



Controlling release from the lipidic cubic phase by selective alkylation

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Abstract

The lipidic cubic phase can be viewed as a molecular sponge consisting of interpenetrating nanochannels filled with water and coated by lipid bilayers. It has been used as a delivery matrix for low-molecular-weight drugs. For those that are water-soluble, release is fast and unregulated. This study seeks to exploit the lipid bilayer compartment as a location within the cubic phase in which to 'hydrophobically' anchor the water-soluble drug. This was accomplished by controlling partitioning into, and thus release from, the aqueous compartment of the cubic phase. Tryptophan was used as a surrogate water-soluble drug and alkylation was implemented to regulate release. By adjusting alkyl chain length, exquisite control was realized. Without alkylation, 20% of the tryptophan was released under standard conditions (infinite sink with a 30-mg cubic phase source at pH 7 and 20 °C) over a period of 30 min (t_{20}). In the case of derivatives with alkyl chains two and eight carbon atoms long, t_{20} values of 3 and 13 days, respectively, were observed. Eliminating the charge on tryptophan completely by alkylation produced a derivative that became irreversibly lodged in the lipid bilayer. The release behavior of the short-chain derivatives was mathematically modeled and parameters describing transport have been obtained. Cubic phase partition coefficients for tryptophan and its derivatives were measured to facilitate modeling. The implications of these findings with regard to the cubic phase and related delivery systems, and to vaccine efficacy are discussed.

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1. Introduction

Drugs are optimally effective when present in what is referred to as the therapeutic concentration range. Above this limit, a drug can be toxic. Below the limit,

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the drug is ineffective. One of the challenges in the area of drug delivery is to design a system that will allow the therapeutic range to be accessed and to be maintained for defined periods. The lipidic bicontinuous cubic phases have been considered as possible drug hosting and delivery matrices with such properties. They can be viewed as molecular sponges where the lipid forms a highly curved, continuous bilayer that separates two interpenetrating, but noncontacting, aqueous nanochannels (Fig. 1).

Studies of controlled release from the cubic phase have used monoolein (also known as glyceryl monooleate, or GMO) or systems with monoolein as the

major component [1]. Monoolein is a monoacylglycerol—a relatively simple neutral lipid consisting of oleic acid in ester linkage to glycerol (Fig. 2A). When dispersed in water, it forms a number of liquid crystalline phases (also known as mesophases) including bicontinuous cubic phases (Fig. 1A). Despite its molecular simplicity, it has a rich mesomorphism, as illustrated in the temperature–composition phase diagram shown in Fig. 2B [2]. Note that, in this system, the cubic Pn3m phase forms in excess water and over a wide temperature range. These properties make it useful as a matrix for drug delivery. Parenthetically, the cubic phase has also proved itself in a related

