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Self-assembling gelling formulation based on a crystalline-phase liquid as a non-viral vector for siRNA delivery

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ABSTRACT

Liquid crystalline systems (LCSs) form interesting drug delivery systems. These include *in situ* gelling delivery systems, which present several advantages for use as self-assembling systems for local drug delivery. The aim of this study was to develop and characterize *in situ* gelling delivery systems for local siRNA delivery. The influence of the components that form the systems was investigated, and the systems were characterized by polarized light microscopy, Small Angle X-ray Scattering (SAXS), swelling studies, assays of their ability to form a complex with genes and of the stability of the genes in the system, as well as assays of *in situ* gelling formation and local toxicity using an animal model. The system containing a mixture of monoglycerides (MO), oleylamine (OAM), propylene glycol (PG) and tris buffer (8.16:0.34:76.5:15, w/w/w/w) was considered the most appropriate for local siRNA delivery purposes. The molecular structure was characterized as hexagonal phase; the swelling studies followed a second order kinetic model and the water absorption was a fast process reaching equilibrium at 2 h. The system formed a complex with siRNA and remained in a stable form. The gel was formed *in vivo* after subcutaneous administration of a precursor fluid formulation in mice and was biodegradable in 30 days. The inflammatory process that took place was considered normal. Therefore, the developed liquid crystalline delivery system shows the appropriate characteristics for use as a local siRNA delivery method for gene therapy.

1. Introduction

Liquid crystals, defined as the state of matter whose symmetric and mechanical properties are intermediate between a crystalline solid and an isotropic liquid (Singh, 2000), have been widely used in the pharmaceutical area due to their ability to form delivery systems, such as nanodispersions and *in situ* gelling systems. These vehicles appear to be potential delivery system for drugs, peptides and genes because they have the ability to incorporate compounds with different solubility, protect them from enzymatic and physical degradation, increase skin penetration and control drug release (Lopes et al., 2006b, 2006a, 2007; Vicentini et al., 2013b).

Liquid crystalline systems (LCSs) are formed from unsaturated monoglycerides (MO), such as monoolein and monolinolein. The ability of the MO to absorb water and to form a viscous liquid crystalline phase, such as a hexagonal or a cubic phase, is well described in the literature and enables the formation of a gel *in situ* (Chang and Bodmeier, 1997c, 1998; Lara et al., 2005; Rizwan et al., 2009). *In situ* gelling delivery systems present several advantages, such as easy administration, simple production, minimal invasiveness (i.e., less painful administration), dose reduction capability and local, sustained delivery (Hatefi and Amsden, 2002).

The importance of *in situ* gelling delivery systems has increased lately because many new macromolecules, such as proteins and nucleic acids, that are easily degraded when administered by conventional routes, have been discovered as a consequence of the development of molecular biology studies and genomic information. These new macromolecules require the development of delivery systems that can protect them and release them in a localized and sustained way (Agarwal and Rupenthal, 2013; Hatefi and Amsden, 2002).

Among the new therapeutic discoveries, gene therapy is promising because it provides a unique way to regulate, repair, replace,

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add, delete or silence a genetic sequence that is identified as beneficial or harmful to the appropriate functioning of the body (Wirth et al., 2013). Thus, gene therapy has the potential to deeply change the way to address and treat both inherited disorders and acquired diseases (Nabel, 2004). In this context, the approach of silencing genes has drawn the attention of many researchers. It can be achieved by different processes. These include RNA interference (RNAi), which inhibits endogenous genes by the degradation of the messenger RNA using corresponding double-stranded RNA. Small interfering RNA (siRNA) is a short double-stranded molecule that can silence the expression of particular proteins through this RNAi process. Compared with other antisense/gene therapies, siRNA presents several advantages, including straightforward synthesis, specificity, potency and robustness. Its site of action is in the cytoplasm and so it presents a smaller risk of promoting toxic effects (Vicentini et al., 2013a).

Although significant progress has been made over the past years, many challenges for effective siRNA therapy must be overcome, including delivery to a specific cell population, efficient gene transfection and avoiding the immune response (Nabel, 2004). siRNA delivery systems have neutralized the negatively charged phosphate backbone of nucleic acids to avoid charge repulsion against the anionic cell membrane; they have also condensed the bulky structure of DNA to appropriate length scales for cellular internalization and protected the nucleic acid from nuclease degradation (Reischl and Zimmer, 2009; Vicentini et al., 2013a). Both viral and non-viral delivery systems have been developed to overcome these problems, showing success in delivering the siRNA to the intracellular environment and inducing RNA interference (RNAi). Non-viral delivery systems, commonly consisting of siRNA incorporated into lipids and polymers, are promising siRNA delivery systems because they are safer than viral vectors; however, efforts should be made to improve the mediation of gene expression by these vectors (Reischl and Zimmer, 2009). One factor that interferes with gene transfer is the administration route. Local delivery of siRNA using non-viral vectors is advantageous because it provides higher bioavailability, efficient targeting of the affected cells and reduced side effects in addition to enabling the use of lower doses (Vicentini et al., 2013a).

Based on these advantages, the development of *in situ* gelling delivery systems for the local release of siRNA was proposed. The delivery system is composed of: MO (Fig. 1a and b present the structure of the two main MO used), which is responsible for the viscous liquid crystalline phase formation, propylene glycol (PG) and 0.1 M Tris buffer (pH 6.5) (tris buffer) that provides fluidity to the precursor formulation and oleylamine (OAM) (Fig. 1c), which is a cationic lipid used to confer a positive residual load to the system and then allow the formation of a complex between the system and the nucleic acids. This complex formation is important to ensure gene transfection and avoid gene degradation (Vicentini et al., 2013a).

The formation of liquid crystals is influenced by the temperature, the characteristics of the lipids and the incorporated drug or additives and the water content of the system (Chang and Bodmeier, 1998; Fong et al., 2009; Gosenca et al., 2013). Depending on the combination of these components, it is possible to obtain different types of liquid crystalline phase (e.g., lamellar, hexagonal or cubic phase). The lamellar phase consists of a linear structure of alternating lipid bilayer with water channels; this phase is less viscous and is injectable; therefore, the lamellar phase can be used for the obtainment of cubic phase due to its ability to absorb excess water from the body fluid, forming a rigid and viscous cubic phase gel. The cubic phase consists of a curved, bicontinuous lipid bilayer extending in three dimensions, separating two congruent networks of water channels; it forms with increasing water content (Larsson, 1989). With an increase in the solvent concentration, a transformation of the solvated molecules from the rod shape (lamellar phase) to a cone shape can occur. Depending on the polarity of the solvating agent and the molecule itself, the transition may result in a hexagonal or a reversed hexagonal phase (Muller-Goymann, 2004). Due to these characteristics, less viscous liquid crystalline systems can be used as *in situ* gelling delivery systems.

