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High throughput preparation and characterisation of amphiphilic nanostructured nanoparticulate drug delivery vehicles

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ABSTRACT

The preparation, characterisation and assessment of drug delivery vehicles is typically a slow and complex process. Here we present a nanostructured nanoparticle system that can be prepared and characterised in a high-throughput fashion. In particular we use phytantriol and Myverol™ to prepare inverse bicontinuous cubic and inverse hexagonal liquid crystalline nanoparticles loaded with 10 commonly used therapeutic agents at increasing concentration. The dispersions are prepared using automated apparatus to create different concentrations and phases using novel protocols. We are able to characterise each stabilised nanoparticle dispersion using a range of methodologies including small angle X-ray scattering, particle sizing and drug partitioning. With this information we are able to assess which drug delivery vehicle is preferred for each drug and at which concentration the drug should be loaded to ensure maximum payload and to retain particle integrity.

1. Introduction

High-throughput screens (HTS), currently in use for drug screening employ a multitude of chemical iterations in order to achieve the best possible balance of a range of parameters, including efficacy, toxicity, and solubility. If delivery vehicles are required to achieve improved pharmacokinetics, then these will typically be brought into play at the latter stages of drug discovery.

High throughput drug discovery techniques make use of complex automated systems and robotics to screen 100,000 s of compounds against a specific target. Preparation and presentation of compounds in such *in vitro* screens are typically performed by using a solvent such as dimethylsulphoxide (DMSO) to achieve solutions of active compounds. This obviously presents a significant problem: poor to low solubility in a solvent will prevent proper evaluation of a class of compounds' efficacy towards a target. In his seminal article Lipinski stated that high throughput screening generates more lipophilic leads, a physical property which can be unfavourable for oral activity (Lipinski et al., 2001). Indeed it has recently been stated that many new drug candidates from drug discovery streams are water insoluble (Rabinow, 2004).

The use of a drug delivery system earlier in the drug discovery pipeline would aid in overcoming bioavailability and solubility hurdles as well as enhancing targeting and cell penetration properties (Rosen and Abribat, 2005). Formulations with lipid can enhance bioavailability as reviewed by Porter et al. (2007). To date, however, the ability to prepare and assess the physical properties of loaded drug delivery vehicles has been restricted. Here we present a 3D nanostructured nanoparticle which can be prepared with automated and standardised high throughput technology for the generation of amphiphilic or hydrophobic drug loaded delivery vehicles. We characterise the structure of the drug loaded delivery vehicle and evaluate the phase behaviour with reference to known structure-property relationships. We anticipate that this approach will expand the breadth of therapeutic opportunities and therefore leads for drug discovery.

The drug vehicles presented are lyotropic liquid crystalline dispersed nanoparticles, sterically stabilised using a block copolymer. Lyotropic liquid crystals form when lipids are added to a polar solvent and can adopt a range of morphologies including 1D flat lamellar structures, 2D inverse hexagonal phases and 3D inverse bicontinuous cubic phases (the nomenclature and structure of these phases are shown in Fig. 1). The physical properties and characteristics of the bulk phases and phase transformations between them have been extensively characterised (Conn et al., 2006, 2008; Dong et al., 2006; Kaasgaard and Drummond, 2006; Mulet et al., 2008, 2009). Here bulk phases are dispersed into sta-

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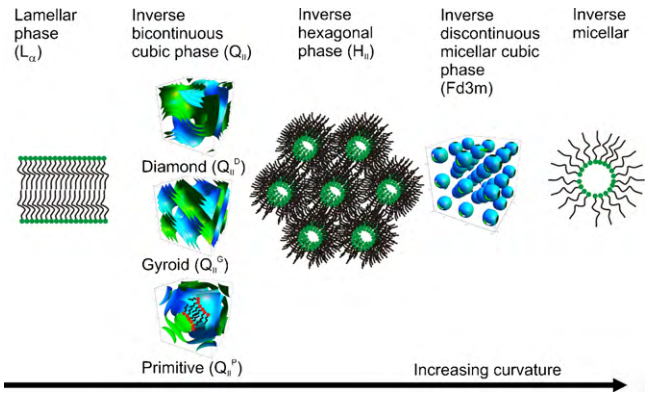

Fig. 1. Inverse phases that can be adopted by lyotropic liquid crystalline systems. From left to right the level of curvature of the system increases from lamellar phase (zero curvature), inverse bicontinuous cubic phase, inverse hexagonal, inverse discontinuous cubic phase and finally the inverse micellar phase.

ble nanoparticles by use of a steric stabiliser. Dispersed lamellar, hexagonal and cubic phases are known as liposomes, hexosomes and Cubosome™s, respectively. The use of the cubic phases in drug delivery technology is gaining in interest as their amphiphilic nature allows the incorporation of a range of drugs (Drummond and Fong, 1999). In addition, inverse bicontinuous cubic phase dispersions can be biodegradable, adaptable to multiple drug sizes, have enhanced physical and chemical stability and may enhance cellular uptake. Controlled release from the cubic phase has already been observed (Shah et al., 2001).

We have studied cubic nanoparticle dispersions made by two different lipids, phytantriol and Myverol™. The main constituent of Myverol™ is monoolein which is a hydrolysis product of triglycerides and is a compound that is generally regarded as safe. Phytantriol is used in cosmetic formulations. A comprehensive review by Kaasgard et al. outlines the phase behaviour of the amphiphilies used here: Myverol™ (as monoolein) and phytantriol (Kaasgaard and Drummond, 2006). The structures of the two lipids and drugs used in this study are shown in Fig. 2. The chosen drugs, apart from indometacin and progesterone, are found on the WHO essential drugs list for minimum medicine needs for a basic healthcare system, listing the most efficacious, safe and cost effective medicines (World Health Organisation, 2010).

