6. Development of novel Nanoparticulate Drug Delivery Systems (NDDS)

6.1. Introduction

To focus on cancer where blatantly cytotoxic drugs with small if any therapeutic margins are classically employed, the issue of specific pharmacokinetic distribution to tumourous tissue is of paramount importance to increase efficacy and decrease systemic toxicity. The great need for novel innovative NDDS is highlighted by the knowledge that many treatment failures are attributed to inadequate drug delivery alone, regardless of the actual effectiveness of the drugs. [98] The use of intelligent delivery systems is imperative to realize the full potential of chemotherapy. DeGeorge *et al*. [84] has identified several advantages afforded by drug delivery systems, including: targeting to tumour; minimization of systemic toxic effects; prolongation of therapeutic drug concentrations; practical administration of highly hydrophobic drugs and membrane transport of highly hydrophilic drugs into the tumour cells.

Of importance is that NDDS can passively or actively compensate for pharmacokinetic profiles that are not conducive to effective therapy. Through the ability to dictate pharmacokinetics and reliant upon stable co-encapsulation of drug combinations, NDDS embody the enabling technology that allows ratio-dependent synergistic FRDC identified *in vitro* to be translated into *in vivo* applications. [99] FRDC consisting of more than two drugs or in combination with biological agents is conceivable.

The ultimate success of any novel NDDS is dependent upon various dynamic factors (many unforeseeable). In truth, these delivery systems are often assembled empirically via convention through identification of what ratio of drug and amphiphile combine well to produce nanoparticles of the desired characteristics (encapsulation efficacy, size and zeta potential) and not through full pre-determined thermodynamic understanding. Drug encapsulation efficacy is

inextricably dependent upon the drugs (structure), the type of amphiphile used and their respective ratios. [80]

The choice of copolymer structure or mixture of amphiphiles used considering the HLB may well need to be reviewed in order to accommodate the concentration of a particular drug/s (drug-excipient compatibility) in aqueous solution. Additional considerations for choice of amphiphile include drug encapsulation efficacy, the attained particle size, zeta potential (electrostatic stability), toxicity as well as the cost and regulatory status.

In this study, a diversified portfolio of NDDS was investigated to increase the probability of success. With due consideration to the stated TPP (Chapter 3) and available resources, the portfolio of NDDS under development included:

A) Riminocelles™

The primary aim of this stage of development was to develop and characterise a passively tumour targeting NDDS that co-encapsulates a synergistic FRDC of PTX and B663 (identified in Chapter 5) at clinically relevant concentrations. Knowing that both PTX and B663 are highly lipophilic, the simplest choice for co-formulation was a micelle with a hydrophobic core.

In terms of composition, Riminocelles can be described as a binary mixed lipopolymeric micellular system (Figure 6.1.) assembled from a mixture (S_{mix}) of the commercially available amphiphiles, DSPE PEG 2000 (Figure 6.2.) and phosphatidylcholine (Figure 6.3.) as the co-surfactant.

Advantageously, such PEGylated diacyl lipids are known to self-assemble at very low critical micellular concentration (CMC) values making them very useful for prolonged systemic circulation - a requirement for successful passive tumour targeting via the EPR effect. [100] Importantly, a low CMC value is required to ensure that the assembled system can sustain the dilution encountered upon IV administration. Lipopolymeric micelles constructed from PEGylated diacyl lipids are

thought to possess superior stability compared to conventional polymeric micelles owing to greater hydrophobic interactions due to the presence of two highly lipophilic fatty acyl chains. [101] Furthermore these amphiphiles are non-toxic and are internationally approved by regulatory bodies for parenteral administration. [102]

As reviewed by Torchilin in 2005 [103] numerous studies have described the assembly and encapsulation of various drugs within micelles constructed from DSPE PEG 2000. Strangely and despite several authors [77, 100, 104, 105, 106] having described the encapsulation of PTX within such micelles (as will be discussed in greater detail), to date there have been no reports concerning *in vivo* efficacy evaluations of PTX loaded DSPE PEG 2000 micelles - In fact, to the extent of the authors literature review, only Tang *et al.* [107] has reported any *in vivo* anticancer efficacy data using DSPE PEG 2000 micelles, in that case loaded with doxorubicin.

However, the *in vivo* passive targeting qualities (longevity within circulation) have been long known and were thought to be well established by Lukyanov *et al* [108] who reported that radio-labelled, drug-free DSPE PEG 2000 micelles possess long-circulating properties (plasma half-life of 2 hours) and to preferable accumulate in tumours compared to muscle. Similarly, Lukyanov *et al*. [109] reported on the increased tumour accumulation by drug-free immuno-micelles (MAb conjugated distally to PEG on the surface of the micelles).

Phosphatidylcholine as a co-surfactant in various proportions has been described as a successful means to increase the encapsulation efficacy of PTX within mixed lipopolymeric micelles. [77, 102, 104] Krishnadas *et al*. [104] identified a molar ratio of 10:1 (DSPE PEG:PC) as the optimal mixed micellular state. The general consensus is that the increased solubility of PTX within mixed (PC) micelles is as result of the higher hydrophobic content as a consequence of two long diacyl chains, [110] although this does not consider the influence of the charged head portion.

Amphiphiles are added to a mixture to reduce the interfacial tension, promoting assembly through hydrophobic interactions and to provide stability through electrostatic and/or steric repulsive forces. Commonly, a surfactant mixture (S_{mix}) is used that allows additional steric flexibility, enabling conformational rearrangements and the formation of spherical droplets preferably as opposed to various liquid crystalline and mesomorphic phases. [111]

In this study, the thin film hydration method was used to encapsulate PTX and B663 within the hydrophobic core of lipopolymeric micelles. The formulation strategy included optimising both the drug: drug ratio as well as the $Drug_{total}$: S_{mix} ratio prior to optimising the amphiphile wt. %, represented as the [S_{mix}] in mg/ml required within the binary system with water to successfully solubilise >1 mg/ml PTX.

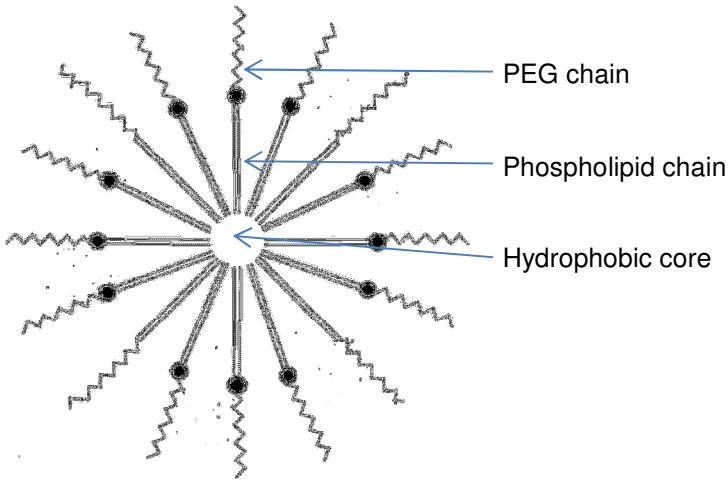

Figure 6.1. Depiction of a lipopolymeric micelle

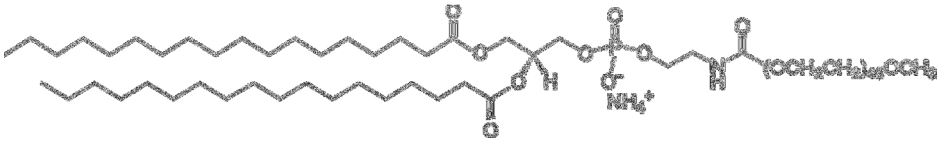

Figure 6.2. Chemical structure of DSPE-PEG 2000

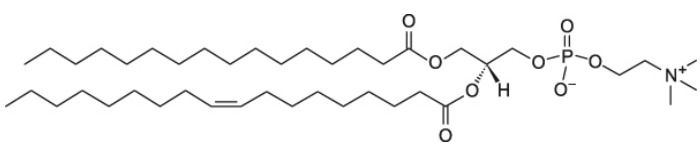

Figure 6.3. Chemical structure of a typical phosphatidylcholine

B. RiminoPLUS™ imaging

A further aim of this study was to explore the development of a multifunctional (theranostic) NDDS for use in diagnosis and treatment. Efforts were directed towards encapsulating Lipiodol (an oil based contrast agent) within a nano-sized, oil-in-water (o/w) emulsion suitable for IV administration that could be used as both an imaging agent and as a carrier of multiple drugs. The RiminoPLUS imaging system is a pseudoternary system (S_{mix}, oil and water) composed of DSPE PEG 2000, phosphatidylcholine, Lipiodol and water.