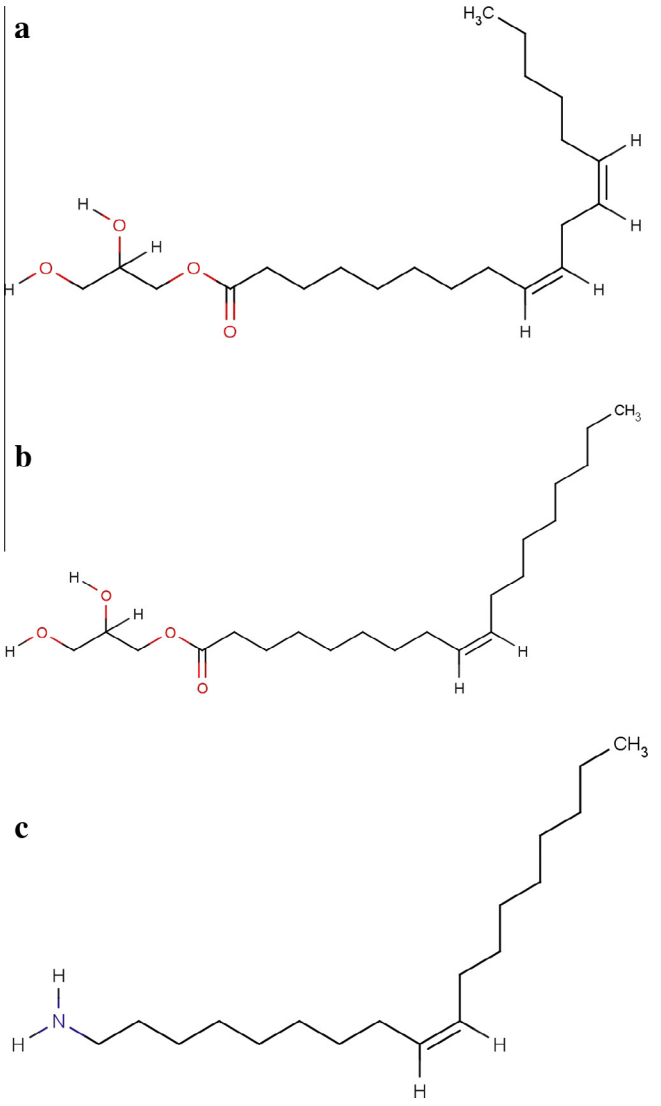

Fig. 1. Chemical structures of (a) glyceryl monolinoleate (monolinolein), (b) glyceryl monooleate (monoolein) and (c) OAM.

Because the components have important effects on the liquid crystalline phase formation, understanding the formation process is important for the development of a delivery system. Each phase has unique characteristics that will influence drug release and, consequently, the therapeutic effect of the drug. In the present study, the systems were characterized by polarized light microscopy, Small Angle X-ray Scattering (SAXS) and swelling studies in addition to studies of complexation with genes, gene stability in the systems, the ability to form a gel *in situ* and local toxicity *in vivo*. These studies were essential to define a potential delivery system for siRNA therapy.

2. Material and methods

2.1. Materials

Monoglycerides (MO, Myverol 18–92 K is composed of 93% of monoglycerides (containing 65% glyceryl monolinoleate, 23%

glyceryl monooleate, 6% monoglyceride (C16), 4% monoglyceride (C18), 1% monoglyceride (C20) and 1% monoglyceride (C18:3)), 6 % of diglycerides and triglyceride following the same fatty acid profile as the MO and other minors (<1%) of free fatty acids, glycerol. They also contain citric acid, ascorbic acid and tocopherols in the ppm range) was provided by Kerry Bio Science (Zwijndrecht, Netherlands); oleylamine (OAM) was purchased from Sigma Aldrich GmbH (Munich, Germany); diethyl pyrocarbonate (DEPC) was obtained from Sigma Aldrich Co. (St. Louis, MO, USA); propylene glycol (PG) was purchased from Labsynth Produtos para Laboratórios Ltda (Diadema, SP, Brazil); and Ultrapure™ Agarose was purchased from Invitrogen™ (Carlsbad, CA, USA). The siRNA was the Silencer Negative Control #1 siRNA (Catálogo #AM4635) obtained from Ambion (Austin, TX, USA). The tube dialysis membrane with a 50,000 Da cut-off was purchased from Spectrum Laboratories, Inc. (Rancho Dominquez, CA, USA).

2.2. Methods

2.2.1. Precursor fluid formulation production

Precursor fluid formulations were formed by mixing molten MO with OAM in proportions of 12:1 and 24:1 w/w. PG was added in proportions of 1:9 to 9:1. The mixture was then agitated in a vortex. Tris buffer 0.1 M, pH 6.5 (tris buffer) pre-warmed at the same temperature as MO (42 °C) was added at concentrations ranging from 5% to 90%. The resulting formulations were kept in closed vials at room temperature.

2.2.2. In vitro self-assembly of precursor fluid formulations

The *in vitro* self-assembly studies were performed by putting 100 μL of the precursor formulation in contact with an excess of water (900 μL) and maintaining the sample at 37 °C.

2.2.3. Characterization studies

2.2.3.1. Polarized light microscopy studies. The precursor fluid formulations and the gel obtained in excess water were macroscopically characterized by visual analysis and examined microscopically under a polarized light microscope (Axioplan 2 Image Pol microscope, Carl Zeiss, Oberkichen, Germany). The precursor fluid formulations were analyzed after 24 h and 7 days, while the gels formed in excess water were analyzed after 24, 48 and 72 h and 7, 10 and 14 days.

2.2.3.2. Small Angle X-ray Scattering (SAXS) studies. The gels obtained in excess water from the chosen precursor fluid formulations were evaluated by SAXS at 24 h after formation. In this study, the analyses were implemented at a wavelength of 0.1608 nm and scattered intensity curves were recorded using a two-dimensional detector with a measurement time of 150 s using the D02A-SAXS beamline at the Brazilian Synchrotron Light Laboratory (LNLS), Campinas, Sao Paulo, Brazil. The samples were placed in a sample holder between Kapton sheets. The results were corrected by a detector response, and the SAXS data were normalized to the transmission for each sample. The scattering from the Kapton sheets in the sample holder was also removed.

2.2.3.3. Swelling studies. The swelling studies were performed by placing the precursor fluid formulations (100 μL) in a dialysis tube membrane with a 50,000 Da cut-off. The membrane was put into excess water (3 mL) and maintained in a thermo-regulated bath at 37 °C. At fixed time intervals (15 and 30 min, 1, 2, 4, 8, 12, 24 and 48 h), the membranes with the samples were removed and the surface of the dialysis membrane was blotted with a piece of fine weave paper to eliminate excess water. The dialysis membrane was then weighed to plot the profile of water absorption. For kinetic swelling studies, the water uptake data were fit according to the first- (Eq. (1)) and second-order (Eq. (2)) kinetics equations (Lara et al., 2005; Rizwan et al., 2009).

$$\ln \frac{W_\infty}{W_\infty - W} = kt \tag{1}$$

$$\frac{t}{W} = \frac{1}{kW_\infty^2} + \frac{t}{W_\infty} \tag{2}$$

where W is the water uptake at time t, W_∞ is the maximum water uptake, k is the rate constant, and $(W_\infty - W)$ is the unrealized water uptake (Lara et al., 2005; Rizwan et al., 2009).

