



A toxicity profile of the Pheroid® technology in rodents

Janke Kleynhans^{a,*}, Dale Elgar^b, Thomas Ebenhan^c, Jan Rijn Zeevaart^{a,d}, Awie Kotzé^b, Anne Grobler^a

^a DST/NWU Preclinical Drug Development Platform, North-West University, Potchefstroom, 2520, South Africa

^b Faculty of Health Sciences, North-West University, Potchefstroom, 2520, South Africa

^c Nuclear Medicine, University of Pretoria, Pretoria, 0001, South Africa

^d Radiochemistry, The South African Nuclear Energy Corporation (Necsa), P.O. Box, 482, Pretoria, 0001, South Africa

ARTICLE INFO

Keywords:

OECD guidelines
Drug carrier system
Genotoxicity
In vivo toxicity
Omega-3-acid ethyl esters

ABSTRACT

The Pheroid® drug delivery system is now on the threshold of progressing into human clinical trials for various patented pharmaceutical applications and a systematic investigation of its toxicological properties *in vitro* and *in vivo* is thus a priority. Colloidal dispersions (nano- and microemulsions) demonstrate the ability to be adapted to accommodate either lipophilic, hydrophilic or amphiphilic drug molecules. The colloidal dispersions investigated during this evaluation has a general size of 200 nm - 2 µm, a zeta-potential of -25 mV and the main ingredient was ethyl esters of essential fatty acids.

The Ames mutagenicity assay was performed on selected *Salmonella thyphimurium* strains TA98, TA100 and TA102. The Ames assay included S9 metabolic activation and no mutagenicity was present during the assay. The effect of acute and subchronic administration on a biological system was investigated in two species of rodent (BALB/c mice and Sprague-Dawley rats). Observations focused on the physical condition, blood biochemical analysis and the haematological profiles. Gross necropsy was performed on all the test animals. Organ weights followed by histopathology of selected organ tissues were recorded.

During the acute evaluation animals showed tolerance of the maximum prescribed dose of 2000 mg/kg (according to OECD guidelines) in two rodent species after intravenous administration (absolute bioavailability). The oral formulation was tolerated without incidents in both acute and subchronic studies. Although valuable baseline safety data was obtained regarding the Pheroid® system, future studies with the entrapped active pharmaceutical ingredients is necessary to provide a definitive safety profile.

1. Introduction

The advantages of colloidal dispersions (micro- and nanoemulsions) as carrier systems include thermodynamic stability, straightforward manufacturing processes and the ability to entrap either lipophilic, hydrophilic or amphiphilic drug molecules. The oil-in-water emulsions are optimal due to a longer shelf-life compared to other nanoparticulate systems as well as having an accepted regulatory status. Furthermore, these structures are not broken down when they are diluted by a biological aqueous phase upon administration [1]. In this investigation the emulsion system evaluated for toxicity can fall in the nanoemulsion category (see for example formulation C evaluated in this study, compared to formulation B) or the microemulsion category based on the method of manufacturing and it is therefore important to clearly indicate size and morphology characteristics as pertaining to a set of safety data. If so desired, the addition of the C20 unsaturated fatty acid

ethyl esters (eicosapentaenoic acid and docosahexaenoic acid) as component during the manufacturing of the emulsion (as done in formulation A) leads to the formation of microemulsion vesicles which can sometimes be more desired in the case of the oral delivery of pharmaceutical ingredients due to increased stability and shelf-life [2]. It is important to note that the incorporation of C20 unsaturated fatty acids in emulsion formulations has been shown to afford cytoprotection in the case of nephrotoxic drugs and therefore inclusion can allow a reduction in toxicity of API's incorporated in emulsion-type systems [3].

The Pheroid® system targets the active pharmaceutical ingredient (API) to the organ of interest (during parenteral or oral administration) in a more effective manner reducing the exposure of healthy tissue. It is a micro- or nanoemulsion and consists of three phases namely an oil-phase (fatty acid based), aqueous-phase and a gas-phase (nitrous oxide gas). The nitrous oxide gas phase is added to the system by saturating both the oil-phase and the water-phase with gas before manufacturing

* Corresponding author.

E-mail address: jankekleyhans@gmail.com (J. Kleynhans).

<https://doi.org/10.1016/j.toxrep.2019.08.012>

Received 27 March 2018; Received in revised form 11 July 2019; Accepted 20 August 2019

Available online 20 August 2019

2214-7500/ © 2019 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

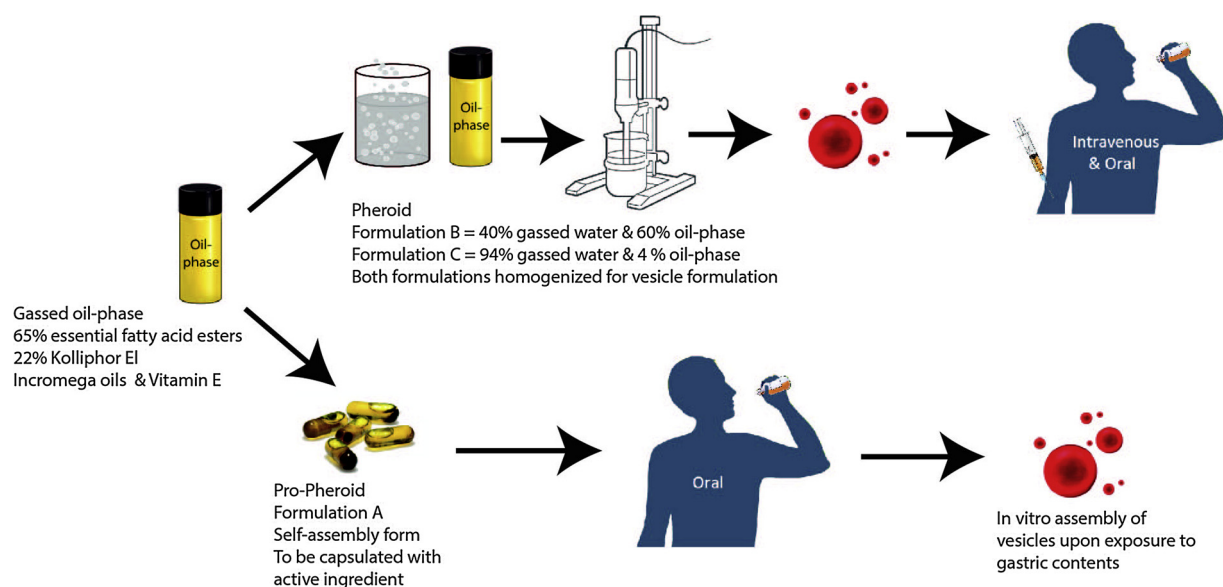


Fig. 1. Description of pro-Pheroid® and Pheroid® technology.

(Fig. 1). When the oil-phase is saturated with nitrous oxide gas, it can be used (without the addition of the gassed water phase) as the self-assembly form of the technology (pro-Pheroid®) which was developed to accommodate pharmaceutical entities labile to moisture. The formation of Pheroid® vesicles and concomitant entrapment in the emulsion vesicles, occurs spontaneously *in vitro* when the pro-Pheroid® is exposed to the gastric contents. For human use, the pro-Pheroid® would be packaged in hard gel liquid capsules. The Pheroid® emulsions are manufactured by adding the required amount of gassed oil-phase (pro-Pheroid®) to nitrous oxide saturated water with the addition of external energy (homogenization) to result in a micro- or nanoemulsion entrapping the API [2,4].

All Pheroid® formulations are manufactured from non-toxic ingredients that is generally regarded as safe (not including the API) in a process designed to be environmentally safe with minimal waste production. The effects on the immune system of the various components used in the manufacturing of the nanoemulsions are presented in Table 1. The individual components of the formulation have been selected to be non-toxic based on individual characteristics.

The aim of this study was to evaluate the safety of Pheroid® technology (both self-assembly macro- and nanoemulsion formulations) taking into account the foreseen applications thereof (Fig. 2). Hereby, genotoxicity was evaluated *in vitro* by the AMES test [15]. The organization for Economic Co-operation and Development (OECD)

guidelines employed in this study are more humane than the LD₅₀ (lethal dose) procedure and incorporates the ethical principles of reduce, refine and replace during toxicity evaluations. This approach avoids using death of animals as an endpoint and instead relies on the observation of clear signs of toxicity at fixed dose levels [16,17].

2. Materials and methods

2.1. Materials

The fatty acids used in the preparation the Pheroid® and the pro-Pheroid® formulations were obtained as vitamin F ethyl ester CLR (CLR Chemisches Laboratorium, IMCD), PEG 400 (Sigma-Aldrich, South Africa), Incromega E3322 and E7010 (Croda Chemicals, South Africa). Other ingredients included dl-Alpha tocopherol (Chempure, South Africa), Kolliphor EL (BASF, Germany), preservatives (methylparaben and propylparaben, Sigma-Aldrich) and the antioxidants (butylatedhydroxyanisole and butylatedhydroxytoluene, Sigma Aldrich, South Africa). The formulations were gassed with nitrous oxide gas obtained from Afrox (South Africa).