Emulsions are semi-stable mixtures of two immiscible liquids with one phase being dispersed as droplets within the other continuous phase. Emulsions are (pseudo)ternary systems consisting of oil, water and surfactant acting as the interfacial stabilizing film. Various possible assemblies can arise from mixing different ratios of S_{mix}, oil and water. [112] Such systems can be mapped through the use of phase diagrams.

In literature a distinction has been made between nanoemulsions and microemulsions both of which are nano-sized and appear translucent [113] (optically isotropic) as result of the particle size being smaller than the wavelength of visible light (below 200 nm). [111] Microemulsions are thermodynamically stable and form spontaneously, whereas nanoemulsions (also referred to as miniemulsions or sub-micron emulsions) are kinetically metastable and generally require considerable energy input for their preparation. [113-117] Kinetically stable nanoemulsions that possess adequate electrostatic and steric stability (interfacial affinity interactions) [118] have been described as approaching thermodynamic stability. [116]

Emulsification methods can be categorized as either low energy or high energy emulsification. Low energy emulsification techniques make use of inherent chemical potential of the components to spontaneously form emulsions as result of phase transitions (inversion from w/o to o/w) produced through changing the systems composition at constant temperature or through altering the temperature

at constant composition - the so called phase inversion temperature method. [119] The preparation method in terms of the order of adding phases (water added to oil phase or vice versa) can influence the final properties of the emulsion obtained. [114] Spontaneous emulsification is dependent upon favourable physicochemical interactions between the specific oil and surfactant components used. [116]

High energy emulsification techniques require the input of external energy for formation. Commonly ultrasonication, high-shear mixing or high pressure homogenisation is used to achieve sub-micron sized emulsions. Importantly, high energy emulsification methods allows for a greater variety in composition as the free energy inherent within a particular system is not required to promote formation [119].

An emulsion for intravenous administration by definition must be stable and maintain particle size upon the dilution encountered after IV injection. For this reason, non-equilibrium (metastable) nanoemulsions which unlike microemulsions can be diluted without change in droplet size are considered preferable. [111, 113, 118, 120]

In this study, the emulsification strategy initially employed the aqueous titration method affording visual observations of phase transitions, consistency and possible spontaneous emulsification (as evidenced by optical isotropy) whilst titrating along various Oil: S_{mix} ratios / “tie-lines” represented in a ternary phase diagram (Figure 6.4.). After dilution to 90% water (w/w) all mixtures (respective Oil: S_{mix} ratios) were ultrasonicated and again visually observed for optical isotropy before conducting thermodynamic stability assessments and size determinations of single phase dispersions only.

The intent was not to exhaustively classify the various conformations (micro and nanostructural characteristics) produced at various component compositions within the ternary phase diagram but rather to quickly and efficiently identify a nano-sized emulsion suitable for parenteral administration. In such a stream-lined approach, special interest was paid only to the water-rich region (in which parenterally

suitable, nanoemulsion formulations are deemed feasible). Following a resource sparing strategy, PTX as the combination drug partner for B663 was not included in these initial experiments.

C. PVP-PVAc polymeric micelles

PVP (Polyvinylpyrrolidone) is a cyclic amine based water-soluble polymer with characteristics similar to PEG. [102, 121] It can be used to impart stealth properties in circulation through immune evasion. Through a strategic alliance with Stellenbosch University, Polymer Science Institute, Ms. N. Bailly developed a B663 loaded PVP-PVAc polymeric micelles under the supervision of Prof. B. Klumperman. The novel amphiphiles synthesized have great potential for further development.

6.2. Materials

Chemicals and reagents

For the pilot study a 1 g sample of LIPOID PE 18:0/18:0-PEG 2000 (1, 2-distearoyl - *sn*– glycerol- 3- phosphatidylethanolamine - *N* - [methoxy (polyeythylene glycol) - 2000] - DSPE PEG 2000), (Figure 6.2.) and 50 g sample of LIPOID S 75-3 Phosphatidylcholine, (Figure 6.3.) were generously provided by Lipoid GmbH (Ludwigshafen, Germany).

Lipiodol® Ultra-Fluid (Guerbet, France) was purchased from Axiam Radiopharmaceuticals (JHB, SA) and stored in the dark at room temperature.

HEPES (4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid) and Sodium salicylate were purchased from Sigma Aldrich and freshly prepared as solutions in water before successive experiments.

6.3. Methodology

6.3.1. Development of Riminocelles™

Assembly procedures:

Stock solutions of DSPE PEG 2000 and PC were typically prepared to 100 mg/ml and 10 mg/ml respectively in $CHCl_3$. PTX and B663 stock solution were made up to 0.5 mg/ml in MeOH. Stock solutions were stored in air tight glassware at -20ºC.

In pilot studies, a checkerboard layout using 96 well plates were used to establish the optimal formulation. B663:PTX (w/w) ratios of 1:0, 5:1, 10:1, as well as $Drug_{total}$: S_{mix} (w/w) ratios of 1:5, 1:10, 1:20, 1:50 were investigated for the optimal composition. The S_{mix} (DSPE PEG 2000: PC) ratio was kept constant at 97:3 (w/w).

To do this practically and simply, the S_{mix} weight used for each of the mixtures was kept constant. A S_{mix} weight of 5 mg was the minimum scale required in order to produce sufficient volume (>1.5 ml) of the desired S_{mix} concentration permitting syringe filtration and servicing subsequent characterization procedures.

Calculated volumes of drug stock solutions were added to individual pre-weighed Büchi flasks to produce each of the desired ratios (Table 6.1.). The solvents were evaporated off under reduced pressure at temperatures below 40ºC using a rotary evaporator. The dried films were desiccated overnight and subsequently weighed to ensure mass balance. Thereafter all of the thin films were hydrated with 1.728 ml of 10 mM HEPES with the aid of a water bath sonicator (Bransonic) to produce a final DSPE PEG 2000 (MM 2806 g/mol) concentration of 1 mM. Finally the dispersions were syringe filtered using a Minisart® SRP (25 mm), 0.2 µm PTFE membrane (EO sterilized) filter to remove unencapsulated drug. In further small scale (sparing expensive PTX) experiments, centrifugation as opposed to filtration was used as an effective means of removing unencapsulated drug.

Table 6.1. Workflow detailing the actual weights of each component used in the pilot study producing the desired ratios

B663:PTX	$Drug_{total}$: S_{mix}				
(w/w)	(w/w)	B663 (µg)	PTX (µg)	DSPE (µg)	PC (µg)
1:0	1:50	100	0	4850.0	150.0
	1:20	250	0	4850.0	150.0
	1:10	500	0	4850.0	150.0
	1:5	1000	0	4850.0	150.0
5:1	1:50	83.3	16.7	4850.0	150.0
	1:20	208.3	41.7	4850.0	150.0
	1:10	416.7	83.3	4850.0	150.0
	1:5	833.3	166.7	4850.0	150.0
10:1	1:50	90.9	9.1	4850.0	150.0
	1:20	227.3	22.7	4850.0	150.0
	1:10	454.5	45.5	4850.0	150.0
	1:5	909.1	90.9	4850.0	150.0

Characterisation procedures:

Drug encapsulation

After assembly and filtration of the various NDDS, a 20 µl aliquot was taken, diluted into linear dynamic range of the instrument with MeOH, transferred to vials and quantified using the optimised and validated LC-MS/MS method (Chapter 8). Both encapsulation efficacy and the percentage of drug weight in the system were determined via the respective calculations:

% Encapsulation = drug encapsulated / drug loaded (w/w) x 100

Drug loading index = total drug encapsulated / (amphiphile + total drug encapsulated) x 100

After preliminary characterization and establishment of the optimal formula, the effect of S_{mix} concentration (within the binary system with water) on the encapsulation efficacy of the two drugs was assessed thus identifying the lowest amphiphile concentration required to effectively solubilize PTX at clinically relevant concentrations (>1 mg/ml). Encapsulation efficacy was assessed at final S_{mix} concentrations of 5, 10, 20 and 40 mg/ml.

Size and Zeta potential

Various formulations were assessed through dynamic light scattering (DLS) / photon correlation spectroscopy and laser Doppler electrophoresis using a folded capillary cell - DTS1060 (Malvern Instruments) to determine the hydrodynamic diameter and the zeta potential respectively using a Zetasizer Nano ZS (Malvern Instruments). Typically 3 independent measurements of 60 seconds or longer were performed per sample to establish measurement repeatability. Dependent on drug loading, typically 50 µl aliquots were diluted further with 0.2 µm filtered, deionised water (typically 2 ml) to ensure that the scattering properties of the sample achieved a suitable intensity as indicated by the average sample count rate.

