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Research paper

Vancomycin ocular delivery systems based on glycerol monooleate reversed hexagonal and reversed cubic liquid crystalline phases

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ABSTRACT

Lyotropic bulk reversed hexagonal and reversed cubic liquid crystalline phases (hexagonal and cubic phases) composed of glycerol monooleate (GM) were used to design the vancomycin hydrochloride's (VHCl) delivery systems aiming to maintain VHCl's therapeutic concentration during 24 h in the eye, locally (as an insert) and/or intravitreally (as a bulk phase injection).

Bulk VHCl's hexagonal and cubic phases were successfully prepared by melted homogenization and solvent evaporation method, and then an insert was prepared.

The structural characteristics of liquid crystalline phases were studied using cross polarized light microscopy and small angle X-ray scattering technique. The presence of VHCl (1–9.5% w/w VHCl solution) did not exhibit any change in the liquid crystalline phase's structure to another liquid crystalline phase, and showed little effect on the lattice parameter of the existing liquid crystalline phase structure.

In order to relate the liquid crystalline phase structure to VHCl's release rate locally into the eye, *in-vitro* release test of an implant has been done using a simulated tear fluid. VHCl's release in the simulated tear fluid from the cubic phase obeyed Higuchi kinetics, with linear VHCl's release versus the square root of time. The hexagonal phase released VHCl in simulated tear fluid significantly slower than the cubic phase. In order to relate the liquid phase structure to VHCl's diffusion intravitreally, *in vitro* release test by the Sirius‘ Subcutaneous Injection Site Simulator (*Scissor*) has been performed. Comparing the release properties by a *Scissor*, the VHCl's cubic phase demonstrated slower permeation through extra-cellular matrix than the VHCl solution.

To evaluate the efficacy of the system investigated, the release properties of VHCl's cubic phase were compared with literature data indicating that the cubic phase could be a potential matrix system in the delivery of VHCl intravitreally during 24 h after intravitreal injection. The release data in the simulated tear fluid indicated that the cubic phase should be further optimized to achieve a therapeutic VHCl concentration locally in the eye during 24 h.

1. Introduction

Endophthalmitis is a purulent inflammation involving both the anterior and posterior segments of the eye resulting in permanent eye damage and loss of sight. It may be endogenous or exogenous, such as acute postoperative, chronic postoperative, traumatic or after intravitreal injections. Postoperative endophthalmitis is one of the most serious complications after cataract surgery with intraocular lens insertation. Intraocular contamination occurs relatively commonly after uneventful cataract surgery and the rate of culture positivity in anterior chamber aspirates ranges 0–46% [1]. It has been revealed that bacteria routinely enter the anterior chamber during cataract extraction. Bacteria may be carried into the eye by irrigation or may adhere to the intraocular lens as it is inserted through the incision. Acute bacterial endophthalmitis represents a true ophthalmological emergency and an effective therapy requires both an immediate and a prolonged treatment with appropriate antibiotics.

Vancomycin hydrochloride (VHCl) is a glycopeptide antibiotic that has been widely used as the primary treatment for infections caused by the methicillin-resistant *Staphylococcus aureus* (MRSA), *Staphylococcus aureus* and other *Staphylococcus* species, reported to be responsible for about 70% of postoperative endophthalmitis [2,3]. The intra-ocular

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irrigating solution of vancomycin used during cataract surgery is not capable to keep an efficient antibacterial level over a sufficient period of time (at least 11 h). VHCl's ophthalmic drops used in the therapy of endophthalmitis are of the conc. 25–50 mg/ml. The standard dosing of VHCl's ophthalmic drops is 1 drop per hour for the first 24 h, then taped gradually according to clinical improvement [4]. Furthermore, VHCl is poorly absorbed from the gastrointestinal tract, so its intravenous administration has been suggested. However, VHCl cannot achieve therapeutic levels in the aqueous humor by this route [5]. Therefore alternative methods must be adopted to prolong local and intracameral vancomycin level and improve the efficacy of this appropriate antibiotic.

The poor availability of drugs into the ocular chamber can be due to the high turnover rate of tears, lower corneal permeation and rapid naso-lachrymal drainage. For enhancing the ocular bioavailability, a variety of formulations have been investigated like gels or hydrogels, *in situ* gelling system [6], polymeric inserts (*Ocusert* [7]) [8], microparticulate polymeric delivery systems [9–13], cubosomes [14]. Hydrogels limit their value due to improper administration that is not reproducible. Gelling systems may cause blurred vision and lachrymation. *Ocusert* revealed a sustained release but it can cause crusting of eye lids, unintended elimination during sleep, and meddling with vision. *In situ* gelling systems showed prolonged result as compared to free solution or suspension. Hachicha et al. [11–13] investigated poly(lactide-co-glycolide) microparticles: they studied the influence of preparation parameters on the particle size, microparticles stability, encapsulation rate, *in-vitro* and *in-vivo* efficacy. Vancomycin-poly(lactide-co-glycolide) microspheres after topical administration in rabbits showed higher and prolonged vancomycin concentration, and increased AUC values (2-fold) with respect to an aqueous solution. However, loading of peptide as VHCl into poly(lactide) and poly(lactide-co-glycolide) microparticles can be problematic owing to their high hydrophilicity. Nanoparticles, like cubosomes are investigated as an effective approach to improve the ocular bioavailability of ketorolac (2.07 folds higher transcorneal permeation, 2.24 folds higher corneal retention regarding to ketorolac solution) [14].

In the last decades, lipids have received considerable attention as an alternative to polymeric system (carrier or coating material) in development of drug delivery systems. Among lipids, the amphiphilic lipids have been generating substantial interest in the design of delivery system for different molecules due to their potential to incorporate molecules of varying physicochemical properties (hydrophilic and lipophilic) and to provide controlled release properties. Amphiphilic lipids form aggregates through a self-assembly process that is driven by the hydrophobic affect when they are mixed with water. Depending on composition and temperature amphiphilic lipids form different liquid crystalline phases: cubic, hexagonal, lamellar. The cubic phase structures built-up either with discontinuous hydrocarbons regions but with continuous water regions in normal micellar aggregates in water or with discontinuous water regions but with continuous hydrocarbon regions in reversed micellar aggregates. The structure of cubic phase is unique and consists of three-dimensional continuous but non-intersecting water channels separated by a lipid bilayer. Hexagonal phase has a two- dimensional periodicity with rod-like aggregates of infinite length packed into a hexagonal lattice. The cubic and hexagonal phases have been recently investigated due to their interesting structural and release properties such as controlled/prolonged drug release. Comprehensive reviews of bulk liquid crystalline phases as drug delivery systems can easily be found in the literature [15–17].

GM is one of the most widely amphiphilic lipid studied in the formation of various lipid liquid crystalline drug formulations. Due to intriguing structure properties of cubic and hexagonal phase following different release properties, both liquid crystalline phase structures were the point of investigation in this study. The aim of this research was to investigate bulk hexagonal and cubic phases in the form of an insert (local application into the eye) or bulk phase injection (intravitreal application) as new ocular VHCl drug delivery systems that are able to maintain VHCl's therapeutic concentration locally and/or intravitreally during 24 h. A significant advantage of such systems is that the matrix material, GM, is a nontoxic, biodegradable and biocompatible material, classified as GRAS (generally recognized as safe) and it is included in the FDA Inactive Ingredients Guide. According to the phase diagram GM/water, it was reported that the GM cubic phase undergoes thermal transition to a hexagonal phase at around 71 °C [18,19]. Therefore, the GM-water system is not able to form a hexagonal phase at room or physiological temperatures. Different approaches could be used to create a reversed hexagonal phase at room or physiological temperatures, i.e. using new lipid materials such as oleyl glycerate, phytanyl glycerate [20,21], by adding oil/hydrophobic substance such as sesame oil [22], sodium oleate [23], R-(+)-limonene [24], tetradecane [24,25], cyclohexane [26], triolein [27], m-xylene, decane, hexadecane [28–31]. Lipophilic molecules are able to alter the liquid crystalline phase structure by integrating into the hydrophobic region on the interfacial film and promoting a more negative interfacial curvature (i.e. higher curvature toward the water region) than a bicontinuous cubic phase, and an inverse hexagonal phase is formed.

Bulk liquid crystalline phases were prepared by melted homogenization and solvent evaporation methods. The specific aim of this study was to establish a ternary phase diagram of glycerol monooleate-(paraffin oil)-vancomycin hydrochloride-water in order to identify hexagonal and cubic phases. In addition, to optimize the formulation regarding water content, to characterize them macroscopically, microscopically, by SAXS and to investigate the release pattern from both liquid crystalline phases.

