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Investigation of diclofenac release and dynamic structural behavior of non-lamellar liquid crystal formulations during in situ formation by UV–Vis imaging and SAXS

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- Intra-articular administration
- Non-lamellar liquid crystalline phases
- Swelling behavior
- time-resolved SAXS
- UV–Vis imaging

**Abstract**

In situ formation of high viscous inverse lyotropic non-lamellar liquid crystalline phases is a promising approach for sustained drug delivery in the joint. The in situ forming process on exposure of two diclofenac-loaded preformulations to aqueous media was characterized with respect to depot size and shape, initial release and structural transitions using UV–Vis imaging and spatially and time-resolved synchrotron small-angle X-ray scattering (SAXS). The preformulations consisted of 10 % (w/w) ethanol, 10 % (w/w) water and a binary lipid mixture of glycerol monooleate (GMO):1,2-dioleoyl-sn-glycero-3-phospho-rac-(1-glycerol) (DOPG) or GMO:medium chain triglycerides (MCT). Upon injection of preformulations into an employed injection-cell containing excess of bio-relevant medium, rapid generation of liquid crystalline depots was observed. UV–Vis images and constructed 2D SAXS maps of the injection-cell showed depots with different shapes and sizes, and features with high nanostructural heterogeneity. More extensive swelling of the GMO:DOPG-based preformulation was observed compared to the GMO:MCT-based preformulation. The UV image analysis found that a higher amount of diclofenac was released in the image area after 20 h from the GMO:MCT depot compared to the GMO:DOPG depot. The injection-cell setup employing UV–Vis imaging and synchrotron SAXS constitutes an attractive approach for evaluating the in situ forming processes of liquid crystalline depots.

**1. Introduction**

In situ formation of depots of inverse lyotropic non-lamellar liquid crystalline phases (LCPs), which have unique and distinct nanostructures, is a promising approach for providing local and sustained drug release (Yaghmur et al., 2013; Li et al., 2018; Yaghmur et al., 2012; Rahnfeld and Luciani, 2020; Hatefi and Amsden, 2002). These depots are formed at the administration site from low-viscous injectable preformulations comprising amphiphilic lipids (such as monounsaturated monoglycerides and glycolipids), which have the propensity to self-assemble into LCPs in response to hydration or change in temperature upon contact with the biological environment (Yaghmur et al., 2013; Shah et al., 2001; Hato, 2001; Kaasgaard and Drummond, 2006). In this context, the formation of high-viscous inverse bicontinuous cubic (Q2) and discontinuous hexagonal (H2) LCPs is an attractive strategy for achieving sustained drug release owing to their stability against water dilution and capability to incorporate drugs with different physicochemical properties (Yaghmur et al., 2013; Shah et al., 2001; Fong et al., 2009; Fong et al., 2016). For instance, injectable preformulations forming LCPs in situ have been proposed for intra-articular administration to manage pain and inflammation associated with joint diseases such as osteoarthritis and rheumatoid arthritis (Li et al., 2018; Mertz et al., 2019; Reeff et al., 2012; Reeff et al., 2013; Mertz et al., 2021; Chen et al., 2015). In addition to sustaining drug release, the formation of a liquid crystalline drug depot in the joint cavity can reduce the needed drug dose and minimize systemic side effects. Thus, the challenge of rapid clearance of small drug molecules from the joint cavity can be overcome by ensuring high local drug concentrations at the site of action for a prolonged period of time (Li et al., 2018; Mertz et al., 2019; Reeff et al., 2012; Reeff et al., 2013; Mertz et al., 2021; Chen et al., 2015; Larsen et al., 2008).

Despite of high interest in injectable in situ forming liquid crystalline...
depots (Li et al., 2018; Chen et al., 2015; Li et al., 2019; Yang et al., 2018), only few studies have in detail described and evaluated how the \textit{in situ} forming process affect the size and shape of the depot, the drug release properties and the involved dynamic structural transitions (Yaghmur et al., 2013; Mertz et al., 2021; Yaghmur and Rappolt, 2012; Yaghmur et al., 2021). Such key attributes should ideally be investigated \textit{in vitro} at biologically relevant conditions mimicking the \textit{in vivo} situation, i.e. using bio-relevant pH and release media volume, introducing tissue relevant constituents, and incorporating an injection step. In case of intra-articular administration, previously reported \textit{in vitro} drug release investigations of LCP drug delivery systems have often employed relatively large release medium volumes ($\geq 10$ mL) (Li et al., 2018; Mertz et al., 2019; Reeff et al., 2012; Reeff et al., 2013), although the human knee joint cavity (largest joint in the body) at normal conditions.

Fig. 1. Illustrations of the applied experimental setups. (A) The injection-cell with the inner dimensions indicated by red arrows. (B) The UV-Vis imaging setup used for monitoring the area of the \textit{in situ} formed LCP depot and diclofenac release upon remote injection of diclofenac-loaded preformulations. (C) The time-resolved synchrotron SAXS setup used for monitoring the dynamic structural transitions at multiple injection-cell positions during the \textit{in situ} formation of the LCP depot.
conditions contains around 0.5 – 2.0 mL synovial fluid (Kang and Im, 2014). It should, however, be mentioned that increased synovial fluid volumes are reported in patients with joint diseases (Kang and Im, 2014; Gerwin et al., 2006). Injection of preformulation into tissue fluid and subsequent in situ formation of the LCP depot is expected to affect the shape of the depot as compared to preformulation injection into simple aqueous solution. However, most reported in vitro drug release studies relevant to the intra-articular administration focused on employment of a well-defined constant interfacial area between the already prepared liquid crystalline depot and the release medium (Li et al., 2018; Reeff et al., 2012; Reeff et al., 2013; Chen et al., 2015). However, in our previous in vitro release study using the commercial Scissor system, the investigated lipid-based preformulations were directly injected into 4.75 mL release media containing major constituents of the synovial fluid, i.e. hyaluronic acid (HA) and albumin (Mertz et al., 2019). This means that the formed liquid crystalline depots varied in size and shape but the applied in vitro setup did not allow for further characterization of depot size and shape.

Real-time investigations of dynamic structural transitions occurring upon exposure of lipid-based preformulations to excess of aqueous media can be performed using synchrotron time-resolved small-angle X-ray scattering (SAXS) (Mertz et al., 2021; Li et al., 2019; Yaghmur et al., 2018). In situ SAXS experiments on the dynamic structural transitions and the nanostructural heterogeneity on modulating the initial drug release properties remains to be investigated.

In the present study, the injection-cell setup was used to combine synchrotron SAXS experiments on the dynamic structural transitions occurring during the in situ generation of LCP depots with initial drug release studies conducted using UV–Vis imaging technology (Fig. 1). The latter utilizes the light absorption of drug substances to create spatially biphasic feature of two coexisting Q2

2. Materials and methods

Glycerol monoooleate (GMO) with a purity ≥ 90 % was purchased from Riken Vitamins Co. (Tokyo, Japan). 1,2-Dioleoyl-glycerol-3-phospho-rac-glycerol sodium salt (DOPG, > 98.0 % phosphatidyl glycerol-Na) was purchased from Lipoid (Ludwigshafen, Germany), and Captex 300 (medium chain triglycerides, MCT) was kindly donated by ABITEC (Columbus, OH, USA). Diclofenac (2,2-dichoroanilino)-phenylacetic acid > 98 %) was obtained from Tokyo Chemical Industry (Tokyo, Japan). Ethanol Absolute > 99.9 % for analysis (WVR International, Soborg, Denmark) was used for preparation of the preformulations and for quantitative analysis of diclofenac in the preformulations. Diclofenac sodium salt (pharmaceutical secondary standard) and hyaluronic acid (HA) sodium salt from Streptococcus equis with an average MW of 1.5–1.8 MDa were purchased from Sigma-Aldrich (Munich, Germany). Sodium dihydrogen phosphate monohydrate and sodium hydroxide were acquired from Merck (Darmstadt, Germany). Demineralized water was used throughout the study.