The following settings were used: Material - PEG polymer; Refractive index (RI) - 1.5; Dispersant - H_2O; Dispersant RI - 1.330; Viscosity (cP) - 0.8872; Measurement temperature - 25℃.

Transmission Electron Microscopy (TEM)

After optimisation of the formulation in terms of drug encapsulation, particle size and zeta potential, the lead formulation was assessed by TEM using a Phillips 301 Multipurpose, 100 kV TEM, equipped with a eucentric goniometer stage and a heating holder. TEM was performed at the Microscopy and Microanalysis Laboratory (University of Pretoria). Briefly, an aliquot of the 1 mg/ml PTX Riminocelle solution was diluted 1:100 in water and a drop placed on a carbon film coated on a copper grid before drying at room temperature and subsequent observation.

CMC determination

Experimentally CMC values are determined by plotting a selected physicochemical property that changes upon micellization (e.g. UV absorption, fluorescence emission, electrical conductivity, viscosity, surface tension etc.) as a function of amphiphile concentration. An abrupt change in the response slope represents self-association and thus indicates the CMC value. Determination of CMC values can thus be ambiguous and dependent upon how sensitive the measured property is to micelle formation. [122]

A UV spectrophotometric method employing the inherent dye quality of Riminophenazines was used to estimate the CMC value:

From $CHCl_3$ stock solutions, a fixed weight (typically 50 µg) of B663 was mixed with various weights (0.0004 – 0.4 mg/ml) of S_{mix} in clean glass test tubes. Solvents were evaporated off under vacuum using a Centrivap at <40℃ and subsequently reconstituted in 10 mM HEPES to a constant volume (typically 4 ml) thereby obtaining a range of S_{mix} concentrations ranging from 0. 1 µg/ml – 100

µg/ml. Each tube was centrifuged, thoroughly vortex mixed and sonicated in a water bath (185 watts at 42 kHz for 10 min) before centrifugation at 10 000 *g* for 10 min at 20ºC. Thereafter 200 µl aliquots of each colloidal dispersion were transferred to a 96 well plate and the absorbance values read at a wavelength of 450 nm (reference 630 nm).

In vitro release profile under sink conditions

As the water solubility of both PTX and B663 is very poor, the use of a hydrotropic agent, sodium salicylate as described by Cho *et al.* [123] was used to maintain adequate sink conditions.

A volume of 100 µl of Riminocelles solution ready for injection (1 mg/ml PTX, 2.5 mg/ml B663) was diluted in 2 ml HEPES and introduced into a dialysis membrane bag (MWCO=6000-8000 Da). The bag was sealed and the experiment was initiated by placing the bag in 100 ml of 1 M sodium salicylate. The release medium was stirred at a constant speed with a magnetic stirrer. At predetermined time intervals of 10 min, 30 min, 1 h, 3 h and 6 h, 0.5 ml samples were withdrawn and replaced with fresh medium so as to assess the stability of the micelles after dilution (mimicking *in vivo* administration). After sample clean-up using SPE to remove the ionization interfering hydrotrope, the validated LC-MS/MS method as described in Chapter 8 was used to quantitate the different drug concentrations in the dialysate to calculate the percentage of each drug released from the micelle formulation.

6.3.2. Development of RiminoPLUS™ Imaging

Drug solubility in Lipiodol

As a first step, the solubility of B663 and B4125 in Lipiodol was determined. From literature, it is known that the solubility of PTX in Lipiodol is approximately 10 mg/ml. [62]

UV spectrophotometry (Perkin Elmer, Lambda 25, UV/Vis Spectrometry) was used to determine the maximum solubility of the two lead Riminophenazines (B4125 and B663) in Lipiodol. One millilitre of Lipiodol oil was saturated with an excess of the respective Riminophenazine powders in clean microreaction vials. Each of the mixtures were thoroughly vortex mixed and ultrasonicated in a water bath for 10 min before being centrifuged at 15 000 *g* for 20 min at 10℃ to remove any unsolubilised material. Complete removal of unsolubilised compound was confirmed by the absence of any drug crystals when an aliquot was viewed under a light microscope at high magnification (400X). Thereafter, triplicate 20 µl aliquots were removed and diluted with MeOH into the linear range of the assay. Linear calibration lines with correlation coefficients of 0.999 were produced for each of the Riminophenazines in MeOH over a range of 1.5-50 µg/ml using a characteristic absorbance wavelength (λ_{max}) of 460 nm.

Aqueous titration method

In the past, the optimal composition/formulation of nano-sized emulsions has been determined rather arbitrarily without adequate control for the influence of composition variables (the ratio of different materials that are mixed). Systematic studies utilising ternary phase diagrams to 'map' the various possible aggregated structures (polymorphism) that can form at various component ratios, as the water content (wt. %) increases, is a convenient means of identifying optimal chemical formulation with minimal expense of material resources and time. Advantageously this method allows for the identification of both spontaneously forming microemulsions and nanoemulsion formed after the input of ultrasonic energy at a pre-specified water content measured as weight percent (wt. %).

As a first step, the density of Lipiodol was calculated gravimetrically by three independent weight determinations of a known volume at ambient room temperature to be on average 1.242 g/ml.

Concentrated stock solutions of DSPE PEG 2000 and PC were typically prepared to 100 mg/ml and 10 mg/ml respectively in $CHCl_3$.

For preliminary studies conducted on a minimum scale, a fine syringe (Series II, SGE Scientific) was used to aliquot 10 µl of B663 saturated Lipiodol (corresponding to 12.42 mg) into each of 9 tubes. At this scale, with the weight of Lipiodol being constant, the calculated total weight of S_{mix} required to represent 9 tie lines (i.e. Lipiodol: S_{mix} ratios) is roughly 240 mg.

So as to assess the influence of co-surfactant, the following S_{mix} (DSPE PEG 2000: PC) ratios: 97:3, 94:6 and 91:9 (w/w) were evaluated. Calculated volumes of each of the three S_{mix} stock solutions were added to each of the 9 tubes to represent the following Lipiodol: S_{mix} ratios: 0.9:0.1; 0.8:0.2; 0.7:0.3; 0.6:0.4; 0.5:0.5; 0.4:0.6; 0.3:0.7; 0.2:0.8 and 0.9:0.1 (w/w). Dependent upon the scale of the specific experiment, the $CHCl_3$ was evaporated off using either a Centrivap (typically) or a rotary evaporator at <40 ℃.

To each specific Lipiodol: S_{mix} ratio, calculated volumes of water were slowly added in 10% increments along the tie lines represented in Figure 6.4. The specified volume of water to be added to each specific tube so as to progress along the designated tie lines (Lipiodol: S_{mix} ratio) was calculated using a simple Excel spreadsheet. Examples of the output is shown in Table 6.2. for the Lipiodol: S_{mix} ratios of 0.9:0.1 and 0.8:0.2.

After each incremental addition of water the respective mixtures were thoroughly vortex mixed. The mixtures were allowed to settle, visually observed and brief qualitative notes made concerning consistency (number of distinct phases, translucency or presence of precipitate).

At 90% wt. water each of the mixtures were ultrasonicated (Biologics, Inc. Model 3000) with a power output of ~ 150 watts at 20 kHz for 5 min (50% pulse - to ensure that the samples did not overheat) using a stepped titanium micro tip (3.81 mm diameter). The samples were observed through the clear door of the sound abating chamber for distinctive translucency changes (optical isotropy) indicating the formation of nanoemulsions. Formulations that were observed to visually phase change from cloudy to translucent after the input of ultrasonic energy were

aliquoted and subjected to multiple freeze-thaw cycles followed by centrifugation at 15 000 *g* for 20 min to assess the colloidal (thermodynamic) stability of the system. Metastable homogenous dispersions (nanoemulsions) were stored in the fridge at 2 - 8℃ and were visually observed daily for phase separation. On a weekly basis samples were further diluted to 99 wt. % water, immediately centrifuged and visually observed for phase separation or precipitate formation. Thereafter, samples were filter sterilized through 0.2 µm syringe filters (as would be required prior to IV administration) and sized via DLS.

As no spontaneous emulsification (indicating microemulsion formation) was seen to occur as titration progressed from water-in-oil (W/O) to oil-in-water (O/W), it was concluded that phase inversion was not a prerequisite in the formation of nano-sized emulsions using the materials tested in this study. Therefore, the direct emulsification method whereby the dispersed phase (oil with S_{mix}) is added to the continuous phase (water) under intensive mixing followed by ultrasonication was successfully used in scaled up production using the identified optimal Oil: S_{mix} ratio to produce metastable nanoemulsions.

