delivery systems as well as evaluate resistance (11). Other assays preformed on erythrocytes include evaluating toxicity including reactive oxygen species (ROS), lipid peroxidation and hemolysis. Hemolysis is the release of hemoglobin into the plasma due to destruction of the membrane of the erythrocytes. Hemolytic activity is utilized during the research procedure to determine the safety of excipients and drugs. Non aqueous formulation can cause hemolysis (12) and is determined by measuring the amount of hemoglobin spectrophotometrically. The milky appearance of these carrier systems can influence the spectrophotometric evaluation of hemolysis.

A technique commonly used to evaluate cellular properties is flow cytometry. Flow cytometry is used to measure different properties of cells individually as they pass through a beam of light. Scattering of light occurs as the particles pass through the light and is collected by a variety of lenses, filters and detectors. This is converted to electric signals and the light scattering and fluorescent properties are given on a variety of graphs. Light scattering is affected by the size, shape and granularity of the cell sized particle. A fluorescent compound is excited by the beam and emits the energy as a photon of light. The fluorescent properties of stained cells combined with light scattering properties can be used to identify certain cell populations that can be defined through gates. This can be used to limit the analysis of a sample to a specific population limiting the amount of background noise (13). This sophisticated, highly sensitive and versatile method is utilized to analyze a variety of cell samples and properties including erythrocytes (14,15) and drug delivery systems (16). Because of the similarity in size between erythrocytes and the lipid drug carriers, it beckons the evaluation of the influence of these systems on the flow cytometric evaluation of *in vitro* efficacy.

Biocompatibility cell assays for flow cytometry include viability (17,18), apoptosis (19,20), ROS (21,22) and lipid peroxidation (22). Drug susceptibility assays against malaria in erythrocytes have been investigated extensively *in vitro* and *in vivo* with flow cytometry (11,23-25). DNA or RNA specific dyes are added to the sample staining the parasite DNA (11) because erythrocytes contain no DNA and is 6.5 – 8.8 µm in size (26). These dyes include Hoechst 33342, SYBR® Green, acridine orange, thiazole orange, DAPI-I, YOYO-1 and propidium iodide (PI) (14,23-25,27-30). PI is membrane impermeant, fixation with gluteraldehyde or formaldehyde is required and binds to DNA and RNA with multiple

washing steps included in the method. It is a reproducible method for accurate stage specific determination of infected erythrocytes (14). All cell types have a degree of autofluorescences (31). Covalent bonds are formed with proteins when cells are fixated with gluteraldehyde leading to autofluorescence (32). Autofluorescences in RBC are observed with gluteraldehyde fixation but showed to have only a small influence on the stained cells (25).

The aim of this study was the evaluation of the influence of liposomes and Pheroid™ vesicles on *in vitro* cellular assays of erythrocytes. The similarity in size and shape of the drug delivery systems and erythrocytes, as well as the autofluorescences of fixed erythrocytes, beckons the evaluation of the biocompatibility of the drug delivery system with certain assays. Very often samples have heterogeneous populations with only some populations of interest. It is therefore necessary to exclude unwanted populations from the analysis. With flow cytometric evaluation, a gate, defining a certain area or population can be used to characterize the events and eliminate noise and unwanted events. The milky appearance of the drug delivery systems will have an influence on the spectrophotometric evaluation of hemolysis.

2. Materials and methods

2.1 Materials

Vitamin F ethyl ester was obtained from CLR (Berlin, Germany), Cremophor® RH40 from BASF (Germany) and DL-α-tocopherol from DSM (Basel, Switzerland). High grade chloroform and methanol were obtained from Rochelle Chemicals (South Africa). The following were purchased from Sigma-Aldrich® (St. Louis, MO, USA): cholesterol, L-α-phosphatidyl choline, RPMI 1640, gluteraldehyde, saponin and Nile Red. Propidium iodide (PI) was purchased from Molecular Probes (Invitrogen®, Breda, The Netherlands). Albumax II was obtained from Gibco® (Invitrogen™, Breda, The Netherlands).