2.2. Preparation of diclofenac-loaded preformulations

Two injectable preformulations loaded with diclofenac at a constant concentration of 18 mg/g were prepared and composed of 72:8:10:10 % (w/w) GMO/DOPG/ethanol/water and 68:12:10:10 % (w/w) GMO/MCT/ethanol/water, respectively. DOPG or MCT, ethanol, and water were added to melted (50 ± 2 °C) GMO in accurately weighed amounts, and the ternary mixtures were heated at 50 ± 2 °C and vortexed for 4–10 min. The obtained mixtures were kept rotating at 37 ± 1 °C in an incubator (Hood TH 30, Edmund Bühler, Bodelshausen, Germany) on a sample rocker (INTELLI-Mixer®, ELMI, Riga, Latvia) until fully mixed. Finally, the acidic form of diclofenac was added and dissolved in the aforementioned clear solutions. The preformulations were stored at room temperature for up to 5 days prior to SAXS measurements. For the UV–Vis imaging studies, the preformulations were kept overnight on the sample rocker at 37 ± 1 °C and prepared one day prior to the experiments.

For each preformulation, the (w/v) diclofenac concentration was determined using UV spectrophotometry (Cary 60 UV–Vis Spectrophotometer, Agilent, Santa Clara, CA, USA). A volume of 30 µL of the preformulation (n = 3) was transferred to a 25 mL volumetric flask and dissolves in ethanol. The solutions were analyzed by measuring the UV absorbance at 279 nm, and the corresponding diclofenac concentrations were determined from a diclofenac calibration curve in ethanol (concentration range 0.005 – 0.032 mg/mL).

2.3. Solubility of diclofenac in prepared preformulations

The two quaternary mixtures, 72:8:10:10 % (w/w) GMO/DOPG/ethanol/water and 68:12:10:10 % (w/w) GMO/MCT/ethanol/water, were prepared as described in section 2.2. The solubility of diclofenac in these mixtures were determined by adding an excess of diclofenac (acidic form) (n = 3) and measuring the concentration of diclofenac in the solutions after agitation on a sample rocker (INTELLI-Mixer®, ELMI, Riga, Latvia). The diclofenac suspensions were agitated overnight at 37 ± 1 °C. After approximately 21 h, the suspensions were vortexed and samples (250–300 µL) were withdrawn. The remaining suspensions were transferred to room temperature (24 ± 0.1 °C) and agitated for 1.5 h where after they were vortexed and samples withdrawn. All samples were centrifuged (10 min at 13500 rpm, MiniSpin Plus, Eppendorf®), Hamburg, Germany), diluted in ethanol and analyzed on the Cary 60 spectrophotometer at 279 nm. The diclofenac content was determined from a diclofenac calibration curve prepared in ethanol (concentration
quartz plates with dimensions of 75 × 10.0 mm² (L × W × D) on both sides (AdValue Technology, Tucson, AZ, USA) and mounted in front of the Actipix® phosphor coated complementary metal oxide semiconductor (CMOS) chip for detection (Fig. 1B). The effective imaging area of the SDi2 was 28 × 28 mm² with a nominal pixel size of 13.75 µm. The system was controlled using the SDi2 data collection Software (version 1.1.9, Pion, Forest Row, UK). Prior to initiation of the experiment, the injection-cell was filled with PBS or 5 mg/mL HA solution, which was preheated at 45 °C for 25 min under magnetic stirring to avoid bubble formation, and fixed in the SDi2 system. A Fluke t3000 digital thermometer (Fluke, Eindhoven, The Netherlands) equipped with a thermocouple probe (RS PRO Type K, RS Components, Corby, UK) was inserted into the aqueous medium inside the cell, allowing continuous monitoring of the temperature. After reaching a stable temperature of 25 ± 2 °C, dark and reference images were recorded.

The experiment was initiated by a remote injection of 300 µL preformulation at 3 mL/min using a 18G blunt needle (1.2 mm × 40 mm, Terumo, Leuven, Belgium) which was placed in the injection port (Fig. 1B). For the first 5 min, images were collected every second followed by collection of an image every 50 s for the remaining experiment. As the initial release was of interest in the present study, the experiments were stopped at 20 h where increased amounts of diclofenac diffused out of the UV–Vis image area. After 20 h, the injection-cell was removed from the SDi2 system and the aqueous medium surrounding the in situ formed LCP depot was mixed. This was done by turning the injection-cell upside down while withdrawing and injecting the medium into the injection-cell several times through the inlet by using a 3 mL syringe (Chirana® T. Injecta, Stará Turá, Slovakia) equipped with a 23G Sterican® needle (0.6 mm × 60 mm, B.Braun, Melsungen, Germany). The injection-cell was reinserted in the SDi2 system and images were recorded every 10 s for 10 min. Finally, the aqueous medium in the injection-cell was collected in a measuring glass to determine the end volume of the aqueous medium. The diclofenac concentration in the collected end sample was determined by spectrophotometry at 279 nm as detailed above. In case the absorbance exceeded 1.2, the sample was diluted 1:4 or 1:5 (v/v) in the relevant medium (PBS or 5 mg/mL HA solution). Calibration curves were prepared from diclofenac solutions in PBS or 5 mg/mL HA solution in the diclofenac concentration range of 0.004 – 0.04 mg/mL.

The UV–Vis imaging studies were performed using a SDi2 instrument (Pion, Forest Row, UK) with dual-wavelength imaging using LEDs at 300 ± 5 and 520 ± 5 nm. Imaging studies were performed by injecting the GMO:DOPG-based or the GMO:MCT-based preformulation into either PBS or 5 mg/mL HA solution (n = 3). For imaging of the in situ formed LCP depots, a custom-made 3D-printed injection-cell with a light path of 4 mm and inner dimensions (Fig. 1A) corresponding to a cell-volume of 4.3 mL was applied. The inlet and outlet of the injection-cell were used for introducing and removing the release medium, respectively. Further, the injection port at the top of the injection-cell enabled injection of the preformulations into the cell (Fig. 1). The injection-cell was covered by a thermocouple probe (RS PRO Type K, RS Components, Corby, UK) was inserted into the aqueous medium inside the cell, allowing continuous monitoring of the temperature. After reaching a stable temperature of 25 ± 2 °C, dark and reference images were recorded.

The diclofenac calibration curves were constructed in PBS and in 5 mg/mL HA solution. Calibration curves were prepared from diclofenac solutions in PBS or 5 mg/mL HA solution in the diclofenac concentration range of 0.004 – 0.35 mg/mL. All standards solutions were prepared by dilution of a stock solution containing diclofenac sodium (3.2 mg/mL) in deionized water. Absorbance values of the diclofenac standard solutions in the injection-cell were determined by UV imaging at 300 nm from images recorded every 10 s for 3 min. The non-linear calibration curves were constructed by fitting the data to the following equation through use of GraphPad Prism version 7.00 (GraphPad Software, San Diego, CA, USA):

\[
\text{Abs} = \text{Abs}_{\text{max}} - (\text{Abs}_{\text{max}} - \text{Abs}_0) \cdot \exp\left(-k \cdot C\right)
\]

where \(\text{Abs}\) and \(C\) are the absorbance and concentration of diclofenac, respectively, and \(\text{Abs}_{\text{max}}\) and \(\text{Abs}_0\) are the maximum absorbance and intercept, respectively.

