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The success of siRNA therapy depends on the development of an effective gene delivery system, which should avoid nuclease degradation and provide cellular uptake and endosomal escape. The in situ gelling delivery system is an interesting delivery system for the local administration of siRNAs. In this system, the self-assembling drug delivery system is based on in situ gel formation from a precursor fluid formulation that can be formed from amphiphilic lipids, such as monoolein (MO), which swell in the presence of water and form various types of lyotropic liquid crystals. In this study, a new non-viral system based on MO, polyethylenimine (PEI), propylene glycol (PG) and 0. 1 M Tris buffer, pH 6. 5 (Tris buffer), which becomes a viscous gel after subcutaneous injection, was proposed for the delivery of siRNA by parenteral administration. The results demonstrated that an appropriate mixture of these components formed precursor liquid-like viscosity formulations that, in the presence of excess water, transformed into a viscous liquid crystalline phase. Additionally, gel formation was a rapid process, as demonstrated by the swelling studies. The incorporation of the cationic polymer PEI into the MO-based systems was necessary for siRNA complexation, and the systems maintained the siRNA in a stable state. The in vitro release studies demonstrated that siRNA release is dependent on the water absorption rate of the formulation. Moreover, in vivo studies showed that the gel can be formed in situ after subcutaneous injection of a precursor fluid formulation in mice and that this gel was biodegradable and biocompatible for 30 days. The precursor fluid formulation composed of MO/PEI/PG/Tris buffer at 7. 85: 0. 65: 76. 5: 15 (w/w/w/w) formed a gel in situ that was characterised as a viscous liquid crystalline phase that delivered the siRNA both locally and sustainably. This study demonstrated the rational development of a biodegradable and biocompatible in situ gelling formulation for the subcutaneous administration of siRNAs, which can be used to administer antisense gene therapy for the treatment of several diseases. Keywords: Liquid crystalline system, siRNA, In situ gelling system, Monoolein, Site-specific delivery, Non-viral gene delivery

## Introduction:

siRNA has garnered much attention as a new approach to treat many human diseases, such as genetic and nongenetic diseases, cancer and viral infections, since 1998, when Fire, Mello and colleagues [1] discovered that double-stranded RNAs can silence target gene expression in the nematode worm Caenorhabditis elegans. However, the clinical use of siRNAs is limited by various challenges specifically related to their delivery. The primary barriers to siRNA delivery are rapid degradation by endogenous enzymes [2], difficult cellular uptake because of high molecular weight (~13 kDa) and negative charge [3]. Therefore, the widespread use of gene therapy for disease prevention and treatment requires the development of clinically suitable, safe and effective drug delivery systems [2, 3]. The essential design criteria for siRNA delivery systems includes neutralisation of the negatively charged phosphate backbone of nucleic acids to avoid charge repulsion against the anionic cell membrane, condensation of the bulky structure to appropriate lengths for cellular internalisation, protection of the nucleic acids from nuclease degradation and promotion of endosomal escape [4]. Based on these concepts, many studies have explored delivery systems for both systemic and local administration to facilitate the therapeutic application of siRNA. Whereas the systemic delivery of siRNAs must contend with challenges in addition to those related to siRNA internalisation, such as interaction of the siRNAs with blood components, uptake by filtering organs, renal clearance and permeation within the tissue [3], local delivery systems for siRNAs can overcome these drawbacks of systemic administration and present with several advantages, including lower doses, decreased risk of clearance, a sustained silencing effect, site-specific delivery and decreased immunostimulation [2, 5]. Among the local administration systems, self-assembling formulations, such as in situ gelling delivery systems, have become an attractive approach for the release of siRNAs [6-9]. These in situ gelling delivery systems have several advantages, such as being minimally invasive and painful compared to implants because they can be injected through a syringe into the body and after injection, they form a localised gel, which prolongs drug delivery in the body and contributes to a dose reduction with a concomitant decrease in undesirable side effects [10-14]. This characteristic is particularly desirable for prolonged therapies in which the compliance and comfort of the patient is critical. The mechanism of achieving solidification in vivo for the in situ gelling delivery systems can be divided into the following four categories: (i) thermoplastic pastes; (ii) in situ crosslinked systems; (iii) in situ precipitation; and (iv) in situ solidifying organogels [11, 15]. The organogels can be composed of amphiphilic lipids, which swell in the presence of water and form various types of lyotropic liquid crystals (LLCs) [11, 16]. The use of in situ gelling systems for gene delivery is fairly new and was first suggested by Krebs et al. [8] in 2009 who developed three different systems to deliver siRNAs based on crosslinked alginates and collagen. Chitosan hydrogel was later shown to deliver siRNAs intratumourally and exhibited significant inhibition of tumour growth in a xenograft model compared to the control [6]. However, although these systems locally delivered the siRNAs using an injectable hydrogel, release of the siRNAs from the gel was not easily controlled. Moreover, the siRNAs were released in a naked form, which have a short in vivo half-life and little gene silencing efficiency [7, 9]. To overcome these drawbacks, other systems have been developed that incorporate or conjugate polyethylenimine (PEI) with the in situ gelling systems [7, 9]. For these systems, gel formation was obtained either by in situ crosslinking or in situ precipitation through stimuli-dependent sol-gel transition. However, the in situ crosslinking and precipitation mechanisms of these systems are harmful to both the living tissue and the encapsulated drug [13]. Therefore, current studies have focused on the in situ gelling system that is formed spontaneously under physiological conditions after the administration of a precursor fluid formulation [17]. Liquid crystalline systems (LCSs) can potentially be used as drug delivery systems because they provide improved drug solubility, relative protection of the solubilised drugs and controlled release of drugs [18-22]. LCSs can be formed by unsaturated monoglycerides, such as monoolein (glyceryl monooleate) (MO) or monolinolein [23]. LCSs are influenced by the presence and ratio of solvents [24] and additives [25] and are classified as lamellar, hexagonal or cubic phase. The cubic phase settles between the lamellar and hexagonal phases and is formed with increasing water content. After the lamellar phase becomes less viscous and injectable, it can be used as a gel precursor because after these compounds come in contact with excess water from the body fluids, they form a rigid and viscous cubic phase gel that promotes sustained release [26]. An increase in the solvent concentration of the lamellar phase may cause the rod shape of the solvated molecules to transform into a cone shape, and depending on the polarity of the solvating agent and the molecule itself, this transition can result in either a hexagonal or reversed hexagonal phase [27]. With these properties, LCSs are exceptional in situ gelling systems [26, 28]. To maintain the advantages of in situ gelling systems and overcome the drawbacks of injectable hydrogels, we propose precursor fluid formulation composed primarily of MO to form an in situ gelling LCS. To the best of our knowledge, this is the first study of an in situ gelling LCS that delivers siRNAs both locally and sustainably. PEI was added to the MO-based precursor fluid formulation, and its influence on the packing parameter of MO and the liquid crystalline phase was evaluated. The optimised precursor formulations were characterised for their ability to complex with siRNAs to maintain stability, as well as the water absorption profile, the in vitro siRNA release profile and their ability to form a liquid crystalline gel after subcutaneous injection in an animal model.