2.2 Preparation of lipid drug carriers

Pheroid™ vesicles were prepared according to the method of Du Plessis *et al*. (9). Briefly, vitamin F and Cremophor® EL were heated to 75°C and left to cool. DL-α-tocopherol was

added and heated to 55°C. Nitrous oxide saturated phosphate buffer saline (PBS) was heated to 75°C. The heated oil phases were added to the water and homogenized at 13 500 rpm until the temperature was below 40°C. The emulsion was shaken using a GFL shaker (GFL Gesellschaft fűr Labotechnik mbH, Germany) until room temperature was reached (9). The film hydration method was used to prepare the liposomes. Cholesterol (1% w/v) and L-α-phosphatidylcholine (1.5% w/v) were dissolved in an appropriate volume of chloroform:methanol (2:1 v/v) solution. The organic solvent was slowly removed under reduced pressure utilizing a rotary evaporator (Heidolph Laborato 4000, Germany) obtaining a thin film of lipids on the inner wall of the flask. A lipid suspension was attained by hydration of the film in PBS (pH 7.4) and swirling with glass beads until all lipids were dispersed. Particle downsizing was obtained by sonication for 5 – 10 minutes at 4°C using a probe sonicator (Hilscher UP 100H Ultraschallprozessor, Germany) (7,33,34).

2.3 Cultivation of *Plasmodium falciparum*

Plasmodium falciparum W2 strain (a generous gift from Prof. P. Smith, Unviersity of Cape Town, Department Pharmacology) was used during the optimization of efficacy evaluation. The strain was maintained in continuous cultures of RPMI 1640 medium supplemented with Albumax II and O^+ human erythrocytes. Cultures were incubated at 37°C in an atmosphere of 5% oxygen, 5% carbon dioxide and 90% nitrogen (35). Ethics approval was obtained from the Ethics Panel of the North West University (NWU-0008-08-S5).

2.4 Fluorescent labeling

A modified method as previously described was used to stain infected erythrocytes (27). Samples in 96 well plates were fixed with 0.025% gluteraldehyde, washed with PBS and permeabilized with 0.005% saponin. Cells were washed with PBS before incubation for 1 hour with PI at a final concentration of 10 µg/ml. Pheroid™ vesicles and liposomes were stained with Nile Red (0.8 µg/ml) at room temperature for 10 minutes (9,36). Simultaneous staining with PI and Nile Red was obtained by adding Nile Red 50 minutes after PI, and left for 10 minutes before analysis.

2.5 FCM measurement

Fluorescence of single cells were measured by a FACSCalibur™ benchtop flow cytometer equipped with a 488nm Argon ion laser linked to Cell Quest Pro Software (2002, Becton & Dickson, Mountain view, CA, USA). Amplification of signals were carried out at logarithmic scale and measurement of events plotted on forward light scatter (FSC), side light scatter (SSC), green fluorescent (FL1) and red fluorescent (FL2). Gating strategy was used to distinguish the erythrocyte population more accurately from unwanted populations. A total of 20000 events as defined by gates, were counted. Samples consisted of 100 µl 2% hematocrit infected erythrocytes (iRBC) or erythrocytes (RBC) in PBS. Pheroid™ vesicles and liposomes were analyzed the same as the erythrocytes. The lipid drug carrier was added to the iRBC before fixation and 100 µl of the final samples was analyzed.

