Development of artemether-loaded nanostructured lipid carrier (NLC) formulation for topical application

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Abstract

NLC topical formulation as an alternative to oral and parenteral (IM) delivery of artemether (ART), a poorly water-soluble drug was designed. A Phospholipon 85G-modified Gelucire 43/01 based NLC formulation containing 75 % Transcutol was chosen from DSC studies and loaded with gradient concentration of ART (100-750 mg). ART-loaded NLCs were stable (-22 to -40 mV), polydispersed (0.4 to 0.7) with d90 size distribution range of 247 to 530 nm without microparticles up to one month of storage. The encapsulation efficiency (EE %) for ART in the NLC was concentration independent as 250 mg of ART loading achieved ~61%. DSC confirmed molecular dispersion of ART due to low matrix crystallinity (0.028 J/g). *Ex vivo* study showed detectable ART amounts after 20 h which gradually increased over 48 h achieving ~26 % cumulative amount permeated irrespective of the applied dose. This proves that ART permeates excised human epidermis, where the current formulation served as a reservoir to gradually control drug release over an extended period of time. Full thickness skin study therefore may confirm if this is a positive signal to hope for a topical delivery system of ART.

Keywords: Malaria; Artemether; Nanostructured lipid carrier; Particle characterization; *Ex vivo* skin permeation; Topical formulation.

Introduction

Malaria is caused by five species of parasites of the genus *Plasmodium* that affect humans (*P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi*). Malaria due to *P. falciparum* is the most deadly form and it predominates in Africa; *P. vivax* is less dangerous but more widespread, and the other three species are found much less frequently [1]. Malaria parasites are transmitted to humans by the bite of infected female mosquitoes of more than 30 anopheline species. Globally, an estimated number of 3.4 billion people were at risk of malaria in 2013, with populations living in sub-Saharan Africa having the highest risk of acquiring malaria. Approximately 80 % of cases and 90 % of deaths were estimated to occur in the WHO African Region, with children under five years of age and pregnant women most severely affected [1, 2].

Malaria is an entirely preventable and treatable disease, provided the currently recommended interventions are properly implemented [1]. These include (i) vector control through the use of insecticide-treated nets (ITNs), indoor residual spraying (IRS) and, in some specific settings, larval control, (ii) chemoprevention for the most vulnerable populations, particularly pregnant women and infants [3-6], (iii) confirmation of malaria diagnosis through microscopy or rapid diagnostic tests (RDTs) for every suspected case, and (iv) timely treatment with appropriate antimalarial medicines (according to the parasite species and any documented drug resistance) [1].

Antimalarial drug resistance is a major public health problem which hinders the control of malaria even at attempting some multistage, multivalented vaccines [7, 8]. Resistance is occurring as a consequence of several factors, including poor treatment practices, inadequate

patient adherence to prescribed antimalarial regimens, widespread availability of artemisinin-based monotherapies and substandard forms of the drug [9, 10]. In recent years, parasite resistance to artemisinins – the key compounds in artemisinin combination therapy (ACTs) – has been detected in four countries of the Greater Mekong subregion: Cambodia, Myanmar, Thailand and Vietnam in agreement with the prediction of the World Health Organization (WHO) 2010 [6]. Most countries where malaria is endemic have adopted the WHO recommendation of ACT for fast and reliable malaria treatment [1, 11, 12]. The five ACTs currently recommended for use by WHO are artemether plus lumefantrine, artesunate plus amodiaquine, artesunate plus mefloquine, artesunate plus sulfadoxine-pyrimethamine, and dihydroartemisinin plus piperaquine. The choice of the ACT is based on the therapeutic efficacy of the combination in the country or area of intended use.

Artemisinin and its derivatives should not be used as monotherapies for the treatment of uncomplicated malaria, due to poor adherence to the required 7-day course of treatment which results in partial clearance of malaria parasites hence, promoting resistance to this critically important class of antimalarials [1, 13]. Artemisinin (Qinghaosu) is a sesquiterpene 1, 2, 4-trioxane (sesquiterpene lactone endoperoxide) isolated from the Chinese medicinal herb qinghao (*Artemisia annua* L.). It has been shown to be an effective antimalarial against chloroquine-resistant strains of *P. falciparum* [14]. This compound and its derivatives, such as artemether (ART), dihydroartemisinin, arteether, and artesunate, are effective against both chloroquine-resistant and chloroquine-sensitive strains of *P. falciparum*, as well as against cerebral malaria [14]. Artemether, a potent rapidly-acting schizonticide is practically insoluble in water. It belongs