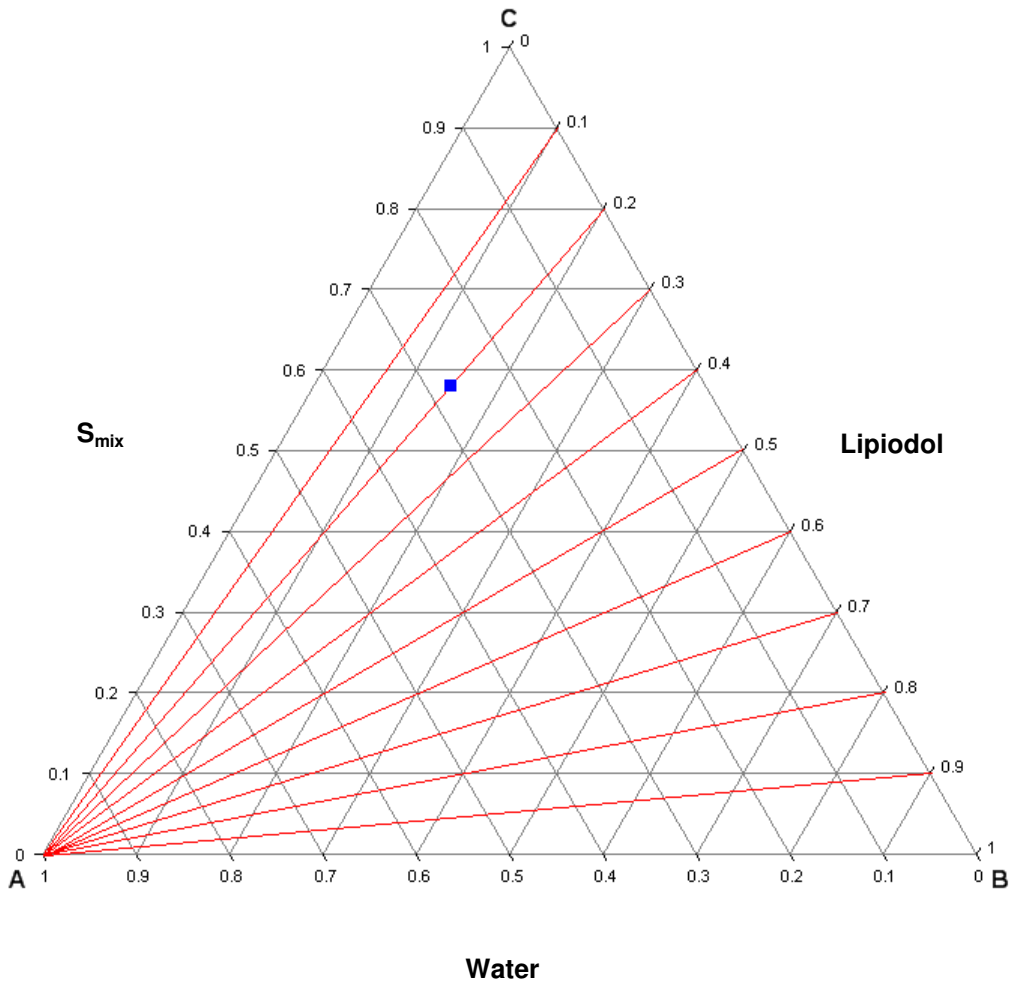

Figure 6.4. Pseudoquaternary phase diagram showing the respective tie / dilution lines (Red), along which titrations were made. A - Water; B - Lipiodol; C - S_{mix}

In a ternary phase diagram all the components (Lipiodol, S_{mix} and Water) add up to 100% of the weight. Titration begins in the upper right side of the triangle with 9 fixed Lipiodol: S_{mix} ratios. Water is slowly added in calculated proportions so as to titrate along the indicated tie lines.

The blue dot in the ternary phase diagram corresponds to a composition (w/w) of: Water - 27%, Lipiodol - 15%, S_{mix} - 58%

Table 6.2. Example calculations (Excel outputs) for dilution along two tie lines (Oil: S_{mix}, 0.9: 0.1 and 0.8: 0.2)

0.9 : 0.1 (Lipiodol : S_{mix})

Oil		S_{mix}		Water			Total wt. of system
wt. %	wt. (mg) [a]	wt. %	wt. (mg)	wt. %	Add µl	*Cumulative* wt. (mg)	
0.900	12.420	0.100	1.380	0.000	0.000	0.000	13.800
0.810	12.420	0.090	1.380	0.100	1.533	1.533	15.333
0.720	12.420	0.080	1.380	0.200	1.917	3.450	17.250
0.630	12.420	0.070	1.380	0.300	2.464	5.914	19.714
0.540	12.420	0.060	1.380	0.400	3.286	9.200	23.000
0.450	12.420	0.050	1.380	0.500	4.600	13.800	27.600
0.360	12.420	0.040	1.380	0.600	6.900	20.700	34.500
0.270	12.420	0.030	1.380	0.700	11.500	32.200	46.000
0.180	12.420	0.020	1.380	0.800	23.000	55.200	69.000
0.090	12.420	0.010	1.380	0.900	69.000	124.200	138.000
0.045	12.420	0.005	1.380	0.950	138.000	262.200	276.000
0.018	12.420	0.002	1.380	0.980	414.000	676.200	690.000
0.009	12.420	0.001	1.380	0.990	690.000	1366.200	1380.000

0.8 : 0.2 (Lipiodol : S_{mix})

Oil		S_{mix}		Water			Total wt. of system
wt. %	wt. (mg) [a]	wt. %	wt. (mg)	wt. %	Add µl	*Cumulative* wt. (mg)	
0.800	12.42	0.20	3.11	0.00	0.00	0.00	15.53
0.720	12.42	0.1800	3.11	0.10	1.73	1.73	17.25
0.640	12.42	0.1600	3.11	0.20	2.16	3.88	19.41
0.560	12.42	0.1400	3.11	0.30	2.77	6.65	22.18
0.480	12.42	0.1200	3.11	0.40	3.70	10.35	25.88
0.400	12.42	0.1000	3.11	0.50	5.18	15.53	31.05
0.320	12.42	0.0800	3.11	0.60	7.76	23.29	38.81
0.240	12.42	0.0600	3.11	0.70	12.94	36.23	51.75
0.160	12.42	0.0400	3.11	0.80	25.88	62.10	77.63
0.080	12.42	0.0200	3.11	0.90	77.63	139.73	155.25
0.040	12.42	0.0100	3.11	0.95	155.25	294.98	310.50
0.016	12.42	0.0040	3.11	0.98	465.75	760.72	776.25
0.008	12.42	0.0020	3.11	0.99	776.25	1536.98	1552.51

[a] 10 µl Lipiodol = 12.42 mg

6.4. Results

6.4.1. Riminocelles

The approach adopted was to first establish the optimal composition with respect to the relative concentrations of the different compounds to be included in the formulation and thereafter to assess the influence of amphiphile concentration on the solubilisation of the drugs considering the minimum concentration requirements and practical (injection volume and infusion time) restrictions when using small animal models.

The thin film hydration method was found to be a simple and effective way to co-encapsulate the FRDC (PTX and B663) within lipopolymeric micelles (Figure 6.5.). After hydration of the various dried films, syringe based filtration using 0.2 um filter cartridges was found to effectively remove any insoluble and thus unencapsulated drug. After quantitation of the respective drug concentrations using LC-MS/MS, the percentage drug encapsulation and the drug loading index were calculated for the various formulations prepared. In addition, the particle size and size distribution as well as the zeta potential were determined for each formulation (Table 6.3).

Due to imperfect encapsulation, final drug ratios different to that which had been loaded were attained. The drug loading index that considered the encapsulation of both drugs simultaneously in one expression revealed three samples to possess a total drug wt. above 10% of the mass of the formulation.

Concerning DLS, the attenuator was generally automatically set to 7 which compensated for high scattering samples while maintaining the count rate within acceptable limits. The mean count rate ranged between 290.4 - 407 kcps. In Table 6.3, the results of cumulants analysis are given as: mean size (Z-average/cumulants mean) of the particles based on signal intensity and Polydispersity index (PDI), which is a dimensionless measure of the extent of the particle size distribution.