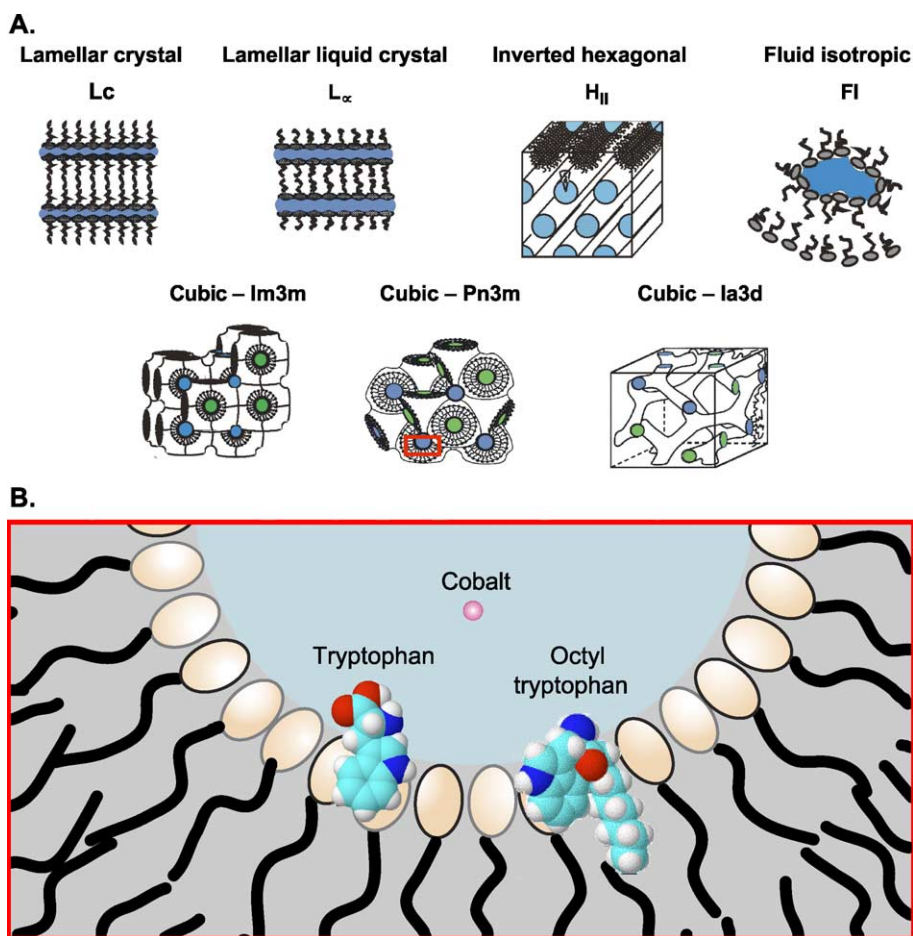


Fig. 1. Cartoon representation of the different phases encountered in the monoolein/water system (A) and the cubic Pn3m phase with additives (B). Individual lipids are shown as lollipop figures with the pop and stick parts representing the polar headgroup and the apolar acyl chain, respectively. The colored regions represent water. Cobalt, tryptophan, and octyl tryptophan are drawn to scale in (B) and are positioned to reflect their probable locations in the cubic phase. The conformation of octyl tryptophan has been drawn according to the results of molecular modeling [26].

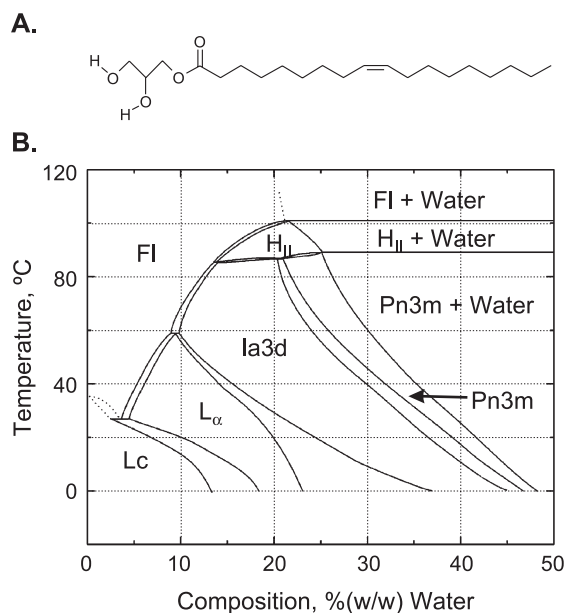


Fig. 2. Molecular structure and phase behavior of the monoacylglycerol, monoolein. (A) The molecular structure of monoolein. (B) Temperature–composition phase diagram of the monoolein/water system determined under “conditions of use” in the heating and cooling directions from 20 °C where the liquid crystal phases are metastable below ~20 °C (redrawn from Ref. [2]).

application: the crystallization of membrane proteins for use in high resolution structure determination [3].

For small, water-soluble drugs, release from the cubic phase is limited by diffusive transport through the pair of convoluted water channels that permeate the phase. Tortuosity contributes to a slowing down of release as does the relatively narrow pore size of the aqueous nanochannels. Nonetheless, release of low-molecular-weight drugs has been shown to be quite rapid [1]. Therefore, if the full potential of the cubic phase as a delivery matrix is to be realized, there exists a need for ways to prolong and control such release.