Despite increasing recognition of this type of vehicle for drug delivery little is known about the effect of the incorporated drug on the structure of the cubic phase. We have therefore designed several novel methodologies for high throughput characterisation of the drug incorporated nanoparticles to assess their structures and levels of drug loading. The main technique used to characterise self-assembled lipid systems is small-angle X-ray scattering (SAXS) providing information not just on the particular mesophase adopted but also on the lattice parameter or unit cell size (a). In this case synchrotron SAXS is required due to the weakly scattering nature of dispersed lipid systems. We have also developed microplate-based spectroscopic assays to assess drug loadings into the particles. Data obtained on the phase behaviour, particle size and drug loading levels of these systems allow us to assess the suitability of each drug-loaded delivery vehicle for HTS drug discovery programmes.

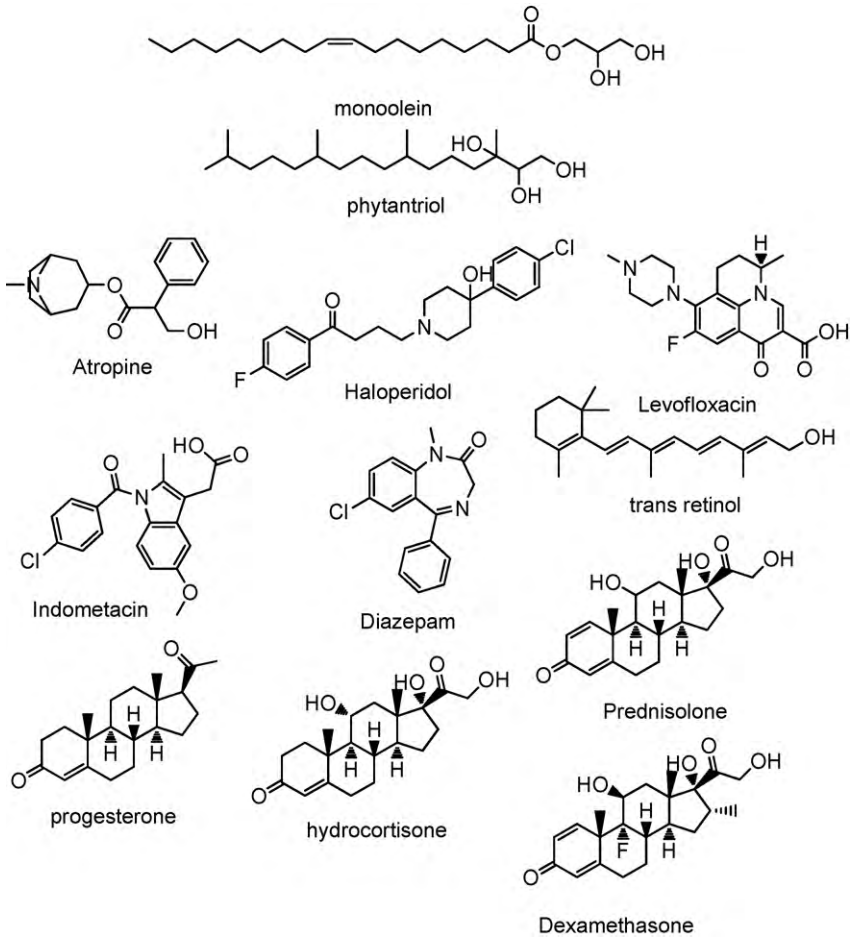

Fig. 2. Amphiphiles and drugs used in the high throughput preparation of a range of nanoparticles. Monoolein is the major constituent of the Myverol™ system used.

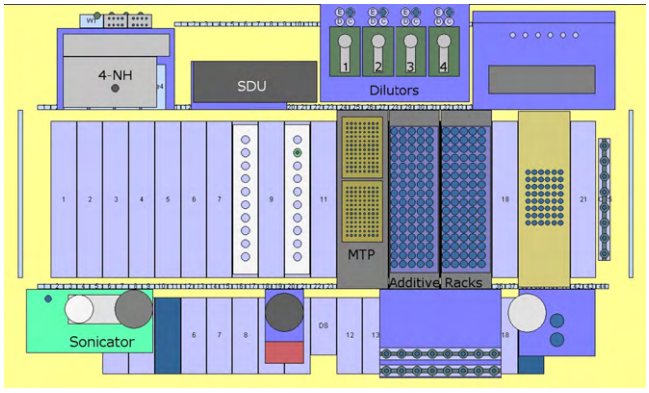

Fig. 3. Typical Chemspeed layout for dispensing solutions. 4-NH: 4 needle head; SDU: solid dispensing unit; MTP: microtitre plate.