All prepared formulations gave particles with bimodal distribution (Figure 6.6.) and were therefore not comparable to other sizing techniques. [124] The first population that accounted for a varied % (intensity) ranged from a particle diameter of 12.37-17.06 d.nm and is consistent with the size range reported by numerous authors for PEGylated phospholipid micelles. [100, 101, 102, 104, 108, 125, 126, 127, 128] The second population that generally accounted for a higher % (intensity) ranged from 124-185.5 d.nm (Table 6.4.). The bimodal distribution is consistent with that reported by others. [128, 129] It should however be stated that this phenomenon is difficult to interpret and could possibly be an artefact of the DLS technique. [130] Population % (intensities) and particulate size were seen to vary in a composition-dependent (drug: surfactant ratio) manner.

All the pilot formulations had a negative surface charge with zeta potentials ranging from -7.1 to -35.1 mV. As the zeta potential of particles in suspension will determine whether the particles within a dispersion will tend to aggregate or not, it is often used as an aid to predict the stability of colloidal systems. The greater the charge (whether positive or negative), the greater is the stability. The zeta potentials obtained are consistent with Wang *et al.* [131] who reported a zeta potential of -25 mV for empty DSPE PEG micelles.

After consideration of the various measured parameters taken together, the formulation adopted for further investigation was a drug (PTX) to drug (B663) loading ratio (w/w) of 1:5 and a total drug to amphiphile loading ratio (w/w) of 1:5. Further characterization studies were only carried out on this lead formulation:

Due to lipid polymorphism, the mean percentage encapsulation for the two respective drugs varied as a function of system composition (amphiphile concentration in water). Encapsulation efficacies for the respective drugs (using the optimised loading ratios) were therefore determined as a function of increasing S_{mix} concentration - The encapsulation of PTX was shown to increase linearly as a function of S_{mix} concentration (Figure 6.7.), consistent with previously reported results [77, 100, 104] and [110] who prepared bile salt/PC mixed micelles.

In contrast, the encapsulation efficiency of B663 steadily declined as the amphiphile concentration was increased. This has a direct effect on the final drug: drug (w/w) ratio achieved. Ultrasonication was shown to have no effect on drug encapsulation efficacy. The minimum S_{mix} concentration required to solubilize >1 mg/ml PTX was found to be 35 mg/ml. The drug encapsulation efficacies at this amphiphile concentration are shown in Figure 6.8. The slight variability of encapsulation was used as an advantage. Repeat preparations produced a range of drug concentrations that when mixed together in calculated proportions could be used to ensure that a precise final PTX:B663 (1:2.5, w/w) concentration is attained.

Morphological characteristics of the final formulation were examined using TEM. TEM revealed well-dispersed, electron dense, micellular particles in the size range of 200 nm (Figure 6.9.). The individual micelles were shown to have a spherical shape and to be of narrow size distribution. The slightly larger particle size suggested by TEM over DLS may be attributed to difference in the physical property that is actually measured (e.g. hydrodynamic diffusion versus the projected area). It is important to understand that no particle sizing technique is inherently more correct as they are dependent upon various parameters. [132] Discrepancies are often observed in the particle size distribution obtained and for this reason it is recommended that particle size analysis be performed using more than one technique. [119]

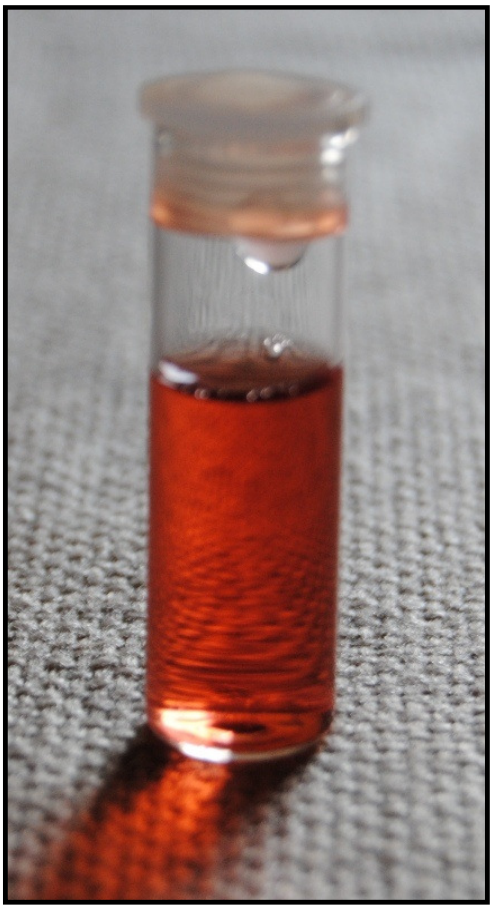

Figure 6.5. Photograph of diluted Riminocelles
The distinctive red colour is evidence that (non-water-soluble) B663 has been solubilised in an aqueous dispersant through encapsulation within micelles.

Table 6.3. Characterisation of various mixed lipopolymeric pilot formulations at a DSPE PEG 2000 concentration of 1 mM in 10 mM HEPES

B663: PTX ratio (w/w)	**1:0**				**5:1**				**10:1**			
$Drug_{total}$: S_{mix} ratio (w/w)	**1:50**	**1:20**	**1:10**	**1:5**	**1:50**	**1:20**	**1:10**	**1:5**	**1:50**	**1:20**	**1:10**	**1:5**
B663 % encapsulation	87.6	92.8	87.6	77.2	87.0	97.0	98.9	92.1	93.5	100.0	89.5	86.1
PTX % encapsulation		*Not applicable*			65.7	59.4	67.5	74.0	58.2	82.7	55.9	70.7
[B663] µg/ml	50.7	134.3	253.5	447.0	42.0	117.0	238.5	444.0	49.2	132.8	235.5	453.0
[PTX] µg/ml		*Not applicable*			6.3	14.3	32.6	71.4	3.1	10.9	14.7	37.2
Final drug ratio	1:0	1:0	1:0	1:0	6.6:1	8.2:1	7.3:1	6.2:1	15.9:1	12.2:1	16:1	12.2:1
Drug loading index	1.72	4.43	8.06	13.38	1.64	4.34	8.57	15.12	1.77	4.73	7.96	14.49
Z-Average Size (d.nm)	175.0	32.2	105.6	37.4	184.9	164.0	95.1	137.6	134.8	117.8	120.6	95.8
PdI	0.22	0.64	0.18	0.14	0.23	0.25	0.25	0.18	0.22	0.20	0.25	0.17
Zeta potential (mV)	-29	-25.6	-31.7	-7.1	-28.5	-31.1	-35.1	-20.6	-31.7	-18.3	-31.9	-10.3

% Encapsulation = drug encapsulated / drug loaded (w/w) x 100

Drug loading index = total drug encapsulated / (amphiphile + total drug encapsulated) (w/w) x 100

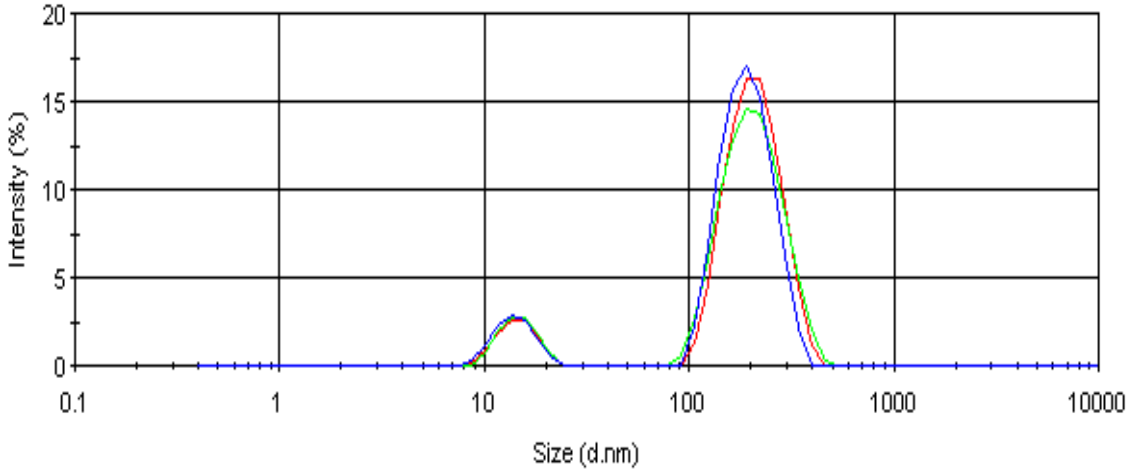

Figure 6.6. Z-average size (hydrodynamic diameter) distribution by intensity demonstrating the bimodal distribution of Riminocelles as determined by Dynamic light Scattering

Table 6.4. Bimodal size distributions of the various pilot formulations at a DSPE PEG concentration of 1 mM in 10 mM HEPES