2.6 Hemolytic activity

Spectrophotometric analysis was carried out in 96 well plates using a plate reader at a wavelength of 540 nm. Samples were centrifuged at 2000 rpm for 8 minutes after which 100 µl of the supernatant was added to a new plate and analyzed. RBC (100 µl) at a hematocrit of 2% were added to each well. For the control plate, a 100 µl of culture medium was added to each well. Control of 100% hemolysis was obtained by adding water to the RBC and was then left to incubate for 1 hour. Determination of the influence of the drug delivery systems were evaluated by determination of the absorbance of the drug carrier in a 1:1 ratio of either Pheroid™ vesicles or liposomes and culture medium. The absorbance of different concentration of the drug delivery systems was determined. Samples of RBC, drug delivery system and culture medium (1:1:2) was analyzed to evaluate if this method can be used to determine the hemolysis of the drug delivery systems over time. The percentage hemolysis was calculated by the following equation:

$$\% \text{ Hemolysis} = \frac{\text{Absorbance of sample}}{\text{Absorbance of 100\% hemolysis}} \text{ X } 100$$

3. Results

3.1 Flow cytometric evaluation – Gating

Distinct gating strategies were used to accurately determine the level of infected erythrocytes. Unwanted populations and background noise can lead to inaccurate determination of parasitemia levels. Different samples, RBC, iRBC, Pheroid™ vesicles and liposomes were analyzed giving distinct populations on a FSC/FL2 dot plot (Figure 1). Dot plots, where each dot represents the size and fluorescent properties of a single cell was used in the gating strategy. Figure 1A shows a representative fluorescent dot plot of RBC used as negative control. Figure 1B represents iRBC and has two distinct populations with different fluorescent intensities. PI stained the DNA of the iRBC resulting in an increase in fluorescent intensity (shift to left on the FL2 axis) separating the RBC (population in lower left quadrant) from the iRBC (population in upper right quadrant). Pheroid™ vesicles as seen in Figure 1C is a single population similar in size and fluorescent intensity to RBC. Liposomes gives a distinctive population overlapping RBC (Figure 1D).

Numerous washing steps between the fixation, permeabilization and staining of the cell lead to a large amount of Pheroid™ vesicles being removed from the sample. This is due to the fact that centrifuging of Pheroid™ doesn't lead to the formation of a pellet but remains in suspension. Liposomes, on the other hand, form a pellet with the RBC leading to liposomes being present in the final sample for analysis. Thus, even though Pheroid™ and RBC has overlapping population, the amount of Pheroid™ present in the final sample is small. An overlay dot plot, as seen in Figure 2A, of the control samples for RBC, iRBC and Pheroid™ vesicles illustrates no difference in size between the Pheroid™ vesicles and RBC. Pheroid™ vesicles has no autofluorescence. Gate 1 (G1) represents the RBC and gate 2 (G2) defines the population of iRBC. Liposomes has a large influence on the final sample resulting in false values. An overlay dot plot (Figure 2B) of liposomes, RBC and iRBC shows that RBC and liposomes has similar characteristics. Liposomes can however be gated to remove all events in G3 from analysis as seen in Figure 3. Gate 4 (G4) is used during the analysis excluding the unwanted events of G3 to more accurately determine the percentage parasitemia.