2. Materials and methods

2.1. Materials

The following drugs were used without further purification: hydrocortisone (Sigma–Aldrich, NSW, AU), atropine (TCI, OR, USA), transretinol (Sigma–Aldrich, NSW, AU), diazepam (Sigma–Aldrich, NSW, AU), prednisolone (Sigma–Aldrich, NSW, AU), dexamethasone (Sigma–Aldrich, NSW, AU), and progesterone (Sigma–Aldrich, NSW, AU), haloperidol (Sigma–Aldrich, NSW, AU), levofloxacin (Sigma–Aldrich, NSW, AU), and indometacin (Sigma–Aldrich, NSW, AU). Solvents used were at least HPLC grade from Merck (Germany). The amphiphiles used for the formation of self-assembled nanoparticles were Phytantriol (DSM Nutritional Products, NSW) and Myverol™ 18–99 K (Bronson & Jacobs, Melbourne) which were sterically stabilised with poloxamer 407 (Sigma–Aldrich, NSW, AU). Steric stabiliser solution of poloxamer 407 was prepared at 7.5 mg/mL using Milli Q deionised water. The samples were prepared in a deep well Microtitre plate 2 mL 96-well plate (Whatman, NJ, USA). For small angle X-ray scattering experiments 40 μL of sample was loaded in 1.5 mm X-ray capillaries (Charles Supper, CA, USA).

2.2. Particle preparation

Drug loaded Cubosome™s were prepared in an automated fashion using a Chemspeed Accelerator™ SLT2 automated synthesis platform (Chemspeed, Switzerland). The layout of the Chemspeed platform is illustrated in Fig. 3. The platform has the capability of utilising a variety of tools however for this protocol only the 4-needle head (4-NH) and the probe sonicator were utilised. The 2 mL microtitre plates (MTP) were mounted in the solvent bottle/MTP rack. The stock solution of the amphiphile (phytantriol or Myverol™) in chloroform was contained in the stock solution rack. Additionally, chloroform stock solutions of the suite of drug additives were loaded in the vial rack in 8 mL vials capped with open lids fitted with PTFE lined septa.

The particle preparation process is outlined in Fig. 4. Initially the stock solution of the amphiphile is added to each of the wells of the microtitre plate, this is followed by the addition of the required amount of drug stock solution (step 1, Fig. 4). Drug concentrations used were 1, 2, 5, 10 and 15 mol% of each sample. The 1 mL dilutors were used for all transfers in this program to increase the accuracy when dispensing small quantities. Multiple aspirations were allowed along with an air gap of 50 μL and an extra volume of 100 μL were used. Between each liquid transfer the needles and lines were rinsed with 900 μL of chloroform. On the completion of the liquid handling step, the microtitre plate is removed from

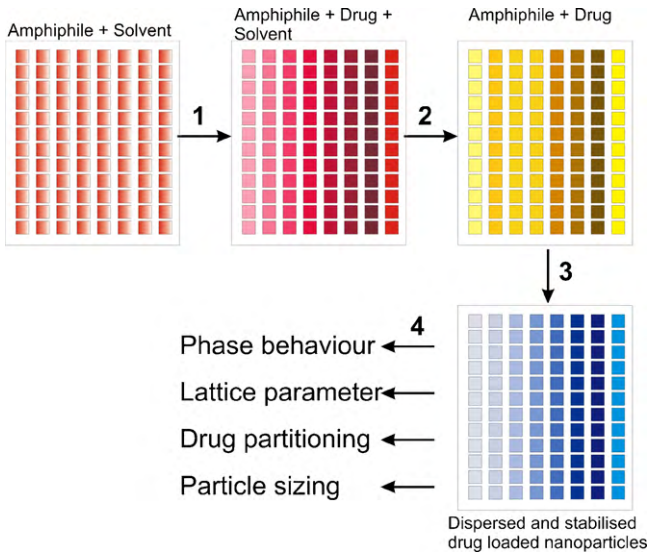

Fig. 4. Schematic depicting nanoparticle preparation. Once the amphiphile in solvent has been dispensed the drug can be aliquoted (step 1) in each well at varying concentrations. All the solvent was removed by vacuum centrifugation (step 2). Once the solvent is removed, water and stabiliser were added to each well and these sequentially sonicated (step 3). Once the samples are dispersed, characterisation of each sample can be performed (step 4).

the Chemspeed™ platform and the chloroform is removed using a Genevac™ centrifugal evaporator (step 2, Fig. 4). To ensure as complete as possible removal of chloroform, the vacuum used was 3 mb and the chamber heated to 35 °C for 2 h. Samples were then stored under vacuum for at least 18 h. Once the chloroform has been removed and 500 μL of steric stabiliser solution added, the plate is again placed on the platform and each well is sonicated in turn (step 3, Fig. 4). The sonicator was set to an amplitude of 80% and a frequency of 0.3 Hz.

2.3. Characterisation

2.3.1. Small angle X-ray scattering/wide angle X-ray scattering (SAXS/WAXS)

The Australian Synchrotron SAXS/WAXS beamline was used to perform scattering experiments. The synchrotron X-ray beam was tuned to a wavelength of 1.0322 Å with a typical flux of 10^{13} photon/s. The 2D diffraction patterns were recorded on a Dectris–Pilatus 1 M detector of 10 modules. The detector was offset to access a greater q-range. Silver behenate ($\lambda = 58.38$ Å) was used as the low-angle X-ray diffraction calibrant for all measurements. A Dectris-Pilatus 200K detector of 2 modules wide angle detector was used to acquire wide angle data.

Diffraction images were analyzed by the IDL-based AXcess software package, developed at Imperial College, London, by Dr. A. Heron (Seddon et al., 2006). The measured X-ray spacings are accurate to within 0.1 Å. The temperature control was achieved in a custom designed high throughput cell capable of holding 34 capillaries with temperature control of approx/0.1 °C. Samples were loaded in 1.5 mm special glass capillaries. The sample cell was mounted on a motorised stage capable of *x*, *y*, *z* motion. Exposure lengths were 1 s for all samples. Sample concentration loaded in capillaries was identical to that found post-sonication by the robot, i.e. 100 mg/mL of amphiphile in solution.