Previous attempts to prolong release from the cubic phase have been made. These included the addition of anionic phospholipids [4] and cationic surfactants [5]. While the release process was slowed, retardation only occurred when the target drug bore a charge that was opposite in sign to that of the cubic phase additive.

In the current study, a different approach is taken. It is one that exploits the very large polar/apolar

interface of the cubic phase (~500 m²/g lipid) and the apolar interior of the lipid bilayer that permeates the phase. The hypothesis being tested is as follows: alkylation of a small, water-soluble drug will cause it to partition into the relatively immobile lipid bilayer of the cubic phase in a chain length-dependent way. As a result, its residency in, and thus release rate from, the aqueous nanochannels and into the bulk aqueous medium can be tailored by adjusting the length of the alkyl chain.

To test the hypothesis, the time-dependent release of a series of tryptophan alkyl esters from the lipidic cubic phase was quantified. Because it is easily monitored spectrophotometrically, tryptophan was used as a surrogate small, water-soluble drug. The series studied included alkyl residues that were 1, 2, 4, 8, and 18 carbon atoms long. An additional derivative was added to the study where both the carboxyl and amino groups of the tryptophan molecule were alkylated. The data show that profound control over release rate can be achieved by adjusting alkyl chain length. While the derivatives are amphiphiles that could potentially destabilize the cubic phase and limit its functioning as a delivery device, X-ray diffraction measurements show clearly that this was not the case. Alkylation has other advantages that make it an attractive derivitizing agent, as discussed.

2. Experimental

2.1. Materials

Monoolein (MW 356 g/mol, lot M-239-MA26-N), diolein (MW 621 g/mol, lot D-236-MA10-K, a mixture of 1,2-diolein and 1,3-diolein), and triolein (MW 886 g/mol, lot T-235) were purchased from Nu Chek Prep (Elysian, MN) and were used as received. L-tryptophan (MW 204.2 g/mol, lot 119H0344), DL-tryptophan methyl ester hydrochloride (MW 254.7 g/mol, lot 043H3252), DL-tryptophan ethyl ester hydrochloride (MW 268.7 g/mol, lot 23H0221), DL-tryptophan butyl ester hydrochloride (MW 296.7 g/mol, lot 99F5013), DL-tryptophan octyl ester hydrochloride (MW 352.9 g/mol, lot 09H0522), oleic acid (MW 282.5 g/mol, lot 60K0703), and cobalt chloride hexahydrate (MW 237.9 g/mol, lot 18F-3536) were purchased from Sigma (St. Louis, MO). *N*-oleoyl-DL-

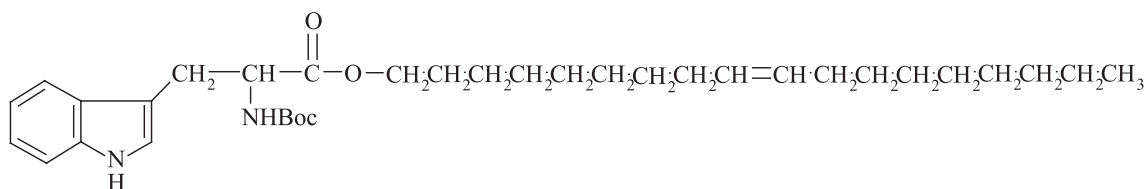
tryptophan ethyl ester (MW 496.7 g/mol, lot 01619PY), the derivatising reagent *N,O*-bis(trimethylsilyl)-acetamide (BSA, lot 39483-1), and the colorimetric reagent 4-(2-pyridylazo)resorcinol (PAR, lot 23403JB) were obtained from Aldrich (Milwaukee, WI). Glycerol (lot 970742) was purchased from Fisher (Fair Lawn, NJ). All solutions were prepared in the following buffer solution: 20 mM *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (TES, lot 121K5449; Sigma) and 0.02% (wt/vol) sodium azide (lot 118H2514; Sigma) adjusted to pH 7.4 using 2 M NaOH. Water was purified by using a Milli-Q Water System (Millipore, Bedford, MA) consisting of a carbon filter cartridge, two ion exchange filter cartridges, an organic removal cartridge, and a final 0.2- μ m filter (Millipak 40, lot F2PN84024; Sterile Millipore).