The supplier for most of the chemicals used in the mutagenicity assay (mutagens 2-acetylaminofluorene, aflatoxin B₁, biotin, histidine, nicotinamide adenine dinucleotide phosphate, glucose-6-phosphate and glucose-6-phosphate dehydrogenase) was Sigma Chemical Co (South

Table 1
Overview of the various components of the Pheroid® delivery system.

	Commercial products	Immune system effects	FDA category	Other effects	Ref
Kolliphor EL (Polyoxyl 35 castor oil)	IV Tacol™ IV Valstar™	Taxol formulation* has risk for acute hypersensitivity reaction	Inactive ingredient	Inactive	[6]
Polyethylene Glycol 400	IV Ativan™ Oral Agenerase®	↓ cytokine production <i>in vitro</i> and <i>in vivo</i>	Inactive ingredient	Inactive	[6–8]
DL-α-tocopherol	IV Amphotericin B®	Immunomodulator ↓ chronic inflammation ↓ Reactive oxidative species	Inactive ingredient	Not to be administered in vitamin K deficiency	[6,9–11]
Essential fatty acids EPA & DHA (Incromega®)	IV Intralipid®	↓ IL-1, IL-2, IL-6 and TNFα	Component in FDA approved intravenous products	Essential for the survival of humans	[12]
Ethyl esters of essential fatty acids (Vitamin F ethyl ester)	IV Lovaza®	↓ cytokine production ↓ nitric oxidase synthase ↓ COX-2 Resolve inflammation	Component in FDA approved intravenous products	Cardiovascular protective effects	[13,14]

*Taxol® is a FDA approved intravenous formulation containing paclitaxel, Kolliphor EL and 50% ethanol.

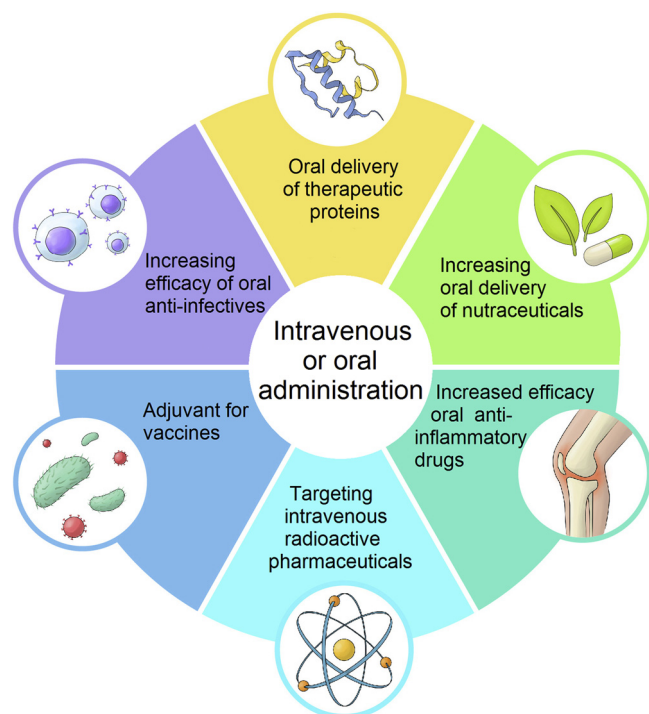


Fig. 2. The current preclinical applications of the Pheroid® and pro-Pheroid® technology under investigation [5,18–26].

Africa). The mutagen cumolhydroperoxide was obtained from Merck (United States), dimethyl sulfoxide was brought from BDH Laboratory suppliers (Kuwait), Bacto® Agar was sourced from Difco® Laboratories (United States) and Oxoid nutrient broth #2 was purchased from Oxoid (United Kingdom).

2.2. Methods

2.2.1. Study design

An illustration of the study design is provided in Fig. 3. Three formulations (pro-Pheroid® formulation A, Pheroid® formulations B and C) used for the different evaluations were modified as described in the OECD guidelines to take into account the route of administration and the unique characteristics required by each [16,17].

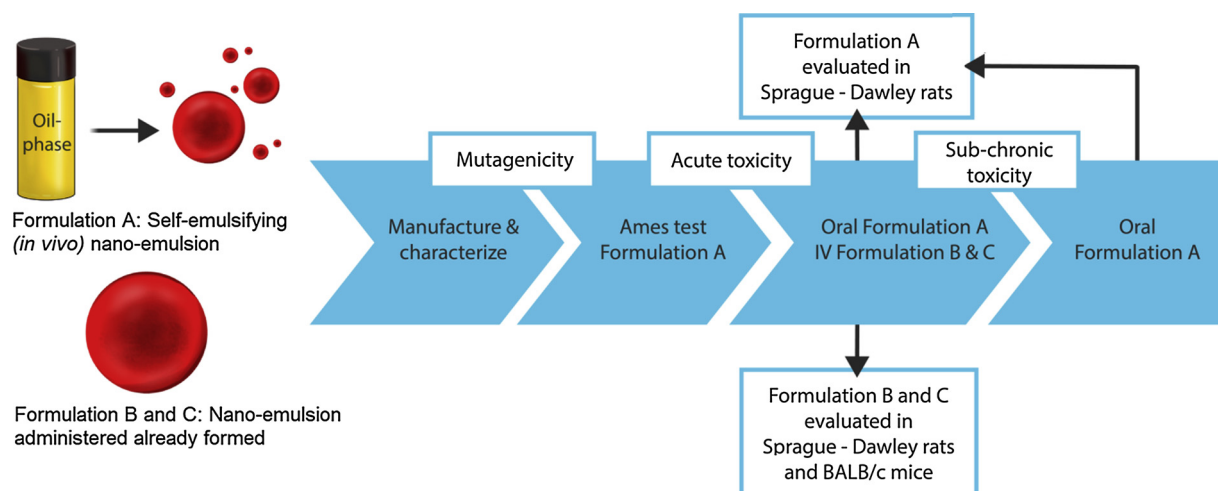


Fig. 3. The study design followed to evaluate the safety of the micro- and nanoemulsion delivery systems (both self-assembly and assembled versions).

2.2.2. Formulation preparation and characterization

The characterization of test formulations is important to ensure repeatability between batches of administered formulations. The particle sizes of the vesicles of the different formulations and the overall distribution were determined by laser diffraction (Malvern Mastersizer Hydro 2000, Malvern Instruments, Worcestershire, United Kingdom) and are indicated as the polydispersity index. Each sample was measured six times and the mean and standard deviation were determined. The Zeta-potential of the samples was measured using the Malvern Zetasizer Nano ZSP (Malvern Instruments, Worcestershire, United Kingdom). Morphological conformation was determined by confocal laser scanning electron microscopy (CLSM, Nikon D-eclipse C1 confocal scanning microscope, United States) as per the method outlined by Slabbert *et al.*. The self-emulsifying formulation was prepared for physical characterization by mixing it with a 0.1 N hydrochloric acid diluent, to simulate the acidic environment of the stomach [27,28]. The presence of bacterial endotoxins in the raw materials was determined using the point-of-use Endosafe®-PTS™ System (Charles River Laboratories, United States).

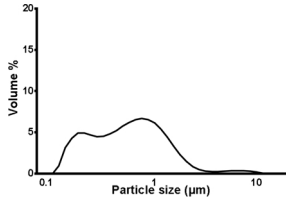
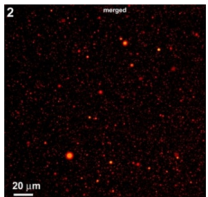
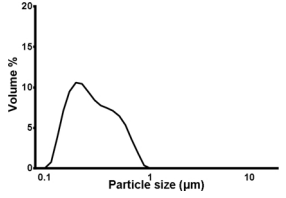
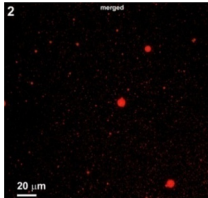
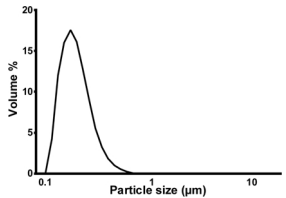
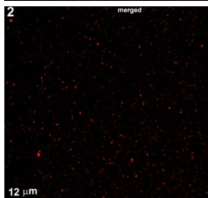
The composition of the test formulations (formulation A, B and C) is provided in Table 2. The different ingredients for each formulation were heated by microwave and added in a stepwise process depending on thermo-stability. All the formulations were subjected to gassing with nitrous oxide for a total of 4 days. For the Pheroid® formulations (B and C), the water gassed with nitrous oxide (± 170 kPa) was mixed with the oil phase by homogenization (13,500 rpm with a HeidolphDixax 600 homogenizer, Labotec South Africa) for 4 min. Prior to intravenous administration, formulations were filtered through a series of filters – the smallest having a pore size of 0.22 μ m to prepare a safe nano-emulsion.