2. Materials and methods

2.1. Materials

Glycerol monooleate (GM) (MONOMULS 90-O 18 PH, 97 w/w % monoglyceride (almost a white paste at room temperature, melting point 35–37 °C) was kindly provided by Cognis GmbH, Monheim am Rhein, Germany, and was used as received. Vancomycin hydrochloride (VHCl) (94.6% purity) was purchased from Alpharma Taizhou Pharmaceuticals Co. Ltd., Zhejiang, China.

Paraffin oil (PO) was provided by Herba Chemosan ACM, Graz, Austria. Triethylamine was of Bio Ultra purity (≥99.5%) (GC) and was bought from Sigma-Aldrich, Chemie, Steinheim, Germany.

Acetonitrile, dichloromethane and tetrahydrofuranol of HPLC grade were supplied by VWR BDH Prolabo, Leuven, Belgium. Water was prepared with Milli-Q® Gradient water purification system (Millipore S.A.S., Molsheim, France) until resistivity was 18.2 mΩ.

2.2. Methods

2.2.1. Phase diagrams

Ternary phase diagrams were investigated to identify the compositions of different liquid crystalline phases at room temperature. To determine the concentration of components for the prevailing range of the hexagonal and cubic phases, ternary phase diagrams were prepared using the water-titration method at room temperature [32,33]. To determine the concentration of components for the prevailing range of the VHCl loaded hexagonal and cubic phases, ternary phase diagrams were generated using the VHCl solution-titration method at room temperature.

The resulting systems were evaluated based on their visual appearance and polarized light microscopy.

2.2.1.1. The phase diagram glycerol monooleate (GM) – Paraffin oil (PO) – Water (W). A ternary phase diagram of the glycerol monooleate (GM)-paraffin oil (PO)-water (W) systems were prepared first by mixing warmed GM at 40 °C (GM is a white, waxy paste, having a melting point

35–37 °C) with PO at ratios of 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, 9:1 (w/w) to a total of 20 g. A specific amount of each mixture was transferred into a glass vial and mixed by a vortex with adding water drop wisely at ratios of 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, and 9:1 (w/w) to a total weight of 2 g. The preparations were homogeneously vortex-mixed for 3 min and then centrifuged at 623*g* for 30 min at room temperature. The samples were allowed to equilibrate for one week prior to liquid crystalline phase identification. The concentrations are given as mass percentages of the representative ingredients. Macroscopic examination at 20 °C included the visual inspection of formulations after one week.

2.2.1.2. The partial phase diagram glycerol monooleate (GM)-paraffin oil (PO) – Vancomycin hydrochloride solution (VHCl). The partial ternary phase diagram of glycerol monooleate (GM)-paraffin oil (PO)-vancomycin hydrochloride solution was prepared first by mixing warmed GM (40 °C) with PO at ratios of 8:2, and 9:1 (w/w) to a total of 20 g. A specific amount of each mixture was transferred into a glass vial and mixed by a vortex adding a VHCl solution (1%, 5% and 9.5% w/w) drop wisely at ratios of 8:2, and 9:1 (w/w) to yield a total weight of 2 g. The preparations were homogeneously vortex-mixed for 3 min and then centrifuged at 623*g* for 30 min at room temperature. The samples were stored for one week prior to liquid crystalline phase identification.

2.2.1.3. The partial phase diagram glycerol monooleate (GM) – Vancomycin hydrochloride solution (VHCl). The partial ternary phase diagram of glycerol monooleate (GM) – vancomycin hydrochloride solution (VHCl) was prepared by mixing warmed GM (40 °C) with VHCl solution (1%, 5% or 9.5% w/w) drop wisely at ratios of 6:4, 6.5:3.5, 7:3, 7.5:2.5, and 8:2 (w/w) to result a total weight of 2 g. The preparations were homogeneously vortex-mixed for 3 min and then centrifuged at 623*g* for 30 min at room temperature. The samples were stored for one week prior to liquid crystalline phase identification. The systems were categorized according to their appearance and light polarizing microscopy.

According to the phase diagram GM-water, the area of investigating the cubic phase falls within the range of approx. 20–42% w/w water [18].

2.2.2. Preparation of the bulk phases

2.2.2.1. Preparation of the hexagonal phase. All operations required in the preparation of the formulations were carried out under aseptic conditions in a class II safety cabinet (Safety cabinet Typ KS12, Thermo Scientific Thermo Electron LED, Langenselbold, Germany). The laboratory glassware used was previously sterilized by steam sterilization according to the European Pharmacopoeia using an autoclave (Autoklav Typ 26, Melag Medizintechnik, Berlin, Germany). The filters used for VHCl solution were 0.22 μm (Sterile Syringe Filter, Cellulose Acetate, VWR International, Vienna, Austria).

GM and PO were warmed at 40 °C. VHCl solution was prepared and filtered with a 0.22 μm membrane filter. A certain amount of VHCl solution was added dropwise into the GM-PO melted mixture under mixing by the Cito Unguator®E 100 homogenizer (GAKO® International GmbH, München, Germany). Formulations were prepared at room temperature, then packaged in glass vials and stored at 20 °C before performing further evaluations.

The hexagonal phase samples without VHCl (e.g. to select an optimized hexagonal phase) were prepared in the same way. In order to select an optimized hexagonal phase, the formulations were prepared with the following water content (in triplicate): 10, 15, 20, 25, 30, 35, 40 and 50% w/w. The samples of hexagonal phase were prepared in triplicate with and without VHCl.

2.2.2.2. Preparation of the cubic phase. All of the operations required in the preparation of formulations were carried out under aseptic conditions as described above (2.2.2.1 Preparation of the hexagonal phase).

VHCl and GM were separately dissolved in water and dichloromethane, respectively. The VHCl solution was filtered with a 0.22 μm membrane filter. The solutions were placed together in a 250 ml glass flask that was then mounted on a Büchi Rotavapor® R-215 (Büchi Labortechnik AG, Flawil, Switzerland). The solvent was then evaporated at 20 °C, under speeds varying from 200 to 280 revolutions per minute (rpm) and pressure ranging from 0.70 to 0.90 bar, until the desired quantity of the final product was obtained. Formulations were filled into glass vials and kept at 20 °C until examination.

The cubic phase samples without VHCl were also prepared in the same way (e.g. to select an optimized cubic liquid crystalline phase). In order to select an optimized reversed cubic phase, the formulations were prepared with the following water content: 20, 25, 30, 31, 33, 35, 37, 40 and 45% w/w.

The samples of cubic phase were prepared in triplicate with and without VHCl.

2.2.3. Preparation of the insert

The insert of hexagonal or cubic phases was prepared from bulk phases using a syringe and with consideration to the size of insert. The inserts were approx. 2 mm in diameter, and approx. 4 mm in length. They were used in the *in-vitro* release test in simulated tear fluid.

2.2.4. Characterization of liquid crystalline phases

2.2.4.1. Polarized light microscopy (PLM). Preliminary inspection of the optical texture of liquid crystalline phase samples was done by polarized light microscopy discriminating isotropic lipid liquid crystalline phases such as the cubic, L_2, and L_3 from anisotropic phases such as the L_α lamellar and the hexagonal (H_{II}). PLM was carried out between crossed polarizers and examined for homogeneity and birefringence.

Samples were inserted between two glass microscope slides and observed with a Zeiss microscope ("Axiovert" 40 CFL, HBO 50/AC, Carl Zeiss, Germany) equipped with a cross-polarizer and attached to a digital Nikon D300 camera (Nikon, Japan) and a PC monitor.

2.2.4.2. Small angle X-ray scattering (SAXS). Small angle X-ray scattering (SAXS) measurements were carried out in order to determine the self-assembled structure in the bulk liquid crystalline phases.

The SAXS equipment consists of a sealed tube X-ray generator (DebyeFlex3000) attached to a SAXSess camera (Anton-Paar, Graz, Austria). The sealed X-ray tube has a Cu-target emitting Cu-Kα radiation (λ = 0.154 nm), and can be operated at a maximum power of 2000 Watt. At the outlet, the X-rays are monochromatized and focused in vertical direction (Multilayer-mirror optics) and then confined by a vertical asymmetric Kratky slit collimation-system.