2.3.2. Dynamic light scattering

A Malvern high performance particle sizer (Wors, UK) was used to acquire particle sizing data. Measurements were conducted at 25 °C, using automated settings in low-volume cuvettes.

2.3.3. Drug partitioning

The five most water soluble drugs were tested to observe drug level partitioning into the cubic phase or aqueous region: hydrocortisone, atropine, diazepam, dexamethasone and prednisolone. Nanoseps 100 K (Pall Life Sciences, Vic., AU) centrifuge filter tubes were used to separate the Cubosome™s from bulk water solvated drug by placing the Cubosome™ dispersion in the filter and centrifuging for 10 min at 80,000 rcf. At least 50 μL was collected for each sample, volumes made up to 100 μL with double distilled water and placed in a UV transparent 96-well plate (Greiner, Germany). The absorbance scans were measured and blank Cubosome™ preparations were used as background to remove any signal from amphiphile or stabiliser monomers. Using calibration curves generated using each drug; a total solubilised amount was calculated.

3. Results

The bulk phase amphiphile drug system was sonicated to form nanostructured nanoparticles in 96-well microplates. A plate of the phytantriol drug delivery vehicles is shown in supplemental Fig. S1. Lyotropic liquid crystalline phase dispersions of phytantriol and Myverol™ are typically milky white. Coloration of the dispersions can be observed on the incorporation of drugs, in particular transretinol at higher concentrations.

Particles sizes measured were in the range of 200–400 nm (a full list of average particle size and polydispersity index for all samples is provided in the supplementary material, Tables S1 and S2).

The first step in the characterisation of the nanoparticles involves establishing the mesophase formed by each dispersion and calculation of the lattice parameter. This is performed using SAXS. Analysis of the scattering data yields the phases present in each dispersion at 25 and 37 °C and generates the trends observed in Fig. 5. We have shown an example of the 2D and 1D diffraction patterns observed for the phytantriol:progesterone system in the supplementary material, Figs. S2 and S3.

Dispersions of phytantriol with poloxamer 407 and no drug additives form nanoparticles with a diamond inverse bicontinuous cubic (Q_{II}^{D}) architecture. We found that increased sonication time had no effect on the adopted phase of the nanoparticle.

Progesterone, haloperidol, diazepam and transretinol were found to have a significant effect on the phase behaviour of phytantriol nanoparticles (Fig. 5). These molecules have the highest water/octanol partition coefficient ($K_{o/w}$) of the drugs tested here. It follows that these will therefore tend to reside in the aliphatic region of the amphiphilic nanoparticles and drive phase changes. The drugs that changed the phase of the phytantriol nanoparticle seem to drive an increase in curvature levels present in the systems by inducing a change in phase from Q_{II}^{D} to inverse hexagonal (H_{II}), fluid isotropic or gyroid inverse bicontinuous cubic (Q_{II}^{G}) phase. Increasing the temperature of the system decreases the drug concentration at which these phase changes are observed. Crystalline peaks were observed for indometacin at higher concentrations indicating that the drug precipitated out of solution and was not completely incorporated in the dispersion.

The batch of Myverol™ used in this experiment once dispersed in the presence of poloxamer 407 typically results in Q_{II}^{D} and inverse hexagonal (H_{II}) coexisting phases at 25 and 37 °C (Fig. 5). Increased sonication times induced some H_{II} phase formation and/or a second inverse bicontinuous cubic (Q_{II}^{P}) phase. This can be attributed to hydrolysis of monoolein to glycerol and fatty acid and/or some degradation of the amphiphile due to the presence of unsaturated chains induced by heat generated during the sonication process. Increased sonication time for Myverol™ leads to a decrease in lattice parameter for hexagonal phase of approximately 5 Å from 120 s sonication to 480 s. The Pn3m phase formed shows small 1–2 Å fluctuations in lattice parameters and the Im3m QIIP shows 4 Å fluctuations. These changes in lattice parameters of the phases formed by the Myverol™ amphiphile are plotted in Fig. S4. It was identified that a sonication time of 300 s limited degradation and achieved optimal dispersion. The Myverol™ based nanoparticles are more sensitive to the presence of drug than phytantriol nanoparticles: more drugs are able to induce a phase change. Indometacin, levofloxacin, progesterone, hydrocortisone, diazepam, prednisolone, transretinol and dexamethasone induce significant changes in the phase exhibited by the drug delivery vehicle. Increasing the levels of drug incorporated within the Cubosome™s creates more significant transformation to the particles' mesophase.

The variety of phase changes observed in the presence of the different drugs provides strong evidence for interaction between the drug and the drug delivery vehicle. Phytantriol appears to be a more robust system, and retains its original phase in the presence of higher levels of drugs than Myverol™.