2.2.3. In vitro toxicity assay

The mutagenic effects of the pro-Pheroid® (formulation A) were tested on *Salmonella typhimurium* strains TA98, TA100 and TA102 according to the method proposed by Maron and Ames (1983) [15]. The assumption is made that due to the fact that the only additional ingredient differentiating pro-Pheroid® from Pheroid® is water, that the data can be extrapolated for all three formulations. Undiluted formulation A (100 μ L per plate) as well as various dilutions with sterile water (20 μ L, 10 μ L per plate) were evaluated. The mutagens used as the positive control were cumol-hydroperoxide (100 ng/plate for TA102), 2-acetylaminofluorene (5 μ g per plate for TA98) and aflatoxin B₁ (10 ng/plate for TA100). Aflatoxin B₁ is a compound known to be metabolised by enzyme oxidase by cytochrome P₄₅₀ of the microsomes located in hepatocytes to human carcinogens [29]. Culture tubes were

Table 2

The specification of the formulations evaluated for toxicity.

Formulation components	Particle Size Distribution	Additional information	CLSM (Morphology)
Formulation A: Oral pro-Pheroid® ● Vitamin F Ethyl Ester (65%) ● Kolliphor EL (22%) ● Polyethylene glycol 400 (10%) ● Incromega E3322 (1%) ● Incromega E7101SR (1%) ● DL-Alpha Tocopherol (1%)		Particle size span: 2.22 μm Median particle size: 0.93 μm Distribution of 80% of vesicles: 0.22 μm–1.7 μm Zeta-potential: -23.3 ± 0.8 mV Endotoxin levels: < 5 EU/ml pH measured: 6.05	
Formulation B: Intravenous Pheroid® ● Vitamin F ethyl Ester (14%) ● Kolliphor EL (5%) ● DL-Alpha tocopherol (1%) ● Nitrous oxide water (40%) ● Saline (40%)		Particle size span: 1.53 μm Median particle size: 0.37 μm Distribution of 80% of vesicles: 0.18 μm–0.65 μm Zeta-potential: -21.1 ± 1.1 mV Endotoxin levels: < 5 EU/ml pH measured: 6.45	
Formulation C: Intravenous Pheroid® ● Vitamin F ethyl Ester (2.8%) ● Kolliphor EL (1.2%) ● DL-Alpha tocopherol ● (0,2%)Nitrous oxide water (96%)		Particle size span: 0.88 μm Median particle size: 0.23 μm Distribution of 80% of vesicles: 0.15 μm–0.33 μm Zeta-Potential -24.2 ± 0.6 mV Endotoxin levels: < 5 EU/ml pH measured: 6.35	

filled with 2 ml of the top agar, 0.1 ml of a fresh overnight culture of one of the strains of bacteria and 0.1 ml of the test formulation. Counting of colonies was performed in the absence (–S9; 0.5 ml water) or presence (+0.5 ml S9) of the metabolic activator S9 – a liver homogenate obtained from Aroclor 1254-induced male Fisher rats, as described by Maron and Ames (1983), to provide cytochrome P₄₅₀ enzyme activity [15]. The addition of P₄₅₀ enzyme activity allows for the prediction of the effect of metabolism on the toxicity of a formulation. The final mixture in each culture tube was transferred onto sterile growth media plates and incubated for 3 days at 37 °C.

2.2.4. In vivo toxicity assays

The animals (Sprague-Dawley rats and BALB/c mice) were obtained from and housed in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) -accredited Vivarium (Department of Science and Technology/North-West University Preclinical Drug Development Platform). The animals were housed in group cages (based on treatment and dose received) with maintenance of an artificial 12-h day / night cycle and *ad libitum* access to a conventional rodent diet and water. Temperature was kept at 21 ± 2 °C and a relative humidity of $55 \pm 10\%$ was maintained prior and throughout the study. The ethical aspects of this study were approved by the AnimCare Committee of the North-West University (NWU-00493-16-A5) and the Ethical Committee of the North-West University (06D01). Animals was selected to weigh within a $\pm 20\%$ of the mean weight of all study animals enrolled of that particular species. All animals were euthanized at the end of the appropriate evaluation time through overdose of iso-flurane, followed by decapitation and a gross necropsy.

All animals underwent blood sampling on which the following haematological parameters were analysed: haemoglobin, white cell count and the red blood cell count. For clinical biochemistry the following parameters were measured: liver function proteins (total serum protein, bilirubin, albumin, globulin), hepatic enzymes (alanine transaminase [ALT], alkaline phosphatase [ALP], aspartate transaminase [AST]), electrolyte levels (sodium, potassium, calcium); indicators of kidney function (urea, creatinine); amylase, glucose and a lipid profile (triglycerides, low-density lipoprotein, high-density lipoproteins and

cholesterol) [11]. After euthanasia, the organs were removed during the gross necropsy in a manner that ensured the integrity of the organs, but also provided for the removal of any irrelevant tissue that could influence the weight. Organs dedicated to histopathology were stored in 10% formalin and embedded in paraffin wax, sectioned at 5 μm and stained with Haematoxylin and Eosin (H & E). After fixation, digital images of the samples were investigated with a model PCM 2000 confocal laser scanning microscope connected to a Nikon TE300 inverted microscope equipped (United States) with a Nikon 60x/1.40 Apo Planar oil objective and/or a Nikon 40X/0.75 dry objective. A Nikon DXM 1200 digital camera (United States) in combination with ACT-1 software was used to capture images. The H & E stained sample was excited with the helium-neon ion and argon ion lasers and a neutral density filter was utilized to obtain distinction between the different regions of the sample. Photobleaching was reduced to the minimum with a pinhole size of $\frac{1}{4}$ airy units.

For the oral acute study, the control group received 300 μL of normal drinking water administered through oral gavage. For the oral evaluation of toxicity formulation A were used. The Sprague-Dawley rats were seven weeks old at enrolment in the study and three treatment groups (n = 20 per group) with equal members of both sexes of animals were composed by random selection. The different treatments were administered through oral gavage and the animals were observed multiple times (with special emphasis during the first 4 h) for clinical signs of adverse events during the first 24 h post administration. The dose of 50 mg/kg of the pro-Pheroid® oil-phase components (not the emulsion) was selected. Due to the fact that this system is the oil-phase only (pro-Pheroid®) and this dose is higher than the upper limit foreseen for administration in any of the applications. The functional assessments performed during these observations included changes in physical condition (skin, fur, eyes, and mucous membranes), cardiovascular systems (respiratory and circulatory), excretion (presence of abnormal urinary symptoms and diarrhoea) as well as behavioural changes. The Humane Endpoints Guidance Document was adhered to during the performance of this study [30]. For the subsequent 14 days these observations were performed daily, as well as daily weighing and food consumption monitoring. Blood samples were collected in EDTA

and lithium-heparin tubes 24 h after the administration of formulation A. Animals were sacrificed on day 14, a gross necropsy was performed on each animal and organ weights (brain, thyroid, heart, lungs, stomach, liver, spleen, kidneys) were determined [14]. The method followed (dosages administered, evaluation time frame and observations performed) is also consistent with current literature [31–34].

The Formulations B and C (Pheroid®) were evaluated during the acute intravenous toxicity assay. All intravenous formulations were evaluated as completely formed emulsions and that due to safety aspects (particle size and risks of embolism); it is not feasible to ever administered pro-Pheroid® intravenously. As per the OECD guidelines, only female Sprague Dawley rats with ages ranging from 8 to 10 weeks were selected, since female rats are generally more susceptible to adverse reactions (unless previous data indicates otherwise for a specific compounds). Animals were randomly selected and acclimatized in study housing for 5 days prior to administration of test formulations. A control group (n = 5) was injected with saline to provide a baseline for general conditions as well as the influence of the injection procedure. A dose of 2000 mg/kg (n = 5) of the final emulsion for each test formulation was evaluated; this is the highest dose prescribed by the OECD guidelines. Animals were monitored 3 times in the first 15 min and hourly thereafter for the first 8 h. Daily monitoring was performed as described for the oral acute study for a span of 2 weeks where after animals were euthanized and a gross necropsy (including measuring of organ weights) was performed. This study was repeated on BALB/c mice (also 2000 mg/kg and n = 5) with blood samples only collected from the rats due to the small blood pool of mice. Organs obtained from all animals (both rats and mice) were preserved in a 10% formalin solution to allow for histopathology if gross necropsy findings or haematological and biochemical analysis were to be deemed abnormal [16].

For the oral sub-chronic study, the control group received 300 µl of normal drinking water administered through oral gavage. For the oral evaluation of toxicity formulation A were used, this is the oil-phase only (not the emulsion). The duration of the subchronic evaluation was 90 days according to the procedure prescribed by the OECD guidelines [17]. Literature also indicates that 90 days is appropriate for repeated dose studies [31–40]. This study was performed on Sprague Dawley rats and only pro-Pheroid® (formulation A - Table 2) was administered. The control group and the treatment group (formulation A) contained 30 animals (male n = 15, female n = 15). Animals were randomly assigned to treatment groups and received 50 mg/kg formulation A as daily dose, diluted to 300 µl with water as treatment, or just the control water (300 µl) through oral gavage. Body-weight determination of the animals as well as functional assessments of overall health was performed prior to enrolment and at least once a week during the span of the study. Food consumption was measured weekly per group cages; additional feed administered between formal weighing times was taken into account. After 90 days, animals were euthanized, organs were removed and whole blood was collected. The rats enrolled in the sub-chronic study group were individually placed in metabolic cages (prior fasted for 12 h) for urinalysis. Urine was collected overnight between days 81–85. The samples were kept cold and analysed the following day

with Multistix™ test strips (Siemens, Germany) and the ClinitekStatus® analyser (Siemens, Germany) for the presence of glucose, bilirubin, ketones, specific gravity, erythrocytes, proteins, pH, nitrites and leucocytes in rat urine (Bayer HealthCare). Differences between the groups of the same gender were analysed by means of a frequency count of parameters. Rat blood samples were obtained by tail vein incision and collected in EDTA tubes (haematological screening), lithium-heparin tubes (clinical chemistry) and SST tubes (blood glucose levels) followed by immediate processing and transfer for the analyses.