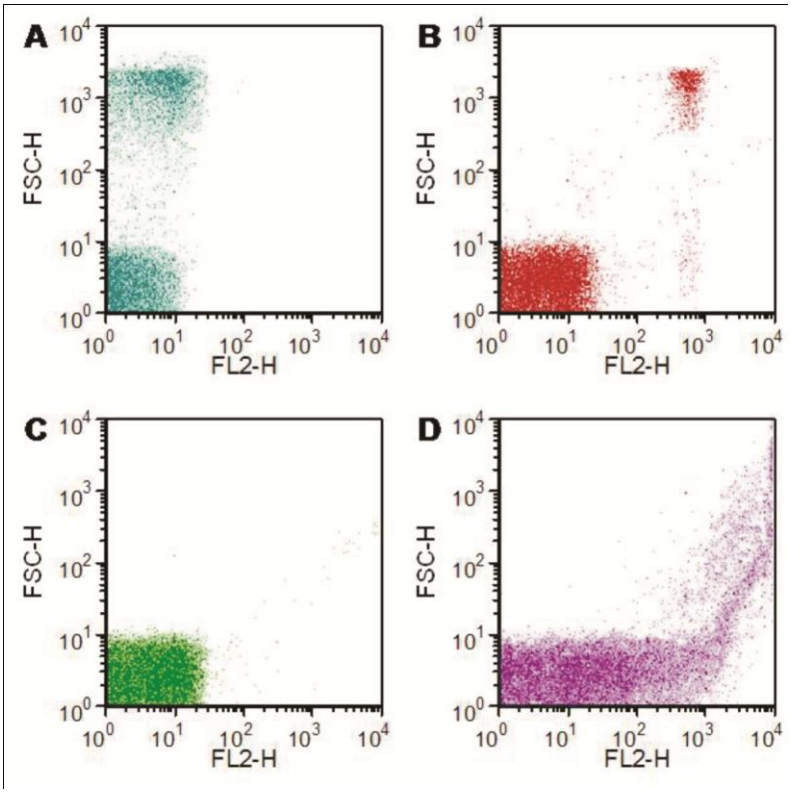

Figure 1 Dot plots of different control samples containing either RBC (A), iRBC (B), Pheroid™ vesicles (C) or liposomes (D).

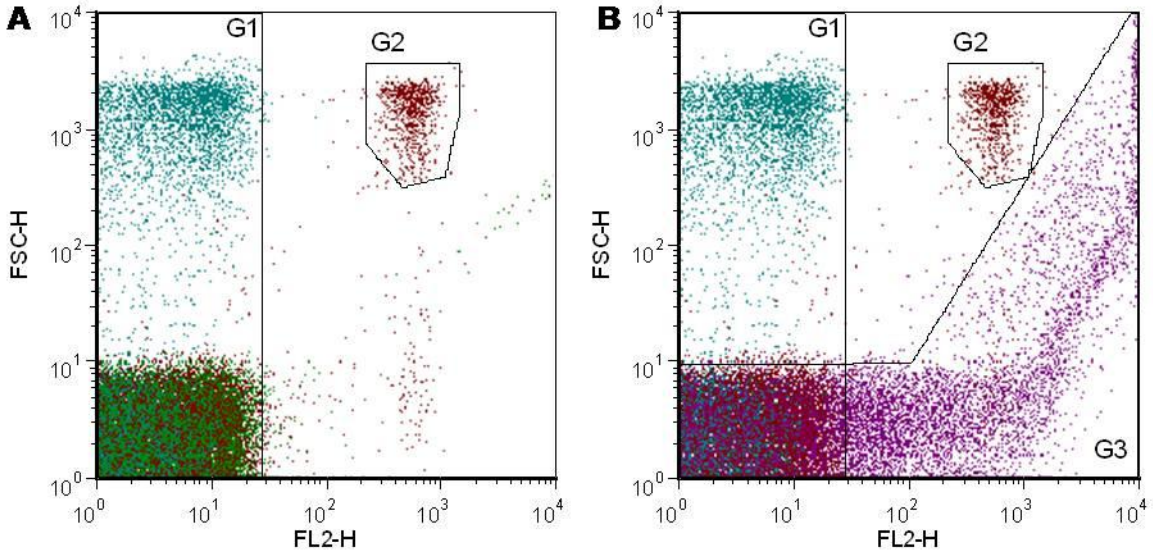

Figure 2 Overlay dot plot of RBC (blue) and iRBC (red) with either Pheroid vesicels (Green) (A) or liposomes (purple) (B). Gates G1 = RBC, G2 = iRBC and G3 = liposomes.