B663:PTX	Drug: S_{mix}	Population 1 (d.nm)	% (Intensity)	Population 2 (d.nm)	% (Intensity)
(w/w)	(w/w)				
1:0	**1:50**	12.7	35.1	124.0	64.9
	1:20	13.7	42.6	158.6	53.3*
	1:10	14.7	55.7	150.4	44.3
	1:5	17.1	78.5	148.5	20.1*
5:1	**1:50**	12.5	20.6	134.1	79.4
	1:20	12.4	22.6	155.2	77.4
	1:10	14.7	46.0	163.6	54.0
	1:5	15.8	59.8	138.2	40.2
10:1	**1:50**	13.7	29.7	138.3	70.3
	1:20	15.7	43.5	167.7	56.5
	1:10	14.9	40.5	185.5	59.5
	1:5	16.9	66.2	157.1	33.8

* Third population >4000 d.nm (suspected dust particles)

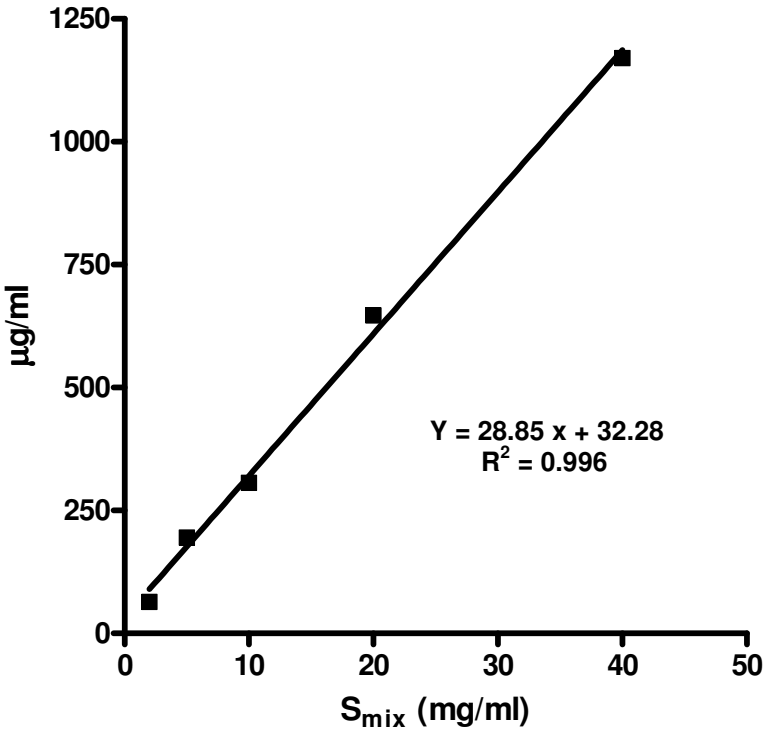

Figure 6.7. Encapsulation of PTX within mixed lipopolymeric micelles as a function of S_{mix} concentration

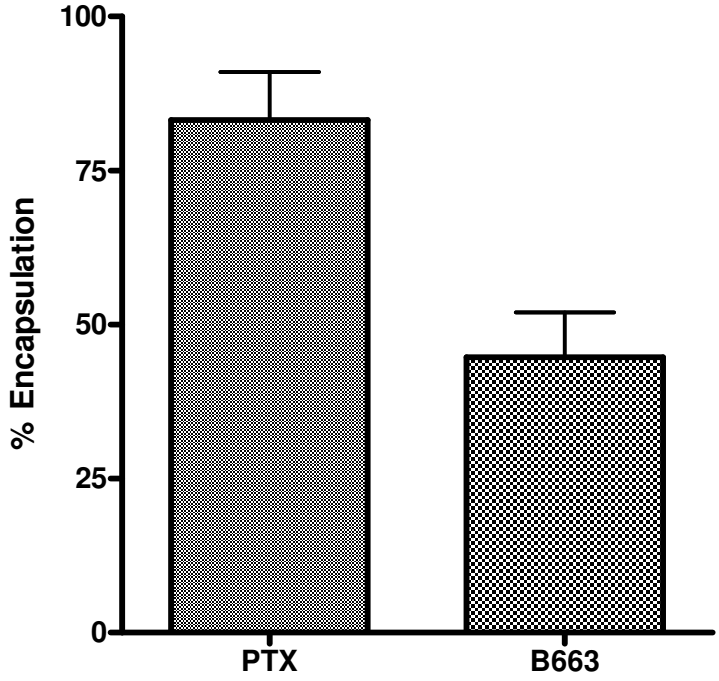

Figure 6.8. Percent encapsulation of PTX and B663 within Riminocelles at a S_{mix} concentration of 35 mg/ml as determined by LC-MS/MS

The inherent dye quality of Riminophenazines was effectively used to determine the CMC value of the S_{mix} using a UV spectrophotometric method (Figure 6.10.). Using the molecular mass for DSPE PEG 2000 of 2806 g/mole an estimated CMC value of $0.178x10^{-5}$ M was obtained. The CMC of micelles formed from DPSE PEG 2000 has been reported as approximately $1.1x10^{-5}$ M as determined using the pyrene assay method. [106, 108] Although it is ill advised to compare CMC values obtained through different techniques, it is not surprising to have attained lowered (improved) CMC values as the incorporation of PC adds to the hydrophobic driving force behind micellization. It was expected that Riminocelles prepared ready for injection can endure greater than 1:7000 dilution in water before micelle dissociation occurs.

In vitro drug release studies were performed under sink conditions simulated through the use of sodium salicylate in the release media (Figure 6.11.). Both drugs were shown to be well retained within the particle. PTX was slowly released over the first hour increasing to approximately 40% PTX release after 6 hours. B663 was steadily released over the 6 hour sampling period to approximately 10% cumulative release. These relative losses support the maintenance of a synergistic ratio between the two drugs.

Drug loaded mixed lipopolymeric micelles demonstrated high integrity after storage for up to 6 months at 4℃ as determined through thermodynamic (centrifugation) tests. No precipitate was observed indicating that the drugs were stably encapsulated. The stability under the recommended storage condition was further attested to independently in a GLP accredited laboratory prior to IV administration in the efficacy study (Chapter 7).

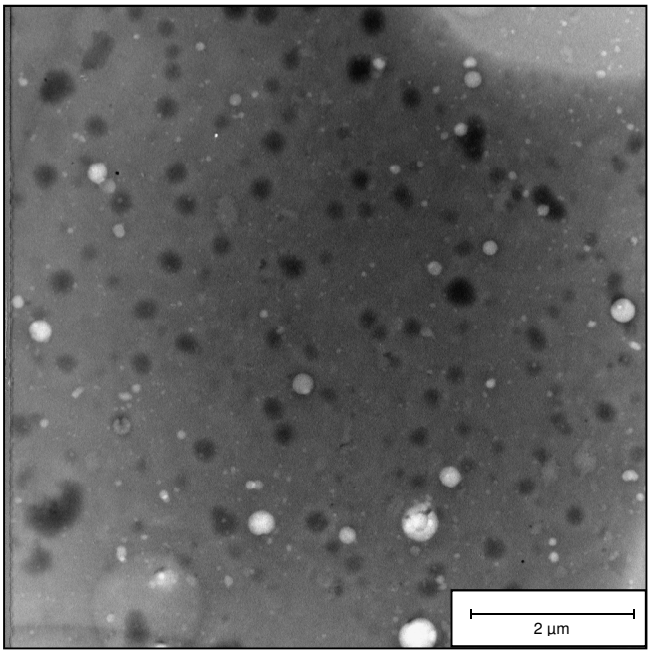

Figure 6.9. TEM image of Riminocelles
Dark spots correspond to the lipopolymeric micelles diffracting the electron beam.