### 2.5.2. UV–Vis image analysis

The imaging files, containing the recorded UV (300 nm) and Vis (520 nm) images, were converted to TIF-format using the ActiPix D100 ImageViewerEditor software (v. 1.0, Paraytec Ltd., York, UK). An in-house developed MATLAB script was employed for analyzing the images. In MATLAB, the images were treated as a matrix of pixel intensities, which were converted to absorbance values by correcting for stray light and electronic noise. The area of the LCP depot in the image area was determined from images obtained at two different wave-LENGTHS: 520 and 300 nm. Two separate binary images were generated from absorbance images at 520 and 300 nm where imadjust function was used to increase the contrast. Pixels (size 13.75 × 13.75 µm²) with an absorbance value ≥ 0.1 (at 520 nm) or an absorbance value of > 1 (at 300 nm) were identified (black pixels; Step 1, Fig. 2). The two binary images for each wavelength were combined to a single binary image (Step 1, Fig. 2), and used for determining the generated LCP depot area. Subsequently, the combined binary image was used to remove the pixels associated with the LCP depot from the corresponding UV absorbance image, thereby defining the part (the pixels) of the image area containing the aqueous medium (Step 2, Fig. 2). The diclofenac calibration curve in the relevant aqueous medium was used to convert the absorbance of these pixels to apparent concentration and the amount of diclofenac was calculated by using the pixel dimensions and the light path. The total amount of diclofenac released in the image area was obtained by summing the amount of diclofenac for each of the pixels. At 300 nm, the total amount of diclofenac in the images (for selected time points between 0 and 20 h) was determined using a conventional for loop function and plotted as a function of time using GraphPad Prism version 7.00. The image files were sorted using a natural order file name sort function from the MATLAB Central File Exchange database (Stephen23, 2021). The absorbance images were shown with contour lines with absorbance changes of 0.2 for visualization of the diffusion patterns.

### 2.6. Swelling experiments

LCP depots were formed in 4 mL vials upon addition of 150 µL diclofenac-loaded (18 mg/g) preformulation (quaternary mixtures of 72:8:10:10 % (w/w) GMO:DOPG/ethanol/water or 68:12:10:10 % (w/w) GMO/MCT/ethanol/water) into 2.15 mL PBS (n = 3). The vials were kept agitated (using an INTELLI-MixTM, ELMI, Riga, Latvia) or non-agitated at 27 ± 3 °C (ProBlotTM Hybridization Oven, Labnet, Edison, NJ, USA) for 20 h. After 20 h, excess PBS was carefully withdrawn from the vials using a pipette, leaving behind the hydrated LCP depot. The vials were weighed without content area after addition of PBS (\(w_1\)) and the preformulation (\(w_2\)). Finally, the vials containing the hydrated LCP were weighed (\(w_h\)). The percent change in mass (\(\%\) W) at 20 h was calculated as:

\[
\% W = \frac{w_h - w_0}{w_2 - w_1} \times 100 \%
\]
weight of the preformulation.

The diclofenac concentration in the excess PBS withdrawn was determined by measuring the UV-absorbance at 279 nm using the Cary spectrophotometer.

2.7. Small-angle X-ray scattering measurements

SAXS measurements were performed at the Austrian SAXS beamline (ELETTRA synchrotron light source, Trieste, Italy) as previously described (Mertz et al., 2021). Static SAXS experiments (25 ± 0.1 °C) were conducted to characterize the structures of the prepared two diclofenac-loaded (18 mg/g) preformulations and their corresponding fully hydrated LCPs. The preformulations were quaternary mixtures consisting of GMO/DOPG/ethanol/water (72:8:10:10 % (w/w)) and GMO/MCT/ethanol/water (68:12:10:10 % (w/w)). As described previously (Mertz et al., 2021), the LCPs were formed upon addition of the preformulations to PBS or 5 mg/mL HA solution, in a volume ratio of 1:15, and kept at room temperature for 21 – 26 h.

Time-resolved SAXS experiments (25 ± 1 °C) were conducted to investigate the dynamic structural transitions on injection of the diclofenac-loaded (18 mg/g) preformulations into PBS or 5 mg/mL HA solution. These dynamic measurements were conducted by coupling synchrotron SAXS to the injection-cell (Fig. 1A, C) as previously described (Mertz et al., 2021). In brief, the cell holder with the injection-cell was mounted on a moveable platform in front of the X-ray beam and 4.3 mL aqueous medium (PBS or 5 mg/mL HA solution) was filled into the injection-cell. Upon 30 s exposure of the aqueous medium, 300 µL GMO:DOPG- or GMO:MCT-based preformulations was injected into the aqueous medium at an injection rate of 3 mL/min (Fig. 1C). Prior to injection, the preformulations were heated to about 40 °C and mixed at room temperature for 10 – 15 min. In the individual experiments, the injection-cell was moved to different horizontal positions in front of the X-ray beam to find positions where the formed LCP depot could be identified. The measurements from the GMO:DOPG-based preformulation studies were obtained using a horizontal position 3 mm to the left of the needle. In the GMO:MCT-based preformulation experiments, a horizontal position 3 mm to the right of the needle or 2 mm to the left of the needle was applied upon injection of the preformulation into PBS or 5 mg/mL HA solution.

In this study, the collected 2D SAXS patterns with distinct scattering rings were azimuthally and radially integrated into one-dimensional (1-D) plots of the scattering function, I(q) versus q. Here, the raw data from the static and time-resolved SAXS measurements were corrected for detector efficiency and background scattering and the detected Bragg peaks were fitted to Lorentzian distributions. The q-value of very weak peaks was read manually. The lattice parameters of the identified Q2 and H2 phases were derived from the SAXS diffraction patterns.

3. Results and discussion

In this work, the influence of the lipid composition on the in situ generated LCP depot shape and size, the initial diclofenac release and the involved dynamic structural transitions during the in situ generation process were investigated by combining UV–Vis imaging and SAXS measurements. All experiments were conducted at room temperature as adaptation to physiological temperature (37 °C) required a temperature control with high accuracy in the SAXS setup, which was not feasible due to the relatively large dimensions of the injection-cell holder (8.0 × 8.0 × 1.6 cm3 (L × W × D)). UV–Vis imaging can be conducted at 37 °C as the SD21 instrument is equipped with heating pads (Bock et al., 2022). However, the present study was conducted as a proof of concept focusing on combining for the first time UV–Vis imaging and synchrotron SAXS experiments. Injections of the two lipid-based
preformulations into phosphate buffer solution at pH 7.4 (PBS) with and without 5 mg/mL hyaluronic acid (HA) were performed. These aqueous media can be considered biologically relevant for the intra-articular administration site as physiological pH of the synovial fluid and one of its major constituents, HA, were used (Kang and Im, 2014; Maudens et al., 2018). The HA solution mimics the viscosity of the synovial fluid and allows for investigations on the effect of the viscosity on the in situ formation process. As simple solutions were preferred in the initial testing of the setup, addition of albumin or other relevant constituents of the synovial fluid previously applied in release studies related to intra-articular drug delivery (Thing et al., 2019; Magri et al., 2019) was not pursued in the present study. The investigated drug, diclofenac (a non-steroidal anti-inflammatory drug), is of interest for intra-articular administration to treat pain and inflammation associated with rheumatoid- and osteoarthritis (Kawanami et al., 2020; Forrest et al., 2002; Thing et al., 2014). The diclofenac loading capacity of the applied GMO:DOPG and GMO:MCT-based preformulations was determined to be 30.9 ± 1.5 and 33.8 ± 1.8 mg/mL at 37 °C, respectively, and 21.6 ± 1.1 and 25.0 ± 0.6 mg/mL at room temperature, respectively. Although the preformulations were prepared at 37 °C, a diclofenac loading of 18 mg/g (below the maximum solubility at room temperature) was used throughout the study to avoid precipitation of diclofenac during the conducted experiments at 25 ± 2 °C. For the UV-imaging experiments, the content of diclofenac in both preformulations was measured to be 18.9 mg/mL (R.S.D < 4 %), which corresponded to an injected amount of 5.7 mg solubilized in 300 µL preformulation.