## Materials and Methods

## Materials

Monoolein (Myverol 18-92 consisting of 67. 5% glyceryl monolinoleate and 18. 7% glyceryl monooleate) was kindly provided by Kerry Bio Science (Zwijndrecht, The Netherlands). Branched polyethylenimine (25 kDa), diethyl pyrocarbonate (DEPC), ethidium bromide and isopentane were purchased from Sigma Aldrich Co. (St. Louis, MO, USA). Propylene glycol (PG) was obtained from Labsynth Produtos by Laboratórios Ltda (Diadema, SP, Brazil). UltrapureTM Agarose was purchase from InvitrogenTM (Carlsbad, CA, USA). The siRNA used was the Silencer Negative Control #1 siRNA (Catalogue #AM4635) purchased from Ambion (Austin, TX, USA). Heparin was purchased from Blausiegel (Cotia, SP, Brazil). Kapton was obtained from E. I. DuPont de Nemours & Co., Inc. (Wilmington, Delaware, USA). Tris(hydroxymethyl)aminomethane was purchase from Merck KGaA (Darmstadt, Germany). Dialysis tubing with a 50, 000 molecular weight cut off was obtained from Spectrum Laboratories, Inc. (Rancho Dominquez, CA, USA). Twelve-well plates and inserts were purchased from Becton Dickinson Labware (Franklin Lakes, NY, USA).

## Preparation and characterisation of formulations

## Precursor fluid formulations

Precursor fluid formulations were obtained by mixing pre-warmed (42° C) MO with PG in proportions of 1: 9 to 9: 1. Immediately thereafter, 0. 1 M Tris buffer, pH 6. 5 pre-warmed to 42° C, was added at concentrations ranging from 5 to 90%. For the systems containing the cationic polymer, PEI was mixed with the MO in the following proportions: 12: 1, 6: 1 and 3: 1 (MO/PEI; (w/w)). The PG was then incorporated under vortex stirring, and the Tris buffer was finally added as described above. The resulting formulations were stored in closed vials at room temperature.

## In vitro gelling formation assay

Homogenous and stable fluid formulations were selected for the in vitro gelling test that was performed by combining 100 μL of these formulations with excess water (900 µL) at 37° C.

## Characterisation of the liquid crystal phases

Both the precursor fluid formulations and the gel formed after contact with excess water were macroscopically characterised by visual analysis and microscopically examined under a polarised light microscope (Axioplan 2 Image Pol microscope, Carl Zeiss, Oberkichen, Germany) after 24 h and 7 days at 25° C.

## siRNA loading and stability in the precursor fluid formulation

To determine the ability of the systems to form complexes with the siRNA, the Silencer Negative Control #1 siRNA (siRNA) was used according to the method of Tran, et al. [29]. The siRNA was added to the precursor fluid formulations at a final concentration of 10 μM. The mixtures were incubated at room temperature for 30 min. A 40 μL aliquot of this mixture or the control (siRNA in DEPC water) was mixed with 10 μL loading buffer. Then, 40 μL of each mixture was electrophoresed on ethidium bromide-stained TAE-based 2% agarose gels at 100 V for 20 min followed by visualisation under UV light. The stability of the siRNA in the precursor fluid formulations was analysed using a heparin polyanion competition assay [30]. For this, siRNAs complexed with the formulation were prepared as described above. To determine the appropriate volume of heparin to promote decomplexation of the siRNA, different volumes (3. 2, 10 and 20 µL) of heparin (5, 000 U. I./mL) were added to 40 µL of the formulations. A volume of 10 µL heparin was sufficient to compete with the siRNA and promote its decomplexation for most systems, with the exception of the MO/PEI 15. 69: 1. 31 (w/w) system, which required 20 µL. Therefore, for the heparin polyanion competition assay, either 10 µL or 20 µL heparin (5, 000 U. I./mL) was added to the formulations with agitation. These mixtures were incubated at 37 °C for 1 h. The solutions were then electrophoresed on agarose gels as described above.