3.2 FCM analysis of iRBC

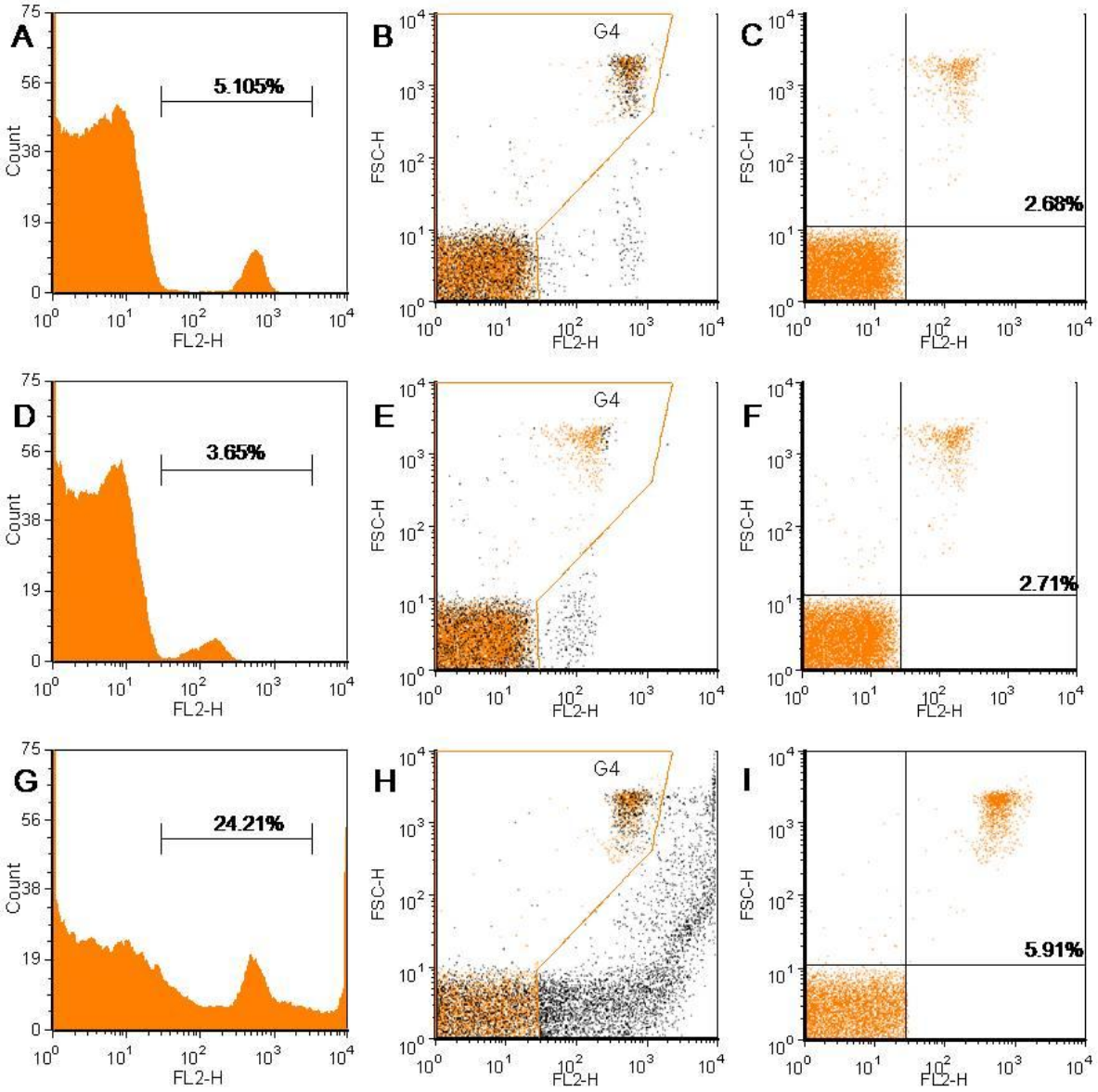

Figure 3 Representative histograms and dot plots of cytometric evaluation of iRBC (A-C) with the addition of either Pheroid™ vesicles (D-F) or liposomes (G-I). Histogram is a representation of RBC and iRBC by the two peaks on the left and right hand side respectively. Background noise (A-F) and liposomes (G-I) as seen in the histogram is gated out of the analysis sample by utilizing G4. Dot plots of G4 (C, F and I) excludes background noise and liposomes form the population analyzed.