2.2. Tryptophan oleoyl ester hydrochloride synthesis

A dry 250-ml round-bottom flask was charged sequentially with 2.0 g (6.5 mmol) of *N* α -(*tert*-butoxycarbonyl)-L-tryptophan (lot 408417-5G; Aldrich), 150 ml of dry dichloromethane (lot 431754; Mallinckrodt Chemicals, Paris, KY), and 1.77 g (6.5 mmol) of *cis*-9-octadecen-1-ol (synthesized from oleic acid). The solution was cooled in an ice water bath and 20 mg (0.16 mmol) of 4-dimethylaminopyridine (lot 18526BO; Aldrich) was added. A solution of 1.36 g (6.5 mmol) of *N,N'*-dicyclohexylcarbodiimide (lot A015265201; Acros Organics, Fair Lawn, NJ) in 15 ml of dichloromethane was then added with stirring over a 5-min period and the cooling bath was removed. The reaction progress was monitored by thin-layer chromatography [TLC; silica gel; eluted with 20% (vol/vol) ethyl acetate in hexanes]. The reaction was considered complete when the reagent no longer appeared on the TLC plate. A

white precipitate of dicyclohexylurea was observed after 15 min. The reaction was stirred for a total of 3 h and filtered. The filtrate was concentrated in vacuo to provide 3.52 g of crude product, which was purified by chromatography over 100 g of silica gel [eluted in sequence with 500 ml of 10% (vol/vol) ethyl acetate in hexanes, 500 ml of 15% (vol/vol) ethyl acetate in hexanes, and 20% (vol/vol) ethyl acetate in hexanes until completion] to provide 2.5 g (69%) of **Derivative 1** as a colorless oil. The nuclear magnetic resonance (NMR) spectra of the derivative were measured at 25 °C using a Bruker 400-MHz NMR. The NMR results are as follows: ^1H NMR (50 mg in 0.5 ml of CDCl_3) δ 0.89 (t, $J=7$ Hz, 3H, CH_3), 1.3 (m, 22H, CH_2 manifold), 1.4 (s, 9H, *t*-Bu), 1.5 (quintet, $J=7$ Hz, 2H, OCH_2CH_2), 2.0 (m, 4H, $\text{CH}_2\text{C}=\text{}$), 3.3 (m, 2H, ArCH_2), 4.0 (m, 2H, OCH_2), 4.6 (m, 1H, CHN), 5.05 (d, 1H, NHBoc), 5.35 (m, 2H, $\text{CH}=\text{CH}$), 6.97 (s, 1H, $=\text{CHN}$), 7.05 (t, $J=8$ Hz, 1H, ArH), 7.15 (t, $J=8$ Hz, 1H, ArH), 7.30 (d, $J=8$ Hz, 1H, ArH), 7.55 (d, $J=8$ Hz, 1H, ArH), 8.10 (s, 1H, NH); ^{13}C NMR (CDCl_3) δ 14.1 (CH_3), 22.6 (CH_2), 25.7 (CH_2), 27.1 (CH_2), 27.2 (CH_2), 28.0 (C), 28.3 (CH_2), 28.4 (CH_2), 29.16 (CH_2), 29.19 (CH_2), 29.28 (CH_2 -2 carbons), 29.25 (CH_2), 29.49 (CH_2), 29.71 (CH_2), 29.74 (CH_2), 31.9 (CH_2), 54.2 (CH), 65.5 (CH_2), 79.7 (C), 110.3 (C), 111.1 (CH), 118.7 (CH), 119.5 (CH), 122.1 (CH), 122.6 (CH), 127.7 (CH), 129.8 (CH), 130.0 (CH), 136.1 (C), 155.2 (C=O), 172.4 (C=O).

To a solution of 1.5 g (2.7 mmol) of tryptophan (**Derivative 1**) in 100 ml of dichloromethane cooled to approximately 10 °C was added 1 ml of methanol followed by dropwise addition of 0.34 ml (0.20 g; 2.7 mmol) of chlorotrimethylsilane (lot 02623TA; Aldrich). The solution was stirred for 3 h and the resulting white precipitate was collected and dried in vacuo for 24 h to afford 1.1 g (83%) of the desired hydrochloride salt (**Fig. 3F**) as a white powder. The



Derivative 1. Molecular structure of the N-Boc blocked tryptophan oleoyl ester intermediate in tryptophan oleoyl ester synthesis.