## Swelling Studies

The swelling studies were performed gravimetrically. The precursor fluid formulations (100 µL) were placed into a dialysis tube (50, 000 Da cut off) and placed in contact with excess water (3 mL), which was maintained at 37° C with a thermoregulated bath. At fixed time intervals (15 and 30 min, 1, 2, 4, 8, 12, 24 and 48 h), each dialysis tube was removed. The surface of the dialysis membrane was blotted with a fine weave paper to remove any excess water and then weighed. The water uptake data were fit according to the first (Eq. 1) and second (Eq. 2) order kinetics equations for swelling studies described in the literature [28, 31-33].(1)(2)where W is the water uptake at time t, W∞ is the maximum water uptake, k is the rate constant and (W∞ - W) is the unrealised water uptake. For the second-order equation, the maximum or equilibrium water uptake (W∞) of the MO can be described from the reciprocal of the slope, and the initial rate of swelling is the reciprocal of the y-intercept of the plot t/W versus t [28, 31-33].

## Small Angle X-ray Scattering (SAXS)

The crystalline structure of the gel formed after 24 h of contact with excess water was determined by SAXS. The analysis was performed by placing the samples in a sample holder between Kapton sheets. The scattering angle measurements were performed at a wavelength of 0. 1608 nm using the D02A-SAXS beamline at the Brazilian Synchrotron Light Laboratory (LNLS), Campinas, Sao Paulo, Brazil. The scattered intensity curves were recorded using a two-dimensional detector with a measurement time of 300 seconds. The results were corrected by detector response, and the SAXS data were normalised by the transmission for each case. It was also removed the scattering from the Kapton sheets in the sample holder.

## In vitro siRNA release from the LCSs

The release studies were performed in a 12-well plate with an insert (1. 0 µm pore size). A 100 µL aliquot of the precursor fluid formulations containing siRNA (10 µM) or control (siRNA 10 µM in DEPC water) was added to the insert membrane. The receiving medium was 1800 µL DEPC water, maintained at 37 °C. All receiving media were removed at specific time intervals (24, 48 h and 7 days) and placed in Eppendorf tubes. The volume removed was replaced with fresh DEPC water. The samples were dried in an Eppendorf Concentrator 5301. The remaining materials were resuspended with DEPC water (60 µL) and divided into two Eppendorf tubes. In one tube, 10 μL heparin (5, 000 U. I./mL) was added for the polyanion competition assay. In the second tube, 10 μL DEPC water was added, and the samples were incubated at 37 °C for 1 h. The siRNA released was qualitatively analysed by agarose gel electrophoresis as described in Section 2. 2. 4. For quantification of the released siRNA, a standard curve (15-0. 9375 µM siRNA) was also electrophoresed. The signal intensities of the standards and samples in each agarose gel were calculated using the NIH ImageJ software (http://rsbweb. nih. gov/ij/).

## In situ gelling and local toxicity in an animal model

Female BALB/c mice (18-20 g) were used to study the in situ gelling ability of the precursor fluid formulations after subcutaneous injection. All animals were maintained under standard environmental conditions (20–24 °C and 12: 12 light-dark cycle) with free access to food and water. The experimental protocol was approved by Ethics Commission for the Use of Animals (CEUA) of the Campus of Ribeirao Preto, University of Sao Paulo, Ribeirao Preto, SP, Brazil (Protocol no. 10. 1. 1749. 53. 8). A 50 µL aliquot of the precursor liquid formulation previously sterilised by membrane filtration (pore size 0. 2 µm) was injected subcutaneously in the dorsum of BALB/c mice. The mice were sacrificed after injection at predetermined time intervals (24, 48 and 72 h and 7, 14 and 30 days), and the gel formation in the subcutaneous region was observed and evaluated as a function of formation time and the time of permanence in the tissue. The presence of gel in the injection site was recorded using a Sony camera model DSC-WX9. For the local toxicity studies, samples of the skin, gel and adjacent tissue in contact with the formulation were removed after the animals treated as described above were sacrificed. The samples were placed under a piece of mouse liver, which served as a support, and immediately frozen in isopentane cooled in liquid nitrogen. The issues were stored at -80 °C until they were sectioned. Histological sections (5 μm) were cut in a cryostat (Leica CM1850) and then mounted on extra-white gelatinised glass slides. The slides were stained with hematoxylin-eosin (H&E) for histological analysis. The slides were observed under a motorised Olympus BX61 microscope attached to an Olympus DP 72 camera.

## Statistical Analysis

The data were statistically analysed with a t-test to evaluate the difference between the water absorption of the different formulations in the swelling studies. The results were considered significantly different when p <0. 05.