to BCS class II and possesses oral bioavailability of ~45 % [15]. The generally recommended oral and parenteral administration, once a day for at least 5 days seems reasonable in view of clinical efficacy. Yet the available marketed dosage forms are tablet, capsule and injections. While the parenteral oily formulation leads to pain on injection plus poor patient compliance, the oral formulations are rapidly but incompletely absorbed, limiting its use in malaria [15]. Still it is widely absorbed (GIT and kidney) distributed and rapidly metabolized and cleared from the body with contra-indication in those with severe liver and kidney diseases, haematopathy (e.g. leucopenia or thrombocytopenia) porphyria. It interacts with drugs that increase the QT interval and has numerous adverse effects such as nausea, vomiting, skin eruption, elevated SGPT and SGOT due to large doses (100 mg b. d). Pharmacokinetics of ART suggests that its clinical efficacy is dependent on the formulation [16, 17]. However, in addition to urgent need for new and effective anti-malarial agents, there is also crucial need to utilize the existing drugs through the concept of novel drug delivery systems with the intention of reducing the dose-induced side effects, while achieving enhanced aqueous solubility, active targeting of diseased tissues, increased bioavailability and above all, patient-friendly dosage regimens to enhance compliance and reduce resistance due to non-compliance. As a result, the skin has been chosen as a route of application to assess the penetration of ART into the living epidermis as a positive signal to hope for transdermal systemic delivery into the bloodstream. This way, the longer acting antimalaria combination which requires shorter dosing intervals will remain orally active while the artemisinin component (ART) will be conveniently applied at once to the skin to synchronize the effect of ACT according to the WHO recommendation. This approach will improve patient

compliance since the extreme nausea-vomitting tendency of ART in the ACT would be taken care of at once.

In view of this, nanostructured lipid carrier (NLC) appear to be an attractive approach for the delivery of highly lipophilic drugs such as ART as NLCs have advantages over all other colloidal systems - SLNs, SLMs, liposomes, nanoemulsions, microemulsions [15-20]. This is because the majority of drugs have higher solubility in liquid lipids (oils) rather than solid lipids. The purpose of an NLC formulation is to produce particles in which the oil is incorporated into the core of the solid lipid and the drug is solubilized in the oily core. This should result in a higher loading capacity, encapsulation efficiency and controlled drug release as the drug dissolves in the oil and simultaneously encapsulates in the solid lipid; which should also lead to slower polymorphic transition and lower crystallinity index (higher stability) [20-23]. The drug of study, ART is photolabile and short-acting, so the major criteria were to find a protective carrier with modified release property as well as good consistency (thickner). Here, Gelucire® 43/01 which is a semi-solid block hard fat (HLB 1) that protects APIs (e.g. ART) sensitive to oxidation, humidity and light was used. Gelucire® 43/01 is also a high melting point lipid for modified release dosage forms, in addition to being a consistency agent (thickner) for topical formulations. Combination of lipophilic and hydrophilic surfactants yields better stabilization of dispersed systems. As a result, Gelucire® 43/01 was structured with a phospholipid, Phospholipon® 85G (P85G 15 %) whereas the liquid lipid (Transcutol® HP) formed 75 % of the entire matrix. High oil content of NLC has been associated with less crystallinity [24]. Artemether-loaded NLCs were prepared and evaluated for *in vitro* and *ex vivo* performances.

2. Materials and methods

2.1. Materials

Artemether was a gift from Ipca Laboratories Ltd. India. Gelucire® 43/01 Pellets (a mixture of mono, di and triglyceride with polyethylene glycol esters of fatty acids was used as a consistency agent, sustained release matrix and protective carrier for the photolabile drug ART), Compritol® 888 pellets and Transcutol® P (liquid highly purified diethylene glycol monoethyl ether was used as solvent for the poorly soluble ART) were kind gifts from Gattefossé, France. Phospholipon® 85G (lecithin fraction enriched with phosphotidylcholine greater or equal to 85% was used as a co-emulsifier, emollient and moisturizer as well as to increase ART skin penetration) was a gift from Lipoid GmbH, Germany. Polysorbates 80 and 20, Macrogol 4000 (surfactants) and sorbitol were obtained from Sigma-Aldrich, USA. Pluronic F68 (BASF, Germany) and Sorbitan monostearate (Span 60, Merck, Germany) were also used. Goat fat (*Capra hircus*) was from a batch prepared in the Department of Pharmaceutics, University of Nigeria, Nsukka (Nigeria). Bidistilled water was used throughout the study.

2.2. Screening of starting materials

2.2.1 Selection of lipids

First of all, thermal properties of all bulk lipids were ascertained by differential scanning calorimetry (DSC Q100 TA Instrument, Germany). Binary lipid mixtures (9:1, 8:2 and 7:3) were obtained from each solid lipid (goat fat, Gelucire 43/01 and Compritol 888 Pellet) with the liquid lipid (Transcutol P) respectively at 90 °C for 2 h. The matrices were cooled for 48 h at room temperature for full recrystallization and afterwards re-investigated by DSC. Secondly, the solid lipids were also mixed at 2:1, 1:1 and 1:2 combinations to modify the crystal properties of each individual lipid. Thirdly, ternary systems were obtained from a combination of two solid lipids

with the oil at ratios of 2:1, 1:1 and 1:2. All matrices were studied by DSC and the mixture that showed the least enthalpy values was selected for further investigation.