The scattering patterns were recorded by a Mythen 1 K (Dectris, Switzerland) Detector (1280 pixels a 50 μm, active length: 6.4 cm). The scattering curves were recorded 10 times 10 s.

The scattered radiation is measured in transmission mode within an angular range (2Q) of 0.1° to ~7° with respect to the incident beam. From the scattering vector one can extract nanostructural information regarding a sample in the range from 1 to 100 nm.

The samples of liquid crystalline phases are filled in a special paste holder where the sample is sandwiched between two foils and sealed vacuum tight. The sample holder is inserted into the sample holder block for temperature control at 25 °C (Peltier element).

The software used was SAXSquant (Anton-Paar, Graz, Austria). The SAXSquant is a comprehensive control/data-acquisition/data-evaluation software for the automated control of camera system components, automated measurements and for producing scattering curves (1D) from detector data.

In the bicontinuous cubic phases, the radius of water channels can be estimated considering water and lipids as two distinct domains.

To get the radius of water channels, one has to first calculate the length of the lipid chain, $L_{lip.}$, as follows [34–36]:

$$\phi = 2A_0\left(\frac{L_{lip.}}{a}\right) + \frac{4}{3}\chi\left(\frac{L_{lip.}}{a}\right)^3 \tag{1}$$

where $A_0 = 3.091$ and $\chi = -8$ for *Ia3d* cubic phase structure, a is the structure parameter as measured by SAXS and ϕ is the lipid volume fraction.

From the known values of lipid chain, $L_{lip.}$, and structure parameter, the radius of the water channels (r) inside the bicontinuous cubic with *Ia3d* symmetry could be estimated as follows by [36]:

$$r = 0.248a - L_{lip.} \tag{2}$$

where a is the structure parameter and $L_{lip.}$ is the length of the lipid.

Thus, the radius of the thickness of water domains is a function of only variables a and ϕ, and the constant $L_{lip.}$.

Calculation of the radius of water channels (r) in reversed hexagonal phase is a little more complex since $L_{lip.}$ is not constant in the repeat cell, due to the packing frustration in the interstitial spaces. The radius of the water channel in the reversed hexagonal phase could be estimated as follows [36]:

$$r = a(1-\phi)^{1/2}\left(\frac{\sqrt{3}}{2}\right)^{1/2} \tag{3}$$

Along a center-to-center edge of the repeated cell, the radius of the water channel in the hexagonal phase is as follows [36]:

$$r = \frac{a - 2L_{lip.,U}}{2} \tag{4}$$

where $L_{lip.U}$ represents the length of the unfrustrated lipid chain, r is the radius of a cylinder of the hydrophilic part, a is the structure parameter measured by SAXS.

2.2.4.3. *HPLC analysis of vancomycin hydrochloride*.

The quality and quantity of vancomycin hydrochloride were analyzed using reversed phase high performance liquid chromatography according to the European Pharmacopoeia (01/2008:1058, corrected 6.0). This method was used on samples extracted from formulations to evaluate the amount of vancomycin hydrochloride in bulk phases (drug content) and released (*in-vitro* drug release tests).

The HPLC system consisted of an HPLC system (1260 Infinity Agilent® Technologies, Germany) equipped with a quaternary pump (1260 Bio Quat Pump, DEAB600659), a degasser, an ALS auto sampler equipped with a G1330B 1290 thermostated column oven, and a diode-array detector set at 280 nm. The separation system was an octadecylsilyl silica gel C18(2), 250 × 4.60 mm (5 µm) column. The equilibration buffer (mobile phase A) consisted of 92% triethylamine buffer (with 6% phosphoric acid, pH = 3.2), 1% tetrahydrofuran and 7% acetonitrile, while the elution buffer (mobile phase B) included 70% triethylamine buffer (with 6% phosphoric acid, pH = 3.2), 1% tetrahydrofuran and 29% acetonitrile. The HPLC system was performed in gradient mode, starting with 0% elution buffer, and, after 26 min, ending with 100% elution buffer. The flow rate was set at 1.0 ml/min. at a temperature of 25 °C. The OpenLAB CDS Rev, C.01.05 software was used for peak integration. The quantification was performed using a standard calibration curve at the investigated concentration range.

2.2.4.4. *Drug content from the bulk hexagonal and cubic phases*.

Drug content was determined in order to make sure that the added amount of VHCl is present in liquid crystalline phase.

A certain quantity of the bulk hexagonal/cubic phases (about 50 mg) was dissolved in approx. 5 ml dichloromethane, and then this mixture was shaken twice with 4 ml of phosphate buffer, pH = 3.2. The thermomixer (Thermomixer *Eppendorf Comfort*, 25 °C, 750 rpm, 2 × 20 min) (Thermomixer *Eppendorf Comfort*, Eppendorf AG, Hamburg, Germany) was used for shaking during the extraction procedure. The upper water layer was taken out and diluted with the phosphate buffer, pH = 3.2 to the appropriate volume. A 0.2 µm cellulose acetate membrane syringe filter (VWR International, USA) was used to filter the solution before HPLC analysis.

Drug content was calculated by the following equation:

$$\%\ drug\ \ content = \frac{amount\ \ determined\ \ by\ \ HPLC\ \ analysis}{theoretical\ \ amount} \times 100 \tag{5}$$

The standard calibration curves for VHCl were linear (the correlation coefficients were > 0.9993) over the studied concentration range (0.01–0.25 mg/ml). The lowest amount of VHCl which has been quantitatively determined corresponded to 0.008 mg/ml. Reproducibility expressed as relative standard deviation was below 1% (0.07–0.54%).

2.2.4.5. *In-vitro vancomycin release test*.

The *in-vitro* VHCl release test of inserts prepared from hexagonal and cubic phases was carried out in freshly prepared simulated tear fluid (pH = 7.4) [37] in 1.5 ml polypropylene tubes (Greiner Bio One GmbH, Frickenhausen, Germany), in triplicate, at 32 ± 0.5 °C, using the Eppendorf Comfort thermomixer (Eppendorf AG, Hamburg, Germany) shaken at 300 rpm. The release media consisted of 1 ml of simulated tear fluid. Tubes were filled with weighed quantities of inserts (10–20 mg) and then 1.0 ml simulated tear fluid was added. Throughout the release studies, an aliquot of 0.2 ml was withdrawn at predetermined time intervals (0, 0.08, 0.17, 0.5, 1, 2, 3, 6, 10, 24 h), centrifuged (Centrifuge Eppendorf 5804R, at 24 °C, 14 000 rpm, 10 min) and analyzed using the above described HPLC method.

The subsequently withdrawn sample was replaced with an equal volume of fresh simulated tear fluid maintained at 32 °C to compensate for the loss due to sampling.

The percentage of VHCl released was calculated based on actual VHCl content. Furthermore, drug release was plotted against the square root of time, as drug release from the liquid crystalline phases has been shown to be mainly controlled by diffusion through the matrix and can consequently be described by the Higuchi diffusion equation as:

$$Q = [D_m C_d (2A - S_d)t]^{1/2} \tag{6}$$

where Q is the mass of the drug released at time t, and is proportional to the apparent diffusion coefficient of the drug in the matrix, D_m, the initial amount of drug in the matrix, A, and the solubility of the drug in the matrix C_d [38].

The slope of the line is proportional to the apparent diffusion coefficient for the drug in a system indicating diffusion as the primary means of drug release.

2.2.4.6. *In-vitro vancomycin release test – Sirius‘ subcutaneous injection site simulator (Scissor)*.

Scissor (Fig. 1) is a new commercially available *in-vitro* tool for subcutaneous/intraocular injection site simulation (Sirius Analytical Instruments Ltd.,/Pion Inc., UK). *Scissor* allows the study of environmental changes that a drug may experience as it transitions from a formulation environment to the human body. The latter is mimicked in terms of pH, temperature, pressure, buffer and tissue composition.

The aim of using this method was to evaluate the permeation properties of formulation(s) in simulated *in-vivo* conditions (to evaluate the formulation with best absorption properties into the eye).