## Results and Discussion

## Characterisation of the precursor fluid formulations by polarised light microscopy

The ability of isotropic liquid and fluid LCSs, such as the lamellar phase, to form a rigid liquid crystalline phase, such as the cubic and hexagonal phases, upon contact with excess water from body fluids have been described in the literature [23, 26, 28, 33, 34]. These systems are promising for local administration and sustained drug release [35, 36]. The addition of components can change the liquid crystalline phase, as demonstrated by several studies [19, 20, 25, 33, 34, 37, 38]. The diversity of factors that alter the formation of LCSs makes it extremely difficult to predict the structure of LCSs. Therefore, phase diagrams must be created experimentally [39]. A ternary diagram or Gibbs Triangle, which functions as a map for navigating between the different phases, is a useful tool to predict the phase behaviour of lipids [39] and was constructed from the macro- and microscopic analysis of systems composed of MO/PG/Tris buffer (Fig. 1A) and MO/PEI/PG/Tris buffer with the MO: PEI ratios of 12: 1 (Fig. 1B), 6: 1 (Fig. 1C) and 3: 1 (w/w) (Fig. 1D). The liquid crystalline structure was characterised macroscopically and by polarised light microscopy at 24 h and 7 days after the formulations were prepared. Fig. 1. Ternary phase diagram of (A) MO/PG/Tris buffer, (B) MO/PEI(12: 1)/PG/Tris buffer, (C) MO/PEI(6: 1)/PG/Tris buffer and (D) MO/PEI(3: 1)/PG/Tris buffer at 24 h and 7 days. As shown in Fig. 1A, at a Tris buffer concentration greater than 70%, a gel in equilibrium with the excess water was obtained, and polarised light microscopy of these systems showed an isotropic structure, which was characterised as cubic phase with excess water. This phase was also observed for Tris buffer concentrations between 50-70% and PG concentrations less than 20%. After 7 days, the formulations present in this region were characterised as isotropic with anisotropic points. In the diagrams Fig. 1B, C and D, the cubic phases with excess water were observed at MO/PEI concentrations of 12, 10 and 6% for 70% Tris buffer, 8, 6 and 4% for 80% Tris buffer and 4, 3 and 2% for 90% Tris buffer. For the proportions of MO/PEI greater than those mentioned above, the formation of a hexagonal phase plus water or the loss of liquid crystalline structure as the proportion of MO/PEI increased was observed. Diagrams A and B also show the presence of a lamellar phase characterised by polarised light microscopy as oily streaks with inserted Maltese crosses. A region of homogeneous isotropic fluid systems under the microscope that were characterised as a liquid isotropic phase, which were not stable for 7 days and transformed into an emulsion or unstable emulsion, was also observed. Diagrams B, C and D show that liquid isotropic systems were formed at concentrations of MO/PEI ranging between 15-65%, 8-65% and 18-48% and Tris buffer less than 15%, 10% and 5%, respectively. Other regions in the diagrams at 24 h were characterised as unstable emulsions and transition phases (a mixture of isotropic, anisotropic and droplet structures) under polarising light microscopy. The constructed diagrams demonstrate that it is possible to obtain MO/PEI-based fluid systems that can transform into viscous LCSs in contact with excess water. Chang & Bodmeier [23] reported that the isotropic liquid phase that shows liquid-like viscosity can be transformed into the cubic phase after injection into the body because the cubic phase is formed in regions with high solvent content, as shown in the present diagrams. This change from a less viscous phase into a cubic structure or rigid gel can be explained by the packing ratio (cpp). The cpp relates the shape of the molecule to properties that influence the curvature of the polar-nonpolar interface, and consequently, the type of aggregate formed. Therefore, it is a useful parameter to predict the mesophase that is preferably formed by an amphiphile. The cpp = vs/aolc, where cpp represents the packing ratio, vs is the hydrophobic chain volume, ao is the polar head group area, and lc is the chain length [40]. After the lc is considered constant, the mesophases are influenced primarily by vs and ao and the addition of others compounds can influence theses parameters, which may result in a change in the structure of the phase formed [34]. The cpp values are usually ~1, > 1 and ≥ 1 for lamellar, hexagonal and cubic phases, respectively [41]. The increase in water allows the polar head groups of MO to move more freely in a perpendicular form relative to the plane of the water layer. These movements promote disorder in the hydrophobic chain of MO, thereby increasing vs. Because interaction of the polar head groups is strong due to hydrogen bonds, their cross section tends to be constant; therefore, the packing factor increases because vs increases whereas ao and lc are constant, thereby facilitating the transformation of a lamellar to a cubic phase [42].