2.2.3.4. Studies of complexation with genes and gene stability. The ability of the precursor fluid formulations to form complexes with genes was evaluated using a control siRNA. The siRNA was added to the precursor fluid formulations at a final concentration of 10 μM. After mixing, the systems were incubated at room temperature for 30 min. The samples were prepared by adding 10 μL of loading buffer to a 40 μL aliquot of this mixture or the control (siRNA in DEPC water). Then, electrophoresis was performed on 40 μL of each sample on ethidium bromide-stained TAE-based 2% agarose gels at 100 V for 20 min followed by visualization under UV light (Tran et al., 2008).

To verify the stability of the siRNA complexed with the formulation, heparin was used as a competitor in a polyanion competition assay (Shen et al., 2011). For this, siRNAs complexed with the formulation were prepared as described above. The appropriate volume of heparin to promote decomplexation of the siRNA was determined previously. It was found that 10 μL of heparin (5000 U.I./mL) was sufficient to compete with the siRNA and promote its decomplexation. Thus, the heparin was added with agitation and these mixtures were incubated at 37 °C for 1 h. The mixtures were then electrophoresed on agarose gels as described above.

2.2.4. In vivo gel formation and local toxicity

The experiments to evaluate the ability of the precursor fluid formulations to form a gel *in vivo* after subcutaneous injection were performed using female BALB/c mice (18–20 g) maintained under standard environmental conditions (20–24 °C and 12:12 light–dark cycle) with free access to food and water. Before injection, the precursor fluid formulations were sterilized by membrane filtration (pore size 0.2 μm). A subcutaneous injection of 50 μL of the precursor fluid formulations was performed into the dorsum of the BALB/c mice. The gel formation, the time of permanence in the tissue and qualitative local toxicity were evaluated through of the sacrifice of the mice at predetermined time intervals (24, 48 and 72 h and 7, 14 and 30 days) after injection. A Sony camera model DSC-WX9 was used to record the presence of the gel in the injection site. The local toxicity studies were performed by removing samples of the skin, gel and tissue in contact with the formulation after the sacrifice of the mice. All the samples were frozen in isopentane and cooled in liquid nitrogen after being placed under a piece of mouse liver used to hold the tissue fixation and section. The tissues were histologically sectioned (5 μm) in a cryostat (Leica CM1850, Leica Microsystems Nussloch GmbH, Nussloch, Germany). Then, the tissues were placed on extra-white gelatinized glass slides and stained with hematoxylin-eosin (H&E). The histological analyses were performed under a motorized Olympus BX61 microscope (Olympus America Inc., Melville, New York, USA) attached to an Olympus DP 72 camera.

This study protocol was approved by the 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).

2.2.5. Statistical analysis

The swelling data were evaluated using a *t*-test to verify the differences between the water absorption of the different formulations. The results were considered significant when $p < 0.05$.

3. Results and discussion

The ability of isotropic liquid and fluid LCSs, e.g., the lamellar phase, to form a rigid liquid crystalline phase, such as the cubic and hexagonal phases, upon contact with excess water from the body fluids has been described in the literature (Chang and Bodmeier, 1997c, 1998; Rizwan et al., 2009; Shah et al., 2001). These systems are promising for local administration and sustained drug release (Fong et al., 2009). A self-assembling MO-based precursor fluid formulation that form gels *in situ* upon contact with body fluids was developed (Borgheti-Cardoso, 2012). In the present study, OAM, a cationic lipid, was added to the precursor fluid formulations to create a system that is able to deliver genes. Cationic lipids have been widely used as gene carriers due to their ability to form complexes with DNA and siRNA, which results in higher *in vitro* transfection efficiency (Ozpolat et al., 2010).

3.1. Phase diagram studies

The phase diagrams of MO/OAM(24:1)/PG/tris buffer and MO/OAM(12:1)/PG/tris buffer are shown in Fig. 2a and b, respectively. The systems were characterized macroscopically and by polarized light microscopy at 24 h and 7 days.

Chang and Bodmeier (1998) constructed different phase diagrams based on MO and demonstrated that fluid formulations were formed in lower water content conditions whereas the cubic phase was obtained in higher water content conditions (Chang and Bodmeier, 1998). In the present study, an isotropic liquid was also observed at tris buffer concentrations of less than 50% and 40%, as shown in Fig. 2a and b, respectively. A viscous liquid crystalline phase (cubic, mixture of cubic and hexagonal phase or hexagonal phase with excess water) was observed when the proportion of tris buffer was higher than 50% (Fig. 2a) and 40% (Fig. 2b). This behavior can be explained based on the critical packing parameter (cpp). The cpp, which is described by the equation $cpp = v_s/a_o l_c$ (where cpp is the critical packing parameter, v_s is the hydrophobic chain volume, a_o is the polar head group area and l_c is the chain length), is a useful parameter to presume the mesophase formed by an amphiphile because the cpp is associated with properties that affect the curvature of the polar-nonpolar interface and the shape of the molecule (Mitchell and Ninham, 1981). The cpp values are commonly $\sim$1, >1 and $\geqslant$1 for lamellar, hexagonal and cubic phases, respectively (Engstrom and Engstrom, 1992). The transformation of a fluid formulation containing MO into a viscous liquid crystalline phase upon contact with excess water occurs due to the effect of the water on the polar head of the MO. As reported by Amar-Yuli and Garti (2005) in their studies with monoolein, the increase in the water content allows the polar head of the monoolein molecule to move more freely, which results in a disorder of the monoolein hydrophobic chain, increasing v_s. The interaction of the monoolein polar head is strong due to the hydrogen bonds. Thus, a_o tends to be constant. Thereby, the cpp increase favors the phase change (Amar-Yuli and Garti, 2005). With these properties, fluid MO-based systems could be formulated for injection; once injected, these systems would be able to form viscous liquid crystalline structures *in situ* that are promising vehicles for the sustained and local delivery of drugs.

From the diagrams, it is also possible to observe that OAM influences the phase formation. This cationic lipid did not influence the common phase formed in excess water content (cubic phase) at lower concentrations. However, at higher concentrations, OAM favors the formation of the hexagonal phase instead of the cubic phase. Several studies evaluating MO or phytantriol-based liquid crystalline systems have shown phase change with the addition of compounds. Liu et al. (2013) demonstrated that charged compounds favors the phase change from cubic phase to lamellar phase (Liu et al., 2013). The same were observed by Engstrom and Engstrom (1992) in their study using lidocaine HCl, while the addition of lidocaine base favors the formation of hexagonal phase (Engstrom and Engstrom, 1992). OAM is a positively charged compound with a small polar head group, therefore, could be expected that it addition might result in lamellar phase, however, hexagonal phase were obtained. This result could be due to the small polar head group of the OAM and might be explained by the cpp. Analyzing the structure of OAM revealed that this cationic lipid favors the formation of the hexagonal phase because the OAM increases the cpp in two ways: (i) the decrease of a_o due to the hydrogen bonds formed between OAM and the MO polar head and (ii) the increase of v_s due to the hydrophobic chain space of the OAM. The increase of v_s by the presence of hydrophobic chain space was also observed by Chang and Bodmeier (1997b) with the addition of oleic acid at the MO system (Chang and Bodmeier, 1997b).