Scissor contains a cartridge (representing the injection site in the human body, such as a vitreous humor) filled with an artificial extracellular matrix (ECM). ECM consists of a hyaluronic acid gel. The cartridge is suspended in a chamber (containing a buffer similar to the interstitial fluid, carbonate buffer) and the formulation components (API and excipients) diffuse through a customized membrane

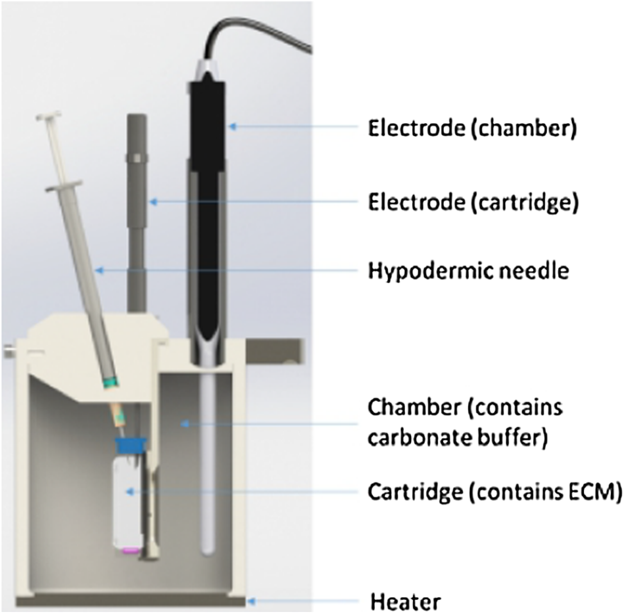

Fig. 1. Schematic of the *Scissor* system chamber featuring: injection cartridge acting as a simulated injection site, pH probe for monitoring the pH inside the cartridge, light source and detector for monitoring aggregation, chamber containing physiological buffer (simulating the body's interstitial fluid) and thermocouple and heater/stirrer.

(molecular weight cut off of 100 000 Da) into the chamber.

The permeation profile of two formulations: the VHCl solution and the cubic phase prepared with the VHCl solution at 9.5% w/w, was investigated by *Scissor*. About 1 g of each formulation was injected into the cartridge (300 mL of carbonate buffer, pH = 7.4) and at the time points: 0; 1; 2; 3; 4; 6; 8; 16; 24; 36; 48, and 72 h an aliquot of 0.1 mL was withdrawn and analyzed using the above described HPLC method. The system was maintained at 32 ± 0.5 °C and the physiological media in the infinite sink was maintained at pH 7.4 by the addition of CO_2.

3. Results

3.1. Phase diagrams

3.1.1. The phase diagram glycerol monooleate (GM) – Paraffin oil (PO) – Water (W)

The aim of generating a phase diagram GM-PO-W was to determine the hexagonal phase area for the selection of appropriate concentrations of GM, PO and W in subsequent liquid crystalline formulation development.

The phase diagram of GM–PO–W (Fig. 2) was done in a wide range of the following components: GM 1–81% w/w, PO 1–81% w/w, and W 10–90% w/w.

The GM-PO-W phase diagram is given in Fig. 2. At room temperature, the system exhibits three different regions: a suspension, an isotropic liquid/gel and a hexagonal phase.

The largest region, the suspension denoted in Fig. 2, extends from the water corner to the PO corner and partly to the GM corner of the phase diagram and belongs to the area: GM 1–63% w/w, PO 1–81% w/w, water 10–90% w/w (black dots in Fig. 2).

At low GM content of up to ~21% w/w, the GM floats in the mixture of high water content (~60–90% w/w) and low PO content (1–32% w/w). These samples are easily resuspended after shaking.

As the concentration of GM increases and water decreases, GM starts to sediment at the bottom and at low PO concentration is easily resuspended. Furthermore, as the concentration of PO increases and the water content decreases, the suspension becomes more viscous and is more difficult to resuspend (GM 3–48% w/w, PO 27–63% w/w, water 10–70% w/w).

Some of these systems (GM 28–49% w/w, PO 12–21% w/w, and water 30–60% w/w) could be described as a suspension gel.

At higher concentrations of PO of 20–81% w/w and lower water content (10–60% w/w), some GM is probably dissolved in PO, as less lipids are noted on the bottom. An isotropic liquid to isotropic gelled

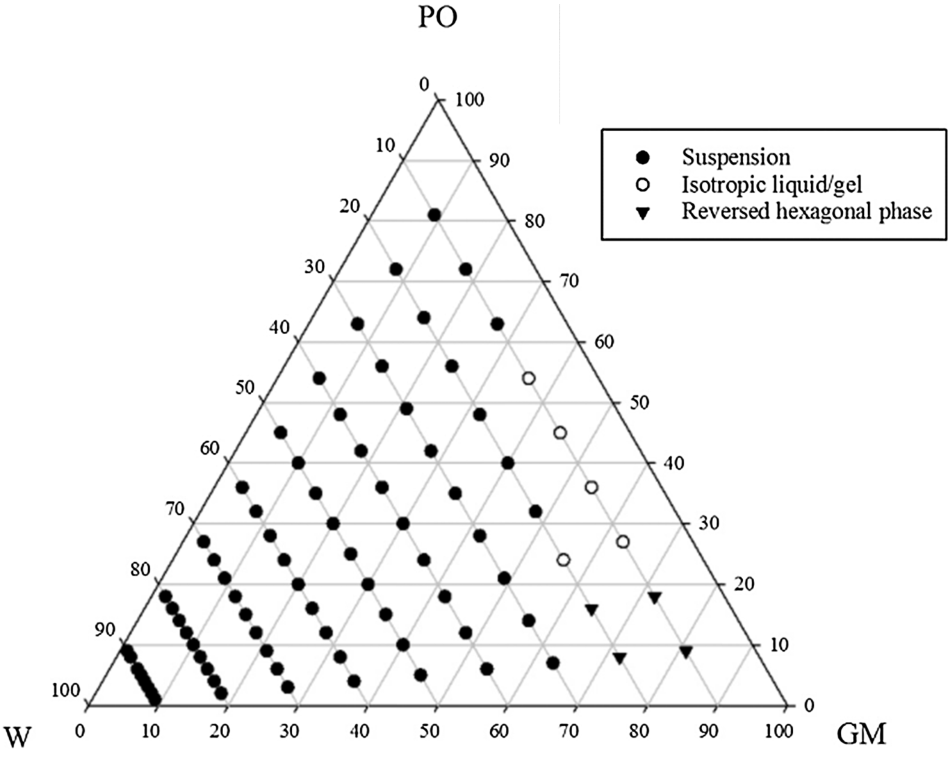

Fig. 2. Ternary phase diagram of formulations prepared from GM, PO and water.

Fig. 3. Polarized light microscopy image of the hexagonal phase. The magnification was 200 ×.

liquid was obtained at the GM 36–63% w/w, PO 24–54% w/w, and at a water concentration of 10–20% w/w.

In the suspension samples with GM 24–40% w/w, PO 6–10% w/w, and water 50–70% w/w, traces of the cubic phase were noted. Close to this area where the cubic phase is observed, suspension samples of GM 27–63% w/w, PO 3–14% w/w, water 30–70% w/w showed traces of the hexagonal phase.

Next to the above mentioned suspension area, the reversed hexagonal phase was observed. It shows a homogeneous semisolid system. The anisotropic hexagonal gel is noted by polarized light microscopy (Fig. 3) in the area GM 64–81% w/w, PO 8–18% and water content 10–20% w/w.

Polarized light microscopy was used to determine the isotropy/anisotropy of the systems and ultimately analyze the presence of anisotropic hexagonal (Fig. 3) or isotropic cubic phases.

The ternary phase diagram in Fig. 2 presenting the appearance of the samples was established seven days after manufacturing. A variety of consistencies was identified in the suspension formulations, from an easily resuspendable suspension, viscous suspension, suspension-gel to solid/paste-like consistencies. Solid ones had a more lipid character, appearing dry and difficult to resuspend. The high consistency was probably due to the low amount of polar liquid component, which was not enough for the swelling or dissolution of the high GM content.

Since cubic phases are optically isotropic and exhibit no birefringence, their formation is simply judged by the optical transparency of the sample.

3.1.2. The partial phase diagram glycerol monooleate (GM)-paraffin oil (PO)-vancomycin hydrochloride solution (VHCl)

The aim of generating the partial phase diagram GM-PO-VHCl solution was to screen the area (composition) representing the VHCl hexagonal phase (Fig. 4).

This partial phase diagram was designed according to the results of the phase diagram GM–PO–W phase diagram (Fig. 2), in which the hexagonal phase appears in the range: GM 64–81% w/w, PO 8–18% w/w, and water 10–20% w/w.

Therefore, the partial ternary phase diagram was developed with GM 64–81% w/w, PO 8–18% w/w, and water content 10–20% w/w (0.1–1.9% w/w VHCl, 9.05–19.8% w/w water).