2.2.5. Statistical analysis

Statistical analyses were performed using Statistica (StatSoft Inc., Germany) and Prism (GraphPad, San Diego, CA, USA). Analyses were performed by one-way ANOVA, with evaluation for normality in distribution. Statistically significant differences were evaluated using the Tukey HSD test or Unequal N HSD test. The Bonferroni test permitted the analysis of weekly bodyweight and food consumption changes within groups. Abnormality in data was analysed with the Dunnett's test or Kruskal-Wallis ANOVA. P-values < 0.05 were considered statistically significant.

3. Results

3.1. Formulation preparation and characterization

The pro-Pheroid® and Pheroid® formulations (A–C) were prepared successfully, and Table 2 provides the product characteristics for each formulation. The medium particle size of A (0.93 µm) was substantially bigger than that of formulation B and C (0.37 µm and 0.23 µm respectively). The largest population of each formulation was measured to be below 2 µm, which is typical for colloidal dispersions (200 nm – 5 µm). The particle size of formulations B and C did not exceed the upper limit of 5 µm deemed as the maximum safe size of individual emulsion drops, for intravenous administration. Formulation A and B follows a bimodal distribution with formulation C demonstrating a unimodal biodistribution, most likely caused by the filtration process. Formulation A had a slightly but not significant decrease in pH when compared to formulation B and C. The filtered formulation (formulation C) has a smaller span of distribution of particles (0.88 µm), a higher zeta-potential (compared to other formulations) and together with the unimodal distribution of particle size this indicates a more uniform vesicle formation throughout.

3.2. In vitro toxicity assay

A pre-incubation assay was performed in the presence of S9 due to the higher sensitivity of this assay. The positive controls added to the test strains (*Salmonella typhimurium* strains TA98, TA100 and TA102) did provide the expected increased histidine positive revertants under control conditions (indicated as statistically different, Table 3). The positive controls therefore provided a good benchmark for positive mutagenicity. The metabolised derivatives of formulation A (pro-Pheroid®) did not induce base-pair or frame shift mutagenesis during

Table 3

The specification of the formulations evaluated for toxicity (indicated as revertants per plate).

	TA 98		TA100		TA102	
	-S9	+S9	-S9	+S9	-S9	+S9
DMSO	36.2 ± 7.0	40.2 ± 8.4	136.4 ± 5.3	140 ± 14.4	581.0 ± 24.2	555.5 ± 60.1
Positive control	38.0 ± 5.8	355.6 ± 82.5 [#]	162.4 ± 30.2	355.6 ± 17.5 [#]	1789 ± 356 [#]	2039 ± 219 [#]
Formulation A 10 µL	26.3 ± 3.2	29.3 ± 7.1	88.3 ± 4.6	81.3 ± 8.1	290.7 ± 2.2	318.7 ± 9.0
Formulation A 20 µL	19.3 ± 5.8	25.0 ± 6.1	95.0 ± 17.4	93.7 ± 21.6	324.0 ± 34.9	313.3 ± 4.5
Formulation A 100 µL	18.7 ± 5.7	29.3 ± 5.5	115.3 ± 6.0	111.0 ± 4.1	327.7 ± 9.3	273.3 ± 8.2

[#]p < 0.001, *p < 0.05, significant different from DMSO control groups for each study.

Table 4The body weights and organ weights (mean $\pm$ SD) of animals during acute and subchronic toxicological evaluations.

	Acute Sprague Dawley rats 2000 mg/kg IV Pheroid®			Acute BALB/c mice 2000 mg/kg IV Pheroid®			Subchronic Sprague Dawley rats 50 mg/kg oral pro-Pheroid®			
	Control (n = 5) ♀	Formulation B (n = 5) ♀	Formulation C (n = 5) ♀	Control (n = 5) ♀	Formulation B (n = 5) ♀	Formulation C (n = 5) ♀	Control (n = 15) ♀	Formulation A (n = 15) ♀	Control (n = 15) ♂	Formulation A (n = 15) ♂
Body weight										
Initial	202.6 $\pm$ 6.6	205.8 $\pm$ 8.9	205.5 $\pm$ 8.6	19.9 $\pm$ 1.3	19.5 $\pm$ 1.4	20.1 $\pm$ 0.6	137.8 $\pm$ 4.6	136.0 $\pm$ 8.4	113.6 $\pm$ 7.2	114.3 $\pm$ 5.3
Terminal	227.8 $\pm$ 3.4	226.4 $\pm$ 7.6	228.5 $\pm$ 8.6	20.8 $\pm$ 0.7	20.2 $\pm$ 0.9	20.1 $\pm$ 0.9	219.6 $\pm$ 12.8	222.1 $\pm$ 12.5	131.6 $\pm$ 10.0	147.6 $\pm$ 23.1
Organ weights as % of final body weight										
Brain	0.7 $\pm$ 0.1	0.7 $\pm$ 0.0	0.7 $\pm$ 0.1	1.8 $\pm$ 0.2	1.6 $\pm$ 0.2	2.0 $\pm$ 0.2	0.6 $\pm$ 0.1	0.5 $\pm$ 0.1	0.7 $\pm$ 0.1	1.2 $\pm$ 0.9
Heart	0.4 $\pm$ 0.1	0.4 $\pm$ 0.0	0.4 $\pm$ 0.0	1.0 $\pm$ 0.2	0.7 $\pm$ 0.2	0.8 $\pm$ 0.1	0.4 $\pm$ 0.1	0.4 $\pm$ 0.0	0.4 $\pm$ 0.0	0.6 $\pm$ 0.4
Kidney (L)	0.3 $\pm$ 0.1	0.3 $\pm$ 0.0	0.4 $\pm$ 0.0	0.9 $\pm$ 0.1	0.9 $\pm$ 0.1	0.9 $\pm$ 0.2	0.5 $\pm$ 0.1	0.5 $\pm$ 0.1	0.4 $\pm$ 0.1	0.6 $\pm$ 0.3
Kidney (R)	0.3 $\pm$ 0.1	0.4 $\pm$ 0.0	0.4 $\pm$ 0.0	1.0 $\pm$ 0.3	0.8 $\pm$ 0.2	0.9 $\pm$ 0.2	0.5 $\pm$ 0.0	0.5 $\pm$ 0.1	0.5 $\pm$ 0.2	0.7 $\pm$ 0.4
Liver	3.3 $\pm$ 0.4	3.5 $\pm$ 0.1	3.5 $\pm$ 0.2	5.8 $\pm$ 0.7	5.3 $\pm$ 0.4	5.3 $\pm$ 0.8	2.3 $\pm$ 0.2	2.3 $\pm$ 0.2	2.1 $\pm$ 0.4	4.1 $\pm$ 1.9
Lungs	0.6 $\pm$ 0.1	0.6 $\pm$ 0.1	0.8 $\pm$ 0.1	1.1 $\pm$ 0.2	0.8 $\pm$ 0.3	1.0 $\pm$ 0.2	0.5 $\pm$ 0.1	0.5 $\pm$ 0.2	0.6 $\pm$ 0.1	1.1 $\pm$ 0.8
Spleen	0.3 $\pm$ 0.0	0.3 $\pm$ 0.0	0.3 $\pm$ 0.1	0.8 $\pm$ 0.2	0.5 $\pm$ 0.1*	0.6 $\pm$ 0.2	0.4 $\pm$ 0.1	0.3 $\pm$ 0.1	0.4 $\pm$ 0.0	0.5 $\pm$ 0.3
Stomach (full)	1.9 $\pm$ 0.5	2.0 $\pm$ 0.2*	1.7 $\pm$ 0.3	1.9 $\pm$ 0.3	1.4 $\pm$ 0.3*	1.1 $\pm$ 0.2*	0.6 $\pm$ 0.2	0.5 $\pm$ 0.1	0.6 $\pm$ 0.0	1.2 $\pm$ 0.7
Thyroid	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	-	-	-	0.2 $\pm$ 0.1	0.1 $\pm$ 0.0	0.2 $\pm$ 0.1	0.3 $\pm$ 0.2

* p < 0.05 significant different from control groups for each study.

this analysis.

3.3. In vivo toxicity assays

3.3.1. Food consumption and condition

No animals (Sprague Dawley rats or BALB/c mice) died or exhibited any adverse events, during any of the 14 day acute (oral and intravenous) or the 90 day sub-chronic evaluations. No abnormal changes in body weight, food consumption, respiration, coat condition, movement and behaviour were present in any of the animals during scheduled examinations in all the studies. The initial and final body weights of animals in different test groups are presented in Table 4.