It is also noteworthy, that prior studies identified a region of sponge phase, which is a liquid phase slightly with higher viscosity and density compared with the isotropic liquids. This phase was identified in the region of the phase diagram containing about 40–30% of water and it presents a typical scattering curve at diffractogram (Alfons and Engstrom, 1998; Evenbratt et al., 2013). According to Alfons and Engstrom (1998), the sponge phase occurs when a water and MO miscible solvents, such as PG and oleic acid, is added (Alfons and Engstrom, 1998). In the present study, a liquid phase was also identified at the region of the phase diagram containing 40–30% of tris buffer, which could be a sponge phase. As this region is not the focus of this work, due to their higher viscosity, SAXS study was not carried out to characterize this system.

It is very important to study the influence of the components that are used to form liquid crystalline structures because they can change the phase formed and consequently change the characteristics of the delivery system. Although the behaviors can be explained by the cpp, it is still necessary to construct a phase diagram, which functions as a map for navigating between the different phases and allows us to better understand the influence of the different components in the formation of the liquid crystalline structure.

3.2. The self-assembly of a precursor fluid formulation in excess water

Precursor fluid formulations characterized as isotropic liquids (the white triangle in the diagrams in Fig. 2) were evaluated by their ability to self-assemble into viscous LCSs in excess water. The viscous LCSs were evaluated macroscopically and by polarized light microscopy. The systems without OAM were used as a control. The gels formed from the systems without OAM were characterized as being in the cubic phase in excess water, and the addition of OAM favors the formation of the hexagonal phase. In the precursor fluids formulation containing MO/OAM (24:1) (w/w), all of the systems formed a gel, whereas the precursor fluid formulations containing MO/OAM (12:1) (w/w) did not form a gel in excess water. The gels formed were characterized as hexagonal phase for 14 days, except the gel formed from the system of MO/OAM/PG/tris buffer (18.24:0.76:76:5 (w/w/w/w)). This gel was characterized as hexagonal phase for 7 days. The gels formed from the system containing MO/OAM 31.2:1.3 (w/w) and the systems with MO/OAM higher than 33.6:1.4 (w/w) were characterized as

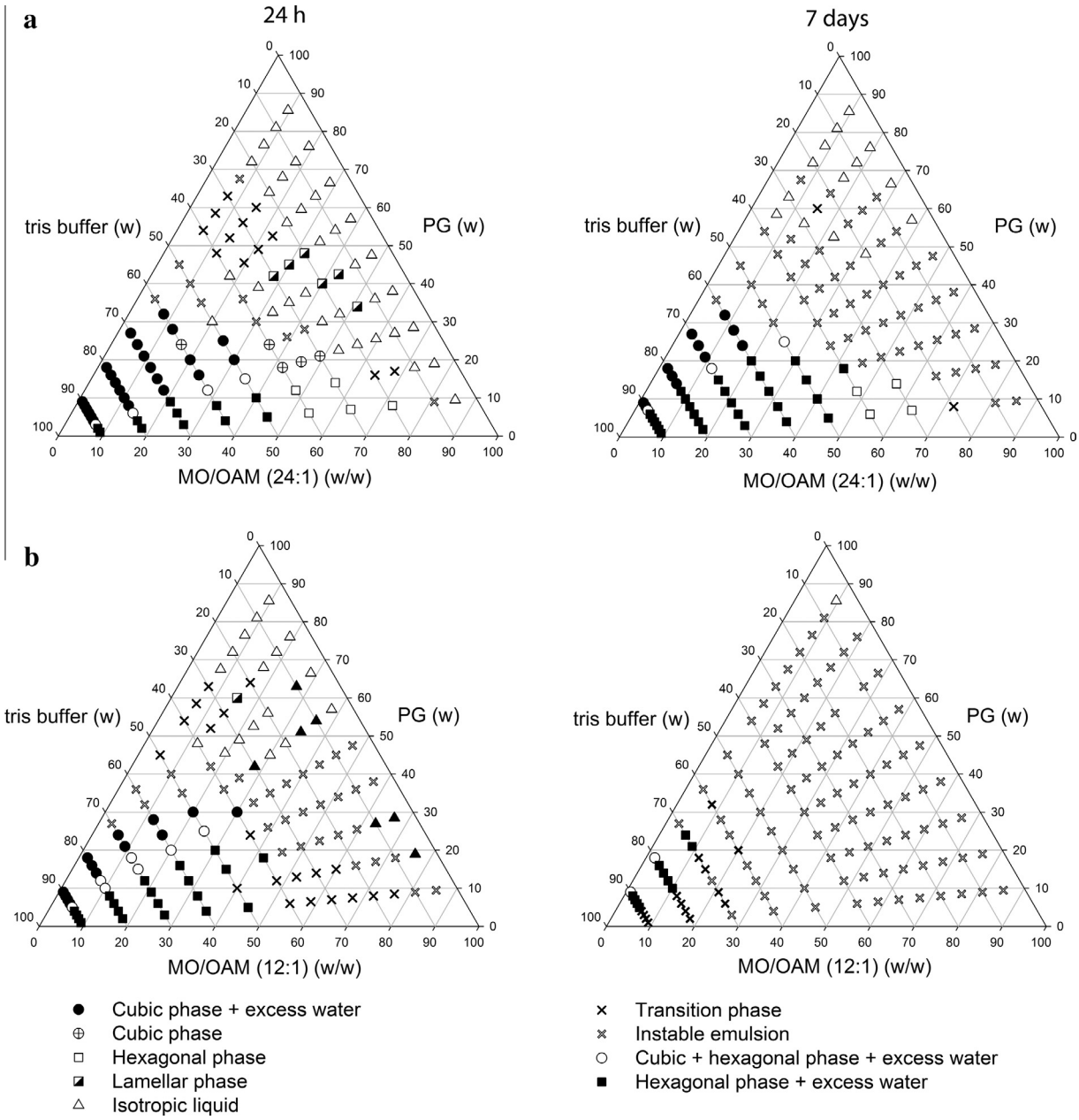

Fig. 2. Phase diagrams constructed after macroscopic and polarized light microscopic analyses. (a) MO/OAM(24:1)/PG/tris buffer (w/w/w/w) after 24 h and 7 days of production. (b) MO/OAM(24:1)/PG/tris buffer (w/w/w/w) after 24 h and 7 days of production.

hexagonal phase at 24 h and then were characterized as a mixture of hexagonal phase and emulsion after 24 h.

3.3. Polarized light microscopy studies

The formation of LCSs can be determined by several methods, such as X-ray diffraction, NMR and polarized light microscopy. Polarized light microscopy is the easiest technique by which to qualitatively identify different phases by their texture (Chang and Bodmeier, 1997c). Therefore, developed formulations were evaluated by polarized light microscopy to construct the diagrams.