2.2.2 Drug solubility assessment

To evaluate the solid lipids' suitable for the study, increasing amounts of ART (10-100 mg) were added to each of the solid lipids and melted under magnetic stirring at 200 rpm (Thermomixer comfort, Eppendorf, Germany) for 2 h at 90 °C. Miscibility and solubility of each system was evaluated for visual homogeneity upon melting and cooling. The samples were allowed to cool at room temperature. To obtain information if recrystallisation of ART occurred from the solid lipids, the samples were investigated using DSC by heating each sample (3-5 mg) from 25 to 150 °C and super-cooling the amorphous melt in sealed aluminium pans while empty aluminium pan served as a reference. Samples were heated from 25 to 150 °C at a heating rate of 10 K/min under constant flushing with nitrogen (10 ml/min). The lipid sample which showed no drug recrystallization and was capable of achieving molecular dispersion of ART was selected for further investigation. For liquid oil, surfactant and co-surfactants, ART solubility was also tested as described above though assessment was limited to visual observation of disappearance of drug crystals in each case by forming a transparent homogeneous system.

2.2.3 Preparation of binary lipid mixtures

Since Gelucire 43/01 emerged as the solid lipid with the least enthalpy upon solubilizing ART, admixtures of Transcutol P and Gelucire 43/01 were prepared to contain increasing concentrations of Transcutol P (25, 30, 50 and 75 %) according to the method of Attama *et al.,* 2006 [24]. The admixtures were prepared by fusion at 90 °C for 2 h and allowed to recrystallize

fully at room temperature. The admixtures were analyzed by DSC. They were further loaded with ART and the thermal property reinvestigated by DSC following the procedure earlier stated.

2.2.4 Preparation of surface-modified lipid matrices

P85G (15 % w/w) was also incorporated as a component of the solid lipid (Gelucire 43/01) and melted with different concentrations of Transcutol P as previously done and the resultant matrices re-characterized by DSC as described above. Samples were heated from -80 to 150 °C with a heating rate of 10 K/min under constant flushing with nitrogen (10 ml/min).

2.2.5 Feasibility of using hot homogenization

Before initiating formulation development studies of ART-loaded NLC, it was considered essential to investigate the thermal stability of ART to determine the feasibility of using hot homogenization to produce the NLC formulations. Thermal gravimetric analysis (TGA) has been previously used and recommended as a simple and rapid method to determine the thermal stability of APIs [25-27]. Consequently, the thermal stability of ART was investigated using a TGA 4000 (Pyris 6 TGA, PerkinElmer, Inc. Waltham, MA 02451 USA). The sample (22.646 mg) was weighed directly into an aluminum oxide crucible pan and heated from 30 to 500 °C at a heating rate of 20 °C /min, under constant purging with liquid nitrogen at a flow rate of 20 ml/min.

2.3 Preparation of NLC

NLCs were prepared by hot homogenization/ultrasonication methods. Briefly, graded concentrations of ART (0.75, 0.5, 0.25 and 0.1 % w/w) were dissolved in a 5 % w/v mixture of the solid (10% Gelucire 43/01 and 15 % P85G) and liquid (75 % Transcutol) lipids at 90 °C. The

aqueous surfactant phase containing Tween 80 (2 % w/v) and sorbitol (4 % w/v) heated to the same temperature were added (dropwise) to the oil phase under magnetic stirring at 1000 rpm for 5 min. The resultant pre-emulsion was subjected to high speed mixing at 28,000 rpm using an Ultra-Turrax T25 homogenizer (Polytron PT 2500 E, Kinematica, USA) for 15 min [28]. The obtained emulsions were subjected to probe sonication at 60 % amplitude for 15 min. Each sample was divided into two portions prior to sealing with paraffin and cooling at room temperature for full recrystallization. After 48 h cooling at room temperature, one part of each sealed sample was frozen at -80 °C prior to lyophilization (Martin Christ, Alpha 2-4 LSC GmbH, Osterode, Germany). The freeze-dried samples were used for solid state characterization whereas the non-freeze dried samples were employed to simulate use condition.

2.4 Physicochemical characterization of particles

2.4.1 Particle Morphology

The microstructure of ART-NLC particles were observed using transmission electron microscope (JOEL 1210 JOEL Inc., Boston, MA, USA). Initially, samples diluted with double-distilled water were deposited on film-coated copper grids and the samples were allowed an overnight drying at room temperature. The dried samples were visualized under TEM.