The majority of drugs studied induce a significant change in the lattice parameter of the cubic phase, indicating that interaction is occurring even with those drugs that do not effect a phase change. Fig. 6A shows the maximum lattice parameter change in the Q_{II}^{D} phase of the phytantriol dispersed nanoparticles. Atropine, a relatively water soluble compound, shows the largest change in lattice parameter with an increase of 9 Å. This effectively means that a decrease in membrane curvature and a potential increase in water pore size occurs. From this one can assume that there is atropine incorporated within the bilayer. Similar increases in lattice sizes are observed with, haloperidol, hydrocortisone, prednisolone and dexamethasone. With the exception of haloperidol these molecules have low $K_{o/w}$ values and appear to drive an increase in lattice parameter. The three corticoid compounds all have similar chemical structures and display related effects on the phase behaviour of phytantriol. This is in contrast to progesterone which has an identical conjugated ring structure but fewer hydroxyl moieties present and therefore higher $K_{o/w}$ coefficient values. Progesterone leads to a decrease in lattice parameter and therefore an increase in bilayer curvature, as well as causing a phase transformation of the nanoparticle. The other drug molecules tested that cause a decrease in lattice parameter are levofloxacin, indometacin, diazepam and trans-retinol. This may be due to increased interdigitation of the aliphatic chains or amphiphile tilt induced by the presence of drug molecules within the bilayer. Fig. 6B shows the change in lattice parameter of two drug moieties: atropine and diazepam. The change observed is quasi-linear.

As for the phytantriol–drug systems, significant variations in cubic phase lattice parameter in the presence of drug molecules indicate drug incorporation within the Myverol™ inverse bicontinuous cubic phase. Fig. 7A shows the maximum lattice parameter change of the Q_{II}^{D} phase of the Myverol™ dispersed nanoparticles. Levofloxacin, a highly hydrophobic antibiotic, induces a large increase in the lattice parameter of the Q_{II}^{D} phase of the Myverol™ dispersion, whilst retaining its original mesophase (Fig. 7B). Diazepam causes a linear decrease in lattice parameter (Fig. 7B), similar to the effect seen in phytantriol. The effect of the drug on the cubic phase curvature can vary with temperature; this effect is evident with the corticoid family highlighted earlier: prednisolone, hydrocortisone and dexamethasone induce an increase in lattice parameter at 25 °C, and a decrease in lattice parameter at 37 °C. The decrease in lattice parameter observed is due to increased chain mobility and therefore splay in the fatty acid region of the bilayer, this may in turn facilitate drug incorporation. The more hydrophobic corticoid, progesterone, does not show an increase in lattice parameter. The other drug molecules tested that cause only a decrease in lattice parameter are: indometacin, haloperidol, diazepam and trans-retinol, again this trend can be

