In situ–Forming Pharmaceutical Organogels Based on the Self-Assembly of L-Alanine Derivatives

Anne-Claude Couffin-Hoarau,[1] Aude Motulsky,[1] Pascal Delmas,[2] and Jean-Christophe Leroux[1,3]

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Purpose. To characterize novel pharmaceutical organogels based on the self-assembly of L-alanine derivatives in hydrophobic vehicles.
Methods. The gelation properties of *N*-lauroyl-L-alanine (LA) and *N*-lauroyl-L-alanine methyl ester (LAM) were investigated in the presence of various solvents. Gel-sol and sol-gel transitions were evaluated by the inverse flow method, and gelation kinetics were determined by turbidimetry. The *in vitro* release kinetics of labeled dextran physically dispersed in the oil-based organogel was assessed in phosphate-buffered saline. *In situ* formation of the implants was evaluated in rats by subcutaneously injecting a solution containing LAM, an oil, and a water-diffusible inhibitor of self-assembly (ethanol).
Results. The LAM-containing formulations showed a hysteretic gelling behavior with transition temperatures between 10 and 55°C. Gelation kinetics exhibited a lag time of 10 and 30 min at 25 and 37°C, respectively. *In vitro*, fluorescein isothiocyanate-dextran was released from the gel in a sustained manner with less than 6% released after 20 days. The addition of ethanol to the LAM/oil mixture inhibited gelation and allowed subcutaneous injection of the solution at room temperature. After injection, ethanol diffusion led to the formation of a solid implant.
Conclusions. Low-molecular weight self-assembling organogelators may allow the preparation of novel *in situ*-forming hydrophobic implants.

KEY WORDS: *in situ* gelation; organogel; self-assembly; sustained release.

INTRODUCTION

In the past decade, several *in situ*–forming implants have been investigated in drug delivery (1–5). Such systems have attracted a great deal of interest, mainly because of their injectability at room temperature (RT) and their ability to form a sustained-release matrix/gel *in situ* (6). In general, the implants are obtained through gelation of an aqueous polymeric solution at body temperature (2) or via *in situ* precipitation of a water-insoluble matrix initially dissolved in an organic solvent (3). A problem frequently encountered with thermosensitive hydrogels is their significant porosity resulting from a relatively high water content, which allows the rapid diffusion of low-molecular-weight hydrophilic molecules. Hydrophilic molecules are indeed better retained in a hydrophobic environment such as that found in organogels. For instance, amphiphilic lipids dissolved in organic solvents form a highly viscous, cubic liquid crystal phase upon injection (7). This gel-forming nature has been used to develop drug depot systems (8). Many studies have reported that some nonpolymeric, low-molecular-mass compounds, referred to as organogelators, can form network structures in hydrophobic solvents as a result of noncovalent interactions (9). Organogelators remain largely unexplored in the field of drug delivery. Murdan *et al.* (10) reported that the non-ionic surfactant sorbitan monostearate forms thermosensitive gels in biodegradable oils. This surfactant could arrange in bilayers and give rise to semisolid gels that exhibit modest depot properties after intramuscular administration.

Recently, Bhattacharya and Krishnan-Ghosh (11) showed that a fatty acid derivative of L-alanine was capable of achieving solvent-specific gelation from a two-phase mixture of water and organic solvent without gelling the aqueous phase. At relatively high temperatures, the system was in the sol state whereas upon cooling, the organic solution solidified into a gel-like structure. The process of self-assembly was mainly attributed to hydrogen-bonding at amide sites and to dimer formation between the free carboxylic groups. A limitation to the potential clinical use of such gels is the requirement of heating the system to the liquid state to incorporate the active compound and inject the solution. This problem could, in principle, be circumvented by adding a small amount of a water-soluble organic solvent (e.g., ethanol), which would disrupt the hydrogen bonds between the organogelator molecules and thus inhibit gelation at RT. Upon injection, selective diffusion of the hydrophilic solvent in the external environment would allow the gelator to self-assemble and generate the network structure. Another method would rely on the hysteretic behavior of some organogelators. This method consists of injecting a system that is liquid at RT and then locally decreasing the temperature below the gelation temperature to solidify the implant. As the temperature at the injection site reverts to body temperature, the system would stay in gel form. In this study, the gelation properties of two L-alanine derivatives (ester and acid forms), dissolved in pharmaceutically acceptable organic solvents, were evaluated. We showed for the first time that not only the ester form of the gelator can induce gelation of these solvents, but also that this compound, as opposed to its acidic counterpart, can display a temperature-dependent hysteretic behavior. Finally, the potential of these organogels for the sustained delivery of hydrophilic macromolecules was addressed, and *in situ* gel formation was demonstrated *in vivo*.