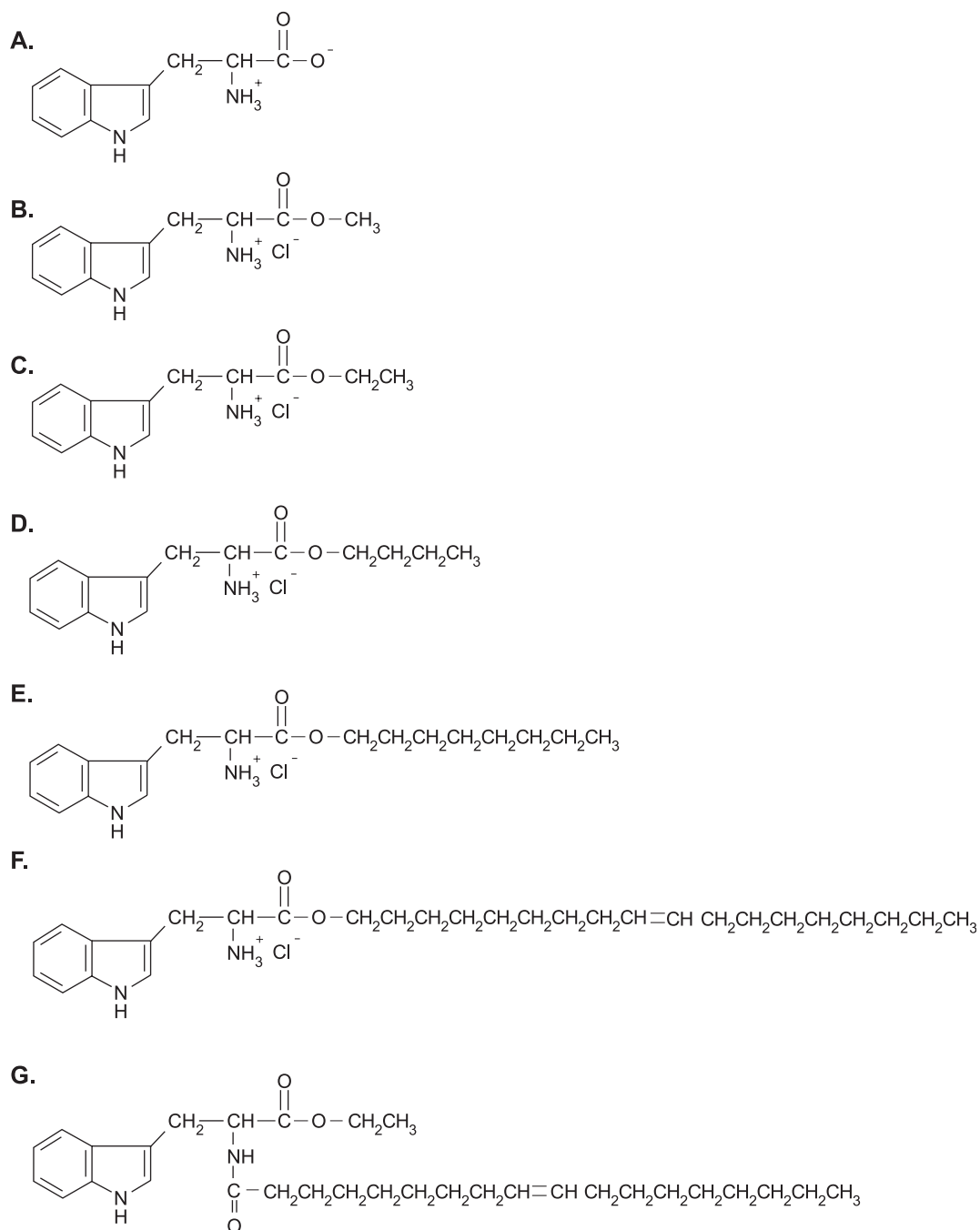


Fig. 3. Molecular structures of tryptophan and its alkyl derivatives used in this study. (A) Tryptophan. (B) Tryptophan methyl ester hydrochloride. (C) Tryptophan ethyl ester hydrochloride. (D) Tryptophan butyl ester hydrochloride. (E) Tryptophan octyl ester hydrochloride. (F) Tryptophan oleoyl ester hydrochloride. (G) *N*-oleoyl tryptophan ethyl ester.