3.3.2. Organ weights, haematology, clinical biochemistry and urinalysis

The organ weights were determined after euthanasia of all the subjects included in the different study groups (Table 4). Note that due to the difficulty of identifying the thyroid of BALB/c mice, this organ was not isolated during the investigation. The only statically significant differences present were in weight of the full stomach during the intravenous acute administration evaluations in both mice and rats, and the spleens of the mice receiving treatment B (Pheroid®).

Hematologic analysis demonstrated no statistical significant changes in blood parameters in any of the treatments (Supplement A). The white blood cell count was however increased in all of the animals (both control and test groups) included in the acute study of the oral administration of formulation A (pro-Pheroid®). The white blood cell counts of all the animals in intravenous acute evaluation and the oral sub-chronic evaluations were normal.

Clinical chemistry indicated a statistically significant decrease in ALT for the animals that received formula C during the acute intravenous evaluation (Fig. 4). Whilst an increase in ALT is associated with liver damage and the destruction of hepatocytes, a decrease is associated with healthy liver function.

Urea was also lower (7.5 ± 0.4 mmol/L compared to the control value of 8.6 ± 0.5 mmol/L) in the animals receiving formulation B (Fig. 5) during the acute intravenous dosing. This is not considered significant in terms of toxicity since an increase is usually associated with malfunction. In the subchronic study group creatinine (Fig. 5) was statistically significantly increased (1.2 fold) for the female test animals, indicating a lowered clearance of creatinine by the kidneys. To evaluate whether test formulation related renal toxicity was present, histopathology and urine analysis was included in this study. This

increase in creatinine was not present in any other test group and all the histopathology reports came back as negative for treatment-related toxicity with no malfunction of organs present. Fig. 6 provides a selection of microscopy images of major organs (including kidney tissue) removed from animals in the formulation A (pro-Pheroid®) treatment group part of the sub-chronic study.

No significant differences were present in the results from urinalysis. All urine samples tested negative for glucose and no significant levels of bilirubin were present in those samples. The levels of ketones, specific gravity of samples, erythrocytes, pH levels, proteins, nitrites and leukocytes in the samples from all test groups and control animals similarly showed normal distribution with no disparities bearing any statistical significance. A full summary of biochemistry and haematological data is referred to in Supplement 2. The frequency table of the results is displayed in Supplement 3.

4. Discussion

The Pheroid® and pro-Pheroid® systems both contain the same oil components, which differ from that of liposomes by the absence of phospholipids and cholesterol. Pro-Pheroid® is a precursor self-assembly form of the system, with the only difference from Pheroid® being the lack of the water phase.

The recently published recommendations by Siegerst and co-workers (2018) were taken into account where a step-by-step evaluation was done on this delivery system [41]. The physicochemical identity (particle size, distribution and endotoxin contamination) was characterized taking into account the constraints of the current drug delivery system with the aim of providing clear parameters for future products for intravenous administration. To provide a reflection of the stability of the formulation, Zeta-potential was measured to ensure that the product is stable and that larger emulsion droplets will not form making it dangerous for *in vivo* administration. The shape and internal structure of the formed emulsions was determined by CLSM. Particle size was measured through both a quantitative analysis (particle size distribution) and visualization (CLSM). The presence of endotoxins as possible impurities was determined. A step-wise increased dose evaluation (as prescribed by the OECD guidelines) was followed to allow for a predictive dosimetry for the evaluated systems.

All the formulations evaluated during this study demonstrated emulsion particles mostly smaller than 1 μ m in diameter with none exceeding 5 μ m. Individual emulsion particles with a size above the

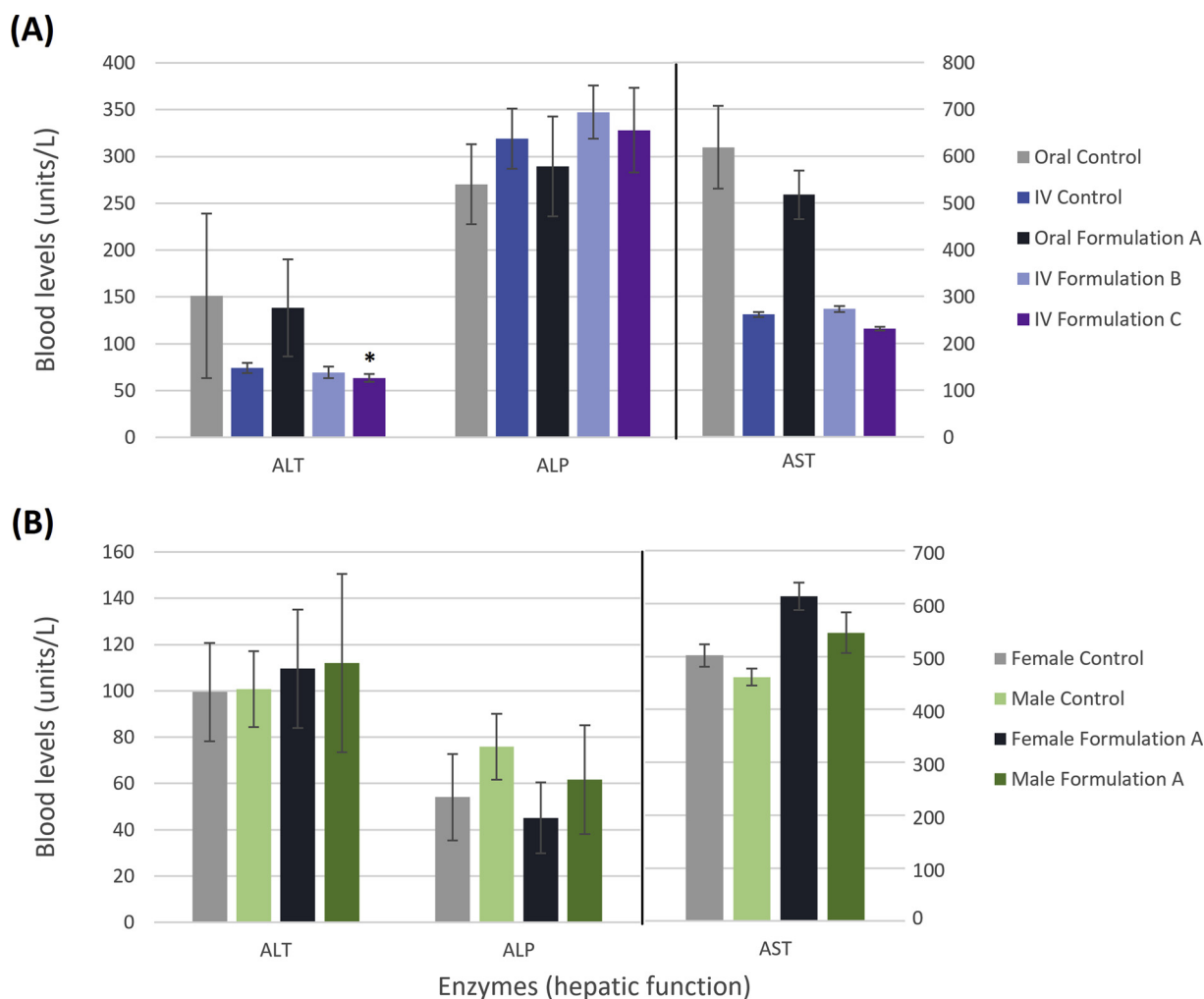


Fig. 4. Hepatic enzyme levels of Sprague-Dawley rats during a) acute oral and intravenous evaluations [female only-male data in supplementary text] and b) oral sub-chronic study (male and female) (* $p < 0.05$ significant different from control group).

safety parameters can cause emboli blocking small blood vessels with lethal results. The total volume of the emulsion administered as well as the concentration of large particles present in these formulations is important contributors to the incidence of adverse events [42]. To focus only on a reduction of particle size will therefore not guarantee the safety of the formulation. Traditionally 5 μm is considered as the upper limit (no more of 0.05% of the formulation may exceed this) allowed for particle size of intravenous emulsion preparations, and is indicated as such in older versions of the British Pharmacopoeia. It was contradictorily determined that commercially available total parenteral nutritional formulations do not always conform to these parameters since it was demonstrated that emulsion droplets of larger diameters (exceeding 7.5 μm) may deform and pass through pulmonary blood vessels depending on their consistency. During this study it was demonstrated that the Pheroid® delivery system allows filtrations by normal 0.22 μm filters and is therefore an uncomplicated system to adapt for intravenous administration. It was postulated by Koster and co-workers (1996) that the individual characteristics or components of the formulation may contribute to the degree of toxicity associated with larger particle size [43]. No agreement on the influence of particle size on adverse events or the exact ranges that intravenous emulsions should abide by, are available in literature [42–44]. Our aim was to restrict the particle size of the intravenous formulations in this study to the smallest possible, economically viable particle size, while concomitantly filtering formulations to provide sterility. Although the Zeta-potential measured for the formulations is lower than the ± 25 mV stated in

literature as beneficial, the formulations did demonstrate sufficient stability based on particle size distributions evaluated over a 7-day period with significant changes measured [45]. We do suggest that the formulations should be filtered just before administration to ensure that the particle sizes are narrowly restricted (below 1 μm) and sterility is maintained. Another option to ensure sterility would be the use of gamma irradiation providing the emulsion is stable during this process. Additionally, the API can be sensitive to degradation by gamma irradiation and this can also be a factor. The pH of the formulations as well as presence of bacterial endotoxins were monitored to ensure adequate formulations for intravenous administration. CLSM demonstrated that satisfactory emulsion formation presenting with internal structure and the correct morphology.