Systems macroscopically visualized as gel in excess water that were isotropic under polarized light microscopy were characterized as cubic phase in excess water, as observed in Fig. 3a. The systems that showed a fanlike structure and birefringence were characterized as hexagonal phase (Fig. 3b). The evaluation of the system by microscopy enabled the observation of other phases, such as the lamellar phase (oily streaks with inserted Maltese-crosses) (Fig. 3c), emulsions (Fig. 3d). The microscopic analysis also allowed the identification of systems that did not form liquid crystalline systems or emulsion, these systems that are not of interest as drug delivery due to not have an defined structure were denominated as Transition phase. The production and characterization of the precursor fluid formulations and the gels formed in excess water was the first part of this study and was useful to understand the influence of the components on the phase behavior of the LCS. To conduct a more detailed characterization of a promising *in situ* gelling delivery system for gene release, the systems with 15% tris buffer (MO/OAM/PG/tris Buffer 8.16:0.34:76.5:15 and 16.32:0.68:68:15 (w/w/w/w)) were chosen for follow-up studies because they presented an appropriate fluidity for a system that should be injected, and because they allow a comparison of the effect of

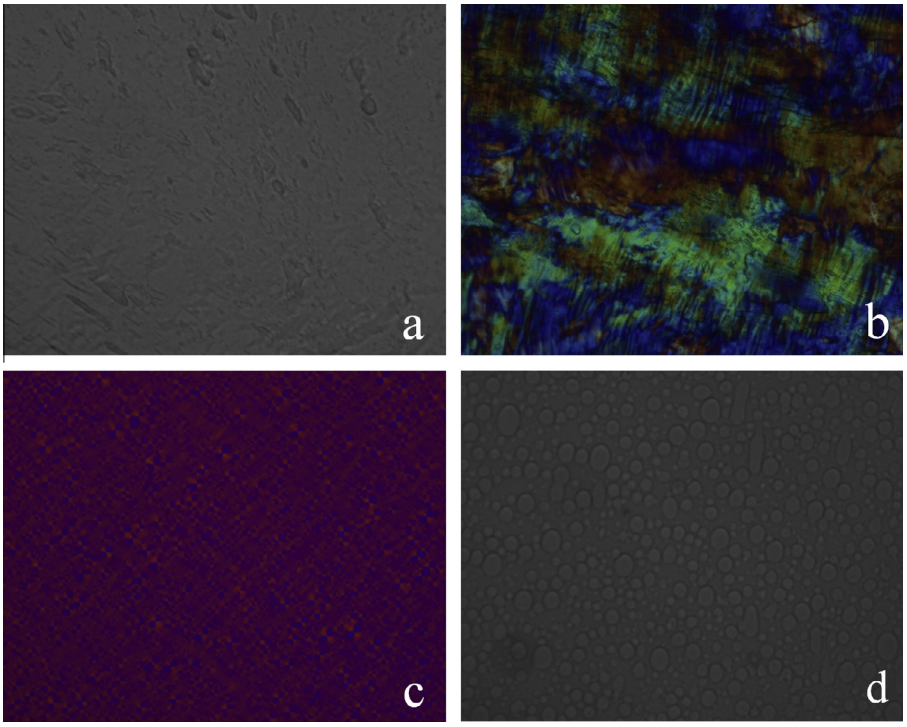

Fig. 3. Characterization of the systems by polarized light microscopy. (a) Cubic phase gel composed of MO/OAM/PG/tris buffer 3.84:0.16:16:80 (w/w/w/w) – 24 h after production. (b) Hexagonal phase gel formed from precursor fluid formulation MO/OAM/PG/tris buffer 8.16:0.34:76.5:15 (w/w/w/w) after contact with excess water – 7 days after production. (c) Lamellar phase gel composed of MO/OAM/PG/tris buffer 30.72:1.28:48:20 (w/w/w/w) – 24 h after production. (d) Emulsion composed of MO/OAM/PG/tris buffer 31.4:2.6:51:15 (w/w/w/w) – 24 h after production.

the two important components on the formation of the delivery system: MO, responsible for gel formation, and OAM, responsible for forming the gene complex.

3.4. Small Angle X-ray Scattering (SAXS) studies

Although polarized light microscopy is an easy way to differentiate liquid crystalline structures, the correct identification can be achieved using alternative identification techniques (Rizwan et al., 2009). Because the structure is the key to the performance of this type of delivery system, gel formed in excess water was also evaluated by SAXS. Dong and Boyd (2011) in their review "Application of X-ray scattering in pharmaceutical science" showed that X-ray scattering is used since the early half of the 20th century for determination of atomic level structure of different compounds and discussed that the SAXS is a fundamental technique to identify the shape, size and internal structure of a drug delivery system such as lyotropic liquid crystals (Dong and Boyd, 2011). In this study, the gel formed from the precursor fluid formulations with and without the presence of OAM and a control siRNA were evaluated. Fig. 4 presents the diffractograms of the intensity versus the scattering vector q acquired for these gels in addition to presenting the ratios between the interplanar distances and the lattice parameter.

As shown in Fig. 4, the addition of OAM favored the obtainment of the hexagonal phase, whereas the systems without the cationic lipid were characterized as the cubic phase. The cubic phases obtained were a mixture of two types of cubic phase: a diamond-type cubic phase (Pn3m space group) and a gyroid-type cubic phase (Ia3d space group). Although the gyroid-type cubic phase is the most typical type of cubic phase, the presence of different compound can change the liquid crystalline structure (Caboi et al., 2001; Chang and Bodmeier, 1997a). Thereby, the presence of two types of cubic phases found in this study may be due to the MO used. The results of the characterization by polarized light microscopy analyses were validated by SAXS.

The addition of different compounds can change the self-assembly of amphiphilic lipids and, consequently, can control the internal structure of the system formed. The self-assembly change can result in a complete phase change or change only the lattice parameter (Phan et al., 2011). In the present work, the addition of siRNA did not alter the phase formed; however, it reduced the lattice parameter for all analyzed gels, except for the gel MO/OAM 8.16:0.34 (w/w). The reduction of the lattice parameter in the presence of siRNA can be explained by its high hydrophilicity, resulting in a competition between MO and siRNA for water molecules. Because the capacity of the siRNA to bind water is higher than the MO, dehydration of the aqueous domain occurs in the organized lipid structure, promoting a reduction in the lattice parameter (Amar-Yuli et al., 2007).

3.5. Swelling studies

A swelling study is essential during the development of new delivery systems because the swelling rate and the resulting LCSs formed by amphiphilic compounds, such as MO, can affect the drug-release kinetics. In this study, the systems without OAM were also used as controls.

As apparent from the graph (Fig. 5a), the systems absorb water quickly and after 2 h reach the equilibrium water content. The rapid water absorption of the systems containing MO agrees with observations from other studies that used different proportions of MO, such as Myverol 18–99 K and monoolein (Lara et al., 2005; Rizwan et al., 2009). The system containing 8.16% MO showed the same water absorption as the system with OAM during the analyzed period, except for the absorption at 15 min; however, the system with 16.32% MO presented different water absorption from the system with OAM after 1 h.