MATERIALS AND METHODS

Materials

Soybean oil, fluorescein isothiocyanate (FITC)-dextran (M_w 9500), L-alanine methyl ester hydrochloride (HCl.Ala-OMe) and lauroyl chloride were from Sigma Chemical Co. (St. Louis, MO, USA) and used as received. Medium-chain triglycerides (MCT) (i.e., Crodamol GTCC-PN) was kindly provided by Croda (Toronto, ON, Canada).

[1] Canada Research Chair in Drug Delivery, Faculty of Pharmacy, University of Montreal, C.P. 6128 Succ. Centre-ville, Montreal, Quebec H3C 3J7, Canada.
[2] Ethypharm Inc., 200 boul. Armand Frappier, Laval, Quebec H7V 4A6, Canada.
[3] To whom correspondence should be addressed. (e-mail: Jean-Christophe.Leroux@umontreal.ca)

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Synthesis of L-Alanine Derivatives

N-Lauroyl-L-Alanine Methyl Ester (LAM)

Triethylamine (2.7 ml, 23.7 mmol, 2.2 eq.) was added to a solution of HCl.Ala-OMe (1.5 g, 10.77 mmol) in 50 ml of chloroform. After 15 min under stirring, lauroyl chloride was slowly added to the reaction mixture. Stirring was maintained for 3 h. The mixture was successively washed with water, a saturated aqueous solution of NaCl, a saturated aqueous solution of $NaHCO_3$, HCl (1 N), and again water. The organic layer was dried ($MgSO_4$), filtrated, and concentrated under vacuum. The resulting colorless oil was purified by crystallization from ethyl acetate/cold hexane (ratio 4/1 v/v) to yield white needles (90%). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 0.88 (t, 3H), 1.26 (m, 16H), 1.43 (d, 3H), 1.63 (m, 2H), 2.21 (t, 2H), 3.73 (s, 3H), 4.61 (qt, 1H), 5.99 (d, 1H).

N-Lauroyl-L-Alanine (LA)

LAM was dissolved in methanol with NaOH (1 N) and the mixture was stirred for 3 h at ~5°C. The reaction medium was acidified in cold conditions upon completion of hydrolysis. The organic layer was washed with water, dried (Na_2SO_4), and concentrated under vacuum. The product was crystallized from a MeOH/hexane (ratio 4/1 v/v) mixture to yield a white solid (85%). ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 0.85 (t, 3H), 1.23 (m, 16H), 1.46 (qt, 2H), 2.07 (t, 2H), 4.16 (qt, 1H), 8.06 (d, 1H), 8.88 (s, 1H).

Determination of Sol-Gel (T_{SG}) and Gel-Sol (T_{GS}) Transitions

Transition temperatures were determined visually by the inverse flow method as previously described (12). The temperature of gel formation (T_{SG}) was noted when the solution did not fall under the influence of gravity when inverted. The reverse transition behavior was characterized by heating the gel in order to determine temperature at which the system starts to flow (T_{GS}). For selected samples, the minimal concentration of ethanol that inhibited gelation at RT was determined. These samples (200 μl) were then deposited over 100 ml of phosphate-buffered saline (PBS) (53 mM Na_2HPO_4, 13 mM NaH_2PO_4, 75 mM NaCl, pH 7.4) in a magnetically stirred volumetric flask, and gel formation was observed visually at 37 ± 0.1°C.

Determination of Gelation Kinetics

The gelation kinetics of samples demonstrating T_{SG} between 45 and 25°C were assessed by turbidimetry using a FL600 microplate reader (Biotek Intruments, Winooski, VT, USA). Briefly, 200-μl samples were deposited in 96-well plates and then heated for 1 h at 55°C. Gelation at 25 and 37°C was monitored by following the increase in turbidity over time at 493 nm.

In situ Gel Formation

LAM was first dissolved in ethanol. Next, soybean oil was added to this mixture. The latter was stirred and heated until a homogeneous solution was obtained. The solution (30% LAM w/v and 18% ethanol v/v) remained stable and liquid at room temperature. It was injected subcutaneously (s.c.) in the dorsal area of male Sprague Dawley rats. After 2 h, 24 h, or 9 days, the animals were anesthetized, sacrificed, and the semisolid gel was extracted from the injection site. Control solutions consisted of pure soybean oil or PBS. All animal care and studies were approved by the Animal Welfare and Ethics Committee of the University of Montreal.