3.1. UV–Vis imaging – Shapes and sizes of in situ formed liquid crystalline depots

In the conducted UV–Vis imaging experiments, the exposure of both diclofenac-loaded preformulations to the aqueous media was associated with a rapid formation of highly viscous liquid lipid crystalline self-assemblies. In agreement with our recent findings (Mertz et al., 2021) and also with previous studies in the literature (Yaghmur et al., 2021; Yaghmur et al., 2011), the formation of these phases is attributed to a fast water diffusion from the continuous aqueous media into the injected preformulations, which is most likely associated with a diffusion of ethanol from these preformulations into the continuous aqueous media. The latter leads to a decrease in ethanol concentration to be below the solubility limits of the used lipids, and enhances therefore their self-assembly in excess water into inverse non-lamellar liquid crystalline phases (LCPs).

Diclofenac absorbs light in the range of 200 – 330 nm (see UV spectrum in Supplementary material Fig. S1) and the LEDs of the SDi2 system allow for UV–Vis imaging at 255, 280, 300, 320, and 520 nm.

Fig. 3. Absorbance maps obtained by UV and Vis imaging at 300 and 520 nm, respectively, for the in situ formation of liquid crystalline depots in 67 mM phosphate buffer pH 7.4 (PBS) or 5 mg/mL hyaluronic acid (HA) solution at 25 ± 2 °C. Images (28 × 23 mm² (L × W)) are shown at selected times after injection of 300 µL diclofenac-loaded (18 mg/g) preformulation composed of 72:8:10:10 % (w/w) GMO:DOPG/ethanol/water (GMO:DOPG) or 68:12:10:10 % (w/w) GMO:MCT/ethanol/water (GMO:MCT). Contour lines indicate absorbance changes of 0.2. Maps from the first experiment (n1) are shown (three experiments were conducted in this study).
Initial measurements at 280 nm (diclofenac absorbance maximum) led to high absorbance values outside the diclofenac calibration curve (data not shown). Thus, a wavelength of 300 nm was selected for quantification of released diclofenac in the image area, where non-linear calibration curves in PBS and 5 mg/mL HA solutions were constructed (Fig. S2). Visualization of the formed LCP depot was possible by monitoring absorbance changes at 520 nm (light obscuration) as diclofenac does not absorb light > 330 nm (Fig. S1). Thus, UV-Vis imaging was performed at 300 and 520 nm for all experiments (n = 3). Absorbance maps are presented at selected time points after injection of the GMO:DOPG- and GMO:MCT-based preformulations into PBS and 5 mg/mL HA solution in Fig. 3 (n = 1, first of three conducted experiments). Additional images providing an estimate of between experiment variability can be found in Figs. S3 - S6. For further visualization, Video S1 and Video S2 (Supplementary Data) show UV-Vis imaging of the depot formation upon injection of the GMO:DOPG- and GMO:MCT-based preformulations, respectively, into 5 mg/mL HA solution.

The shapes of the formed LCP depots were clearly seen from both the Vis and UV images, whereas diclofenac release from the LCP depots was observed from the UV absorbance maps at 300 nm (Fig. 3). Depending on the lipid composition of the investigated preformulation, it was evident that the in situ generated LCP depots varied in size and shape. Upon injection of the GMO:DOPG-based preformulation, the LCP depot was formed in the center of the injection-cell, whereas injection of the GMO:MCT-based preformulation resulted in a LCP depot situated at the top of the cell and around the injection needle (Fig. 3). These observations can most likely be explained by the lower viscosity of the GMO:MCT-based preformulation (as noticed during handling) compared to that of the GMO:DOPG-based preformulation. The viscoelastic properties of the two formed LCP depots are also expected to be different. To this end, Mezzenga and co-workers have previously shown that each distinct liquid crystalline phase, including inverse cubic Pn3m phase and inverse H2 phase, has a specific rheology signature (Mezzenga et al., 2005).

Fig. 3 shows that the GMO:MCT-based preformulation formed depots that cover a smaller part of the imaging area as compared to those generated from the GMO:DOPG-based preformulation. To gain further insight into the swelling behavior attributed to water uptake and potential solubilization of additives present in PBS and HA solution during the in situ formation process, changes in the cross-sectional area of the LCP depots based on image analysis (see section 2.5.2) are plotted (Fig. 4). Clear differences in the extent of swelling were observed between the GMO:DOPG- and GMO:MCT-based preformulations. The cross-sectional area covered by the hydrated GMO:DOPG depots, 20 h after preformulation injection, amounted to 207.7 ± 41.8 and 216.3 ± 14.0 mm² in PBS and 5 mg/mL HA solution, respectively (Fig. 4). However, substantially lower cross-sectional areas of 109.8 ± 14.6 and 100.3 ± 1.6 mm² were found 20 h after injection of the GMO:MCT-based preformulation into PBS and 5 mg/mL HA solution, respectively (Fig. 4).

From these areas, the volumes of the LCP depots were calculated assuming that the thickness of the depot at all positions was equal to the depth of the injection-cell (4 mm). The percentage increase in the volume (% V) due to swelling 20 h upon injection of both preformulations is reported in Table 1. It is worth noting that the GMO:DOPG-based preformulation has taken up a relatively large amount of water (~180 % increase in volume). Here, it should be mentioned the inclusion of charged phospholipids (such as DOPG) to fully hydrated GMO is associated with an enhanced water uptake (enlargement of the water nanochannels of the inverse cubic Pn3m phase), and the induction of direct cubic Pn3m-Im3m phase transition in a concentration-dependent manner (Fong et al., 2016; Zhai et al., 2020; Tyler et al., 2015; Brassett et al., 2017). In contrast to the highly swollen GMO:DOPG depots, a significant lower water uptake was observed for the GMO:MCT-based preformulation (Table 1), which is attributed to the slightly lower GMO content (68 % versus 72 % (w/w)) in the preformulation as well as to the presence of MCT. Addition of hydrophobic excipients, such as MCT, to GMO-based systems in excess water have been shown to induce Q2 - H2 phase transitions (Fong et al., 2016; Martiel et al., 2015; Mei et al., 2017) as described in more detail in section 3.4.

For both preformulations, no significant difference in the size of the LCP depot area was found when replacing PBS by 5 mg/mL HA solution.