The pro-Pheroid® component did not demonstrate mutagenic effects in the AMES test. Formulations containing similar fatty acid ingredients in literature also were void of mutagenicity [46–48]. Another component of the delivery system, alpha-tocopherol, also demonstrated no mutagenicity in a study by Karekar and co-workers [49]. The testing for mutagenicity in the presence of cytochrome P450 enzymes (S9) demonstrated that both the original system as well as any breakdown products are safe bearing no effect on the structural integrity of cellular DNA.

The highest dose prescribed by OECD guideline 420 (2000 mg/kg) was reached during the acute evaluation of the intravenously administered formulations B and C, with no adverse events identified. During intravenous administration to BALB/c mice and Sprague Dawley

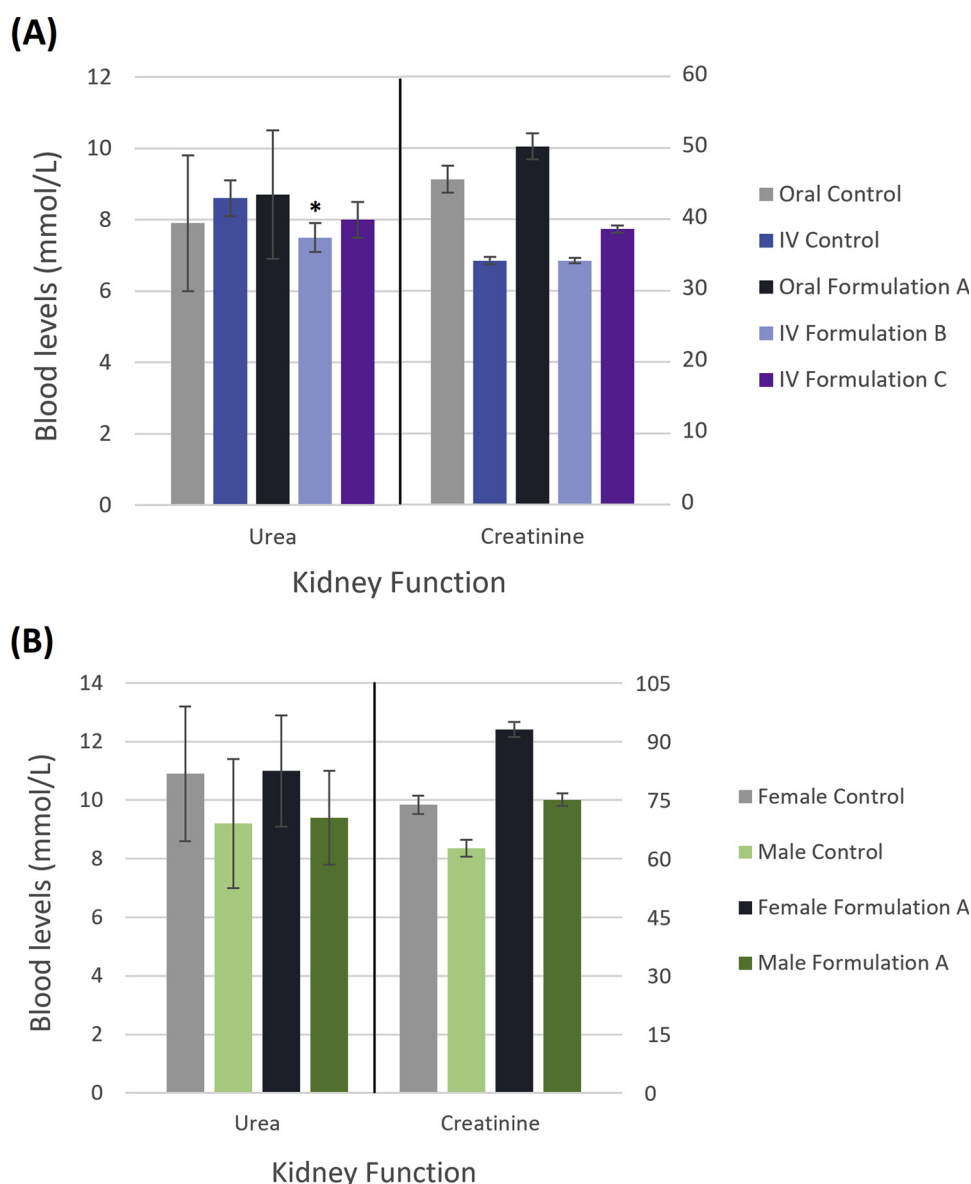


Fig. 5. Urea and creatinine levels of Sprague-Dawley rats during a) acute oral and intravenous evaluations [female only-male data in supplementary text] and b) oral sub-chronic study (male and female)(*p < 0.05 significant different from control group).

rats, particular care was taken to monitor animals intensely for acute shock symptoms due to intravenous administration and animals were unhindered by this administration. Animals were in good health during all the scheduled examinations of both the acute and sub-chronic studies; final body weights in all the groups were characteristic for these animal species. During these studies the aim was mainly to compare the parameters of the control groups with that of the test groups and not comparing them with literature values. It was demonstrated that housing, breeding and biological rhythm of animals housed in animal facilities has a large influence on haematology and blood chemistry levels [50]. Our studies therefore relied on the differences between the control groups and the test groups to increase accuracy, although none of the parameters evaluated was abnormal to the extent that indicates disease or distress.

The following differences were noted when compared with the control values.

- (1) The increased white blood cell count in control and test groups of the acute oral study (pro-Pheroid®) was determined not to be related to treatment with the systems. This phenomenon was present in all four groups evaluated during the study and not the tests groups only. Furthermore, it was not present in any of the acute intravenous study test groups (with the formulation being 100% bioavailable) or the sub-chronic study (with continuous administration). No other significant changes in haematology were present.
- (2) The decrease in ALT demonstrated as statistically significant during the blood chemistry analysis of acutely intravenously treated animals (Pheroid® formulation C) is not associated with any disease process. A positive correlation between lowered cholesterol and ALT also exist, with high levels of cholesterol and fatty liver disease associated with high levels of ALT. It is notable, that the system incorporates essential fatty acids, which is well known for the treatment of distorted lipid profiles.
- (3) The decrease in cholesterol levels was therefore not an unexpected result, which was present in the male group of the oral acute study-although it would be unwise to assume this to be a therapeutic effect.
- (4) The statistical significant increase in creatinine levels in the sub-chronic study for the female treatment group is noteworthy. To investigate this phenomena, histopathology examinations as well as