A limiting factor for the VHCl concentration in this ternary system was the high viscosity of the VHCl solution above about 10% w/w VHCl. Due to this, we have only information on the phase equilibria in the concentration region below 10% w/w VHCl solution. Thus, three VHCl concentrations, 1%, 5% and 9.5% w/w (corresponding to 0.2–3.8% VHCl), were incorporated in the above mentioned composition of GM and PO. All three VHCl solutions, 1%, 5% and 9.5% w/w, incorporated into the previously described composition of GM and PO, showed the structure of the hexagonal phase, as confirmed by polarized light microscopy (Fig. 4). The points demonstrating the hexagonal phase composition using different VHCl concentrations (1%, 5% and 9.5% w/w) are overlapped in a ternary diagram (Fig. 4).

3.1.3. The partial phase diagram glycerol monooleate (GM) – Vancomycin hydrochloride solution (VHCl)

The aim of generating the partial phase diagram GM-VHCl solution was to confirm the structure of the cubic phase in the presence of VHCl in the area where the pure cubic phase appeared (Fig. 5).

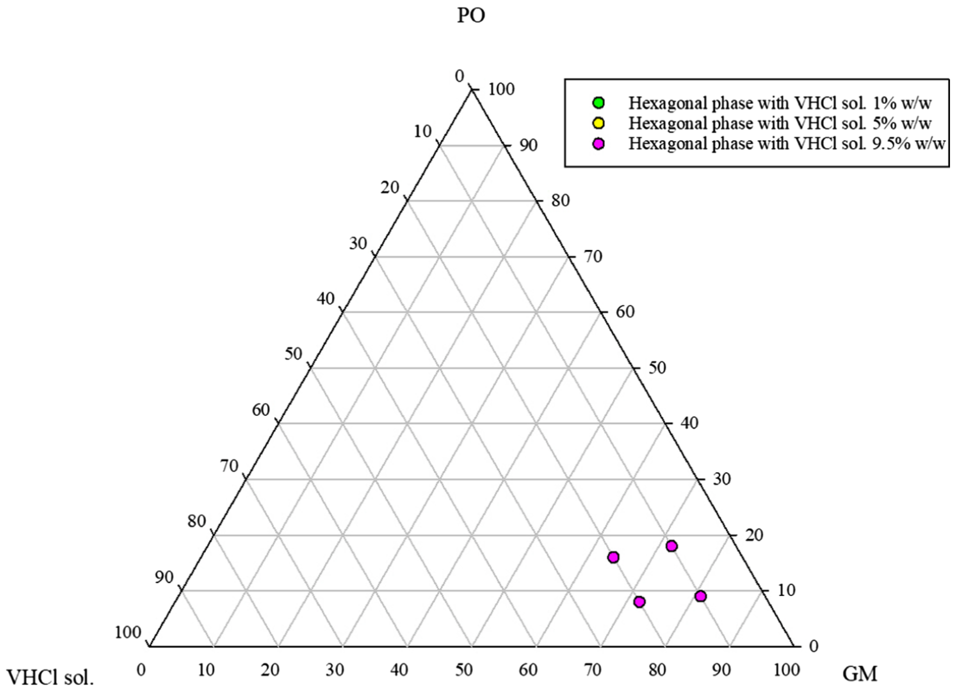

Fig. 4. Partial ternary phase diagram of formulations prepared from GM, PO, and VHCl sol. 1, 5 and 9.5% w/w.

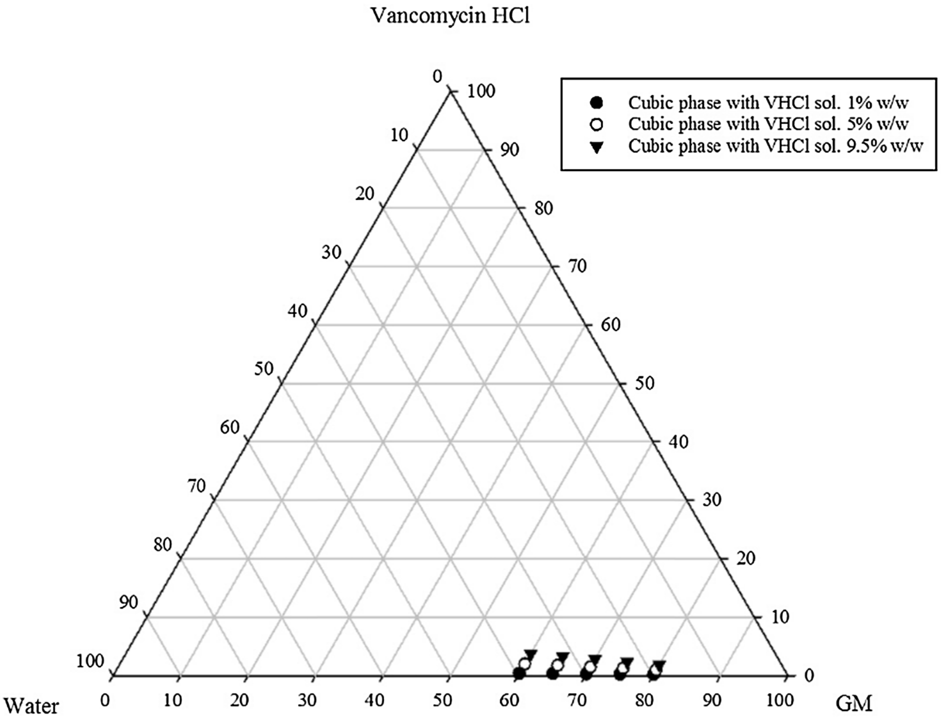

Fig. 5. Partial phase diagram made of formulations made up of GM, and VHCL solutions: 1, 5 and 9.5% w/w.

In the range of GM 60–80% w/w, all samples prepared with the VHCl solution showed a cubic phase structure since these samples were optically isotropic when viewed under polarized light (Fig. 6).

3.2. Preparation of liquid crystalline phases

3.2.1. Preparation of the hexagonal phase

3.2.1.1. Optimization of the hexagonal phase. The optimization of both phases, hexagonal and cubic, was done with consideration to the water content. It is known that both structures, hexagonal and cubic phases, swell in water and are in equilibrium with an excess of water.

Fig. 7 demonstrates the structure parameter behaviour in the GM-PO-W hexagonal phase at different water percentages. At lower water content, from 10 to 30% water w/w, there is more or less of a linear increase of the structure parameter corresponding to the phase behaviour below saturation. At full hydration conditions, the structure parameter is constant and the hexagonal phase exists in equilibrium with excess water. The water content, represented as maximum solubilization, corresponds to the crossing of these two behaviours. Our SAXS results of samples with different water content: 10, 15, 20, 25, 30, 35, 40 and 50% w/w water, indicate that the maximum swollen hexagonal phase structure was obtained with a minimum of about 28% w/w water in the formulation of hexagonal phase.

3.2.2. Preparation of the cubic phase

3.2.2.1. Optimization of the formulation of the cubic phase. The cubic phase was also optimized regarding water content to avoid a burst release. The evolution of the structure parameter in these phases is demonstrated in Fig. 7.

As illustrated in Fig. 7, GM was found to form the pure *Ia3d* cubic phase at lower water content (up to 30% w/w) and the pure *Pn3m* cubic phase from ~38% water w/w. Within the range, both cubic phases, *Ia3d* and *Pn3m*, exist together.

The structure parameter (Fig. 7) in the *Ia3d* cubic phase was increased with water content, but the system had not been saturated yet. However, in the range of pure *Pn3m* cubic phase existence, the structure parameter was constant and the system saturated leading to a smaller negative curvature. The pure *Pn3m* phase appears close to the water saturation of the GM. Later our results will show that the incorporation of VHCl caused shrinkage of the structure leading to decreased water capacity. In order to make sure that no excess water in the phases is used in further experiments, water content of 33% w/w was chosen.

Following the optimization process of hexagonal and cubic phases, the formulations of the hexagonal and cubic phases were prepared according to the optimized formula and VHCl loading at three different concentrations: 1, 5 and 9.5% w/w VHCl.

3.3. Influence of VHCl on the structure of liquid crystalline phases

3.3.1. Influence of VHCl on the structure of the hexagonal phase

The influence of VHCl on the structure of the hexagonal phase was done by SAXS on the optimized formulation of the hexagonal phase (Table 1). Table 1 shows that the structure parameter of the hexagonal phase decreased as vancomycin concentrations increased (1, 5 and 9.5% w/w vancomycin hydrochloride), indicating that the structure of hexagonal phase was shrunk.