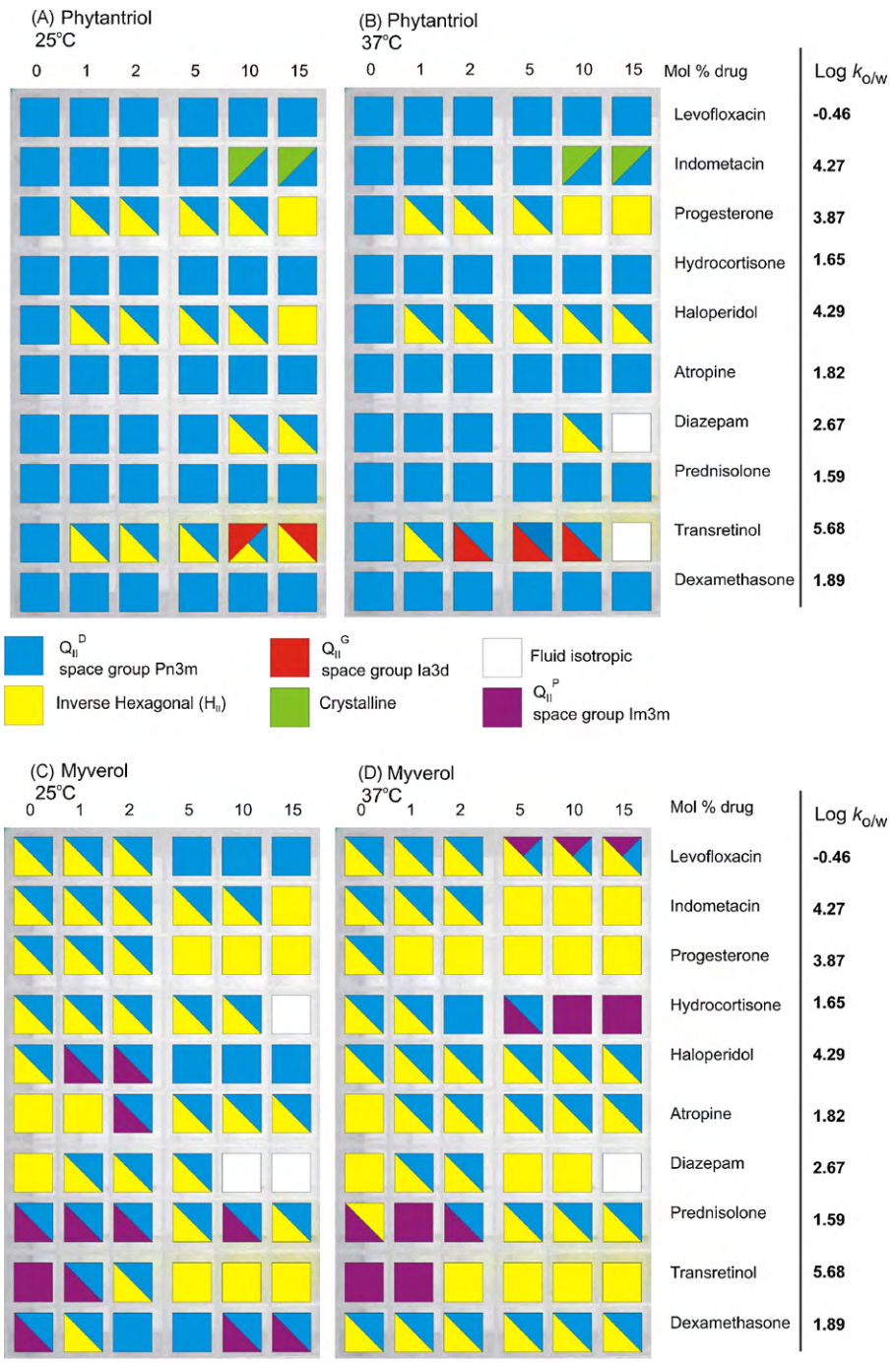

Fig. 5. Effect of drug incorporation for phytantriol and Myverol™ dispersions at 25 and 37 °C. The 0 mol% drug concentration column was subjected to increasing sonication time from 120 to 180, 240, 300, 360, 420, 450, 480, 540 and 600 s down the first column. Phytantriol-drug samples were sonicated for 420 s whilst Myverol™-drug samples were sonicated for 300 s. Log $K_{o/w}$ were obtained from the online database of the Sangster Research Laboratories (http://logkow.cisti.nrc.ca/logkow/).

linked to a high $K_{o/w}$ coefficient. Atropine shows a small increase in lattice parameter at 25 °C and a more significant decrease at 37 °C. Levofloxacin shows an increase in lattice parameter which is the opposite to its behaviour with phytantriol. We postulate that this may be caused by differential packing within the bilayer with the Myverol™ chains having their splaying motion reduced by the presence of levofloxacin.

3.1. Drug partitioning

The aqueous media was separated from the nanoparticles and the level of solvated drug (as opposed to drug which is incorporated within the nanoparticle) tested using UV absorbance. Only the five most water soluble drugs (low $K_{o/w}$), namely hydrocortisone, atropine, diazepam, prednisolone, and dexamethasone could be tested using this methodology as they permitted effective calibration curves to be developed. As highlighted earlier, the presence of precipitated drug could be observed by the presence of crystalline peaks in the scattering data. This precipitation effect was only observed for indometacin incorporated at 10 and 15 mol% with phytantriol. Significant changes in lattice parameter of the cubic phase in the presence of incorporated drug, along with the absence of these peaks for most systems, indicate that the majority of unsolvated drug is associated with the nanoparticles.

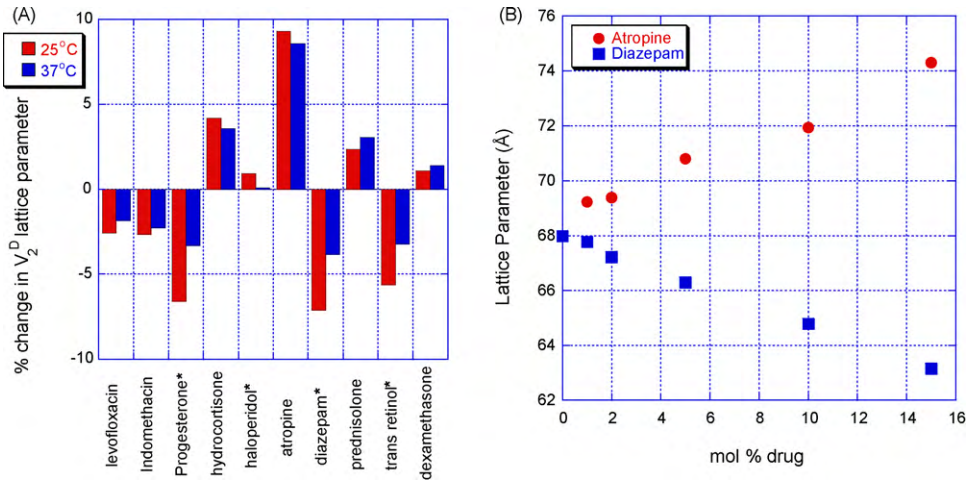

Fig. 6. (A) Representation of maximum lattice parameter changes induced by drugs observed for the Q_{II}^D phase of phytantriol dispersed nanoparticles at 25 and 37 °C. *Labels drugs that induced a phase change. (B) Lattice parameter of Q_{II}^D phytantriol dispersion changes for two representative drugs: atropine and diazepam at 25 °C.

The amount of solvated drug remaining after the formation of the nanoparticles varied significantly between drugs and with concentration of the drug. Atropine was found to have a significant proportion in solution at lower drug concentrations, but as the atropine concentration increased so did the relative percentage incorporated in the nanoparticles. Atropine and prednisolone are more easily incorporated in the Myverol™ nanoparticles compared to the phytantriol dispersion at 1, 2, and 5 mol% concentrations. Hydrocortisone, diazepam and dexamethasone all incorporate at high levels in both the phytantriol and Myverol™ nanoparticle system as shown in Figs. 8 and 9.

4. Discussion

It is evident that cubic phase lipidic nanoparticles can act as drug carriers for a range of therapeutic drugs. The ability to not only produce, but more importantly to characterise these nanoparticles in a high throughput methodology is an important advance in developing self-assembled drug delivery vehicles and opens many potential applications. For example 120 distinct samples were prepared in a standardised operation and characterised at different temperatures at the SAXS beamline at the Australian synchrotron in under 2 h. It will allow us to develop methodologies to incorporate drug libraries with differential loading into the colloidal dispersions. The introduction of drug delivery vehicles in the high throughput drug discovery path may thus occur earlier due to their automated preparation, adding value to libraries and extending the range of compounds that can be tested. These nanoparticles are also compatible with current efficacy or toxicity assays. Creation of nanoparticles using this technique makes them directly suitable for screening purposes due to the range of distinct materials that can be created in short time periods. Typically sonication protocols run overnight result in the preparation of two 96-well plates.