During analysis of control samples, gates were drawn up to define certain populations. Events in G3 was unwanted resulting in defining a certain population by G4. G4

represents the RBC and iRBC of the heterogeneous population. Analysis of iRBC after PI staining showed two distinct peaks (Figure 3A). Each peak represents either the RBC (left peak) or iRBC (right peak) population as seen in Figure 1B. When the events analyzed were restricted to G4, eliminating background noise a more accurate parasitemia of 2.68% were calculated (Figure 3B and C). Addition of Pheroid™ to the 2.68% iRBC (Figure 3D) showed a parasitemia of 3.65% compared to 2.71% (Figure 3F) when noise is gated out. No difference between Pheroid™ vesicle samples and iRBC are seen after the gating strategy was followed. Addition of liposomes to the 2.68% iRBC an increase to 24.21% when no gate is used as seen in Figure 3G was observed. The percentage parasitemia increased to 5.9% due to the presence of liposomes in the sample when liposomes where excluded through gating (Figure 3H-I). No difference should be observed between the control of iRBC and the samples containing either Pheroid™ vesicles or liposomes.

3.3 Hemolytic activity

To determine the influence of the drug delivery systems on the spectrophotometric evaluation different concentrations of the drug delivery system were added to the RBC. Absorbance values of the different drug carrier system with and without RBC are shown in Figure 4. The higher Pheroid™ vesicle concentration showed higher absorbance values thus having a greater effect on the reading. Pheroid™ vesicles with centrifuging stays in suspension leading to an increase in absorbance value with an increase in Pheroid™ vesicle concentration. Liposomes seemed to have a lesser effect on the absorbance value but also show an increase with an increase in liposome concentration. Correlations between samples with and without RBC is r^2=0.9937 and r^2=0.8492 for Pheroid™ vesicles and liposomes respectively.

By subtracting the absorbance value of the drug delivery system with RBC sample from the experimental sample value according to concentration lead to a value representing the hemolytic activity. An increase in hemolytic activity was observed at an increase in drug delivery system concentration as seen in Figure 5. Pheroid™ vesicles also showed an increase in hemolysis compared to liposomes.

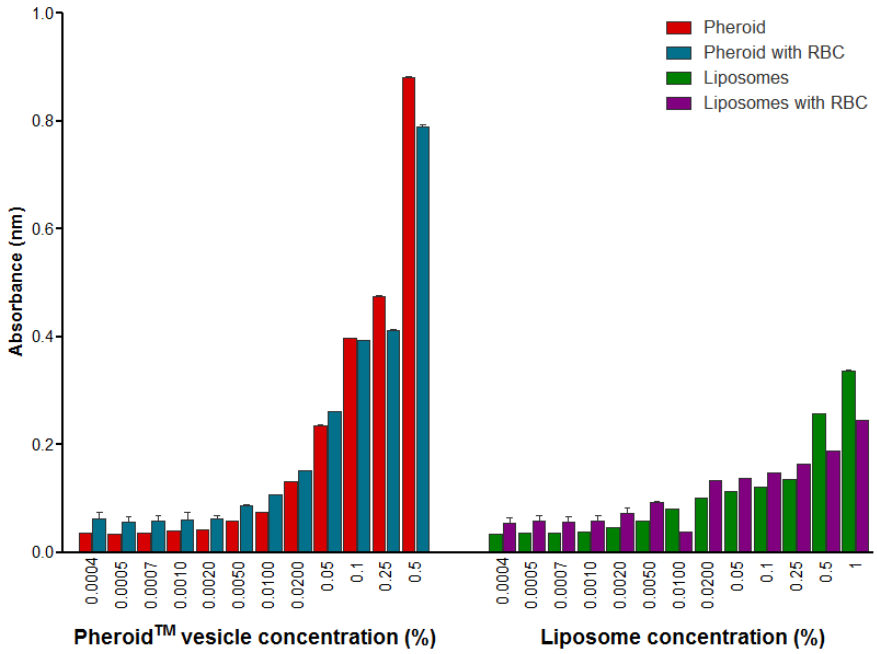

Figure 4 Absorbance values as obtained from Pheroid™ vesicles and liposomes with and without RBC. Each bar represent the mean ± SEM of triplicate analysis.