3.3.2. Influence of VHCl on the structure of cubic phase

Influence of VHCl on the structure of the cubic phase was investigated by SAXS on the optimized formulation of the cubic phase (Table 1).

When VHCL was loaded at 3 different VHCl concentrations into the optimized formulation of cubic phase, the structure of cubic phase was preserved (Table 1). However, the preserved cubic phase corresponded to the *Ia3d* symmetry.

In addition, it was demonstrated that by slightly increasing the VHCl concentration, by 1–9.5 % w/w, the structure parameter of the cubic *Ia3d* phase decreased, hence the cubic *Ia3d* phase shrank. The same effect, a decrease of a structure parameter, was noted for the vancomycin hexagonal phase (Table 1).

It would be to expect that vancomycin HCl will increase the solvation of GM glycerol group, more water attraction center would be formed following by the cubic *Ia3d* phase structure's swelling. On the opposite, the cubic *Ia3d* phase structure's shrinkage has been

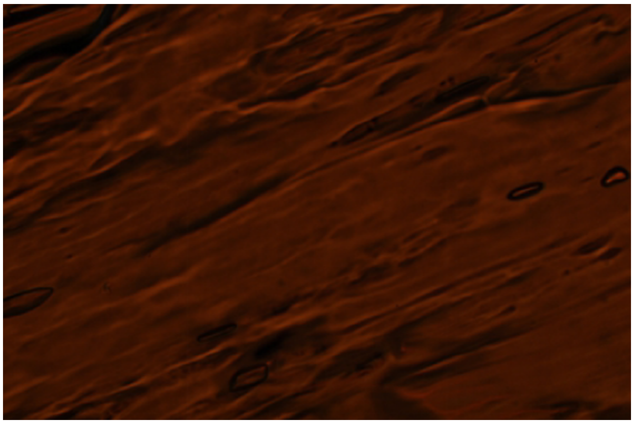

Fig. 6. Polarized light microscopy image of the cubic phase. The magnification was 200 ×.

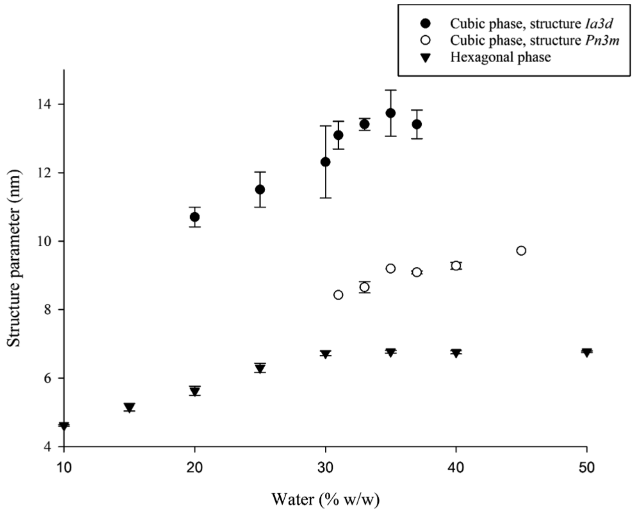

Fig. 7. Change in the structure parameter in hexagonal phase and cubic phases as a function of concentration of water at 25 °C.

Table 1
Structure parameter determined by SAXS in VHCl hexagonal and cubic *Ia3d* phase formulations at different VHCl concentrations (1–9.5% w/w) at 25 °C.

VHCl solution, % w/w	structure parameter* [nm]	
	Hexagonal phase	Cubic *Ia3d* phase
1	6,677 ± 0,019	13,684 ± 0,019
5	6,583 ± 0	12,883 ± 0,409
9.5	6,538 ± 0,023	12,528 ± 0,109

* Structure parameter is expressed as the mean values ± standard deviation.

demonstrated. It is assumed that the cubic phase structure's shrinkage is due to a lowering of the pH, as the vancomycin stable solution has a pH 3–5.

3.4. Drug content

From the pharmaceutical viewpoint, the drug content is an important issue with respect to use insert of liquid crystalline phase as drug carriers, which is a momentous appraisal items for preparation technology.

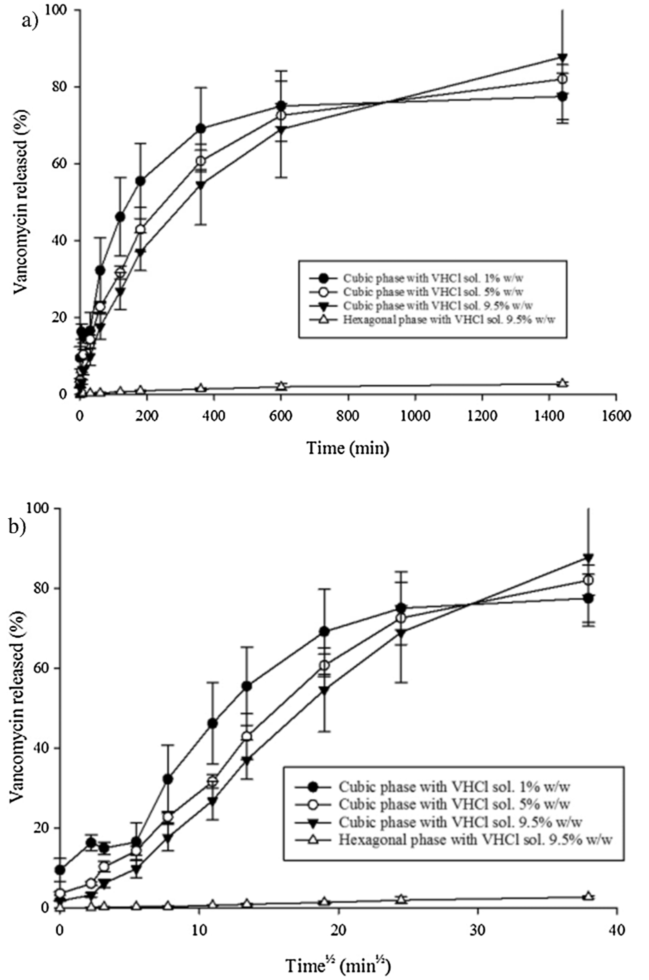

Fig. 8. *In-vitro* release of VHCl in simulated tear fluid at 32 °C from the hexagonal and cubic phases expressed in: (a) % VHCl released vs. time (min) and (b) % VHCl released *vs.* time½ (min$^{1/2}$).

The hexagonal and cubic phases had drug content ranging from 95.4% to 100.6% of the added amount of VHCl (data not shown). VHCl encapsulation was complete in both liquid crystalline phases, hexagonal and cubic phases. Therefore, the methods of preparation developed, melted homogenization for the hexagonal phase, and solvent evaporation for the cubic phase allowed the incorporation of VHCl without any degradation or loss of material. Furthermore, throughout the entire period of analysis, i.e. max. 3 months after preparation, no degradation of VHCl in the hexagonal and/or cubic phases was observed by HPLC.

3.5. In-vitro vancomycin release test

In-vitro membraneless release method was developed to study the release of VHCl from VHCl's hexagonal and cubic phases locally into the eye (into the simulated tear fluid).

Fig. 8a and b show the release of VHCl from cubic and hexagonal phases into the simulated tear fluid. The initial rate of VHCl release from the cubic phase was so high that 42.92 ± 5.7% of VHCl (cubic phase with 5% VHCl) released in 3 h. Thereafter, a slow release was observed and 82.08 ± 3.0% of VHCl (cubic phase with 5% VHCl solution) released in 24 h. In addition, Fig. 8a and b shows that VHCl's release from the hexagonal phase is very slow, significantly slower than from the cubic phase, with 2.73 ± 0.3% VHCl released after 24 h.

3.6. In-vitro vancomycin release test – Sirius‘ Scissor

In-vitro release test – Sirius‘ *Scissor* was developed to study the release of VHCl from VHCl's cubic phase in the eye, in a simulated

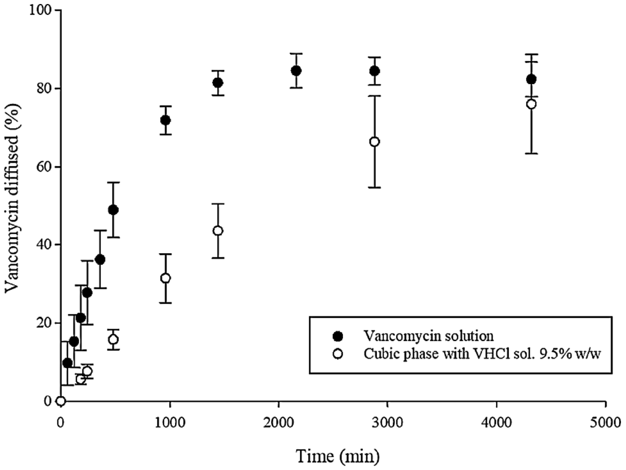

Fig. 9. VHCl diffused from its cubic phase in comparison to VHCl solution in the Sirius‘ *Scissor* system. The data represent average (n = 3) ± S.D.

intravitreal administration (Fig. 9).