Table 1

<table>
<thead>
<tr>
<th>Description</th>
<th>GMO:DOPG</th>
<th>GMO:MCT</th>
</tr>
</thead>
<tbody>
<tr>
<td>% W (non-agitated)</td>
<td>173 ± 28</td>
<td>28 ± 3</td>
</tr>
<tr>
<td>% W (agitated)</td>
<td>160 ± 18</td>
<td>39 ± 4</td>
</tr>
<tr>
<td>% V (SD2)</td>
<td>177 ± 56</td>
<td>188 ± 19</td>
</tr>
</tbody>
</table>

N.A.: Data not available as the depot and 5 mg/mL HA solution could not be fully separated.

a Composition of the GMO:DOPG preformulation: 72:8:10:10 % (w/w) GMO/DOPG/ethanol/water loaded with 18 mg/g diclofenac.

b Composition of the GMO:MCT preformulation: 68:12:10:10 % (w/w) GMO/MCT/ethanol/water loaded with 18 mg/g diclofenac.

Fig. 4. A) Cross-sectional areas of the in situ formed LCP depots during 20 h UV-Vis imaging experiments at 25 ± 2 °C (n = 3). B) Zoom-in of the cross-sectional areas in the first 15 min after injection of the preformulations. The GMO:DOPG-based (squares) and GMO:MCT-based (triangles) preformulations were injected into 67 mM phosphate buffer pH 7.4 (open symbols) or 5 mg/mL hyaluronic acid solution (closed symbols), respectively.
However, the volume increase (% V) was associated with relatively large standard deviations when employing PBS as compared to 5 mg/mL HA solution (Fig. 4). The relative standard deviations employing the HA solution were in average 8.5 % and 2.1 % for the GMO:DOPG- and GMO:MCT-based preformulations, respectively. These results indicate that the HA solution may be a suitable release media for in vitro release testing of in situ forming LCP drug delivery systems as it provides acceptable variations in the generated depot area even with varying depot shapes.

A slower swelling of the GMO:DOPG-based system was observed as compared to that of the system composed of GMO:MCT. Fig. 4 shows an increase in the LCP depot area up to 2 – 3 h upon injection of the GMO:DOPG-based preformulation, whereas the LCP area was almost constant throughout the experiments conducted by using the GMO:MCT-based preformulation. This is most likely attributed to the high viscous coexisting Q2 phases, formed from the GMO:DOPG-based preformulation, retarding a fast water diffusion into the depot.

The results, given as a percentage volume change (% V), of the UV–Vis imaging studies were compared to mass changes (% W) obtained in swelling experiments based on a separation approach as previously used for similar systems (Mei et al., 2017; Mei et al., 2018). The obtained mass changes are reported in Table 1 for the swelling studies on exposure of the preformulations to excess PBS. Swelling experiments in 5 mg/mL HA solution were unsuccessful due to lack of optimal phase separation. The volume changes obtained from the UV–Vis imaging experiments were in good agreement with the obtained mass changes from the traditional swelling studies when comparing the two preformulations. However, the results also showed variable water uptake depending on the agitation conditions (Table 1). Overall, the results indicate that the UV–Vis imaging experiments using the employed method can be used as a tool for evaluating the swelling behavior of in situ forming LCP depots, and the method may be beneficial for studies using viscous release media, such as HA solutions.

### 3.2. UV–Vis imaging - initial diclofenac release from in situ formed liquid crystalline depots

From the UV-absorbance maps, obtained at 300 nm and depicted at Fig. 3, the initial diclofenac release from the depots and the diffusion in the release media (PBS and 5 mg/mL HA solution) were visualized during the in situ forming process. The UV–Vis imaging allowed for monitoring diclofenac release in the immediate vicinity of the newly formed depot. Here, apparently, the depot shape dictated the observed diclofenac concentration gradients. For the GMO:MCT depot, which was formed at the top of the injection-cell, a high diclofenac concentration was initially observed around the depot, and diffusion of diclofenac towards the bottom of the injection-cell was seen over time (Fig. 3). In comparison, the diclofenac release from the GMO:DOPG depot occurred from the surface of the depot positioned at the center of the injection-cell and diffusion of diclofenac into the surrounding release media was seen in all directions (Fig. 3). In the time period from 5 to 20 h, a higher absorbance of diclofenac was clearly observed in the images of the GMO:MCT depots compared to those of the GMO:DOPG depots as visualized in Fig. 3 (and Figs. S3 - S6) by the observed color changes and obtained contour lines.

The amount of diclofenac released from the depots generated from both GMO:MCT- and GMO:DOPG-based preformulations into the release medium in the image area was calculated using the image analysis process (section 2.5.2), and depicted for the 20 h studies in Fig. 5. Immediately after injection of the preformulations, diclofenac was detected in the medium close to the formed depots. At 20 h, the amounts of diclofenac quantified in the image area corresponded to 1.7 ± 0.3/1.4 ± 0.2 % and 2.6 ± 0.1/2.6 ± 0.3 % (w/w) of the injected amount in PBS/HA solution for the GMO:DOPG- and GMO:MCT-based preformulations, respectively. The apparent release profiles can be described by a fast release at 0–1 h, followed by a decline in the release rate, which was more pronounced for the GMO:DOPG depots (Fig. 5). The different diclofenac release profiles for the two formulations might partly be ascribed to differences in the depot position and shape, leading to different diffusion patterns. Further, the significantly different depot sizes (Fig. 4) of the two LCPs affected the volume ratio between them and the surrounding release medium in the image area (as seen in Fig. 3). This may result in a lower amount of released diclofenac at the end of the experiments, when investigating the release from the GMO:DOPG depot as compared to that from the GMO:MCT depot. Thus, the results showed that the initial drug release behavior in the current in vitro setup, employing different medium compositions and volumes relevant for intra-articular drug delivery, were highly affected by the in situ generated depot shape and size.

For the in situ formed GMO:DOPG depot, a significantly higher amount of diclofenac (p < 0.05) was released in the image area using PBS as release medium compared to 5 mg/mL HA solution in the time period from 2 min to 8 h. After 8 h, no significant difference in the diclofenac released amount was seen between the two release media (Fig. 5A). In comparison, no significant difference was found in the apparent release profile for the GMO:MCT depot formed in PBS and 5 mg/mL HA solution (Fig. 5B). It is still worth noting that a slightly faster diclofenac release at an early stage was observed in PBS (Fig. 5B). Further studies are needed to elucidate whether the higher initial diclofenac release in PBS is a result of higher diclofenac diffusivity in PBS as compared to the 5 mg/mL HA solution or not.

![Fig. 5. Amount of released diclofenac in the image area from the in situ formed LCPs in 67 mM phosphate buffer pH 7.4 (open symbols) and 5 mg/mL hyaluronic acid solution (closed symbols) as obtained from the analysis of UV–Vis imaging SDI2 experiments at 25 ± 2 °C. A) Release profiles for the two GMO:DOPG-based depots generated from the preformulation (72:8:10:10 % (w/w) GMO/DOPG/ethanol/water), and B) release profiles for the two GMO:MCT-based depots generated from the preformulation (68:12:10:10 % (w/w) GMO/MCT/ethanol/water).]
At the end of the UV-Vis imaging experiments, the absorbance maps (Fig. 3 at 20 h) clearly revealed the presence of diclofenac concentration distributions in the image area, which indicated that the system was far from equilibrium. To obtain an absorbance value, reflecting the average concentration of diclofenac in the release medium at the end of each experiment, the release medium inside the injection cell was mixed and an average UV absorbance was measured. The obtained corresponding average diclofenac concentrations were not significantly different from the diclofenac concentrations in the collected release media as determined by UV-spectrophotometry (Table 2). Thus, the employed UV-imaging method appears to be suitable for determination of the average diclofenac concentrations in the release medium at the end of such experiments. Based on these diclofenac concentrations, the released amount of diclofenac to the total volume of the release medium (collected and measured at the end of the experiment) was calculated to be 0.24 ± 0.03 mg (4.3 ± 0.3 % (w/w)) and 0.32 ± 0.04 mg (5.5 ± 0.7% (w/w)) in PBS at 20 h for the GMO:DOPG- and GMO:MCT-based depots, respectively. In comparison, the obtained amounts of diclofenac in the image area at 20 h (Fig. 5) constituted approximately half of the total amounts released as the remaining diclofenac diffused out of the image area. To this end, the image area constituted about half of the injection-cell area. Thus, the very low amounts of diclofenac released (< 6 % at 20 h) and the image area only covering a part of the release medium area indicate that the current setup, where drug release is monitored at non-sink and stagnant conditions, is unable to investigate drug release characteristics in all parts of the long-term release process. For in situ forming drug delivery systems, however, it is of interest to characterize the real-time drug release in the initial stage in the immediate vicinity of the depot during the depot formation process.