In vitro Release Kinetics

FITC-dextran (~10 mg) was dispersed at RT in soybean oil (~800 mg) containing 30% (w/v) LAM or LA. The samples were deposited in 2-ml vials and allowed to gel overnight at 4°C. Then, 1 ml of PBS was added, and the system was incubated under stirring at 37°C. At the allotted time intervals, 0.5-ml aliquots were withdrawn and replaced by the same amount of fresh buffer prewarmed at 37°C. The amount of FITC-dextran released was measured by spectrofluorimetry (λ_{ex} = 492 nm, λ_{em} = 518 nm) on an Aminco-Bowman Series 2 fluorimeter (Spectronics Instruments Inc., Rochester, NY, USA).

RESULTS AND DISCUSSION

The gelating properties of LAM and LA were evaluated in soybean oil and MCT; two solvents approved for parenteral administration. Figure 1A shows the T_{SG} and T_{GS} of LA as a function of organogelator concentration. At RT, gelation occurred at relatively low concentrations (2%) in both oils.

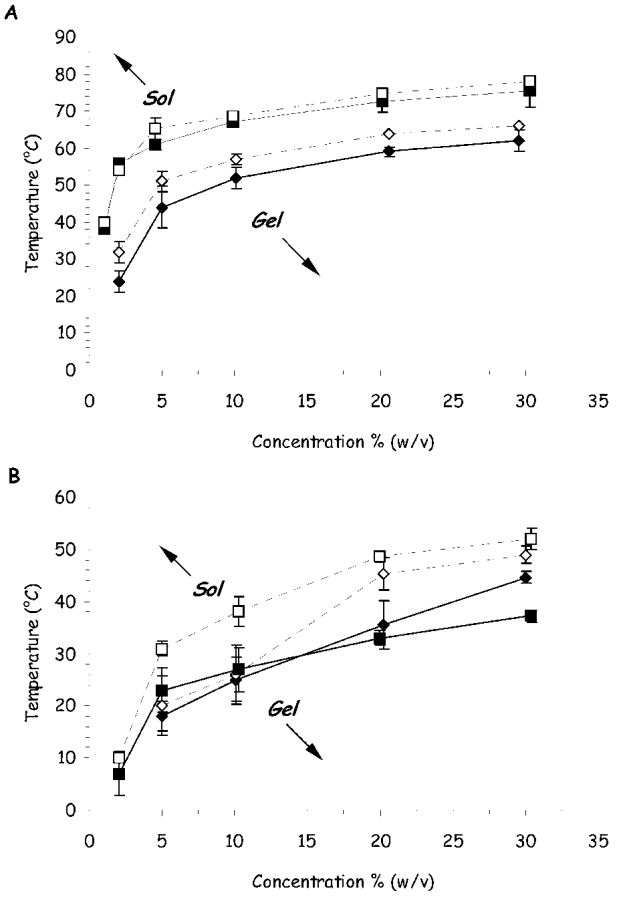

Fig. 1. Sol-gel transition (closed symbols, solid line) and gel-sol (open symbols, dashed line) of (A) LA and (B) LAM in soybean oil (diamonds), MCT (squares). Mean ± SD (n = 3).

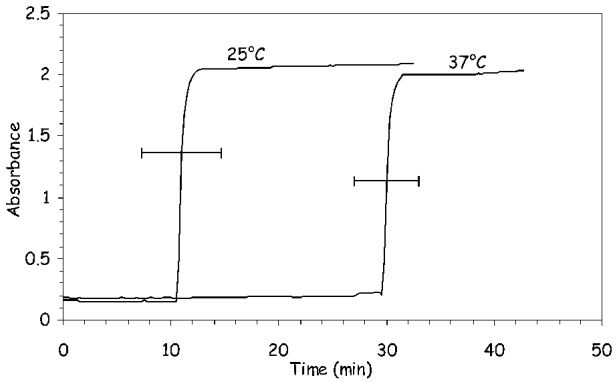

Fig. 2. Gelation kinetics at 25 and 37°C for 20% (w/v) LAM in soybean oil. SD is shown on the rising part of the curve (n = 3).