2.4.2 Particle size and polydispersity index

The mean diameter and polydispersity index of the NLCs particle loaded or not with ART were measured using a Zetasizer Nano-ZS (Malvern Instruments, Worceshtire, UK) equipped with a 10 mW He-Ne laser employing the wavelength of 633 nm and a backscattering angle of 173° at 25 °C. All samples were diluted with a fixed amount of double-distilled water to obtain a suitable scattering intensity, before Photon correlation spectroscopic (PCS) analysis, and were

then placed in a 10 mm diameter cell. Particle size analysis was performed using Mie theory with the refractive index and absorbance of lecithin at 1.490 and 0.100 respectively.

2.4.3 Zeta potential analysis

The zeta potential of NLC formulations was determined via electrophoretic mobility measurements using a Zetasizer Nano-ZS (Malvern Instruments, Worceshtire, UK). The zeta potential was calculated applying the Helmholtz–Smoluchowski equation (n = 3).

2.4.4 Particle size distribution by Nanoparticle tracking analysis (NTA)

For all NTA analysis, all samples were diluted 1:7000 with bidistilled water. NTA was performed with digital microscope LM10 HS System (NanoSight, Wiltshire, UK). The diluted samples were injected with sterile syringes (BD Discardit II, New Jersey, USA) into the sample chamber equipped with a 640 nm diode laser (red). All samples were measured in a single shutter and gain mode for 90 s with manual shutter, gain, brightness, and threshold adjustments at room temperature. The video images of the particles, moving under Brownian motion, were captured and analyzed by the NTA 2.0 image analysis software NanoSight LM10 HS (NanoSight Ltd., Minton Park, Amesbury, Wiltshire SP47RT, UK), 635 nm laser with a high sensitivity camera (EMCCD). Three measurements of each newly injected, sample were performed. The mean size and SD values obtained by the NTA software were based on the arithmetic values calculated with the sizes of all particles analyzed by the software to determine the D10, D50, D70 and D90 as number median diameters size distributions.

2.5 Degree of crystallinity and polymorphism

To evaluate the degree of crystallinity and polymorphism of NLC formulations, sufficient quantity of NLC formulation enough to contain at least 3-5 mg of ART was weighed in aluminum pans, heated from 25 to 150 °C at 10 °C /min under constant flushing with nitrogen (10

ml/min). The DSC parameters, such as temperature onset, maximum peak, and enthalpy, were generated.

2.6 Entrapment efficiency (EE) and drug loading (DL)

The encapsulation efficiency (EE) of ART-loaded NLC was determined by ultrafiltration using Vivaspin filter tubes (Vivaspin, Germany), which consisted of a filter membrane with a molecular weight cut-off of 10,000 at the base of the sample donor chamber. Two milliliter aliquot of undiluted sample of ART-loaded NLC was placed in the upper chamber and the sample recovery chamber was fitted below the membrane in the lower compartment. The unit was closed and centrifuged at 11,000 rpm for 2 h at 30 min intervals using a centrifuge (Model 420 R Rotina Hettich, Germany). The principle behind this process was to separate the aqueous phase by filtering it through the membrane into the recovery chamber. The filtrate was appropriately diluted with acetonitrile: water (90:10) and the amount of ART in the aqueous phase was estimated by a validated RP-HPLC. The EE and DL were calculated from the following equations:

$$EE\ (\%) = \frac{Real\ ART-loading}{Theoretical\ ART-loading}\ x\ 100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots....(1)$$

$$DL\ (\%) = \frac{Amount\ of\ encapsulated\ ART}{Total\ weight\ of\ the\ particles \in the\ formulation}\ x\ 100\ \ldots\ldots\ldots\ldots\ldots\ldots.(2)$$

2.6.1 HPLC method for artemether

HPLC determination of ART was performed using a Dionex P680 HPLC pump (ASI-100 automated sample injector) equipped with UV/VIS detectors operating at 208 nm (210 and 214

nm). Samples were chromatographed on a stainless steel C18 reverse phase column (250 x 4.0 mm) packed with 5 µm particles (Lichrospher® 100 RP-18). Elution was conducted with a mobile phase of acetonitrile:water (90:10) at a flow rate of 40 µl/min at 40 °C for 5 min. A calibration curve was plotted for ART in the concentration range of 5-10 µg/ml. A good linear relationship was observed between the concentration of ART and the peak area of ART with a percentage correlation coefficient of 99.7904 %. The required studies were carried out to estimate the precision and accuracy of the HPLC method.

2.7 Stability study

NLC formulations were stored at room and refrigeration temperatures for one month. At different time intervals of 1, 6, 15 and 30 days, the average particle size, pdi and ZP were redetermined. Samples were estimated in triplicates. Statistical analysis of the data was performed using Student's t-test. A probability of less than 0.05 ($P<0.05$) was considered significant.