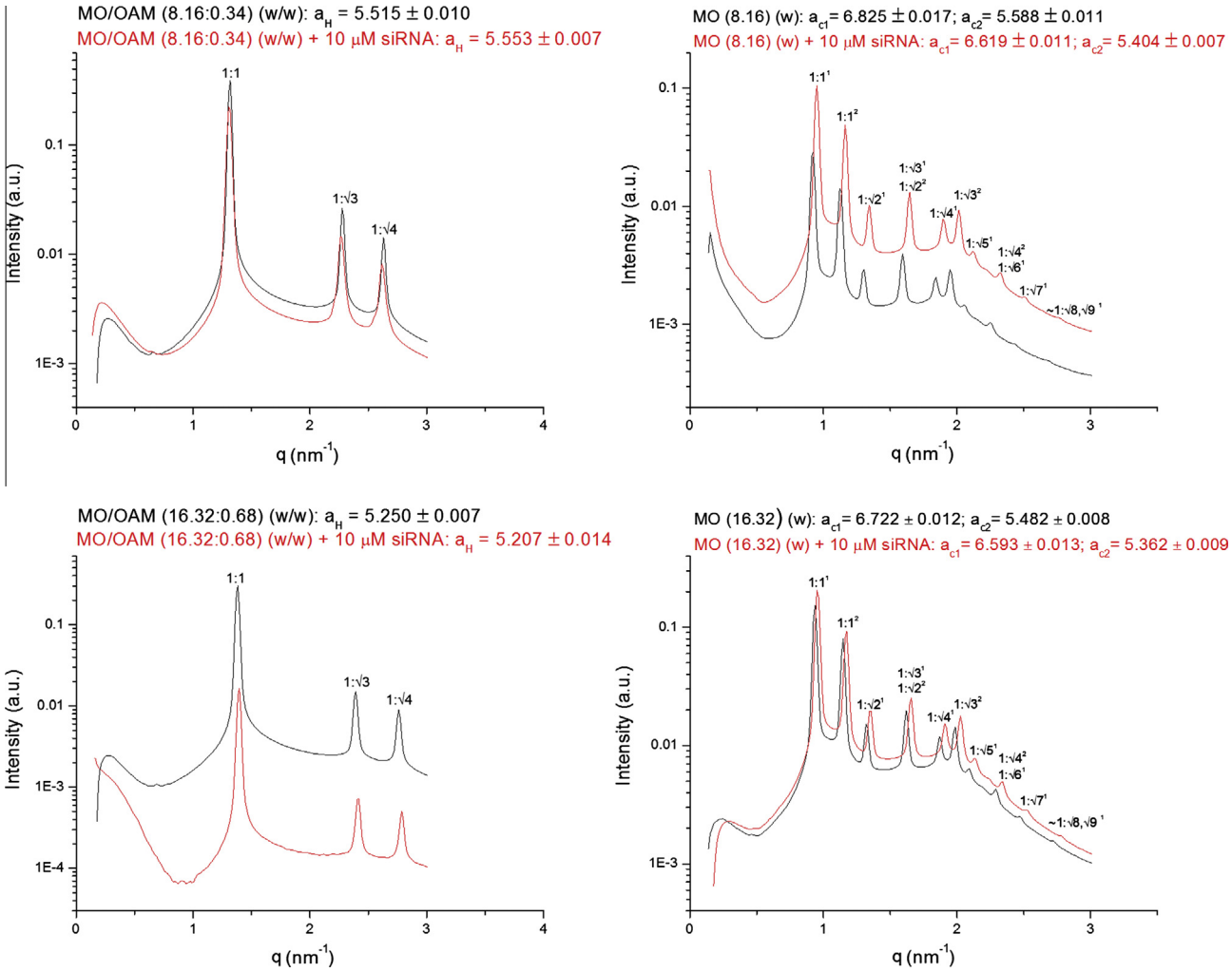

Fig. 4. SAXS results for the gels obtained from precursor fluid formulations composed of MO/OAM/PG/tris buffer (w/w/w/w) and control (MO/PG/tris buffer (w/w/w)) with and without addition of 10 μM siRNA. The graphics show the ratio between the interplanar distances and the lattice parameter (a_H: lattice parameter of the hexagonal phase; a_c: lattice parameter of the cubic phase).

Comparing the water absorption of the systems containing 8.16% and 16.32% of MO, it was observed that there were differences in water absorption for 30 min and after 48 h. For systems containing MO/OAM 8.16:0.34 and 16.32:0.68 (w/w), there were differences after 24 h. From these results, it was observed that, although there are differences in water absorption during some analyzed periods, the water absorption of the system is quite similar. This finding is consistent with the literature, where it is shown that the initial water content determines the water absorption, e.g., samples with a higher initial water content absorb less water than those with a lower initial water content, because they are almost fully hydrated (Lara et al., 2005). In this way, our systems that have the same initial water content should absorb similar amounts of water.

Fig. 5b shows the swelling kinetics. The water absorption followed a second order kinetic model, which means that at any time *t*, the swelling rate is directly proportional to two factors. First, the swelling rate is proportional to the amount of unrealized water uptake. As the system absorbs water, an expansion of the amorphous domain occurs and results in an increase of the stress on the crystalline domains that maintain the semi-crystalline network. These crystalline domains with higher tension resist further swelling. Thus, the resistance of the amorphous domains increases as the swelling becomes more extensive and the rate decreases. Second, the swelling rate is proportional the area of the regions that have not interacted with water at time *t*, but will be swollen (Schott, 1990). Similar results were found by other authors in their studies using different proportions of MO (Lara et al., 2005; Rizwan et al., 2009).

Table 1 shows the two swelling kinetics parameters: initial rate of absorption and maximum water uptake. Maximum swelling capacity of the systems containing MO/OAM 8.16:0.34 and 16.32:0.68 (w/w) obtained experimentally was in close agreement to the calculated value of 0.17 and 0.29 g/g using Eq. (2), respectively. The same was observed for the control of 8.16% MO; however, values obtained experimentally (0.19 g/g) for the control 16.32% MO were slightly higher than the calculated value (0.16 g/g). The initial rate of swelling was also calculated and the results showed that the initial rate of swelling increased as the content of MO decreased.

3.6. Studies of complexation with genes and gene stability

To be effective for gene delivery, a system should be able to form a complex with the gene to avoid its degradation, facilitate cellular uptake and endosomal escape (Whitehead et al., 2009). The OAM is a cationic lipid that was used in these systems to confer a positive residual and form a complex with the gene.