NMR results for the final product are as follows: ^1H NMR (50 mg in 0.5 ml of DMSO-d_6) δ 0.84 (t, $J=7$ Hz, 3H, CH_3), 1.2 (m, 22H, CH_2 manifold), 1.4 (quintet, $J=7$ Hz, 2H, OCH_2CH_2), 1.98 (m, 4H, $\text{CH}_2\text{C}=\text{}$), 3.25 (dd, $J=14$, 7 Hz, 1H, ArCH_2), 3.37 (dd, $J=14$, 7 Hz, 1H, ArCH_2), 3.98 (m, 1H, CHN), 4.15 (t, $J=7$ Hz, 2H, OCH_2), 5.30 (m, 2H, $\text{CH}=\text{CH}$), 6.95 (t, $J=8$ Hz, 1H, ArH), 7.05 (t, $J=8$ Hz, 1H, ArH), 7.25 (s, 1H, $=\text{CHN}$), 7.33 (d, $J=8$ Hz, 1H, ArH), 7.50 (d, $J=8$ Hz, 1H, ArH), 8.75 (broad s, 3H, NH_3), 11.18 (s, 1H, NH); ^{13}C NMR (50 mg in 0.5 ml of DMSO-d_6) δ 14.2 (CH_3), 22.4 (CH_2), 25.4 (CH_2), 26.6 (CH_2), 26.9 (CH_2), 27.0 (CH_2), 27.7 (CH_2), 28.58 (CH_2 , more than one carbon), 28.66 (CH_2), 28.72 (CH_2), 28.82 (CH_2), 29.08 (CH_2), 29.13 (CH_2), 31.25 (CH_2), 52.7 (CH), 65.4 (CH_2), 106.4 (C), 111.5 (CH), 117.9 (CH), 118.4 (CH), 120.9 (CH), 124.8 (CH), 126.9 (CH), 129.53 (CH), 129.56 (CH), 136.2 (C), 169.3 (C=O); MS (electrospray) $\text{C}_{29}\text{H}_{46}\text{N}_2\text{O}_2\text{Na}$: calculated 477.3451, found 477.3454. Estimated purity of the final product, as determined by ^1H and ^{13}C NMR, was in excess of 99%.

2.3. Sample preparation for release experiments

Stock solutions of tryptophan, tryptophan methyl ester, tryptophan ethyl ester, and tryptophan butyl ester were prepared in buffer at a concentration of 9.4 mg/ml. The cobalt chloride solution used was 10.5 mg/ml. These provide for a cubic phase loading of ~ 10 mmol additive/mol lipid. The cubic phase was prepared by mechanically mixing molten monoolein (melted at 40°C) with stock additive solutions using a syringe-based mixing device, as described [6]. Briefly, molten monoolein was weighed (~ 25 mg) in a 100- μl Hamilton gas-tight syringe. This was connected via a 14-mm-long coupler (22 gauge) to a second 100- μl Hamilton gas-tight syringe containing the additive solution. The latter volume was adjusted to give a lipid dispersion with $\sim 39\%$ (wt/wt) solution. Homogenization was achieved by passing the contents of the syringes back and forth many times through the coupler at room temperature ($\sim 22^\circ\text{C}$). Properly mixed samples were colorless and transparent. Tryptophan octyl ester, tryptophan oleoyl ester, and *N*-oleoyl-tryptophan ethyl ester are insoluble or sparingly soluble in water. Accordingly, they were introduced into the mix by first dissolving them in molten

monoolein (fused at 40°C). The solution was then mechanically homogenized with buffer, as described above.