## In vitro gelling formation assay

All precursor fluid formulations characterised as isotropic liquids (white circles in Fig. 1) were analysed, and the gels obtained in the in vitro gelling test were characterised macroscopically and by polarised light microscopy. All gels obtained from the formulations of MO/PG/Tris buffer were characterised as isotropic for 7 days. This analysis of the systems without incorporation of PEI was important to evaluate the influence of this cationic polymer on the formation of liquid crystalline structures. The gels obtained from the precursor fluid formulations with PEI (white circles in Fig. 1B, C and D) showed different characteristics. At lower concentrations of PEI, the gels were characterised as cubic phase, at middle concentrations, the gels obtained were hexagonal phase, and at higher concentrations, the formation of liquid crystalline phases does not occur. The change of liquid crystalline phases obtained due to the presence of PEI may be explained by the cpp. PEI may interact with the polar head of the MO through hydrogen bonds, thereby promoting a decrease of ao that favours hexagonal phase formation. Changes of liquid crystalline phases in the presence of hydrophilic additives were also observed by Mezzenga et al. [41], who demonstrated that glucose, a hydrophilic compound, may have changed the ao through of strong hydrogen bonds, such that the curvature of the lamellar phase was reduced by the presence of glucose to become a cubic phase or similarly, the curvature of the cubic phase was reduced to become a hexagonal phase. However, excess concentrations of PEI harm the production of LCSs because the increase of hydrogen bonds between this polymer and MO can disturb the organisation of the structures in liquid crystalline systems. After the selection of precursor fluid formulations that formed gels in contact with excess water, a detailed in vitro gelling test was performed to characterise the gel formed in excess water for 24, 48, 72 h and 7, 10 and 14 days macroscopically and by polarising light microscopy. The gel obtained from the formulations containing 5. 57, 6. 46, 7. 85 and 15. 69% MO remained stable for up 24 h after contact with excess water. Moreover the systems containing 7. 85 and 15. 69% MO visually showed higher gel volumes. Because PEI is cytotoxic depending on its molecular weight, structure and concentration [43], lower proportions of PEI were also tested from the chosen formulations (Table 1). The precursor fluid formulations obtained were characterised as fluid and homogenous systems at 24 h. Again, it can be observed that higher PEI concentrations can influence the liquid crystalline phase formed. Notably, all formulations containing lower polymer concentrations did not affect the structure of the formed gel, similar to formulations without this polymer that remained as a cubic phase. Table 1Characteristics of gels formed from precursor formulations with excess water as determined by polarised light microscopy at 24, 48 and 72 h and 7, 10 and 14 days.

## siRNA loading and stability in precursor fluid formulations

Cationic polymers, such as PEI, have been described for use for nucleic acid delivery because of their ability to form non-covalent complexes with nucleic acids. PEI is a water-soluble synthetic polymer that is either linear or branched, with a high cationic charge density at physiological pH because of its free amine groups [44, 45]. PEI is the most studied cationic polymer for non-viral gene delivery [46]. Formation of a complex with siRNA using PEI is necessary for protection from degradation by endonucleases [47] and to avoid the problems of repulsion by the cellular membrane, which has a residual negative charge. It also decreases the size of the nucleic acids due to condensation of the molecule, thereby facilitating internalisation by the cell [44, 46, 48]. Therefore, electrophoresis was performed to verify the ability of the developed MO/PEI-based systems to form complexes with siRNA. Fig. 2A shows that all MO/PEI-based formulations were able to form complexes with siRNA, even those with reduced concentrations of PEI, contrary to the control formulations without PEI, which allowed the release of siRNA from the systems in the agarose gel. These results demonstrate the importance of the presence of PEI in the precursor fluid formulations for the incorporation of the siRNA. Based on these results and those observed in the in vitro gelling formation assay, the formulations containing 7. 85 and 15. 69% MO were chosen for the following studies because it is expected that these formulations, which have highest gel volume, will have a longer time of permanence in the tissue and may sustain siRNA release for an extended period of time. The following studies were performed with the lowest and highest concentrations of PEI. With these MO and PEI compositions, it was possible to evaluate the influence of the amounts of both the main lipid component responsible for gel formation (MO) and the siRNA complexing agent (PEI). The stability studies of the siRNA complexes were performed using heparin, a competition agent for PEI. After treatment with heparin, the complexed siRNA in the formulation was released in stable form, as observed in Fig. 2B. Fig. 2. (A) Loading efficiency of the precursor fluid formulations with different percentages of MO and PEI. (B) Evaluation of siRNA stability in different precursor fluid formulations using a heparin polyanion competition assay. Control: 10 µM siRNA in DEPC water. Hep: Heparin.