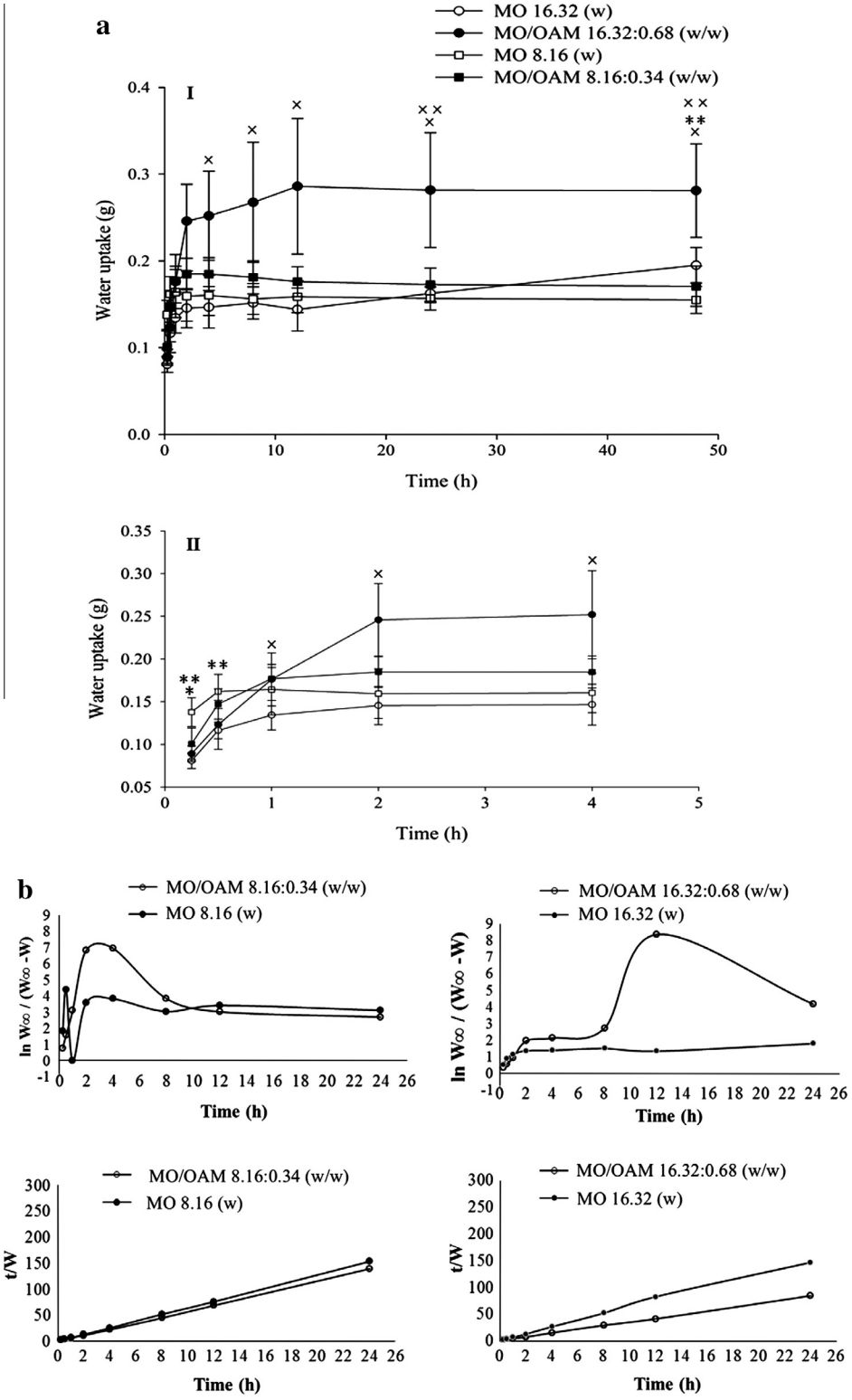

Fig. 5. (a) Plots of water uptake by the dialysis tubing with precursor fluid formulations containing MO/OAM/PG/tris buffer and control (without OAM) expressed as a function of time. Water uptake: (I) over the swelling studies period (48 h) and (II) over the first 4 h. Statistical analysis: *t*-test ($n = 5$, ±E.P.M.). $^{*}p < 0.05$ compared to the formulation MO 8.16 without OAM. $^{x}p < 0.05$ compared to the formulation MO 16.32 without OAM and $^{xx}p < 0.05$ comparison between systems containing MO/OAM and $^{**}p < 0.05$ comparison between systems without. (b) Swelling kinetics of different formulations with OAM and their controls, $\ln W_{\infty}/(W_{\infty} - W)$ versus time (first order kinetics) and t/w versus time (second order kinetics).

Considering that some studies showed that systems formed with cationic lipids have limited effectiveness due to their toxicity (Ozpolat et al., 2010), systems with a lower percentage of OAM were also evaluated. The ability of the systems to complex with genes was evaluated by electrophoresis (2% agarose gel at 100 V for 20 min and visualized under UV) using a control siRNA. Fig. 6a shows that the systems composed of a higher OAM concentration were able to complex with siRNA, whereas the systems

Table 1
Swelling kinetic parameters of the precursor fluid formulations evaluated at 37 °C calculated from swelling data using a second order equation (Eq. (2)).

Precursor fluid formulation	Initial rate of absorption (g/g h)	Maximum water uptake (g/g)	Correlation coefficient
MO/OAM/PG/Tris Buffer 8.16:0.34:76.5:15 (w/w/w/w)	2.65	0.17	0.999
Control of the sample MO/OAM 8.16:0.34 (w/w)	5.26	0.16	0.999
MO/OAM/PG/Tris Buffer 16.32:0.68:68:15 (w/w/w/w)	0.53	0.29	0.999
Control of the sample MO/OAM 16.32:0.68 (w/w)	0.42	0.16	0.998

with lower OAM proportions could not. In light of this observation, the systems were selected according to their ability to form a complex with siRNA. Thus, the systems containing MO/OAM 8.16:0.34 and 16.32:0.68 (w/w) were chosen.

In the next characterization step, these systems were evaluated for their ability to form a complex and not degrade the siRNA. For this, heparin was used for polyanion competition. When heparin was added, it competed with the siRNA and promoted its release. The results (Fig. 6b) showed that the siRNA remains stable after forming a complex in the systems.

3.7. In vivo gel formation and local toxicity

The self-assembling properties of precursor fluid formulations to form a gel were also evaluated *in vivo*. For this purpose, 50 μL of the formulation was injected subcutaneously using a syringe

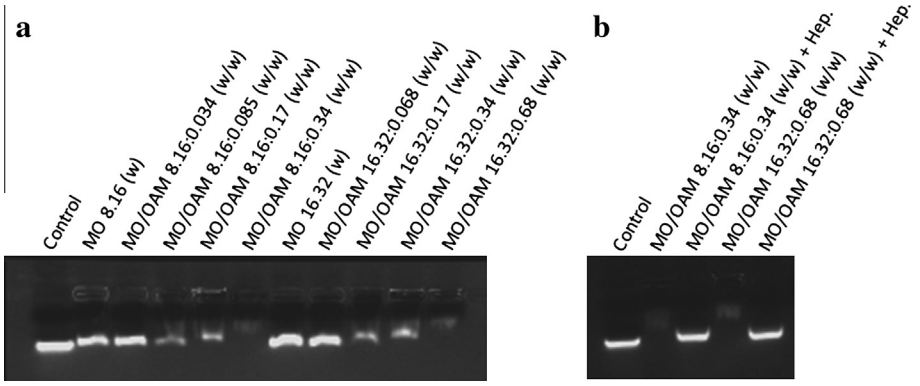

Fig. 6. (a) Ability of precursor fluid formulations with different percentages of MO and OAM to complex with genes. (b) Evaluation of gene stability in precursor fluid formulations using heparin for polyanion competition. Control: 10 μM siRNA in DEPC water. Hep: Heparin.