2.8 *Ex vivo* skin permeation study

Ex vivo skin permeation study was performed using a Franz diffusion cell. The effective diffusion area was 1.767 cm^2, and the receiver chamber had a capacity of 12 ml. The excised human epidermis from abdomen of female donor was mounted between the donor and acceptor compartments of the diffusion cell. Formulation weights of 1.13, 1.20 and 1.00 g containing 28, 30 and 25 mg of ART respectively were applied to maintain a constant concentration in the donor compartment which was occluded during the whole experiment. The acceptor compartment was filled with ethanol/water mixture (50:50) and continuously stirred by a stirring bar at 50 rpm. The whole assembly was fixed in an incubator at 32 °C. Samples (400 µl) were withdrawn at

different time intervals and analyzed for drug content by HPLC at 208 nm UV-VIS absorption. Sink condition was maintained and the receptor phase was replenished with equal volume of fresh acceptor phase at each sample withdrawal. Amount of drug permeated per unit area of the skin was plotted against time.

3.0 Results and discussion

3.1 Screening of lipids

Table 1 shows the thermal properties of the bulk lipids, binary and ternary matrices. All binary and ternary matrices melted at lower temperatures than their corresponding bulk matrices. Lower enthalpies were generally obtained from matrices containing a mixture of solid and liquid lipid rather than two solid lipids. Worthy of note was the combination of Gelucire 43/01-Transcutol P at a ratio of 7:3 which melted with an initial enthalpy of 1.395 J/g and lastly at 11.13 J/g. The visual assessment of homogeneity of this matrix upon melting and cooling showed changes in the appearance and/or texture of the resultant mix that was different from that of the individual lipids. Upon melting a higher concentration of this lipid mixture at a ratio of 70:30, the enthalpy obviously became 4.31J/g. When ART was melted in it, the enthalpy value drastically reduced as the concentration of ART increased, such that the 5 mg ART-containing matrix melted at 7.563 J/g while the 10 mg ART counterpart melted at 2.031 J/g. This agrees with earlier reports that lipid matrices with a certain degree of disorder are considered to be ideal for formulation of nanoparticulate lipid carriers due to their high active ingredient payload capacity [24].

3.1.2 Drug-lipid solubility determination

To further confirm the preliminary selection of the lipid matrix (Gelucire 43/01-Transcutol P), DSC assessed ART solubility at 10-100 mg in individual solid lipids (goat fat, compritol 888 pellets and Gelucire 43/01) and liquid lipid (Transcutol P) prior to nanostructured lipid matrix of Gelucire 43/01-Transcutol 70:30 as well as phospholipid-modified nanostructured lipid carriers [28]. It is known that employment of two surfactants of lipophilic and hydrophilic nature yields better stabilization of dispersed systems [28]. All lipids were able to solubilize up to 100 mg of ART. The melting peak of ART was 86.96 °C, which means that drug degradation during NLC preparation (90 °C) would most likely not occur (Table 2). The DSC results of matrices containing different concentrations of Transcutol (25, 30, 50 and 75 %) showed concentration-dependent (75>50>30>25) decreases in enthalpy of matrices in the order of 0.5124<0.8062<4.341<15.67 J/g respectively. The enthalpy values were reduced similarly, which indicated the presence of less crystalline lipidic structures in the mixtures especially in the 75 % Transcutol composition. However, the 75 and 50 % Transcutol-containing matrices melted with initial high enthalpy values due to formation of unstable polymorphs which at higher temperature of 73.05 and 50.47 °C melted into their respective stable forms with lower enthalpies (0.5124 and 0.8062 J/g). Since there was no peak due to the drug in all matrices, it also suggests that the drug was molecularly dispersed in the matrices. The magnitude of both these changes was greater as the amount of drug increased.

3.1.3 Feasibility of using hot homogenization

Due to dearth of data describing the thermal stability of ART, the present study first of all attempted to ascertain the effect of use of a relatively high processing temperature (90 °C) as well as to find out if the drug was thermolabile. Therefore, TGA was used to establish the thermal stability of ART by measuring the loss in weight of a pure ART sample as a function of

increasing temperature, and this weight loss was then correlated to the thermal stability of ART. TGA has been previously used to determine the thermal stability of various drugs [25, 26]. TGA profile of bulk ART showing the percentage weight loss of ART as a function of increasing temperature (30 to 500 °C at a heating rate of 10 °C/min) at a constant rate is shown in Fig. 1 (data in Table 3). In fact, the small initial weight loss of 1.70 % in the temperature range between 30 °C and 175 °C could be attributed to water loss because ART is slightly hygroscopic according to the manufacturer's instruction [29]. However, ART appears to decompose in a process involving two major stages as the temperature to which the drug sample was exposed exceeded 175 °C (Table 3). The idea was to investigate whether ART would be thermolabile when exposed to temperatures between 86 – 90 °C, which is the temperature range the molecule melts at and would be exposed to during NLC production. Since ART did not exhibit significant weight loss upon exposure to such simulated high temperature then the proposed processing temperature (90 °C), appeared feasible; hence, ART-loaded NLC could be prepared using hot melt homogenization method.