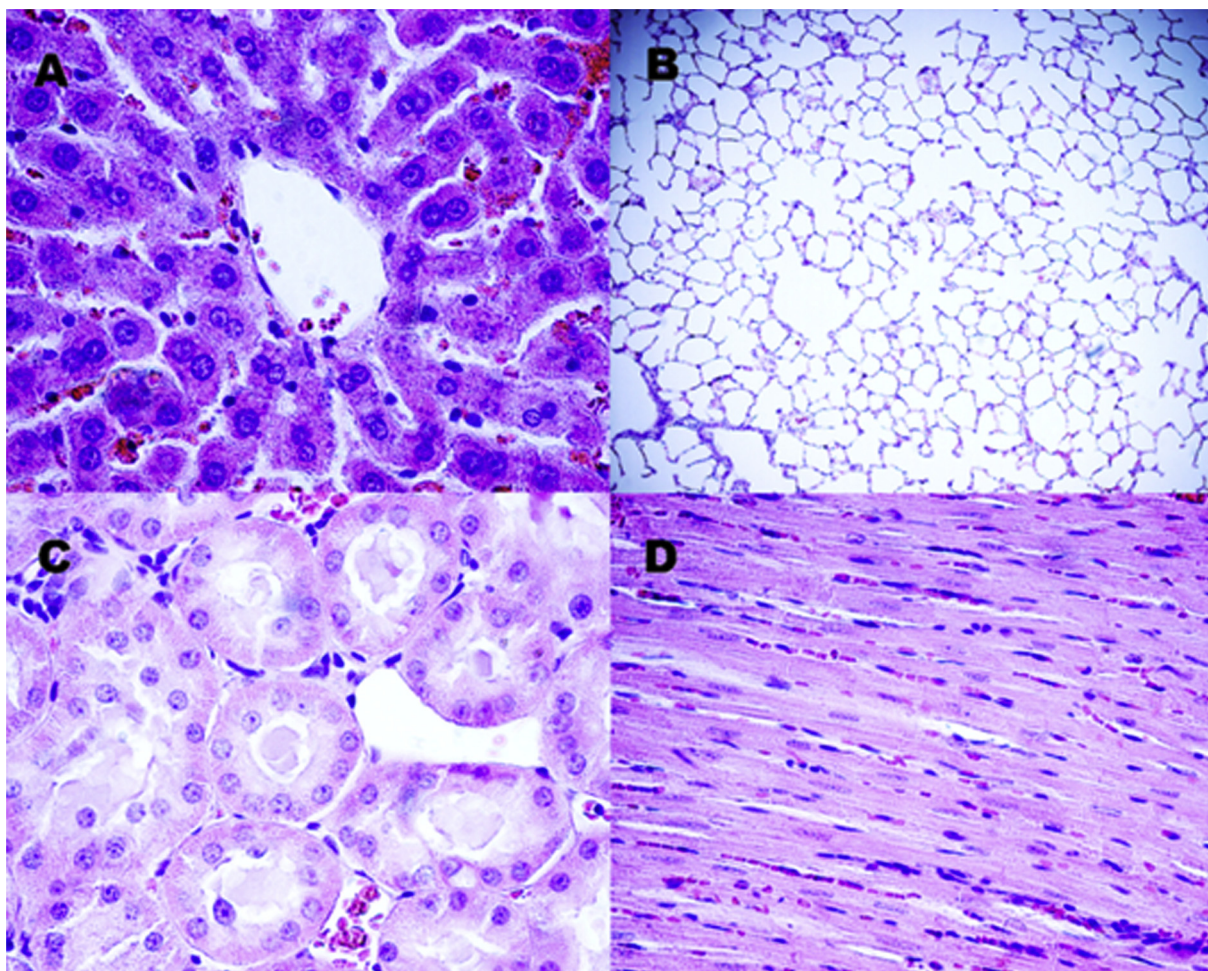


Fig. 6. Light microscopy images of organs from the sub-chronic treatment group (pro-Pheroid®) with a) liver b) lung c) kidney and d) heart. All tissues were stained with Haematoxylin and Eosin.

the urine analysis were performed, which are indicators of structural integrity. Both urine analysis and morphology demonstrated normal function. There was an absence of any pathology related differences in organ weights determined after termination of animals. No treatment related pathology was identified as present in any of the histopathology examinations of removed organs.

Emulsion type systems containing essential fatty acids has been proven to be non-toxic and biocompatible and even sometimes have the ability to negate the toxicity that is imposed by the API's packaged in them [51,52]. There are also additional oil-phase components (such as a *Silybum marianum* extract proposed by Kalantari et al) that can be added to enhance the hepatoprotective effects of emulsions in the case of particularly toxic compounds which clearly demonstrates the adaptability of these systems [51,53].

5. Conclusion

This evaluation did not identify any risk factors present for toxicity during oral or intravenous administration of the tested formulations during acute or repeated dosing. The maximum dose tested in Sprague Dawley rats and BALB/c mice was 2000 mg/kg of Pheroid® formulations B and C and 50 mg/kg of the pro-Pheroid® formulation A. It is important that normal precautions for intravenous safety of emulsions (particle size, bacterial endotoxin measurement and sterility) have to be adhered to. Future studies might be needed to determine the sub-chronic adequateness of the intravenous administered formulations

should the need arise for multiple intravenous administrations. It would also be good scientific practice to evaluate the toxicity and safety pharmacology of the API incorporated in the Pheroid® or pro-Pheroid® system in a larger animal model following the envisioned route of administration. The effect of the system on the developing animal foetus should be evaluated prior to administration during pregnancy and lactation.

It is hypothesised that the Pheroid® delivery system will inherently contribute less toxicity (compared to other lipid-based drug delivery systems) based on the non-toxic ingredients used during manufacturing. Due to the non-rigid characteristics of emulsions and in particular also Pheroid® (when compared to liposomes for instance) that allows for the may deformation and passage through smaller blood vessels, it is envisioned that this system will allow a higher safety margin than other lipid-based drug delivery systems.

It is also critically important to evaluate the toxicity of the drug delivery system with the selected pharmaceutical entities entrapped to ensure that alterations in biodistribution and possible slow release mechanisms brought about by the system does not alter the toxicity profile of the pharmaceutical ingredient itself in a negative way.

Funding

Funding was received from the National Research Foundation (NRF) of South Africa (grant number SFH 14070573914), as well as the Nuclear Technologies in Medicine and Biosciences Initiative (NTEMBI). Opinions and conclusions arrived at, are those of the authors and not

necessarily to be attributed to the NRF and NteMBI. No conflict of interest was created by receiving this funding.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Acknowledgements

Figs. 2 and 3 of this article was drawn by scientific illustrator Cristina Sala Ripoll (www.cristinasalaripoll.com). Scientific editing was done by The Expert Editor (<https://experteditor.com.au>). The authors would also like to thank Prof. Faans Steyn (Statistical Consultation Service NWU) for assisting with statistical analysis. Assistance with the characterization of the formulations was provided by Dr. Matthew Glyn and Mr. Lesley Masetle. Laboratory Animal Technicians involved in this study was Mr. Cor Bester, Mr. Kobus Venter and Mrs. Antionette Fick of the DST/NWU PCDDP Vivarium are thanked.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.toxrep.2019.08.012.