## Swelling Studies

The swelling rate and resulting LCSs formed by swelling amphiphiles, such as MO, can affect drug release kinetics; therefore, a swelling study is essential during the development of new delivery systems [28]. Fig. 3A presents the plots showing the increased weight of the dialysis tubing containing the selected formulations expressed as a function of time. The swelling kinetics parameters (Wo: initial rate of absorption; W∞: maximum water uptake) are summarised in Fig. 3A. In this experiment, the systems without PEI were used as controls. Notably, for all tested formulations, the water uptake initially increased rapidly and after a few hours, this absorption stabilised and reached the equilibrium water content. The rapid swelling indicated that the formation of a viscous cubic or hexagonal phase is a rapid process, consistent with other studies [28, 31, 33]. As observed in Fig. 3A, formulations with higher PEI concentrations have a greater water uptake. The statistical analyses of the results showed that the formulation composed of MO/PEI at 7. 85: 0. 65 (w/w) absorbed more water than the formulation of MO/PEI at 7. 85: 0. 065 (w/w) (p <0. 05 from 1 h) and the formulation without PEI (p <0. 05 from 30 min), whereas the system with the lowest proportion of PEI (MO/PEI at 7. 85: 0. 065, (w/w)) had the same water uptake as the formulation without PEI for 8 h. The systems composed of MO/PEI at 15. 69: 1. 31 (w/w) have a water uptake rate higher than formulations with lower and without PEI from 2 and 1 h (p <0. 05), respectively. Additionally, lower concentrations of PEI (0. 131%) in the systems containing 15. 69% MO exhibited the same absorption of water as the system without PEI during the entire period evaluated. For both systems containing different MO concentrations, the presence of the highest PEI concentration promoted the greatest water absorption. This behaviour can be explained by the high cationic charge density of PEI at physiological pH due to its protonable amino group, which induces an osmotic gradient [44]. The greater water uptake can also be demonstrated by analysing the maximum water uptake (W∞) determined by the swelling kinetics. The formulations with MO at proportions of 7. 85 and 15. 69 without PEI absorbed 0. 10 and 0. 09, whereas the systems with lower PEI proportions absorbed 0. 17 and 0. 11. The system with higher PEI concentrations had a maximum of water uptake of 0. 37 and 0. 25 g water/g of formulation. The swelling of the systems with and without PEI followed second order kinetics (Fig. 3B) because a linear relationship was obtained when the swelling data were plotted as t/w versus t according to Eq. 2. This result is consistent with other studies that evaluated the MO water absorption profile [28, 31, 33]. Fig. 3. (A) Plots of water uptake by the dialysis tubing with different formulations expressed as a function of time. Water uptake by (I) over the swelling studies period (48 h) and (II) over the first 2 h. \* p < 0. 05 (t-test) (n= 5, ± E. P. M.). Swelling kinetics parameters: Wo (initial rate of absorption; g water absorption / g formulation x hour); W∞ (maximum water uptake; g water absorption / g formulation). (B) Swelling kinetics of different formulations with PEI and their controls, t/w versus time (second order kinetics) and ln W∞ / (W∞ - W) versus time (first order kinetics). Notably, these results may deviate from what occurs in biological fluids because the method used to determine the swelling kinetics influences the results as demonstrated by Lee et al. [32]. However, these methods represent tools to analyse the systems in mimetic physiological conditions and also allow for the observation that PEI interferes with LCS formation.

## Small Angle X-ray Scattering (SAXS)

The ability to load, release drugs and interact with biomembranes is closely associated with the inner structure and additives present in the LCSs [25]. In the present studies, the gels obtained from the precursor fluid formulations were analysed by SAXS to determine the internal structure of LCSs through calculation of the interplanar distances value d from Bragg´s law. These distances were then correlated with the Miller indices of structure symmetry. We evaluated the effects promoted by the addition of 10 µM siRNA and PEI to the liquid crystalline structure. Fig. 4 shows plots of the intensity versus the scattering vector q obtained for the gels formed from the precursor fluid formulations with or without PEI and siRNA. The diffraction peaks allowed us to determine the lattice parameters and to identify the liquid crystalline phase of the samples as shown in Table 2. Fig. 4. Intensity versus q plots from the SAXS measurements of gels obtained from the precursor fluid formulations of MO/PG/Tris buffer and MO/PEI/PG/Tris buffer with and without siRNA. Table 2Ratio, lattice parameter average and structure of liquid crystalline phase determined by SAXS. The structure of the gels formed without PEI or siRNA was characterised by polarised light microscopy as cubic phase, which is consistent with the SAXS results. The structures exhibited multiple peaks indicative of a diamond type cubic phase (Pn3m space group) and gyroid type cubic phase (Ia3d space group). The gyroid cubic phase is the most common type of cubic phase [37]; however, the presence of different components can affect the type of liquid crystalline structure [34, 37]. Therefore, the mixture of types of cubic phases observed in this study may be due to the materials used. The MO used to prepare the formulations was Myverol® 18-92, which consists primarily of 67. 5% glyceryl monolinoleate and 18. 7% glyceryl monooleate [34]. Consequently, the presence of these components may promote the occurrence of two different types of cubic phases. The addition of components in liquid crystalline phases can manifest as either complete phase changes or alterations in the lattice parameter [49]. The lattice parameter represents the distance between the inner cores of the ordered micelles of the liquid crystalline phase; therefore, we obtained important information regarding the internal structure of the liquid crystals [50]. The polarity and molecular structure of the added compound determined whether it is located at the polar interface or the non-polar region of the lipid layer, which affects the packing ratio [37]. The increase in the lattice parameter value can be due to (i) the structure hydration or the presence of hydrophilic molecules in the aqueous domains, which results in its enlargement, and (ii) the presence of amphiphilic molecules partitioned between the apolar tail and polar head groups of the MO [50-52], suggesting that the molecules added to the system are located internally in the liquid crystalline structure. A reduction in the lattice parameter value suggests the dehydration of aqueous domains [50], and the maintenance indicates the presence of hydrophobic molecules located in the hydrocarbon chains of MO or the adsorption of molecules on the surface of the reversed micelles [52]. In this study, PEI added at low concentrations (MO/PEI at 7. 85: 0. 065 and 15. 69: 0. 131 (w/w)) did not alter the liquid crystalline structure compared to formulations without PEI, but it did promote an increase in the lattice parameters as observed in Table 2. This increase suggests that PEI, a hydrophilic molecule, is located in the aqueous domain as previously proposed by the packing ratio analysis. However, higher proportions of PEI resulted in a phase change. The formulations composed of MO/PEI at 7. 85: 0. 65 (w/w) was determined to be a mixture of cubic phase with an interstice of hexagonal phase, and the formulations composed of MO/PEI at 15. 69: 1. 31 (w/w) were characterised as a mixture of cubic phase with hexagonal phase. The correct identification of the liquid crystalline phase can be achieved using different identification techniques [33]. The difference between the results obtained with polarised light microscopy, which characterised the formulations as hexagonal phase only, and SAXS can be due to the birefringence of the hexagonal phase observed under polarised light microscopy that can disguise the isotropic behaviour of the cubic phase. The impact of the interaction of the siRNAs with the LCSs was also observed during the SAXS evaluation. The lattice parameters of the samples were reduced with the addition of siRNA, which can be explained by the high hydrophilicity of the siRNA. This makes the siRNA compete with the MO for water molecules. The ability of the siRNA to bind water is most likely higher than the MO; therefore, this competition leads to MO dehydration, thereby reducing the effective size of the MO head groups, which is reflected in the decrease of the lattice parameter value. This effect was also observed in another study with the addition of a highly hydrophilic polymer (hydrophilic-lipophilic balance = 17) to liquid crystalline system composed of MO [50].