The phase transition temperatures increased with concentration and reached a plateau above 10%. The minimal gelation concentration at RT was at least one order of magnitude higher than that found in nonpolar organic solvents such as aromatic and aliphatic hydrocarbons (11). Indeed, relatively polar solvents like biodegradable oils can develop hydrogen bonding with the organogelator, resulting in an increase in the minimal gelation concentration. These systems did not displayed a hysteretic behavior since the T_{GS} and T_{SG} curves were almost superimposed.

In the case of LAM, T_{SG} occurred at much lower temperatures than for LA. Indeed, most formulations remained in the sol state at body temperature (37°C) (Fig. 1B). Esterification of the carboxylic acid group probably interfered with dimerization of the organogelator and thus perturbed self-assembly. However, this compound exhibited strong hysteretic behavior with T_{GS} exceeding T_{SG}, in some instances by almost 20°C. This means that, once gelled, a particular system can remain in the gel state at body temperature even though T_{SG} is below 37°C. Hysteresis was thermodynamic in nature and not kinetically driven, as the gelled samples did not revert to the sol state even when stored several days above T_{SG}. Hysteresis is a common property in first-order phase transition and can be attributed to the presence of 2 minima in the free energy diagram (13).

The systems were also characterized with respect to their gelation kinetics, and typical curves obtained by turbidimetry with a plate reader are presented in Fig. 2. In order to be commercially viable, an *in situ* gelling formulation should stay