The additive-loaded cubic phase was transferred to a home-built sample holder (an acrylic or Plexiglass disc: outer diameter, 7.8 mm; thickness, 0.68 mm; sample-holding volume, ~ 30 μl ; exposed sample surface area, 0.48 cm^2). A spatula was used to fill the holder with the cubic phase and to produce a uniformly flat, exposed surface. At point of loading, a small aliquot of the cubic phase sample was taken and analyzed by X-ray diffraction for phase identification and microstructure characterization. The initial additive content of the cubic phase was calculated by knowing the concentration of the additive solution used to prepare the cubic phase and the weight of the cubic phase in the sample holder.

2.4. Release experiments

To quantify the release of tryptophan and its alkyl derivatives from the cubic phase, a multicompartiment dialysis cell was used. A schematic of one such cell is shown in Fig. 4. It has two compartments: the source, corresponding to the cubic phase sample holder (described in Section 2.3), and the sink, consisting of a chamber with 1 ml of buffer. The two compart-

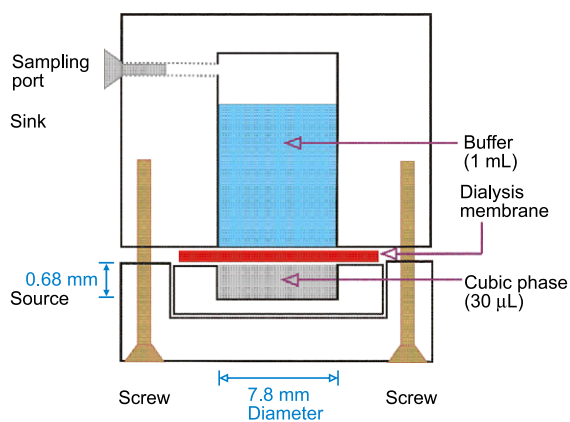


Fig. 4. Schematic of the dialysis cell used in this study (not drawn to scale). The acrylic cell has two compartments: the source that houses the cubic phase sample and the sink consisting of a block that accommodates a 1-ml buffer reservoir. The two are separated by a dialysis membrane and are held together by screws. The sink reservoir is accessible by means of a screw-capped sampling port.

ments are separated by a dialysis membrane (mixed cellulose esters, 110 μm thick, 8 μm pore size; lot H2AN07696; Millipore). Each experiment made use of a reference sample cell (cubic phase only) and triplicate sample cells (additive-loaded cubic phase). Throughout the experiments, the dialysis cells were gently shaken on a Mistral Multi-Mixer (Lab-Line Instruments, Melrose Park, IL) in an incubator (Model 815; Precision Scientific, Chicago, IL) at 20 °C. A measurement consisted of removing the entire 1-ml solution from the sink and immediately replenishing it with 1 ml of fresh buffer. The concentration of tryptophan additives in the sink solution was determined by measuring absorbance at 280 nm (cell path length 1 cm, calibrated with standards of known concentration and a measured tryptophan absorptivity of 5600 $\text{M}^{-1} \text{cm}^{-1}$) in a UV–visible spectrophotometer (Uvikon XL; Bio-Tek Instruments, San Diego, CA). Cobalt ion was assayed colorimetrically, as described [7]. Measurements were made at timed intervals for 14 days with the exception of the studies involving cobalt, tryptophan, and *N*-oleoyl-tryptophan ethyl ester, which were measured for 12 h, 2 days, and 7 days, respectively. The results are reported as the fraction of additive released versus time. At the end of the release experiments, the dialysis cells were taken apart and the lipid dispersion was sampled for phase characterization by X-ray diffraction. The aqueous content of the lipid dispersions at the end of the release study was determined gravimetrically using a microbalance (R200D; Sartorius, Edgewood, NY), as described [6].