References

- [1] I. Solé, C. Solans, A. Maestro, C. González, J.M. Guitiérrez, Study of nano-emulsion formation by dilution of microemulsions, *J. Colloid. Interfaces Sci.* 376 (2012) 133–139.
- [2] J.D. Steyn, L.H. Du Plessis, A.F. Kotzé, Nasal delivery of recombinant human growth hormone: in vivo evaluation with Pheroid technology and N-Trimethyl chitosan chloride, *J. Pharm. Pharm. Sci.* 13 (2010) 263–273.
- [3] G.A. Bonaterra, F. Wakenhut, D. Röhlein, M. Wolf, B.R. Bistran, et al., Cytoprotection by omega-3 fatty acids as a therapeutic drug vehicle when combined with nephrotoxic drugs in an intravenous emulsion: effects on intraglomerular mesangial cells, *Toxicol. Rep.* 1 (2018) 843–857.
- [4] J.D. Steyn, L. Wiesner, L.H. Du Plessis, A.F. Grobler, P.J. Smith, W.-C. Chan, et al., Absorption of the novel artemisinin derivatives artemisone and artemiside: potential application of Pheroid technology, *Int. J. Pharmaceut.* 414 (2011) 260–266.
- [5] Grobler, A.F., Zeevaart, J.R. 2015. Pharmaceutical composition. (Patent, WO 2015/063746 A1).
- [6] R.G. Strickley, Solubilizing excipients in oral and injectable formulations, *Pharm. Res.* 21 (2004) 201–230.
- [7] B.Q. Li, X. Dong, S.H. Fang, J.Y. Gao, G.Q. Yang, H. Zhao, Systemic toxicity and toxicokinetics of a high dose of polyethylene glycol 400 in dogs following intravenous injection, *Drug. Chem. Toxicol.* 34 (2011) 208–212.
- [8] G.L. Ackland, A. Gutierrez Del Arroyo, S.T. Yao, R.C. Stephens, A. Dyson, N.J. Klein, et al., Low-molecular-weight polyethylene glycol improves survival in experimental sepsis, *Crit. Care Med.* 38 (2010) 629–636.
- [9] H. Kappus, A.T. Diplock, Tolerance and safety of vitamin E: a toxicological position report, *Free Radic. Biol. Med.* 13 (1992) 55–74.
- [10] E. Reiter, Q. Jiang, S. Cristen, Anti-inflammatory properties of α - and γ -tocopherol, *Mol. Aspects Med.* 28 (2007) 668–691.
- [11] C.A. McCary, H. Abdala-Valencia, S. Berdnickovs, J.M. Cook-Mills, Supplemental and highly elevated tocopherol doses differentially regulate allergic inflammation: reversibility of α -tocopherol and γ -tocopherol's effects, *J. Immunol.* 186 (2011) 3674–3685.
- [12] U.N. Das, Essential fatty acids: biochemistry, physiology and pathology, *Biotechnol. J.* 1 (2006) 420–439.
- [13] S.Y. Park, R. Seetharman, M.J. Ko, D.Y. Kim, T.H. Kim, M.K. Yoon, J.H. Kwak, S.J. Lee, Y.S. Bae, Y.W. Choi, Ethyl linoleate from gargic attenuates lipopolysaccharide-induced pro-inflammatory cytokine production by inducing heme oxygenase-1 in RAW264.7 cells, *Int. Immunopharmacol.* 19 (2014) 253–261.
- [14] M.J. Hubler, A.J. Kennedy, Role of lipids in the metabolism and activation of immune cells, *J. Nutr. Biochem.* 34 (2016) 1–7.
- [15] D.M. Maron, B.N. Ames, Revised methods for the salmonella mutagenicity test, *Mutat. Res.* 113 (1983) 173–215.
- [16] OECD, OECD Guidelines for Testing of Chemicals No 420: Acute Oral Toxicity-Fixed Dose Method, OECD, Paris, 1992.
- [17] OECD, OECD Guidelines for Testing of Chemicals No 408: Subchronic Oral Toxicity - Rodent, 90 day study, OECD, Paris, 1992.
- [18] L.H. Du Plessis, J. Lubbe, T. Strauss, A.F. Kotzé, Enhancement of nasal and intestinal calcitonin delivery by the novel Pheroid fatty acid based delivery system, and by N-trimethyl chitosan chloride, *Int. J. Pharmaceut.* 385 (2010) 181–186.
- [19] L.H. Du Plessis, E. Marais, M. Faruq, A.F. Kotzé, Applications of lipid based formulation technologies in the delivery of biotechnology-based therapeutics, *Curr. Pharma. Biotechnol.* 15 (2014) 659–672.
- [20] Meyer, P.J. Inventor. Administration media for analgesic, anti-inflammatory and anti-pyretic drugs containing nitrous oxide and pharmaceutical compositions containing such media and drugs. Patent: 1996, WO97/17978.
- [21] J. Kleynhans, P. Grobler, A. Grobler, C. Bester, M. Sathekge, J.R. Zeevaart, Enhancing the bioavailability of ^{99m}Tc -MIBI in myocardial perfusion imaging by utilizing the Pheroid technology drug delivery system, Poster session presented at the International Conference on Integrated Medical Imaging in Cardiovascular Diseases. Contribution ID: 103, International Atomic Energy Agency, Vienna, 2016.
- [22] J. Kleynhans, A.F. Grobler, T. Ebenhan, M.M. Sathekge, J.-R. Zeevaart, Radiopharmaceutical enhancement by drug delivery systems: a review, *J. Cont. Release.* 287 (2018) 177–193.
- [23] G.E.E. Krause, A.F. Grobler, J.P.D. Goldring, Comparing antibody responses in chickens against plasmodium falciparum lactate dehydrogenase and glycer-aldehyde-3-phosphate dehydrogenase with Freund's and Pheroid adjuvants, *Immunol. Invest.* 44 (2015) 627–642.
- [24] D. Le Roux, P.B. Burger, J. Niemand, A. Grobler, P. Urban, Fernandez-Busquets, et al., Novel S-adenosyl-L-methionine decarboxylase inhibitors as potent anti-proliferative agents against intraerythrocytic plasmodium falciparum parasites, *Int. J. Parasitol. Drugs Drug. Resist.* (2014) 28–36.
- [25] L.H. Du Plessis, K. Govender, P. Denti, L. Wiesner, In vivo efficacy and bioavailability of lumefantrine: evaluating the application of Pheroid technology, *Eur. J. Pharm. Biopharm.* 97 (2015) 68–77.
- [26] L.H. Du Plessis, A.C. Van Niekerk, M.M. Maritz, A.F. Kotzé, In vitro activity of Pheroid vesicles containing antibiotics against plasmodium falciparum, *J. Antibiot.* 65 (2012) 609–614.
- [27] C. Slabbert, L.H. Du Plessis, A.F. Kotzé, Evaluation of the physical properties and stability of two lipid drug delivery systems containing mefloquine, *Int. J. Pharmaceut.* 409 (2011) 209–215.
- [28] L. Grobler, A. Grobler, R. Haynes, C. Masimirembwa, R. Thelingwani, P. Steenkamp, et al., The effect of the Pheroid delivery system on the *in vitro* metabolism and *in vivo* pharmacokinetics of artemisone, *Expert. Opin. Drug. Met. Toxicol.* 10 (2014) 313–325.
- [29] O. Peña-Rodas, R. Martinez-Lopez, R. Hernandez-Rauda, Occurrence of Aflatoxin M₁ in cow milk in El Salvador: Results from a two-year survey, *Toxicol. Rep.* 5 (2018) 671–678.
- [30] OECD, OECD guidance document on the recognition, assessment, and use of clinical signs as humane endpoints for experimental animals used in safety evaluation, OECD, Paris, 2000.
- [31] T. Manaharan, S. Chakravarthi, A.K. Radhakrishnan, U.D. Palanisamy, In vivo toxicity evaluation of a standardized extract of Syzygium aqueum leaf, *Toxicol. Rep.* 1 (2014) 718–725.
- [32] S. Gopi, J. Jacob, K.Y. Mathur, Acute and subchronic oral toxicity studies of hydrogenated curcuminoid formulation “CuroWhite” in rats, *Toxicol. Rep.* 3 (2016) 817–825.
- [33] U. Saleem, S. Amin, B. Ahmad, H. Azeem, F. Anwar, et al., Acute toxicity evaluation of aqueous ethanolic extract of Saccharum munja Roxb. Roots in albino mice as per OECD 425 TG, *Toxicol. Rep.* 4 (2017) 580–585.
- [34] R. Sundaram, M. Karuppaiah, P. Shanthi, P. Sachdanandam, Acute and sub acute studies of catechol derivatives from Semecarpus anacardium, *Toxicol. Rep.* 5 (2018) 231–239.
- [35] J. Jacob, A. Amalraj, C. Divya, S. Janadri, P.M. Manjunatha, et al., Oral toxicity study of sports nutritional powder in Wistar rats: a 90 day repeated dose study, *Toxicol. Rep.* 5 (2018) 497–503.
- [36] Y. Pei, R. Wei, J. Sun, H. Gao, Safety assessment of freeze-dried powdered Cassiae Semen: evaluation of chronic toxicity (26-week) in Sprague-Dawley rats, *Toxicol. Rep.* 4 (2017) 143–150, <https://doi.org/10.1016/j.toxrep.2017.02.003>.
- [37] S.-H. Moon, D. Kim, N. Shimizu, T. Okada, S. Hitoe, H. Shimoda, Ninety-day oral toxicity study of rice-derived γ -oryzanol in Sprague-Dawley rats, *Toxicol. Rep.* 4 (2017) 4–9.
- [38] M. Majeed, K. Nagabhushanam, S. Natarajan, S. Bani, A. Pandey, S.K. Karri, Investigation of repeated dose (90 day) oral toxicity, reproductive/developmental toxicity and mutagenic potential of “Calebin A”, *Toxicol. Rep.* 2 (2015) 580–589.
- [39] K. Berge, B. Roberson, L. Burri, Safety assessment of Surperba™ krill powder: Subchronic toxicity study in rats, *Toxicol. Rep.* 2 (2015) 144–151.
- [40] T. Katsumata, T. Ishibashi, D. Kyle, A sub-chronic toxicity evaluation of a natural astaxanthin-rich carotenoid extract of Paracoccus carotinifaciens in rats, *Toxicol. Rep.* 1 (2014) 582–588.
- [41] S. Siegrist, E. Cörek, P. Detampel, J. Sandström, P. Wick, J. Huwyler, Preclinical hazard evaluation strategy for nanomedicines, *Nanotoxicol.* 13 (2019) 73–99.
- [42] K. Hörmann, A. Zimmer, Drug delivery and drug targeting with parenteral lipid nanoemulsions – a review, *J. Control. Release.* 233 (2016) 85–98.
- [43] V.S. Koster, P.F.M. Kuks, R. Langer, H. Talsma, Particle size in parenteral fat emulsions, what are the true limitations? *Int. J. Pharmaceut.* 134 (1996) 235–238.
- [44] R.C. Mehta, L.F. Head, A.M. Hazrati, M. Parr, R.P. Rapp, P.P. DeLuca, Fat emulsion particle-size distribution in total nutrient admixtures, *Am. J. Hosp. Pharm.* 49 (1992) 2749–2755.
- [45] I. Roland, G. Piel, L. Delattre, B. Evard, Systematic characterization of oil-in-water emulsions for formulation design, *Int. J. Pharmaceut.* 263 (2003) 85–94.
- [46] L.M. Arterburn, K.D. Boswell, T. Lawlor, M.A. Cifone, H. Murli, D.J. Kyle, In vitro genotoxicity testing of ARASCO® and DHASCO® oils, *Food. Chem. Toxicol.* 38 (2000) 971–976.
- [47] S. O'Hagan, A. Menzel, A subchronic 90-day oral rat toxicity study and in vitro genotoxicity studies with a conjugated linoleic acid product, *Food. Chem. Toxicol.* 41 (2003) 1749–1760.
- [48] R.A. Matulka, O. Noguchi, N. Nosaka, Safety evaluation of a medium- and long-chain triacylglycerol oil produced from medium-chain triacylglycerols and edible

- vegetable oil, *Food Chem. Toxicol.* 44 (2006) 1530–1538.
- [49] V. Karekar, S. Joshi, S.L. Shinde, Antimutagenic profile of three antioxidants in the Ames assay and the *Drosophila* wing spot test, *Mutate. Res.* 468 (2000) 183–194.
- [50] J.P. Braun, M. Aktas, H. Lefebvre, A.G. Rico, P.L. Toutain, Clinical enzymology for the assessment of organ damage: interspecific differences, *Comp. Haematol. Int.* 3 (1993) 27–32.
- [51] A. Kalantari, A. Salimi, H. Kalantari, J.E. Brooji, I. Rashidi, et al., The hepatoprotective effect of livergol microemulsion preparation (nanoparticle) against bromobenzene induced toxicity in mice, *Toxicol. Rep.* 6 (2019) 444–448.
- [52] G.A. Bonaterra, F. Wakenhut, D. Rothlein, M. Wolf, B.R. Bistrian, et al., Cytoprotection by omega-3 fatty acids as therapeutic drug vehicle when combined with nephrotoxic drugs in an intravenous emulsion: effects on intraglomerular mesangial cells, *Toxicol. Rep.* 1 (2014) 843–857.
- [53] S. Ghosh, S. Ghosh, P.C. Sil, Role of nanostructures in improvising oral medicine, *Toxicol. Rep.* 6 (2019) 358–386.