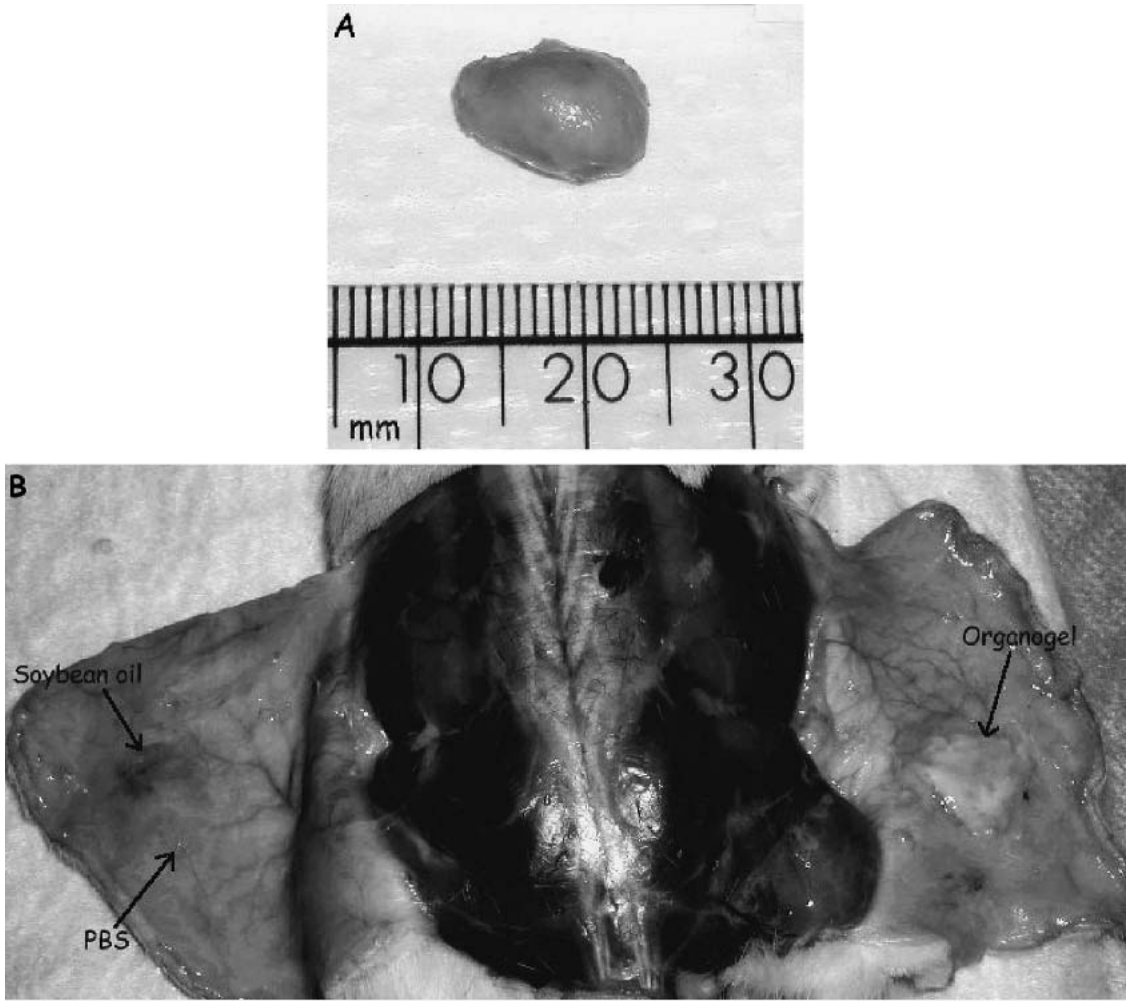

Fig. 3. *In vivo* demonstration of the *in situ* gelation of soybean oil/LAM/ethanol solution after s.c. injection into rats. (A) Implant excised 9 days after administration; (B) injection sites of the organogel, soybean oil, or PBS 24 h after administration.

in the sol state at RT and solidify within minutes after injection. At 25°C, the gelation kinetics generally exhibited a lag phase of approximately 10 min after which gelation proceeded rapidly. Lag time increased at 37°C, reflecting the fact that gelation is primarily driven through H-bonds. The addition of ethanol inhibited gelation at RT. For instance, the formulation containing 14% ethanol and 20% LAM in soybean oil stayed in the sol state at RT and turned into an opaque gel within 2 min when placed in 100 ml of PBS at 37°C. In prewarmed PBS, gelation was faster than in the plate reader due to a more efficient heat transfer. This suggests that it is possible to prepare a formulation that will be in the sol state at RT and gel *in situ* following injection. This formulation was indeed injected s.c. into rats. Gel formation was evident *in vivo* 2 h after injection. A macroscopic observation of the injection site 2 h, 24 h, and 9 days after administration revealed no great difference in the gel's integrity. Subcutaneous gel implants had bean-like shapes and remained quite morphologically intact upon excision (Fig. 3A). In the absence of LAM, soybean oil was cleared rapidly (<24 h) from the injection site (Fig. 3B).

Under the *in vitro* release kinetic conditions, LA emulsified the soybean oil in PBS, resulting in the loss of gel integrity. In contrast, the release kinetics could be carried out with LAM because the latter is more hydrophobic and did not diffuse in the aqueous phase. FITC-dextran (1.3% w/w in gel) was released very slowly with less than 6% released after 20 days (Fig. 4). *In vivo*, one might anticipate that the release of hydrophilic drugs would primarily be controlled by gel erosion/enzymatic degradation. Attempts will be made in the future to address this issue, investigating the influence of overall organogelator hydrophobicity on release kinetics.

In conclusion, the following results demonstrated in the current study that the ester form seems more favorable for the preparation of a depot formulation. It exhibits hysteretic behavior and also stays confined in the organic matrix, thus maintaining the integrity of the gel over time. As for other lipid parenteral formulations (14), the drug could be either dispersed in the matrix or first dissolved in water and then emulsified. One advantage of organogels over other lipid implants is the absence of possible polymorphic transformation upon storage which has been reported for solid lipid particles (15). Thus, *in situ* gelling, oil-based formulations may open new avenues for minimally invasive and site-specific sustained drug delivery. *In vivo* studies with entrapped low-molecular-weight peptides are currently underway to assess the depot properties of these novel organogels.

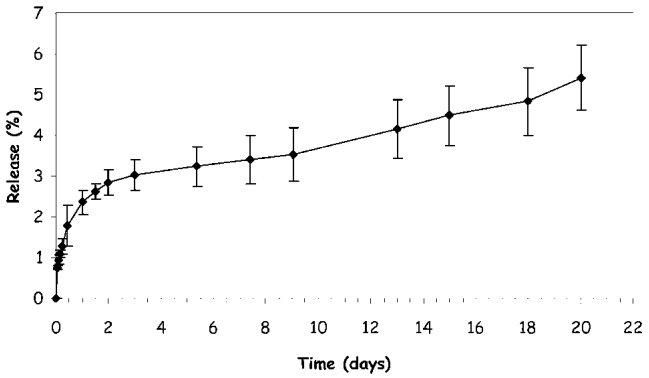

Fig. 4. Release profile of FITC-dextran (M_w 9500) from a 30% (w/v) LAM/soybean oil gel in PBS at 37°C. Each point represents the mean value ± SD (n = 3). The area of the gel exposed was 0.64 mm^2.

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