Efforts are being undertaken to develop new amphiphilic lipid self-assembly materials for drug delivery (Sagnella et al., 2009, 2010). In turn as the knowledge of drug interactions with different lipid dispersions increases it will be possible to establish models to predict the structure–property relationship of drug molecules on amphiphilic liquid crystalline dispersions. Such models will have the benefit of estimating ideal drug loadings. We observed an increasing partition coefficient with increased drug loading indicating that drug uptake within the nanostructures increases at increased drug loading levels. This is further evidenced by phase and lattice parameter changes within the self-assembly systems. The ability of the liquid crystal to adapt its phase and lattice parameter, with retention of self-assembly behaviour, is crucial to this capability. This can be observed

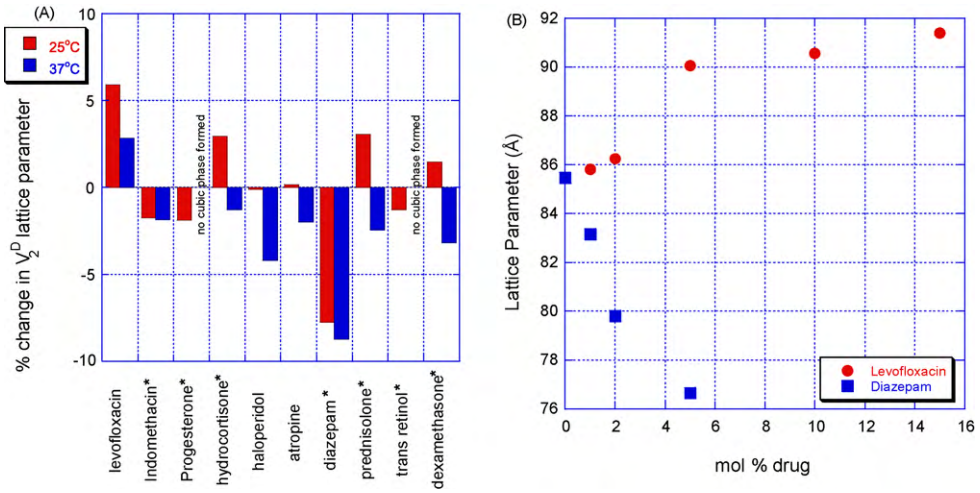

Fig. 7. (A) Representation of maximum lattice parameter changes induced by drugs observed for the Q_{II}^D phase of Myverol™ dispersed nanoparticles at 25 and 37 °C. *Labels drugs that induced a phase change. (B) Lattice parameter of Q_{II}^D Myverol™ dispersion changes for two representative drugs: levofloxacin and diazepam at 25 °C.