The initial diclofenac release characteristics of the two in situ forming LCP depots, differing with respect to lipid composition, were compared. The reported diclofenac concentrations in the release media (Table 2) showed that the GMO:DOPG depots provided significantly lower diclofenac concentrations as compared to the GMO:MCT depots, which were in agreement with the obtained release profiles. As described above, the difference between the two depots might be attributed to less release medium available for drug release in the case of the GMO:DOPG depot due to the larger depot size. However, the slightly higher GMO content in the GMO:DOPG-based preformulation (72 % (w/w)) as compared to the GMO:MCT-based preformulation (68 % (w/w)) may also contribute to a lower diclofenac release due to the preferential interactions of this drug with GMO at the lipid-water interface, as discussed in previous studies (Efrat et al., 2008). Moreover, alterations in the structural features of the in situ generated depots (as discussed below in sections 3.3. and 3.4) and their viscosities on varying the lipid composition may influence the released amount of diclofenac at the end of the experiments (as indicated from the apparent diclofenac release profiles presented in Fig. 5).

The findings from the diclofenac release studies were supported by results from separate swelling studies (at agitation and non-agitation conditions). The latter showed significantly higher diclofenac concentrations 20 h after adding the GMO:MCT-based preformulation to PBS compared to those obtained with the GMO:DOPG-based preformulation (Table 2). The swelling studies clearly demonstrated the impact of the agitation on the total released diclofenac amount as significantly higher amounts of diclofenac were found on employing agitation conditions.

In the injection-cell setup coupled to UV imaging, the in vitro release studies were performed at stagnant conditions. The UV-images showed concentration gradients as they developed around the depot over time (Fig. 3). The diclofenac build up in the immediate vicinity of the depot decreases the rate of diclofenac release. It is of interest in future development of the in vitro release testing method to focus on including flow conditions in the system.

3.3. Structural characterisation of diclofenac-loaded preformulations and corresponding liquid crystalline phases by static SAXS measurements

The SAXS patterns of both GMO:DOPG- and GMO:MCT-based preformulations loaded with 18 mg/g diclofenac and their corresponding LCP depots formed in situ upon exposure (for 21 – 26 h) to PBS or 5 mg/ml HA solution at 25 ± 0.1 °C are shown in Fig. 6. Consistent with our recent report (Mertz et al., 2021), the SAXS patterns for these two preformulations (Fig. 6, A1 and B1) were characterized by a single broad peak at a q value of 1.73 and 1.77 nm⁻¹ for the GMO:MCT-based and the GMO:DOPG-based preformulations, respectively, indicating the formation of inverted-tube micellar solutions (L2 phases) with a corresponding distance of 3.64 and 3.54 nm, respectively. It is worth noting that a very weak peak was also detected at q of 1.25 nm⁻¹ in the SAXS pattern of sample B1, indicating most likely the presence of traces of unidentified liquid crystalline phase. On exposure of both preformulations to PBS and 5 mg/ml HA solution, alteration in their lipid composition was associated with a huge impact on the structural features of the in situ formed LCP depots, as seen in PBS (Fig. 6, A2 and B2) and 5 mg/ml HA solution (Fig. 6, A3 and B3).

It is worth noting that the LCP depots formed from the GMO:MCT-based preformulation (Fig. 6, B2 and B3) were characterized as triphasic features (coexistence of three hexagonal (H₂) phases, where at least two characteristic peaks were identified for each coexisting H₂ phase). The corresponding lattice parameters of these three phases generated in PBS (marked in black, red and blue in Fig. 6) were 6.43 ± 0.02, 6.41, and 6.24 ± 0.01 nm, respectively; whereas their values were 6.41 ± 0.06, 6.04 ± 0.01, and 5.68 nm in 5 mg/ml HA solution. The formation of such H₂ phases seen on exposure of the MCT-containing preformulations to the aqueous medium was expected and in line with previous findings on the inclusion of hydrophobic additives (e.g., MCT, n-tetradecane, or a poorly water-soluble drug) to mono- and diunsaturated monoglycerides such as glycerol monooleate (Yaghmur et al., 2012; Mertz et al., 2019; Mei et al., 2017; Guillot et al., 2006; Boley et al., 2004).

Table 2

<table>
<thead>
<tr>
<th>Deposit</th>
<th>Medium</th>
<th>Diclofenac concentration in release medium at 20 h (mg/ml)</th>
<th>UV-Vis imaging experiment</th>
<th>Swelling experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Inside injection-cell</td>
<td>Collected sample</td>
</tr>
<tr>
<td>GMO:DOPG</td>
<td>PBS</td>
<td>0.073 ± 0.008</td>
<td>0.077 ± 0.002</td>
<td>0.045 ± 0.003</td>
</tr>
<tr>
<td>GMO:MCT</td>
<td>PBS</td>
<td>0.090 ± 0.011</td>
<td>0.094 ± 0.006</td>
<td>N.A.</td>
</tr>
<tr>
<td>GMO:MCT</td>
<td>HA</td>
<td>0.081 ± 0.003</td>
<td>0.082 ± 0.005</td>
<td>0.108 ± 0.005</td>
</tr>
</tbody>
</table>

N.A.: Data not available.

*Mertz et al., 2021* formed in situ from the 72:8:10:10 % (w/w) GMO:DOPG:ethanol/water preformulation loaded with 18 mg/g diclofenac.

*Mei et al., 2017* formed in situ from the 68:12:10:10 % (w/w) GMO:MCT:ethanol/water preformulation loaded with 18 mg/g diclofenac.
formed upon exposure to excess PBS (A2 and B2) or 5 mg/mL HA (A3 and B3) generated from the GMO:MCT-based preformulation, leading to pres (w/w) GMO/MCT/ethanol/water) (B1) preformulations containing 18 mg/g GMO/DOPG/ethanol/water) (A1) and the GMO:MCT-based (68:12:10:10 % w/w) GMO:MCT formulation, diclofenac loading was also found to differ in structure, respectively (Fig. S7, C1 and C2). For the 68:12:10:10 % (w/w) GMO:MCT-based formulation, diclofenac loading was also found to differ from previously reported neat cubic Pn3m phase (with a small lattice parameter of 9.91 nm) generated upon exposure of GMO to excess PBS (Yaghmur et al., 2021). This was attributed to the presence of the negatively charged phospholipid, DOPG, at the GMO-water interface, which resulted in an enlargement of the hydrophilic nanochannels of the inverse cubic Im3m and Pn3m phases, as discussed in our recent study (Mertz et al., 2021) and consistent with previous reports (Zhai et al., 2020; Tyler et al., 2015; Brasnett et al., 2017). In contrast, the inclusion of MCT in the GMO-based depot was associated with a decrease in water uptake as discussed above. Thus, the results confirmed the observed different swelling behavior on exposure of both GMO:DOPG-based and GMO:MCT-based preformulations to PBS or HA solution as described employing the UV–Vis imaging method (Figs. 3 and 4).