3.2 Particle morphology

Images of the shape and surface morphology of NLC formulations in aqueous systems were studied by TEM (Fig. 2a) and Fig 2b (SEM of optimized formulation). There was nanoparticle aggregation in the blank formulation (image 2E) and in 500 mg ART-loaded formulation (image C). This could be due to insufficient drying time of the sample during sample preparation prior to TEM analysis. The particles therefore contained a mixture of spherical and non-spherical (anisometric) shapes. Single particles of the ART-free formulation (E) appeared more cuboid (image F) while the ART-loaded nanoparticles in images A, B and D were spherical. The shape of solid lipid nanocarriers has been reported to be dependent on the purity of the lipids used [23] and particles prepared from highly pure lipids are usually more cuboid in nature [30],

whereas those obtained using chemically polydispersed lipids are typically spherical [31]. The lipid matrix used consisted of a mixture of solid lipids (Gelucire 43/01 and P85G) and liquid lipid (Transcutol), which suggests that the lipid matrix was chemically polydispersed as well as highly pure.

Even though expected that only spherical particles would be present in all formulations (and not only in A, B and D), it should also be realized that the polymorphic nature of the lipid matrices used could determine the shape of the particles. So that particles which existed in the stable β-modification assumed anisometric shapes, whereas those that existed in the metastable α-polymorphic forms appeared spherical in nature [32, 33]. This agreed with literature as the shapes of solid lipid nanocarriers established using TEM may be spherical [34] or non-spherical [35, 36]. In summary, TEM images reveal spherical particles for ART-loaded batches containing different concentrations of ART (0.1, 0.25 and 0.75 g) corresponding to images A, B and D; perhaps suggesting that the nanoparticles in these formulations existed in the α-polymorphic form. Blank formulation (image E) and 0.50 g ART-loaded batch (image C) comprise of spherical and non-spherical particles, which suggests that the nanoparticles in these formulations coexist as the α- and β-polymorphic forms. However, the degree of crystallinity and polymorphic nature of particles in all formulations were further confirmed by DSC.

3.3 Particle size, polydispersity index and zeta potential

Homogenization followed by ultrasonication has been reported [28]. Use of surfactant (Tween 80) and co-surfactant (P85G) of hydrophilic and lipophilic natures yield better stabilization of the disperse system [28]. Pre-formulation studies with different concentrations of surfactant (Tween 80) showed that lower surfactant concentrations yielded larger particles while higher concentration of Tween 80 favored lower particle size, narrower size distribution (within

nanometer range) and better long term stability of lipid nanoparticles (data not shown). However, to avoid toxicity issues, moderate surfactant concentration of 1 % w/v has been employed in the present study to obtain small enough stable particles. Meanwhile use levels of 1.5 – 2 % Tween 80 has been reported in literature [28]

Particle characterization of NLC dispersions was essential to ensure the production of stable product of suitable quality. One day following production, PCS data showed that the mean particle size of ART-free NLC was 1506 nm while ART-loaded NLCs containing 750, 500, 250 and 100 mg of ART had particle sizes of 1145, 533, 497 and 591 nm respectively. Increase in the amount of ART in the NLC formulations did not have much influence on the particle size as well as polydispersity indices (0.9, 0.9, 0.6, 0.6 and 0.7). This agrees with an earlier report that stated that loading concentrations of *trans*-retinoic acid had no influence on the particle size of SLN [38]. It invariably suggests that entrapment and loading efficiency of ART would be less at higher than lower concentrations.

In the present study, the lipid content of NLC formulation was set at 5 % w/v. ART concentration was varied from 100 to 750 mg to estimate the effect of this increasing drug concentration on encapsulation and loading efficiency. Particle size was independent of drug-loading, in order words, ART-loaded formulation containing 0.25 g of ART had the least particle size at all measurement times of 1, 3 and 6 days post formulation (497.2, 379.0, 249.0 nm) respectively (Tables 4, 5). This also confirms that %EE and DL of ART into NLC would be independent of initial drug concentration, as confirmed by Table 4. It appeared that drug encapsulation and loading were more favorable at lower concentrations (100-250 mg) than at higher concentrations (500-750 mg). In other words, the 250 mg ART-containing NLC formulation had the highest %EE of approx. 61 % whereas the 750 mg ART-containing NLC had