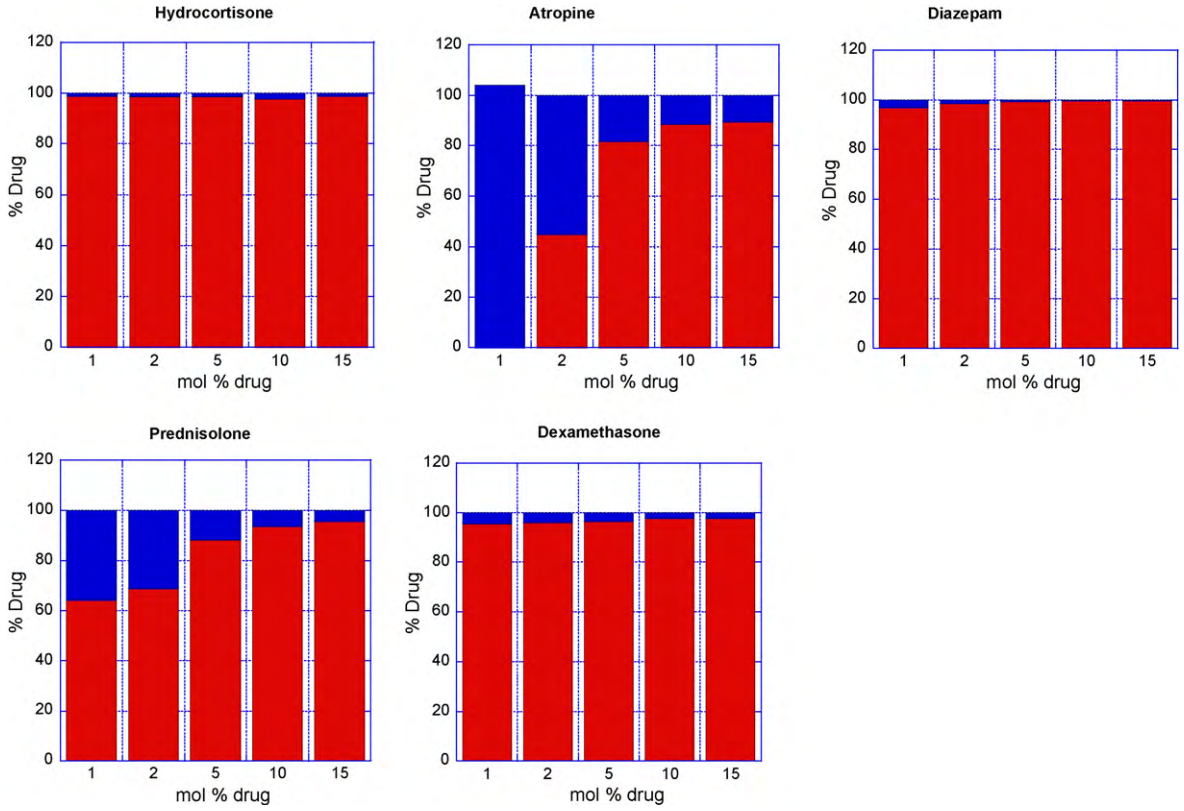

Fig. 8. ( ) Solvated drug, ( ) In mesophase drug. Incorporation of drugs in phytantriol-based nanoparticles as a function of drug concentration. Proportion of the drug population in the excess water phase is shown in blue and red represents drug in the dispersed lyotropic liquid crystalline phase. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

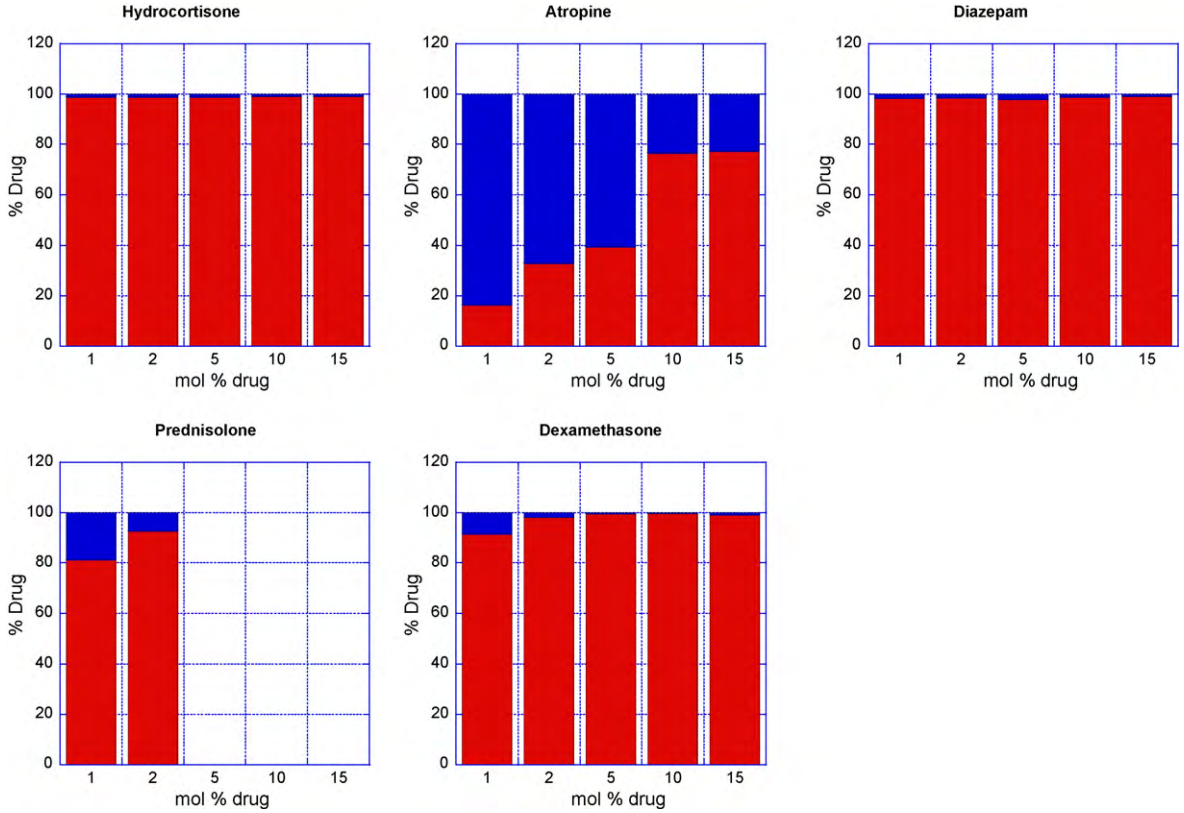

Fig. 9. ( ) Solvated drug, ( ) In mesophase drug. Incorporation of drugs in Myverol™-based nanoparticles as a function of drug concentration. Proportion of the drug population in the excess water phase is shown in blue and red represents drug in the dispersed lyotropic liquid crystalline phase. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

from the partition coefficient against concentration data shown in Figs. 8 and 9.

Using a drug carrier does increase the complexity of the system. One needs to be aware of the effect of lipidic nanoparticles on transporter function, intracellular drug presentation, and generation of immune response. They do however have the ability to be multifunctional (loaded with several therapeutic agents) and targeted. The dispersions of the lyotropic liquid crystalline phases also bear clear advantages over the bulk phase analogues for drug delivery. Their low viscosity makes them suitable for drug screening as they can be handled by automated instruments. Additional functionality can be added to the nanoparticles in the form of active targeting of receptors and loading with imaging labels (PET, MRI). They also address the problem of highly potent and very hydrophobic compounds that often result from drug development. Another of their key advantages is the ability to prepare the drug delivery scaffold loaded with active compounds in a 96-well format which allows for parallel processing and automation.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpharm.2010.05.029.

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