## In vitro siRNA release from LCSs

For the effective delivery of siRNAs, it is essential that the siRNA is released complexed with PEI to avoid siRNA degradation and to facilitate its cellular uptake [44, 46]. To determine whether the siRNA is released in complex with the PEI, heparin was used as a competition agent to promote siRNA decomplexation. Fig. 5A shows the qualitative evaluation of the siRNA released from the precursor fluid formulations by agarose gel electrophoresis. The precursor fluid formulations composed of MO/PEI at 7. 85: 0. 65 and 15. 69: 1. 31 (w/w) released the siRNA complexed with PEI because the siRNA was visualised only in the samples with heparin, indicating that these systems may have potential for gene therapy. The percentages of released siRNA were determined using the ImageJ program (Fig. 5B). The siRNA in DEPC water (control) released 97. 96% at 24 h and reached 109. 34% at 48 h. The gel obtained from the formulation composed of MO/PEI at 7. 85: 0. 65 (w/w) released 25. 32% at 24 h and reached 40. 34 and 43. 59% siRNA complexed with PEI at 48 h and 7 days, respectively. The formulation composed of MO/PEI at 15. 69: 1. 31 (w/w) released only 2. 66% at 48 h. The gels from the precursor fluid formulations composed of MO/PEI at 7. 85: 0. 065 and 15. 69: 0. 131 (w/w) did not release siRNA during the analysis period. The difference in the release profile can be explained by the difference in water absorption. It has been reported that matrices that absorb more water would provide a rapid diffusion and more rapid release than matrices with low water absorption. This is attributed to an increase of channels available for release of drugs with increasing water content [33]. In this in vitro release study, the gel obtained from the precursor fluid formulation composed of MO/PEI at 7. 85: 0. 65 (w/w), which absorbed more water in the swelling studies, released siRNA faster than the systems that absorbed less water. Another factor that may have contributed for this behaviour is the presence of different liquid crystalline phases in this gel. Fig. 5. (A) Release of siRNA from different formulations at 24, 48 h and 7 days as determined by qualitative analyses using agarose gel electrophoresis. (B) Release of siRNA from the control (white), gel obtained from the precursor formulations of MO/PEI/PG/Tris buffer at 7. 85: 0. 65: 76. 5: 15 (grey) and 15. 69: 1. 31: 68: 15 (black) (w/w/w/w) analysed by Image J. After 24 h, the % of siRNA released shown in the graphic is cumulative. SC: Standard curve. Control: siRNA in DEPC water (10 µM).