The aqueous medium composition (PBS vs HA solution) did not affect the structural features of both GMO:DOPG- and GMO:MCT-based depots (Fig. 6): identical self-assemblies were in situ generated in both aqueous media. As the structural features are identical, the observed faster initial diclofenac release from the GMO:DOPG depot in PBS as compared to that in HA solution (Fig. 5A) is most likely not attributed to their hydrated nanostructures under equilibrium conditions. However, we do not exclude possible alterations in the dynamic structural transitions under initial conditions on exposure of both preformulations to PBS as compared to HA solution (see section 3.4).

3.4. Position- and time-dependent structural alterations of in situ formed liquid crystalline depots

For gaining further insights on the in situ forming process of the LCP depots, time-resolved investigations were conducted by coupling synchrotron SAXS with the injection-cell. Fig. 7 presents the SAXS diffraction patterns, indicating the detected structural alterations and phase transitions, after injection of the GMO:MCT-based preformulation into PBS and 5 mg/mL HA solution. They were obtained at the following two specific positions: 4.5 mm below the top of the injection-cell and 3 mm to the right of the needle (Fig. 7A), or 2 mm to the left of the needle (Fig. 7B). In a good agreement with our recent findings (Mertz et al., 2021), fast water uptake was associated with a rapid evolvement of inverse non-lamellar liquid crystalline phases (within the first 2 min) as indicated from the SAXS patterns shown at different selected time points (Fig. 7). At the monitored injection-cell positions, the hydration-triggered L2 → H2 phase transition occurred, however, traces of L2 phase were still detected up to 1164 s after injection of the preformulation into PBS (Fig. 7A). The transition to inverse LCPs (including an intermediate cubic Pn3m phase and newly formed H2 phase) was faster in HA solution as the L2 phase vanished 24 s after preformulation injection (Fig. 7B). Here, penetration of HA into these liquid crystalline depots may contribute to such a different behavior in PBS as compared to HA solution. In excess PBS, traces of coexisting L2 Pn3m phase (phase identification was based on the appearance of 2 – 3 peaks of its characteristic peaks that were very weak) started to evolve 84 s upon injection of the preformulation, and was present for the rest of the experiment (Fig. 7A). In addition, two coexisting H2 phases started to develop (marked with black and magenta in Fig. 7A). The lattice parameters of these two coexisting H2 phases at the end of the experiment (at 2004 s) were 5.87 ± 0.05 and 6.53 nm, respectively. Here, the lattice parameter of the latter H2 phase is comparable with the aforementioned lattice parameters of the coexisting H2 phases formed ~ 25 h after addition of the preformulation to excess PBS at 25 °C (Fig. 6, B2). On exposure of the same preformulation to the HA solution (Fig. 7B), few very weak peaks characterizing an intermediate cubic Pn3m phase with symmetry (a double diamond cubic phase) (Mertz et al., 2021). For the Im3m Q2 phases, the calculated lattice parameters in PBS and 5 mg/mL HA solution were 16.31 ± 0.03 nm and 16.15 ± 0.06 nm, respectively, whereas the values were 12.85 ± 0.08 nm and 12.75 ± 0.04 nm, respectively, for the coexisting Pn3m Q2 phases (Mertz et al., 2021).

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The aqueous medium composition (PBS vs HA solution) did not affect the structural features of both GMO:DOPG- and GMO:MCT-based depots (Fig. 6): identical self-assemblies were in situ generated in both aqueous media. As the structural features are identical, the observed faster initial diclofenac release from the GMO:DOPG depot in PBS as compared to that in HA solution (Fig. 5A) is most likely not attributed to their hydrated nanostructures under equilibrium conditions. However, we do not exclude possible alterations in the dynamic structural transitions under initial conditions on exposure of both preformulations to PBS as compared to HA solution (see section 3.4).
relatively short lifetime were initially detected and disappeared after 444 s. A coexisting $H_2$ phase started to evolve and develop 24 s upon preformulation injection into the solution, and a transition from the biphasic cubic $Pn3m/H_2$ feature to a neat $H_2$ phase was completed at 444 s (Fig. 7B). The lattice parameter of this $H_2$ phase only changed slightly (on increasing the elapsed time from 144 to 3504 s), and was found to be 6.33 ± 0.005 nm after 3504 s (the end of the experiment). In comparison, the three coexisting $H_2$ phases (including the two $H_2$ phases with comparable lattice parameters) were identified for the depot formed ~ 25 h after injection into the HA solution at 25 °C (Fig. 6, B3).

Here, it is important to take into account that differences in the structural features of the in situ generated depots may occur on varying the position for acquiring SAXS data in the injection-cell (Mertz et al., 2021). Thus, position-dependent dynamic structural transitions on exposure of both preformulations to PBS (or HA solution) were investigated and discussed below. In addition, the observed differences in the structural features (including the calculated lattice parameters of the identified inverse non-lamellar liquid crystalline phases) found at the end of the dynamic structural transition experiments in the injection-cell (Fig. 7) and in the static measurements (Fig. 6) can be attributed to the employment of different experimental conditions. In particular, stirring conditions may facilitate a faster hydration and influence the structural characteristics of the LCP depots formed in situ.

The time-resolved SAXS experiments were performed in three vertical positions (p0, p1 and p2, see section 2.7) at 4.5, 6.5 and 8.5 mm, respectively, below the top of the injection-cell. However, the LCP depots formed in situ from the GMO:MCT-based preformulation were only detected in position p0 in PBS and in position p0 and p1 in 5 mg/mL HA solution as the depot was formed in the upper part of the injection-cell. This was consistent with the UV–Vis absorbance maps (Fig. 3). The dynamic structural transitions during the depot formation in 5 mg/mL HA solution in position p1 were comparable to those observed at position p0 (data not shown). For the GMO:DOPG-based preformulation, the structural transitions of the LCP depot formation could be followed in all three measured injection-cell positions. The findings in 5 mg/mL HA solution was previously reported (Mertz et al., 2021). A transition from a micellar solution ($L_2$ phase) to coexisting inverse bicontinuous cubic $Pn3m$ and $Im3m$ phases was found and involved a short-lived intermediate lamellar ($L_{α}$) phase (disappearance after 5 min of its occurrence) (Mertz et al., 2021). In the present study, similar dynamic transitions were seen in PBS after injection of the GMO:DOPG-based preformulation (data not shown). An intermediate $L_{α}$ phase was observed in the first 2 min, and a biphasic feature of two coexisting $Q_2$ phases with $Im3m$ and $Pn3m$ symmetries with lattice parameters deviating only slightly from the previously determined values in 5 mg/mL HA solution was identified. Such slight structural alterations are most likely attributed to differences in hydration level in the generated LCP depots, and penetration of HA into these phases may influence their lattice parameters.