The concentration-time profiles of the VHCl solution showed 81.40 ± 2.52% VHCl diffusional release into the chamber during the first 24 h, while the VHCl's cubic phase (loaded with 9.5% w/w VHCl solution) showed a release of 43.50 ± 5.67% during the same period of time. The VHCl's cubic phase showed a total diffusion of 75.97 ± 10.36% into a carbonate buffer after 3 days.

4. Discussion

In the last several decades, amphiphilic lipid-based liquid crystalline phases have been widely investigated as drug delivery systems [17,39,40]. This is due to their highly ordered nanostructure, which showed interesting release properties of different molecules. Most of the investigated GM's liquid crystalline phase is a cubic phase used to reach a sustained release of incorporated drug. However, other phases, such as hexagonal phase is more promising to achieve controlled and sustained release properties and have become more interesting in recent years.

The bulk liquid crystalline phases, hexagonal and cubic phases, investigated here are used as a precursor to prepare an ophthalmic insert system for local application in the eye. To this day, there are no data in the literature to design an ophthalmic insert system from GM bulk liquid crystalline phases. Hydrophilic molecules like VHCl could be easily dissolved in the water phase and incorporated into this system. Different structural and *in vitro* release properties of the hexagonal and cubic phases - inserts were investigated in this study trying to maintain VHCl's therapeutic concentration in the eye (locally and intravitreally – after intravitreal injection of liquid crystalline phase) during 24 h.

At the beginning of the study, significant effort was invested into preparing homogeneous bulk liquid crystalline phases, especially the bulk cubic phase. This could be explained by its high viscosity and difficulties in achieving adequate wetting properties. Our trials showed that the most suitable method to prepare a hexagonal phase is a melted homogenization method, and a solvent evaporation method to prepare a cubic phase.

The area of appearance of the cubic and hexagonal phases was constructed from the phase diagrams (Figs. 2, 4 and 5) following their identification by visual appearance and polarized light microscopy (Figs. 3 and 6).

Polarized light microscopy was used after the preparation of liquid crystalline phases for the first screening of liquid crystalline phases. PLM can spot the difference between an anisotropic structure (hexagonal or lamellar phase) (Fig. 3), and an isotropic one (cubic phase) (Fig. 6) [17].

SAXS is the most recognized method to investigate the structural features of liquid crystalline phases, both in their bulk and dispersed form [41]. SAXS is able to identify a certain type of a self-assembled structure and the corresponding space group using the position and intensity of the diffraction peaks [41]. In our study, SAXS was used to identify the structure of the bulk liquid crystalline phase during bulk liquid crystalline phase optimization (Fig. 7). Optimization of both bulk liquid crystalline phases was necessary to avoid structure swelling and burst release on exposure to physiological fluid (e.g. tear fluid). This data is also very important for the incorporation of hydrophilic molecules, such as VHCl, which are included in the aqueous phase. So, the maximum water content in both phases was set up precisely, using the structure parameter obtained by SAXS (Fig. 7). A linear increase in the structure parameter was noted in the example of the hexagonal phase, as the water percentage increased until saturation (Fig. 7). The constant value of the structure parameter obtained by further increasing water content indicated that the structure was saturated. The maximum water percentage of 28% w/w water, as visible on Fig. 7, was chosen in the formulation of the hexagonal phase to avoid the burst release during contact of the hexagonal phase with a release medium. Similar water content data for the appearance of a saturated hexagonal phase was provided by Salonen et al. [24].

The optimization of the cubic phase was a little more complex as there are two cubic phases, *Ia3d* and *Pn3m*, which are pretty viscous. As described, below the saturation concentration, only *Ia3d* or both *Ia3*d and *Pn3m* existed. However, the constant value of the structure parameter of *Pn3m* indicated that the structure was saturated. A total of 33% of water was taken as the minimal water concentration at which the structure is saturated (Fig. 7). Appearance of different cubic phases in the GM water system at different water percentages agrees well with earlier findings [18,19,24,42–44]. Some small discrepancies have been observed by different GM samples, probably due to the different purity of the GM samples, leading to slightly different water-imbibing properties [19].

Except the liquid crystalline phase's identification and optimization, SAXS was used to study the changes in the structure parameter of our formulations (hexagonal and cubic phases prepared with VHCl) depending on the different VHCl loading (1–9.5% w/w) (Table 1). Both phases demonstrated the structure's shrinkage, as the structure parameter decreased with the VHCl concentration. Due to its hydrophilic properties, it was to expect that VHCl would increase the solvation of GM glycerol group and therefore to induce the swelling of liquid crystalline phase structure. It is assumed that the cubic phase structure's shrinkage might be due to a lowering of the pH, as the vancomycin stable solution has a pH 3–5.

Furthermore, opposite to our expectations, the cubic phase in these trials revealed the *Ia3d* structure. According to the optimization trials, we expected a *Pn3m* structure, which normally appeared in equilibrium conditions. The structure of the cubic phases consists of two interpenetrating water channels surrounded by the glycerol monooleate. However, there are differences in the architecture of water channel junctions in the *Ia3d* and *Pn3m* cubic liquid crystalline phases. In an *Ia3d* structure, the repeating unit in the whole architecture creates two three-way junctions, while in a *Pn3m* structure the repeating unit forms two four-way junctions [45]. The structure of a cubic *Pn3m* phase probably corresponds to a water channel volume ratio higher than in an *Ia3d* phase structure.

The effect of the structure's shrinkage for the lipid's reversed cubic liquid crystalline phase has been observed in the literature, e.g. by increasing temperature [18,35,36] in the presence of the peptide gramicidin S [46], salt and phosphate-buffered saline [47], polar amino acids [48], by adding a hydrophilic polymer to a glycerol monooleate/water system [49], and by adding Transcutol/ethanol [50].

From the values of composition and the structure parameter, one can determine the characteristic length scale within which water is confined to a corresponding structure. These values are interesting from a theoretical point of view in discussing structure properties such as release data. Using Eqs. (3) and (4), the radius of water channels in the hexagonal phase was: placebo 1.25 nm (without VHCl), 1.23 nm with 1% VHCl w/w, 1.25 nm with 5% VHCl w/w, 1.27 nm with 9.5% VHCl w/w.

Our SAXS data indicated that VHCl loaded in the cubic and hexagonal phases did not cause a transition into other liquid crystalline phases. VHCl in the concentrations investigated, 1–9.5% w/w, has no significant structural effects in our system. Furthermore, it is possible that the vancomycin hydrochloride molecule is able to penetrate deeper into the interface region of the liquid crystalline phase and interacts with the inner parts of the GM molecules, including carbonyl groups, ester moiety, and lipophilic parts, because of its amphiphilic character.

In order to relate the nanostructure of a hexagonal and cubic phase to VHCl release rate locally into the eye or intravitreally, *in-vitro* release tests were done in simulated tear fluid and extra-cellular matrix (Sirius's *Scissor*). Results from *in-vitro* release test in simulated tear fluid (Fig. 8a and b), indicated a quick initial release of VHCl from the cubic phase followed by a slower VHCl release. Upon initial contact of the cubic phase with simulated tear fluid, the VHCl contained in the thickness of the cubic phase close to the gel/water interface would readily release into the bulk simulated tear fluid. This would account for the initial fast release. As time elapses, VHCl in the utmost gel thickness would be exhausted, the VHCl concentration would develop in the simulated tear fluid, and VHCl would begin to diffuse at a deeper thickness. Under these circumstances, the concentration difference of VHCl between the gel/insert and the simulated tear fluid would decrease and the travel length of VHCl to diffuse out would increase. This might explain the slower release at the later stage.