## In situ gelling and local toxicity in an animal model

The subcutaneous injection of the precursor fluid formulations using a syringe and a 26 gauge needle (0. 45 X 13 mm) was straightforward and well-tolerated in the mice. Therefore, the precursor fluid formulations developed in this study may be a new method of minimally invasive and local drug delivery. To determine the influence of the components in the in vivo gel formation, precursor fluid formulations containing 7. 85 and 15. 69% MO without PEI were selected to evaluate the influence of the amount of MO on in situ gel formation and local toxicity. These precursor fluid formulations gelled in situ upon injection. Fig. 6A shows the gel formed in vivo and its subcutaneous location in mice. Fig. 6B shows the subcutaneous presence of the gel and it degradation over several days for both formulations. Moreover, the gel obtained from the precursor fluid formulation containing 15. 69% MO had a larger volume than the gel formed by the formulation containing 7. 85% MO. In this study, the gel formed from 7. 85 and 15. 69% MO maintained their integrity for at least 14 days, and at 30 days, a gel was not observed in the subcutaneous tissue. Degradation of the gels formed in situ occurs by lipolysis caused by different types of esterases that are present in the tissues [26, 53]. To understand the degradation process of the gels formed in situ, it is important to note that the hydrolysis of MO in glycerol and oleic acid is a slow process that can be accelerated by lipases [37]. Moreover, in addition to the trigger elements, such as pH, biological fluids, light and/or temperature, both the process of gel formation and degradation depend on the components of the formulation. An in situ gelling system based on chitosan decreased the in situ gel time of 10 days to 3-4 days with the addition of insulin [54]. Because the components can affect the gel formation and degradation, the system containing the highest PEI concentration (MO/PEI at 15. 69: 1. 31 (w/w)) was also evaluated. Despite the observation that the gel formation process for the system containing MO/PEI at 15. 69: 1. 31 (w/w) was the same observed in the systems without PEI, the degradation was slower because at 30 days, traces of gel were observed in the subcutaneous tissue. Fig. 6. (A) In vivo gel formation: (I) Precursor fluid formulation injected subcutaneously. (II) Localisation of the gel in vivo in BALB/c mice. (III) H&E staining of skin with gel. (B) Gelling kinetics of systems with MO/PG/Tris buffer at 7. 85: 76. 5: 15. 65 (w/w/w) and 15. 69: 68: 16. 31 (w/w/w) administered subcutaneously in BALB/c mice at 24, 48 and 72 h and 7, 14 and 30 days. Gel formation is indicated by the arrow. The pictures were taken using macro mode without zooming, 10 cm from the subject (f-stop: f/2. 6, exposure time: 1/250 s, exposure index: ISO-100). There are examples of toxic substances that cause severe side effects, such as skin bleeding, after subcutaneous injection [55]. Therefore, qualitative toxicity studies were performed in mice. The skin and gel with sufficient surrounding tissue were excised for evaluation of the local tissue response by histology using H&E. Saline was injected as a control and no inflammatory process was observed. siRNA (10 µm) in saline was also evaluated and no inflammatory process was observed. Histological analysis was performed for 30 days after subcutaneous administration of the precursor fluid formulations containing 7. 85 and 15. 69% MO without PEI and MO/PEI at 15. 69: 1. 31 (w/w) and showed that the dermis and epidermis characteristics were maintained after the subcutaneous injections. However, tissues around the gel were affected and inflammatory cells were recruited to the sites. After 24 h of subcutaneous administration, the presence of polymorphonuclear cells was observed. After 7 days, a predominance of mononuclear cells, such as macrophages, was observed for all studied formulations. After 14 days, fibroblasts and collagen fibres were visualised in the tissue around the gel formed from precursor formulations of 7. 85% MO, promoting tissue repair. However, higher amounts of MO (15. 69%) retarded the tissue repair process, which was achieved only after 30 days. In the presence of PEI (MO/PEI at 15. 69: 1. 31 (w/w)), fibroblasts and collagen fibres were not observed during the analysis period. The toxicity analyses indicated that MO had the most influence on the damage in the tissue because 7. 85% MO showed a faster tissue repair and less inflammatory cell recruitment than 15. 69% MO. PEI, a component considered an irritant in this study, had little influence on the toxicity because the systems with and without PEI had similar toxicities. This is somewhat intriguing because MO alone is not toxic [39], and a possible explanation is that the gel volume formed after in situ gelling may exert pressure on the tissue and recruit more inflammatory cells. The small influence of PEI on local toxicity may be due to its internal localisation in the liquid crystalline phase of the subcutaneously formed gel. The inflammatory process promoted by gel formation of the system containing 7. 85% MO can be considered a normal inflammatory process because migration of inflammatory cells occurred and the tissue was regenerated after several days. Therefore, systems containing 7. 85% MO can be appropriate for subcutaneous delivery because the inflammation was not severe, the tissue regenerates and the animals do not lose function or weight during the experiments, indicating that the inflammation was well-tolerated. Several studies have obtained similar results, and also considered their matrices to be appropriate for use in humans [56, 57]. The systems developed in this study may be a new approach for the delivery of molecules such as siRNA because they act as a depot that can protect the siRNA against degradation and can control the release of the loaded drug. Additionally, the systems are minimally invasive and biodegradable.

## Conclusions

All the parameters evaluated in this study were required for the rational development of an in situ gelling liquid crystalline system for gene therapy. The present study demonstrated that a proper combination of MO, PEI, PG and Tris buffer can form interesting liquid crystalline phases in situ for use as gene delivery systems. These systems are promising for local administration because they are less viscous and injectable and come in contact with excess water from body fluids to form viscous liquid crystalline phases that promote the sustained release of drugs. Additionally, the results showed that incorporation of the cationic polymer PEI into the previously developed MO-based systems allowed complexation with siRNA, which is necessary for the effectiveness of the proposed systems as nucleic acid carriers and to maintain the siRNA in a stable state. Moreover, the swelling study indicated that the formation of the liquid crystalline phase is a rapid process, rendering these systems even more attractive because they avoid the possible " burst effect". The release studies demonstrated that siRNA release is dependent on the ability of the formulation to absorb water. The in vivo studies showed that the gels can be formed in situ and that they are biocompatible and biodegradable. These studies were necessary to define a promising formulation with many advantages, such as minimal invasiveness and localised and sustained release of siRNA complexed with PEI. Based on these results, the formulation MO/PEI at 7. 85: 0. 65 (w/w) appears to be the most suitable delivery system developed. Additional studies will be pursued to verify the in vivo release profile and gene silencing efficiencies of this formulation.