29 % EE. Meanwhile all particles were within nanometer size ranges even though polydispersity index (0.4 to 0.7) indicated broad distribution that would result in particle growth and physical instability. Surprisingly, upon storage, the particles became smaller in size (Table 5). For all formulations, a decrease in particle size was observed within 1 week of preparation as well as 3 weeks of formulation for the reconstituted freeze-dried samples. Since there were decreases in particle size rather than increase in particle growth, it perhaps meant that the particles were physically stable. This was therefore confirmed by the zeta potential measurement which showed that the particles well existed above -30 mV, as those recommended (ZP values of ≤ -30) for aqueous nanoparticle dispersions to be considered stable [39] both as fresh dispersion or freeze-dried products. This suggests that the surface properties of the particles in all formulations were not altered during the 1-month storage period and, therefore, the stability of the NLC formulations can hereby be inferred. Relating stability to NLC batch which had the highest EE of ART (optimized formulation), it became most evident that the particles of this formulation were quite spherical, almost monodispersed (PDI 0.4) upon storage and existed well above -30 mV. To validate data obtained from PCS technique, nanotracking of particles was done to obtain direct qualitative view of the samples under analysis in order to rule out the discrepancy in PDI and presence of large particles.

3.4 NTA

The NTA data showed that the d90 value for un-loaded NLC formulation was 530 nm whereas those of ART-loaded NLCs containing 750, 500, 250 and 100 mg of ART were respectively 247, 362, 346 and 299 nm (Table 6). This confirms that microparticles were not present in the NLC dispersions. Once again, an increase in the amount of ART added to the formulation had no effect on the NTA data; thus suggesting low drug loading at higher

concentration of ART. PCS and NTA data agreed that sizes of the particles in all NLC formulations remained within the nanometer range following storage for 1 month at 25°C. For polydisperse samples, the NanoSight system exhibits significant advantages over photon correlation spectroscopy (dynamic light scattering technique) in that the size distribution profile obtained is based on separate but simultaneous analysis of each and every particle seen during the analysis period. The NanoSight systems therefore, visualizes in real time and in liquids the paths taken by these particles under Brownian motion over a suitable period of time (e.g. 90 s) while the trajectory described for each particle is tracked for as long as it is visible or until it crosses with that of an adjacent particle [40]. The rate of the movement is related solely to the viscosity of liquid, temperature, and size of the particles. Each particle is simultaneously, but separately visualized and tracked from frame to frame by particle tracking image analysis software [41]. This is in contrast to the data obtained by PCS in which a large ensemble of particles are measured and from which only intensity weighted (Z-average) is obtained.

The rate of the particle movement is related to a sphere equivalent hydrodynamic diameter and calculated through a variation of the Stokes-Einstein equation. NTA operates for particles from about 10 to 1000 nm in diameter depending on type of particle. Analysis of particles at the lowest end of this range is possible for particles composed of materials with high refractive index, such as gold or silver. The upper size limit is restricted by the Brownian motion of large particles; because a large particle moves very slowly with diminished accuracy. Viscosity of the solvent also influences the movement of particles, and too plays a part in determining the upper limit for a specific system. For medium sized particles such as obtainable in the present study, nanoparticle tracking analysis measurements were suitable. Yet the

formulation could be improved to obtain physically stable particles by using high pressure homogenization instead of ultrasonication in the production of the particles.

3.5 Degree of crystallinity and polymorphism

To gain insight into the thermal behavior of ART prior to NLC formulations, DSC analysis of pure ART coarse powder and ART-loaded (optimized) lipid matrices were done to determine the degree of crystallinity and polymorphism therein. The DSC profile of ART obtained showed a melting peak of 86.96 °C with an enthalpy of 87.51 J/g. Upon super-cooling the amorphous melt, ART recrystallized to a large extent.

DSC parameter of aqueous NLC formulations measured following storage at 25°C for a period of 2 weeks are summarized in Table 7. Generally, the onset temperatures and peak maxima (MP, 42.14-49.29 °C) of the NLC formulations were all higher than that of the bulk lipid material (Gelucire 43/01) which showed an onset and MP of 30.98 °C and 38.71 °C respectively. However, NLC formulations achieved far lower enthalpies than their corresponding drug-containing matrices (Table 2) before formulation as well as the pure drug sample (98.33 J/g). In addition, all formulations had melting endotherms indicating that the particles slightly recrystallized and that there were no supercooled melts present in all formulations. Low recrystallinity indices (RI, 8.05, 2.23, 3.44, 2.40 and 4.37 %) also confirmed that 75 % Transcutol content greatly disorganized the matrices (blank NLC and ART-loaded NLCs containing 0.75, 0.50, 0.25 and 0.10 g of ART) respectively. It has been established that irrespective of the lipid matrix and/or the type of surfactants used to ensure thermodynamic stability of solid lipid nanocarries, that the major factor affecting the thermal behavior of solid lipid-based carriers is the particle size. In other words, the onset temperature, melting peak, and melting enthalpy of triacylglycerol-based carriers are directly proportional to the size of the particles [42].