Opposite to the VHCl release pattern from the cubic phase, VHCl's release from the hexagonal phase into the simulated tear fluid was very slow. Our release result clearly demonstrates that VHCl's release is governed mostly by the structure of the liquid crystalline phase. The two-dimensional symmetry of the hexagonal phase with the closed water channels provided a complex diffusion pathway for a controlled release of the entrapped molecule [49]. However, the cubic phase with a fascinating and complex three-dimensional structure and open water channels is responsible for the quicker diffusion rate than in the hexagonal phase.

When VHCl's release data is plotted as percent released versus the square root of time, the data show a linear relationship, particularly at early times, indicating that diffusion is the dominant release mode [17,39,51].

Additionally, our results (Fig. 8a and b) demonstrated that there is no complete vancomycin release from the cubic phase insert. After 24 h, 82.08 ± 3.0% of VHCl (cubic phase with 5% VHCl solution) was detected in the release medium. An incomplete drug release from GM liquid crystalline phase has been reported previously [19,39,52,53]. Chang and Bodmeier observed on the example of propranolol and chlorfeniramine maleate their incomplete release from the GM matrix system describing this as drug binding [54]. However, this was later described as a bilayer participation [16]. Kim et al. demonstrated that max. 80% of H_2O_2 had been released from the GM cubic phase at different temperatures [19]. They also reported oxidation of the unsaturated hydrocarbon chain by H_2O_2 and hydrolysis of the ester bond between the glycerol moiety and the aliphatic chain [19]. Our results of incomplete VHCl release could be supported by VHCl's participation in the bilayer. VHCl, as an amphiphilic molecule, is able to trigger its close arrangement to the interfacial layer leading to the expansion of the cubic phase. However, this was not represented by our SAXS experiment investigating the influence of the VHCl concentration on the structure parameter of the cubic phase (Table 1).

In order to evaluate the efficacy of our VHCl insert system locally into the eye (results of the *in vitro* test into simulated tear fluid), literature data were examined. One drop (0.05 ml) of 25 mg/ml VHCl ophthalmic solution (drops) taken locally each hour corresponds to 1.25 mg VHCl [4]. Considering that the average tear turnover rate is 20%/min as the main elimination principle (ocular absorption < 5% of the dose), this will result for the 25 mg/ml solution locally used in the eye in a local concentration of 0.048 µg/ml of VHCl after 60 min. This theoretically means that the local effective VHCl concentrations could be > 0.048 µg/ml in 1 h. The minimal inhibitory concentration (MIC) for VHCl for *Staphyloccocus epidermis* and most microorganisms ranges 1–5 µg/ml. Following the same calculation (20%/min tear turnover) and release pattern our cubic phase insert (9.5% w/w VHCl) was able to achieve the VHCl concentration of 0.163 ng/ml in 1 h locally in the eye. This value indicates that the cubic phase insert should be further optimized to achieve the therapeutic concentration in the eye locally in 1 h/24 h. The mass of the above inserts was in the range 10–20 mg. However, optimizing the mass (size) of the insert should lead to the desired therapeutic VHCl concentration in the eye at the desired time.

Recently, intravitreal administration has attracted more interest as a route of administration in some ocular diseases, especially when it comes to vancomycin therapy [55,56]. Consequently, there is a need for platforms that can successfully mimic the intra-ocular environment and allow for a better understanding of the behavior of formulations aimed at this delivery route. Sirius' *Scissor* was evaluated as an useful tool to simulate the intraocular environment [57,58].

In vitro release test by the Sirius' *Scissor* (Fig. 9) demonstrated that after the cubic phase injection the VHCl diffusional release of 43.50 ± 5.67% during the first 24 h was achieved. The VHCl diffusional release of 75.97 ± 10.36% has being revealed after 3 days. By the comparison with the reference VHCl's solution, the VHCl's cubic phase revealed a slower diffusional release (VHCl's cubic phase 43.50 ± 5.67%, VHCl'solution 81.40 ± 2.52%) during the first 24 h.

An extra-cellular matrix is a complex matrix consisting of a hyaluronic acid gel [57]. VHCl diffusion in the simulated intraocular tissue might be affected by the increased viscosity of the ECM and an interaction with molecules in the extracellular matrix (e.g. due to VHCl's electrostatic effect).

Transport of a guest molecule through such matrices is regulated by a variety of mechanisms, such as steric exclusion and electrostatic interactions [57,59]. There is evidence that this transport is a complex interplay between the interactions and diffusing molecule(s). For molecules significantly smaller than the extracellular matrix/intraocular tissue pore structure, electrostatic effects are the most important determinant for diffusion rates. For molecules with a size similar to the pore size of the extracellular matrix/intraocular tissue, steric hindrance is significant and the charge of the particles is negligible in determining the diffusion rate.

In order to evaluate the efficacy of our VHCl cubic phase (results of *in vitro* test by a *Scissor*), literature data were taken into account. Ferencz et al. demonstrated that after intravitreal VHCl injection (solution), the VHCl concentration found 48 h after injection ranged between 25.05 and 182.36 µg/ml [60]. As our VHCl's cubic phase system was able to achieve 61 µg/ml VHCl in a simulated intravitreal tissue after 48 h (Fig. 9), we can conclude that our delivery system might be effective *in-vivo* after intravitreal injection.

Additionally, the Sirius' *Scissor* system has the potential to evaluate the behaviour of different pharmaceutical formulations in simulated *in vivo* conditions with regards to their performance after injection. In addition, when it comes to pharmaceutical formulations, the rate and mechanism regulating the transport rate of active molecules may be dependent on the excipients present in the formulation [57,59]. The influence of different excipients in the VHCL insert was not part of our study.

The VHCl hexagonal and cubic phases investigated in this study have been demonstrated to possess the following advantages:

- able to avoid structure swelling in the eye, as the systems were already optimized and prepared at max. water content

- controlled release of an hydrophilic molecule, VHCl from a highly ordered, viscous liquid crystalline systems, stable in excess water, with a quick release from the cubic phase compared to a very slow release from the hexagonal phase; the release is controlled by the phase's microstructure and not by viscosity like in many other traditional gel systems
- comparing the efficacy of our VHCl's insert system locally into the eye (*in vitro* vancomycin release test into the simulated tear fluid) with the literature, we demonstrated that our delivery system should be further optimized to be effective *in vivo*
- comparing the efficacy of our VHCl's cubic phase (injection) intravitreally (*in vitro* vancomycin release test Sirius‘ *Scissor*) with the literature, it has been demonstrated that our delivery system might be effective *in vivo*
- safety and biocompatibility of GM. GM is a naturally occurring product of the digestion of triglycerides in the gastrointestinal tract. Being composed of well tolerable and physiological glycerol monooleate, these drug delivery systems are expected to be well tolerated, compatible and non-toxic. GM is classified as GRAS (generally recognized as safe) material and it is included in the FDA Inactive Ingredients Guide.

One of the disadvantages of the systems investigated is that glycerol monooleate is prone to degradation in the eye by esterase. Glyceride lipids are degraded by esterase to form diglycerides, monoglycerides and fatty acids leading to changes in nanostructure and release properties. Degradation products of glycerol monooleate can influence the structure and thus change the release properties. Additionally, the tolerability and local toxicity of the degradation products are unknown and will need to be determined. An additional disadvantage of our system could be in the strict release properties governed by the type of the liquid crystalline phase. In an attempt to create desirable release properties depending on certain external factors, lipid packing might be changed to alter the phase structure of the liquid crystalline phase. The most common external factors influencing the lipid packing are pH, ionic strength, pressure, temperature, the presence of additives. In our next study, one of these approaches is used to further investigate the structure – release relations of liquid crystalline phases.

5. Conclusions

In this study, VHCl's hexagonal and cubic phases were successfully prepared from GM by a melted homogenization and solvent evaporation method. At the VHCl concentration tested, 1–9.5% w/w, the original liquid crystalline phase's nanostructure was preserved, with a small decrease in structure parameter (SAXS) observed. Furthermore, our results demonstrated that the hexagonal and cubic phases are able to control the rate of VHCl's release. The hexagonal phase with closed water channels displayed a significantly slower release of VHCl than a reverse cubic phase with an open water channel.

A convenient intravitreal VHCl concentration could be obtained *in-vitro* by the intravitreal injection of present VHCl's cubic phase. The insert prepared from VHCl's cubic phase should be further optimized regarding its mass (size) to be able to deliver the effective dose of VHCl locally into the eye.

It can be concluded that the developed formulations could be an excellent substitute to conventional eye drops. However there is need to further optimize the mass/size of insert to achieve the desired VHCl's concentration locally and to perform more finding in terms of *in-vivo* results to make it clinically viable.

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