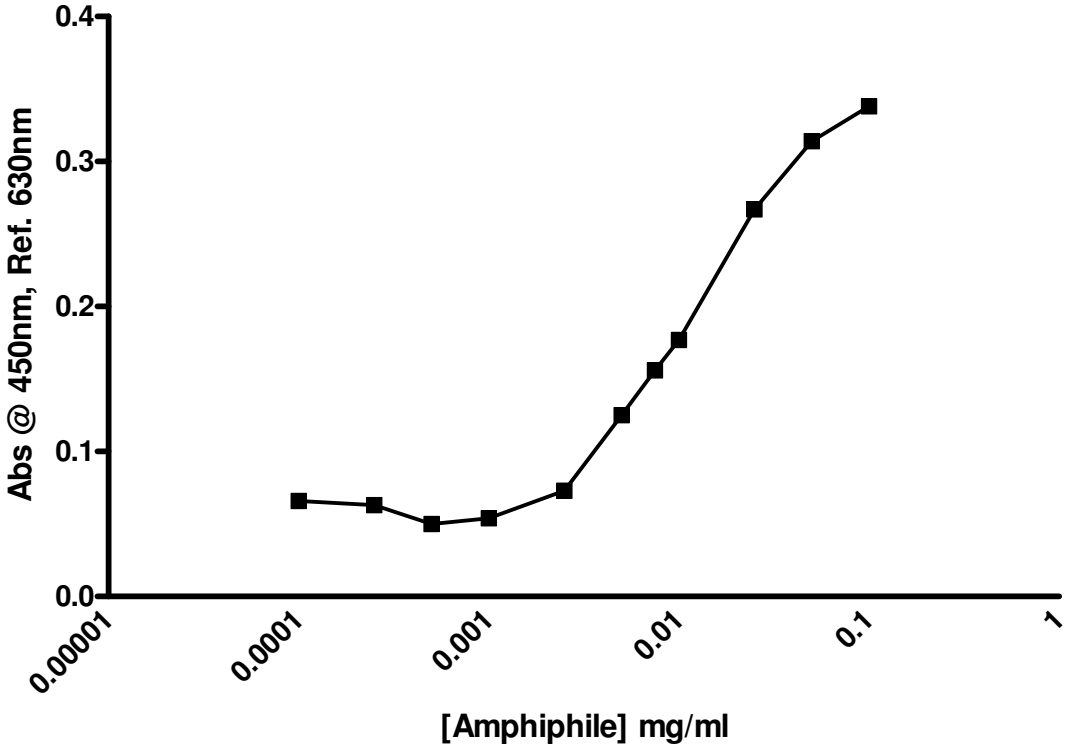

Figure 6.10. Determination of the critical micellular concentration (CMC) of the S_{mix} using the dye micellization method. Semi-log graph representing the approximate CMC value of Riminocelles.

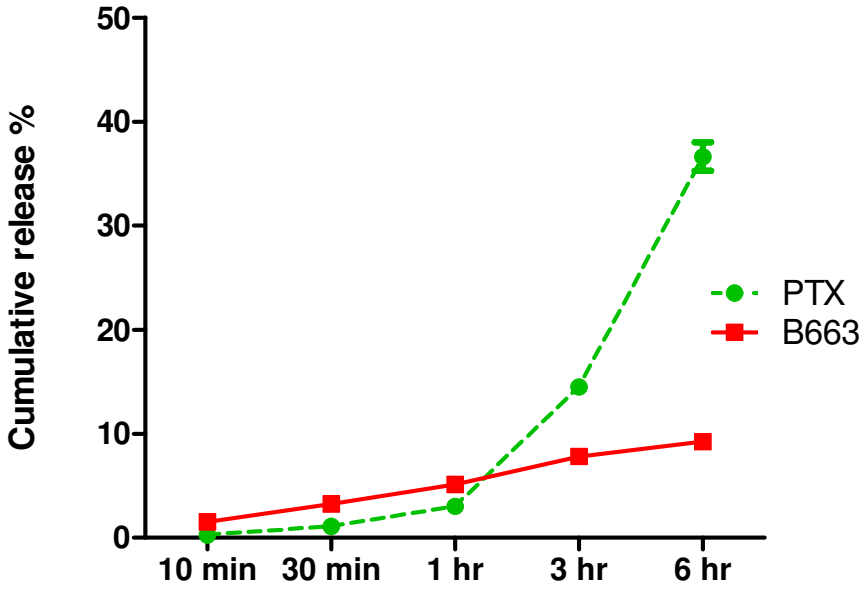

Figure 6.11. Typical release profiles of PTX and B663 from Riminocelles in 1 M sodium salicylate at ambient room temperature

6.4.2. RiminoPLUS Imaging

After a 200 fold dilution of drug saturated Lipiodol using pure MeOH, the solubility of B663 and B4125 in Lipiodol was measured using a calibrated UV spectrophotometric method and found to be roughly 7 mg/ml and 4 mg/ml respectively.

An efficient stream-lined approach was adopted with the intent of quickly formulating a Lipiodol-core, nano-sized emulsion satisfying the requirements for a ready to inject IV solution. Although phase inversion naturally occurred from W/O to O/W as aqueous titration proceeded, no spontaneous nanoemulsification was observed as evidenced by optical isotropy. Nor could spontaneous emulsification be achieved in pilot experiments for any of the compositions through repeated heating to in excess of 90℃ followed by rapid cooling in ice water (phase inversion temperature method). Although various different associated structures (lipid

polymorphisms) were assumed to form during transition, so as not to detract away from the study's aim, the nature of these intermediate phases were not investigated and merely qualitatively visually observed.

Prior to ultrasonication, all ternary systems (oil, surfactant, water mixtures) appeared opaque to different degrees (Figure 6.12.); colloidal dispersions were centrifuged to assess the degree of homogeneity and complete solubility. Pellets of different colours and quantities were observed for different compositions demonstrating the heterogeneity of the various mixture systems.

After ultrasonication at 90% water (w/w) all mixtures were again visually inspected. Several compositions notably the Lipiodol: S_{mix} ratios of 6:4, 5:5 and 4:6 appeared optically isotropic after ultrasonication (Figure 6.13.). These single phase translucent compositions were subjected to three repeated freeze-thaw cycles followed by centrifugation. The Lipiodol: S_{mix} ratio of 4:6 appeared to be thermodynamically stable as no pellet was evident after prolonged centrifugation.

A reproducible monodisperse population with a Z-average size of 101.2-105.1 d.nm and a PdI of <0.22 for the Lipiodol: S_{mix} ratio of 4:6 was attained (Figure 6.14.A.). Nanoemulsions produced with varying proportions of PC as a co-surfactant were shown not to influence the particle size nor stability (Figure 6.14. B & C). This dispersion was shown to maintain particle size for 7 days when stored at 2-8℃ despite possessing approximately zero electrophoretic mobility (zeta potential). Neither freeze thaw cycle nor ultracentrifugation produced phase separation for a period of approximately 2 weeks, after which degradation through Ostwald ripening and/or coalescence was evident that could be mitigated through re-sonication. Filtration of the dispersion through 0.2 µm syringe filters did not influence the particle size.

Figure 6.12. Flattop scanned image of the 9 Lipiodol: S_{mix} ratios at 0.90% water, visually indicating the difference in consistency
From right to left – 0.9:0.1 to 0.1:0.9 (Lipiodol: S_{mix})

Figure 6.13. Photograph of RiminoPLUS demonstrating the homogeneity and optically isotropic nature (visual perspective) of the nanoemulsions (roughly 100 nm). The dispersion is being held horizontal.