In the present study, sorbitol which acted as a cryoprotectant exhibited a melting peak at 102.52 °C with an enthalpy of 1.792 J/g. All NLC formulations melted in two stages. The initial event was indicative of recrystallization of an unstable polymorph, and the latter event showed the melting of the most stable form. It was generally observed that there was complete absence of drug peak in all ART-loaded NLC formulations. All thermal properties were independent of drug concentration. The slightly increased onset temperature of 49.29 °C (which was highest in the ART-free NLC batch) and the much reduced enthalpies in addition to the broad and less-intensive endothermic melting peaks (with no peak due to the drug) for all ART-loaded formulations, could be attributed to a decrease in the degree of crystallinity and a change to the more stable polymorphic form of ART. The enthalpy of the ART-free NLC formulation was 0.075 J/g whereas those of ART-loaded NLC were 0.021, 0.038, 0.028, 0.04 J/g corresponding to 700, 500, 250 and 100 mg concentrations of ART respectively. This is most likely to have resulted from high oil content (75 % Transcutol P); hence a lower matrix crystallinity [24]. Since the thermal property was dose-independent, it implies that higher drug encapsulation efficiency would most likely occur in matrices loaded with smaller concentration of ART rather than NLC containing the highest ART loading [24, 33]. In this regard therefore, the DSC result hereby agrees with and confirms the results of particle characterization (PS, PDI and ZP) by photon correlation spectroscopy as well as EE of ART which mostly favored the 250 mg ART-loading in the NLC. It could be henceforth stated that the 250 mg ART-loaded NLC formulation (≅61 % EE) is the optimized formulation followed by 100 mg ART batch (≅52 % EE). Therefore *ex vivo* studies would be carried out only on the optimized formulation containing 250 mg ART since confirmatory stability and drug content have been affirmed.

3.6 *Ex vivo* skin permeation experiment

The skin permeation profile is shown in Fig. 3. There was gradual skin permeation of artemether from the nanostructured lipid carriers detectably at 20 h and with sustained permeation over 46 h. Amount of ART permeated varied with dose of formulation over time (Fig. 3) but overall at 46 h, ~26 % cumulative amount of ART (Fig. 4) had permeated irrespective of dose [43-45]. A control experiment employing only ART in solution was impossible due to insolubility of ART in water. Also there was no marketed transdermal formulation of ART which could have been used as alternative. Yet, the experiment has at least demonstrated that ART can permeate human excised epidermis. This can be seen as a positive signal to hope for a controlled release topical delivery system of ART, which may be devoid of the drug's extensive nausea-vomiting effect which majorly account for patient non-compliance aside from some contra-indications. A considerable inter-individual variation in the pharmacokinetics of oral ART has been reported [46, 47]. In the treatment of malaria, the therapeutic concentration of ART must be maintained throughout at least 3-4 cycles of schizongony (7 days) in order to achieve a radical cure. In other words, a minimum therapeutic concentration of 50 ng/ml (trough level) is expected to be maintained throughout the period of dosing. Since the optimized ART-loaded NLC could release 3-6 μg/ml of ART within 46 h, it is expected to provide minimum steady state blood concentration of ART necessary to obtain sufficient treatment. However, further study on full thickness skin is hoped to complete the proof of concept.

Conclusion

The *ex vivo* permeation result presented here indicates permeation of ART through human excised skin which is known to mimic permeation *in vivo* [48-50]. There was gradual and sustained permeation of ART through the skin into the acceptor phase (50:50 ethanol-water). By implication, more advanced skin studies could be conducted on full thickness skin to understand

the penetration of ART through different skin layers. Since ethanol has acted as a permeation enhancer in the Franz diffusion cell experiment, it is expected that its inclusion in reformulation of ART-NLC into semi-solid forms such as film/patches or gels, would enhance drug permeation. These semi-solid forms are thought to increase use convenience and room temperature stability of the resultant topical regimen in malaria endemic tropical areas of the world where storage at 4-8 °C may not readily be guaranteed. In this case, the upper left arm may be the desired skin area to secure the dosage form for extended periods of time in order to achieve a sufficient high artemether concentration in the blood.

Acknowledgement

Dr. P. O. Nnamani is grateful to TWAS-DFG for funding the research visit to Helmholtz-Institute for Pharmaceutical Research Saarland (Ref. 3240263814) . She greatly appreciates the mentorship of Prof. Dr. Claus-Michael Lehr and his team on this visit. We are grateful to Gattefossé, France for gift samples of Gelucire® 43/01 Pellets, Compritol® 888 pellets and Transcutol® P. We also thank Lipoid GmbH, Germany for Phospholipon® 85G sample.

Conflict of interest

The authors declare no conflict of interest concerning the work.

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