It is worth gaining insight into the structural features of the in situ generated LCP depots by acquiring SAXS data at various positions in a selected regime of the injection-cell. For this purpose, 2D spatial maps were constructed from SAXS patterns, which were recorded at 70 different positions in a selected part of the injection-cell close to the injection needle. The map depicted in Fig. 8, presents an overview of the
Fig. 8. A) A 2D spatial map of the scanned area of the injection-cell and constructed from SAXS patterns recorded at 70 different positions at 59 min after injection of the GMO:MCT-based (68:12:10:10 % (w/w) GMO/MCT/ethanol/water) preformulation to 5 mg/mL hyaluronic acid (HA) solution (25 ± 1 °C). B) The structural features (H2 phases and two coexisting Q2 phases with Im3m and Pn3m symmetries) and their corresponding lattice parameters (in nm) as determined at various positions in the in situ formed depot situated at the upper part of the injection-cell (the area is marked with a red rectangle on the map presented in (A)).

structural features detected 59 min upon injection of the GMO:MCT-based preformulation into 5 mg/mL HA solution. This figure allows for a clear-cut differentiation between liquid crystalline rich- and HA solution-rich regimes. At positions, where the LCP depot was observed (Fig. 8A), the phases were identified and their lattice parameters were calculated through analysis of the individual SAXS diffraction patterns (Fig. 8B). Fig. 8A clearly shows that the LCP depot was formed in the upper part of the scanned area of the injection-cell close to the injection site in line with the observations in the UV-Vis images (Fig. 3). Depending on the injection-cell measurement position, the recorded distinct SAXS diffraction patterns indicated structural heterogeneity, where different inverse non-lamellar LCPs were detected in a spatial-dependent manner (Fig. 8). For instance, weak Bragg peaks pertaining to Q2 phases of Im3m and Pn3m symmetries along with characteristic peaks of two coexisting H2 phases were observed in the SAXS pattern taken at 6 of the 7 positions in the top row of the injection-cell. In contrast, only a single H2 phase was detected in the remaining positions of the liquid crystalline-rich regime (Fig. 8B). The coexisting phases in the upper part of the in situ formed LCP depot may indicate that the LCPs were more rich in GMO, i.e. consisted of a lower amount of MCT compared to the LCPs in the three rows below (Fig. 8B). A LCP depot with heterogeneous structural features was also seen when the GMO:MCT-based preformulation was injected into PBS (Fig. S8). However, in the constructed map, a single broad peak at q ≈ 1.5 nm⁻¹, indicating the formation of L2 phase with most likely a very limited water uptake, was identified from the diffraction patterns recorded at the two upper rows of the injection-cell. In contrast, H2 phases were identified in the formed depot at the remaining positions (Fig. S8). Taking into account that the aforementioned transitions from L2 to different inverse LCPs were mainly driven by hydration, the map (Fig. S8) clearly
demonstrated that the accessibility level of the preformulation to water plays an important role in modulating the structural features of the \textit{in situ} generated depots. Thus, a higher water uptake in the liquid crystalline-rich regime was most likely the main reason behind the appearance of H$_2$ phases as compared to significantly lower water uptake at the two upper rows of the injection-cell (a water-poor regime). Thus, the heterogeneous features of the LCP depots indicate that a redistribution of the involved lipids and water is occurring during the \textit{in situ} forming process of the LCP depot upon injection of the preformulation into the aqueous medium. To this end, a potential effect of ethanol accommodation in the nanochannels of the LCPs on their structural features should be considered as discussed in our recent report (Mertz et al., 2021).

The constructed 2D spatial maps of the injection-cell upon injection of the GMO:DOPG-based preformulation are presented in Supplementary Material (Fig. S9), when using PBS as release medium. In our recent study (Mertz et al., 2021), similar maps were constructed on exposure of the preformulation to 5 mg/mL HA solution. In both aqueous media, a biphasic feature of coexisting Q$_2$ phases with the space groups of Im3m and Pn3m was formed, however, their lattice parameters varied on changing the measurement positions. For instance, the lattice parameters of the Q$_2$, Im3m and Pn3m phases formed in PBS were in the range of 15.03 – 18.83 nm and 11.74 – 14.23 nm, respectively (Fig. S8).

The obtained results from the spatially and time-resolved SAXS experiments confirm the fast formation of the LCP depots in the injection-cell upon injection of the two diclofenac-loaded preformulations into biological relevant media (PBS and HA solution). The observed variations in the structural features of the generated LCP depots at different positions and the alterations seen in the sizes and shapes of the depots complicate further assessment of a potential link between the structural features of the generated depots and the initial diclofenac release properties. The employed setup, where the injection-cell is coupled to synchrotron SAXS, provides an useful tool for studying dynamic structural transitions during the \textit{in situ} formation of the LCP depots at conditions similar to \textit{in vitro} release testing experiments and with relevance for the intra-articular delivery.

4. Conclusion

The present study showed for the first time that a combination of UV–Vis imaging and spatially and time-resolved SAXS measurements through use of an injection-cell setup can provide detailed insight into the \textit{in situ} forming process of depots of non-lamellar liquid crystalline phases (LCPs) intended for intra-articular drug delivery. The setup was advantageous for monitoring the rate of depot formation, and characterizing its size and shape. In addition, investigations on the initial drug release in the vicinity of the formed depot can be combined with both SAXS experiments at static conditions as well as real-time SAXS measurements for studying time- and position-dependent dynamic structural transitions during the \textit{in situ} depot formation.

Results from the UV–Vis imaging and time-resolved SAXS measurements showed a fast generation of the LCP depots upon injection of the two diclofenac-loaded preformulations into bio-relevant aqueous media (PBS or HA solution). The GMO:DOPG-based and GMO:MCT-based preformulations formed \textit{in situ} LCP depots with structures of coexisting Q$_2$ phases with Im3m symmetry and Pn3m symmetries and H$_2$ phases, respectively. The obtained UV–Vis images enabled quantification of the formed depot sizes during the \textit{in situ} forming process, and allowed for a distinction between the depot sizes generated from the two different preformulations. The highly swollen LCP depots generated from the GMO:DOPG-based preformulation compared to the GMO:MCT depots were in agreement with the different structural features of the two depots. Local concentration distributions of released diclofenac in the immediate vicinity of the depots were visualized from the UV-images and initial amounts released were quantified in a well-defined area of the injection-cell. The observed difference in the apparent release profiles of the two depots (GMO:DOPG- and GMO:MCT-based self-assemblies) was most likely attributed to the detected different shapes and sizes of the formed depots as evident from UV–Vis imaging findings. In addition to structural features, a complex interplay among other factors (including rheological characteristics, depot size and shape characteristics and diffusion rates of water and ethanol) seem to play an important role in modulating the drug release properties from \textit{in situ} generated LCP depots. Thus, further investigations are needed to fully elucidate the relationship between release properties and structural features of the two LCP depots. Results from the UV–Vis imaging and SAXS experiments showed no or limited effect by the presence of HA in the aqueous medium on the shape, size, structural features and initial diclofenac release. The position-dependent SAXS measurements allowed for construction of 2D spatial maps, which confirmed the analysis of the UV–Vis images with respect to depot shape and further revealed variations in the structural features of the LCP depots at different positions.

The current injection-cell setup employing a combination of UV–Vis imaging and synchrotron SAXS constitutes an attractive tool for characterization of \textit{in situ} forming LCP formulations intended for an intra-articular administration. Further development of the setup should focus on adaptation to long-term release testing.

CRediT authorship contribution statement

Nina Mertz: Conceptualization, Data curation, Formal analysis, Software, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing, Project administration. Frederik Bock: Data curation, Formal analysis, Software, Visualization. Jesper Østergaard: Conceptualization, Investigation, Methodology, Writing – review & editing. Anan Yaghmur: Conceptualization, Investigation, Methodology, Resources, Writing – review & editing. Susan Weng Larsen: Conceptualization, Investigation, Methodology, Writing – original draft, Writing – review & editing, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

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