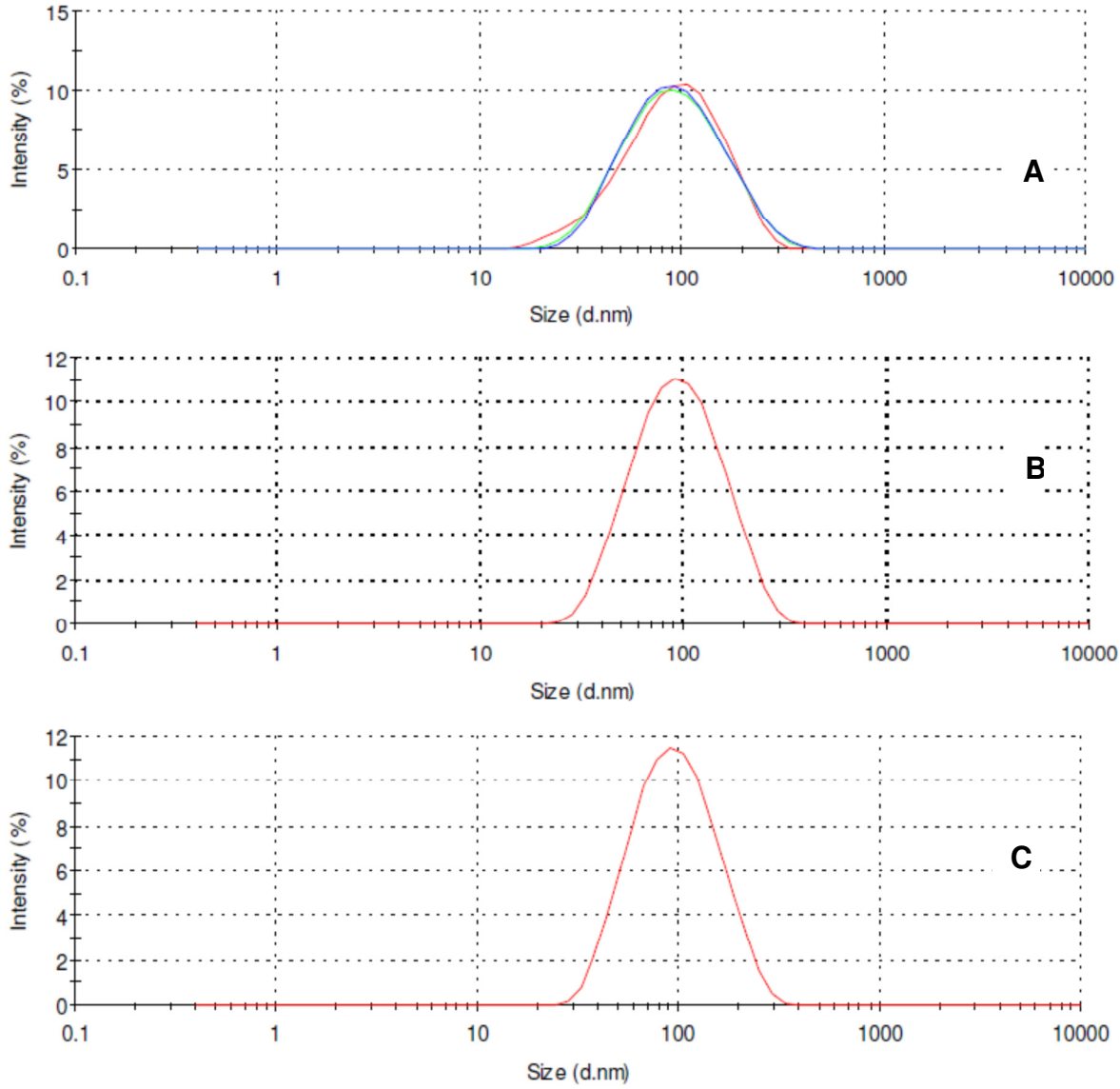

Figure 6.14. Size distribution by intensity of various RiminoPLUS formulations with a Lipiodol: S_{mix} ratio of 4:6

A - S_{mix} of DSPE PEG 2000: PC (97:3); B - S_{mix} of DSPE PEG 2000: PC (94:6); C - S_{mix} of DSPE PEG 2000: PC (91:9)

6.5. Discussion and conclusion

Phospholipids were first conjugated to PEG in 1984 when Sears [133] coupled carboxy-PEG and pure soy phosphatidylethanolamine via an amide linkage. Subsequently, lecithin and PEG modified phospholipids have become the most commonly used emulsifiers for parenteral application [119] and are widely used in various registered pharmaceutical dosage forms. Although other hydrophilic polymeric blocks and mixes thereof could be used as efficient steric barriers provided that they are biocompatible, PEG still remains the hydrophilic block of choice used to form the corona of many particulate drug delivery systems.

Interest in nanotechnology and its biomedical application, particularly with respect to intelligent dosage form design, has exploded in recent years. [134] NDDS assembled with biocompatible high molecular weight lipopolymeric amphiphiles offer the advantage of direct access to the blood stream, rapid onset of action and improved targeting.

NDDS are a platform in which drugs can be targeted to specific sites through surface functionalization with targeting moieties and specific disassembly responses (triggers). DSPE PEG 2000 with folate or specific antibody conjugation on the distal portion of the hydrophilic block have been synthesised and are commercially available. Therefore, both Riminocelles and RiminoPLUS could be “upgraded” to include active targeting mechanisms with ease.

Riminocelles™

The experimental approach used to optimise the binary system (Riminocelles) was very effective at achieving the desired objective with minimal resource expenditure. For all the pilot studies, only 60 mg of S_{mix} was required. This drug delivery system was optimised with respect to composition to obtain the desired particle characteristics thought pre-requisite for exploitation of the EPR effect of solid tumours.

The use of DSPE PEG 2000 and PC proved to be an efficient means of solubilising the poorly water soluble drugs, PTX and B663. The thin film hydration and filter method was reproducible, at various scales of synthesis (2 ml - 100 ml) to produce particles of reproducible size and encapsulation efficacy. This preparation method could foreseeably be scaled up to provide enough Riminocelle for clinical studies if required.

The fluctuation in B663 encapsulation efficacy as a function of amphiphile concentration is thought to be due to different conformations of aggregate structures that form at different points along an isothermic binary (lipid-water) phase diagram influencing the degree of solubility. [135, 136]

Through proportionally mixing replicate preparations, the inherent variability of encapsulation (~10% standard deviation) was used to prepare the final sterile formulation of 1 mg/ml PTX and 2.5 mg/ml B663 (with a controlled ratio) for *in vivo* toxicity, efficacy and pharmacokinetic studies. Such clinically relevant concentrations of PTX were solubilised at a low amphiphile concentration of 35 mg/ml. This methodology would require stricter standardisation, adhering to GMP in preparation for clinical trials.

Previous studies using D-α-tocopheryl polyethylene glycol 1000 succinate (TPGS) as a co-surfactant with DSPE PEG 2000 (in a 1:1 molar ratio) have reported that 3 - 5 mg/ml of PTX have been attained but at amphiphile concentrations in excess 100 mg/ml. [106]

Gao *et al.* [100] reported a PTX incorporation of 1.5 wt. % into DSPE PEG 2000 micelles. Gao *et al.* [77] reported a PTX incorporation of 3 wt. % into DSPE PEG/PC (1:4) mixed immunomicelles (with surface conjugated 2C5 MAb). In this study, a PTX incorporation of 2.86 wt. % together with 7.14 wt. % B663 in mixed lipopolymeric micelles was attained.

In vitro release studies under sink conditions suggests that the novel NDDS retains sufficient drug for effective drug delivery following the dilution experienced after IV

administration. It should however be stressed that this study did not attempt to model the *in vivo,* protein rich, plasma environment. Further studies with such lipopolymeric micelles should be evaluated for drug release in the presence of plasma proteins to mimic the *in vivo* environment.

Riminocelles loaded with PTX and B663 were independently observed (by Charles River Laboratories - Chapter 7) to be stable (without precipitation) and suitable for intravenous injection for periods in excess of 6 months when stored at 2 - 8℃.

In summary, the ready-to-inject, sterile Riminocelles nanoparticulate co-formulation possesses good particle characteristics including: physical size, electrostatic stability, CMC and stable drug retention, which collectively suggest suitability for passive tumour targeting after IV administration.

The novel feature of this NDDS in comparison to currently used PTX formulations (Taxol and Abraxane) is the combined use of B663 as a chemosensitizing agent. The use of a FRDC encapsulated within NDDS is expected to offer greater efficacy against a wider range a drug resistant solid tumours and to reduce systemic toxicity.

RiminoPLUS™

The inner core of the nanoemulsion used for the manufacture of the RiminoPLUS formulation is composed of Lipiodol that has proven X-ray imaging capabilities and has been shown to solubilise diverse chemotherapeutics. It is thought that once released at the tumour site, drug saturated Lipiodol will remain within the tumour interstitium (and potentially increase intracellular concentrations) for prolonged periods [56], facilitating maximum neoplastic cell kill while facilitating diagnostic CT imaging.

The solubility of the B663 and B4125 in Lipiodol (7 mg/ml and 4 mg/ml respectively) being well below that of PTX (solubility ~10 mg/ml) is not conducive to attain the synergistic combination ratios identified *in vitro* as the drug ratio at

maximal encapsulation would be at levels where antagonistic effects are observed (Chapter 5). A possible solution may be to make use of alternative iodinated oils that have greater solubility for these Riminophenazines.

B663-loaded, kinetically metastable nano-sized (~100 nm) emulsions suitable for parenteral administration were successfully assembled with the aid of pseudoternary phase diagrams by ultrasonication at the water rich region (90% water) of the ternary phase diagram. Further dilution to 99% water prior to size determination using DLS was intended to plausible match the dilution effect experienced after IV injection. The particle size attained is thought perfect for passive tumour accumulation after either IV or intra-arterial injection.

This nanoemulsion system was only stable for a short period of ~1 week. As many nanoemulsion systems do not possess long-term storage stability [117] this was not deemed to be a failed formulation. This nanoemulsion could still be of clinical utility and additional co-surfactants such as Polysorbate 80 could be used to improve the shelf life, as has been reported. [137]

The approach employed in preparing the nanoemulsion was simple and employed a minimum scale of material (10 µl Lipiodol). Future endeavours will benefit from the use of the created Excel spreadsheet that facilitates quick calculation of titration volumes in 10% increments. The advantage of the aqueous titration method is that the entire ternary phase diagram (for a particular Oil, S_{mix} combination) can be “mapped” using only 9 samples and that both microemulsion (formed spontaneously) and nanoemulsions (requiring energy input) can be identified.