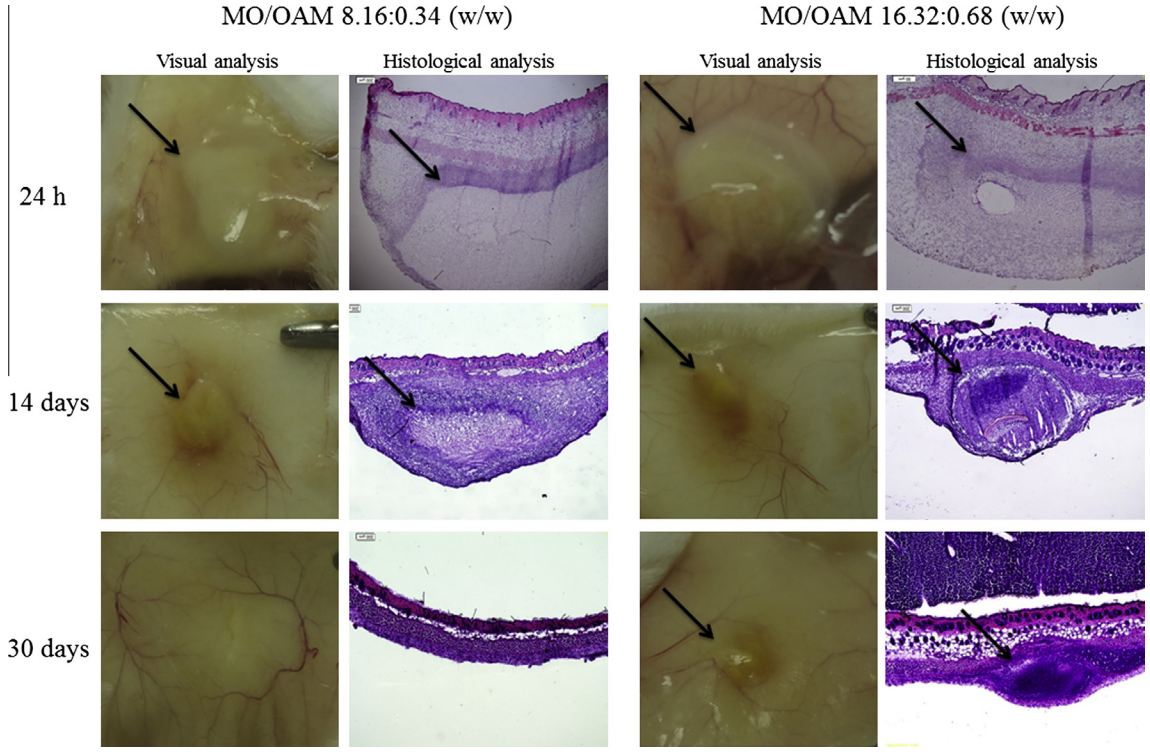

Fig. 7. Visual and histological analyses of *in vivo* gel formation from precursor fluid formulations containing: MO/OAM 8.16:0.34 and 16.32:0.68 (w/w) at 24 h and at 14 and 30 days. The arrow indicates the gel formation. The pictures of the visual analyses were taken using macro mode without zooming, 10 cm from the subject (f-stop: f/2.6, exposure time: 1/250 s, exposure index: ISO-100). The pictures of the histological analyses were taken using a motorized Olympus BX61 microscope (Olympus America Inc., Melville, New York, USA) attached to an Olympus DP 72 camera (40× magnification).

and a 26 gauge needle (0.45 × 13 mm) in female BALB/c mice. After the injection of the systems containing MO/OAM 8.16:0.34 and 16.32:0.68 (w/w), the mice were sacrificed after a predetermined period and the site of the injection was observed. Fig. 7 shows the presence of gel 24 h after the injection, which remains for 14 days for the system MO/OAM 8.16:0.34 (w/w) and for 30 days for the system MO/OAM 16.32:0.68 (w/w). Saline and the systems without OAM were used as controls. The gels formed from the systems without OAM have the same permanence profile as those from the system MO/OAM 8.16:0.34 (w/w).

To evaluate the local toxicity, the gel and the surrounding tissue were removed after the sacrifice of the animals and stained with H&E for histological analysis. The qualitative histological analyses showed that a normal inflammatory process took place in the tissue surrounding the gel, while the dermis and epidermis remained intact. In the tissue surrounding the gel, the presence of polymorphonuclear cells was observed after 24 h. After 7 days, a prevalence of mononuclear cells, such as macrophages, was noticed. At 14 days, fibroblasts and collagen fibers were detected. The presence of fibroblasts and collagen fibers indicate tissue repair. Although the injected precursor fluid formulations promoted some inflammation, could be observed that the systems with a higher percentage of MO with and without OAM showed a slightly stronger inflammatory process. This response could be explained by the gel volume because the systems with higher MO produced a higher volume of gel *in vivo* that could result in greater pressure in the tissue and thus recruit a greater number of inflammatory cells (Ricci et al., 2005). The inflammation process promoted by these systems seems to be more related to the physical presence of the gel in the tissue than to a possible toxicity of the system components. The histological analyses also showed the presence of the gel in the tissue (Fig. 7).

These *in vivo* studies were essential to prove the ability of a MO/OAM-based precursor fluid formulation to form *in situ* viscous LCSs after absorbing water from the bodily fluids. The developed systems, mainly that with 8.16% of MO, may be considered appropriate because they induce a normal inflammation process that disappears in several days, and the tissue is regenerated. Thus, the present study has developed and characterized promising systems for the local delivery of siRNA. Future studies will be conducted to characterize the release of nucleic acids by these systems and the promotion of the desired therapeutic effect.

4. Conclusions

In this study, it was demonstrated that LCSs could be used as promising local delivery systems for siRNA. By associating the self-assembly approach and the ability to complex with nucleic acids, the present work showed that LCSs can be a potential non-viral vector for gene delivery. Local delivery can be possible due to the capacity of the MO-based precursor fluid formulation to absorb water from the subcutaneous tissue and form viscous liquid crystalline gels. The presence of OAM enabled these gels, characterized as hexagonal phase, to form a complex with a nucleic acid. These are important conditions for the success of gene delivery.

Among the developed systems, the system containing MO/OAM/PG/tris buffer 8.16:0.34:76.5:15 (w/w/w/w) appears to be the most appropriate for use in gene therapy because it transformed quickly *in situ* into a non-toxic hexagonal-phase gel. The present work demonstrates a self-assembly formulation based on LCSs to be a potentially useful non-viral vector for gene therapy mediated by siRNA.

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