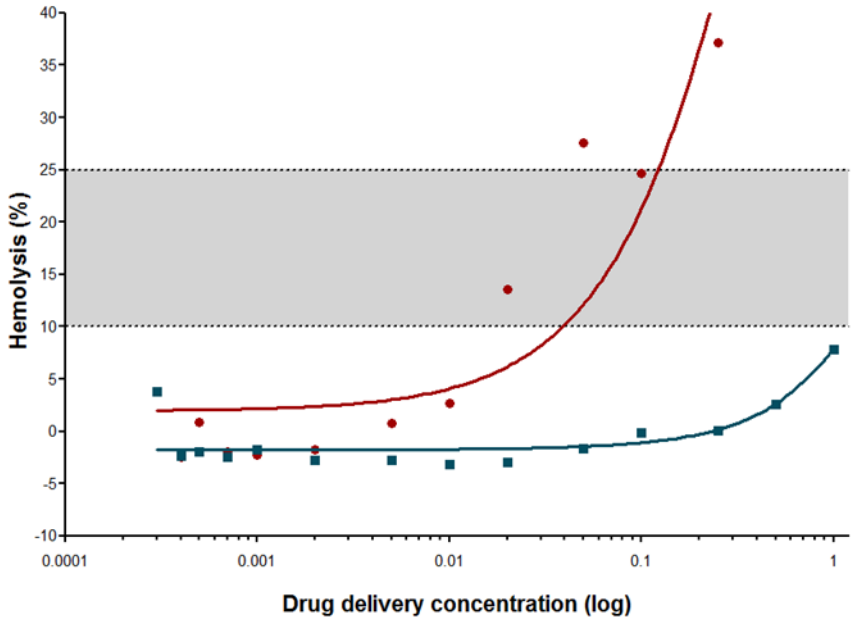

Figure 5 Hemolytic activity of Pheroid™ vesicles (red) and liposomes (blue) after 24 hours incubation of the drug delivery system with RBC. Measurements under 10% are non-hemolytic and above 25% are hemolytic Each point represent the mean ± SEM of triplicate measurement. Data was transformed to X=LogX, normalized between 0% and 100% after which the data was fitted with sigmoidal doses response curve.

4. Discussion

To determine the biocompatibility of the drug delivery system on the biological system *in vitro* assay, among others is a good indication. A limitation of biocompatibility testing is the lack of sufficient assays. It is necessary to determine the influence of the drug delivery systems on the assays to ensure the accurate determination of biocompatibility. Because of the similarity in size between the RBC and drug delivery systems and autofluorescence of the RBC, the influence should be determined. Efficacy results obtained displays graphs where iRBC are clearly separated from the RBC with two distinct peaks in a FL2 histogram. Distinct drug delivery system populations do not overlap with the iRBC. Samples containing Pheroid™ vesicles do not have an influence on the parasitemia levels. In contrast, liposomes contributes to the parasite levels producing false readings. It is therefore necessary to obtain an accurate reading to adapt the liposome parasitemia. It is possible to gate only the population of interest decreasing the amount of unwanted events and background noise resulting in more accurate analysis. To obtain accurate readings with liposomes, the percentage parasitemia is multiplied by a constant values (data not shown). Although the PI assay requires cells to be fixated and permeabilized, the method was shown to be accurate in the determination of parasite levels for Pheroid™ vesicles. Liposome efficacy can be determined with additional data analysis steps.

The milky appearance of Pheroid™ vesicles and liposomes has an influence on the spectrophotometric evaluation of hemolysis. Results of the optimization of the hemolytic activity assays shows a direct correlation exists between the absorbance values and drug delivery system concentrations. It is possible to eliminate the influence of the drug delivery system by subtracting the values as background noise. An accurate reading to determine the hemolytic activity can be obtained for both Pheroid™ vesicles and liposomes

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