2.5. Partition coefficient

2.5.1. Experimental measurement

The distribution of tryptophan and of its three shortest alkyl derivatives between the lipid bilayer and the aqueous compartment of the cubic phase at equilibrium was determined experimentally as a partition coefficient (P). P is defined [8]:

$$P = \frac{[X]_{\text{bilayer}}}{[X]_{\text{aqueous}}} \quad (1)$$

where $[X]_{\text{bilayer}}$ and $[X]_{\text{aqueous}}$ are the molar additive concentrations in the cubic phase bilayer and aqueous channel compartments, respectively. P was

measured by incubating together the additive solution and a bolus of cubic phase, and following a net loss of additive to the bolus indicating a preferential partitioning into the apolar compartment. Measurements were carried out in semimicro UV-transparent disposable cuvettes (path length 1 cm; BrandTech Scientific, Essex, CT) such that the concentration of additive in the aqueous medium above the cubic phase could be monitored directly in situ. Cubic phase [$\sim 40\%$ (wt/wt) buffer] was prepared as in Section 2.3 and a known amount (~ 30 mg) was placed in the bottom of the cuvette. To this was added 1 ml of additive solution (0.315 mM tryptophan, methyl tryptophan, ethyl tryptophan, or butyl tryptophan) and the cuvettes were capped and sealed with parafilm. Throughout the equilibration, samples were gently shaken (Mistral Multi-Mixer) at 20 °C (incubator Model 815; Precision Scientific) in the dark. Periodically, cuvettes were transferred to the Uvikon spectrophotometer, where the absorption spectrum from 400 to 250 nm of the aqueous solution above the cubic phase was recorded. Absorption at 280 nm was used to calculate additive concentration, as noted previously. Equilibrium was reached in ≤ 4 days. All samples were prepared in triplicate. Controls that were devoid of additive and/or cubic phase were run.

To calculate P according to Eq. (1), it is assumed that the previous measurement of additive concentration in the aqueous medium in equilibrium with the cubic phase is also a measure of $[X]_{\text{aqueous}}$, and that water inside the cubic phase has the exact same characteristics as that in the bulk aqueous medium. $[X]_{\text{bilayer}}$ can be determined by mass balance.

2.5.2. Estimated values

In addition to determining P experimentally, use was made of the so-called KOWWIN program (<http://www.syrres.com/esc/kowwin.htm>) to calculate octanol/water partition coefficients ($P_{\text{o/w}}$) for the additives used in this study. The program is statistically based and weighted depending on the identity of the atoms and groups of atoms (fragments) that make up the target organic [9]. A molecular structure or a CAS registry number is all that is required as input. When experimental $P_{\text{o/w}}$ values are available, they, too, are reported along with the estimated value. The correlation coefficient

between estimated and experimental values is 0.95 based on 13,000 compounds, which covers 15 decades in $P_{o/w}$.

2.6. X-ray diffraction

X-ray diffraction was used to characterize the phase properties of lipid dispersions before and after the release measurements, as described [10]. Briefly, samples were transferred to 1-mm-diameter quartz capillaries (Charles Supper, Natick, MA), flame-sealed, and glued with Extra Fast Setting Epoxy (Hardman, Belleville, NJ). Diffraction measurements were made using a two-beam port rotating anode X-ray generator (18 kW, RU-300; Rigaku USA, Danvers, MA) operating at 42 kV and 220 mA, generating copper K_{α} X-rays (1.542 Å, nickel-filtered). Sample-to-detector distance (~333 mm) was determined using silver behenate (d_{001} =58.4 Å) [11]. Measurements (30-min exposure) were made in a temperature-regulated sample holder at 20 °C.

2.7. TLC

TLC was used to monitor the chemical stability of the lipid during the course of the release study. Thus, cubic phase consisting of hydrated monoolein was carried through a typical release protocol at 20 °C and samples were taken for analysis after 1, 7, and 22 days. After overnight vacuum drying (1.5 mTorr, 20 °C), the samples were dissolved in chloroform. Standards were prepared in chloroform also. Plates (Adsorbosil Plus; Alltech, Deerfield, IL) were prerun twice with chloroform/methanol (10/1 by vol) and lipid was applied to the plates with a Wiretol pipette (Alltech). Four development solvents (chloroform/acetone, 96/4 by vol; hexanes/ethyl acetate/acetone, 50/25/25 by vol; hexanes/ethyl acetate/acetone, 73.5/1.5/25 by vol; chloroform/methanol/water, 65/25/4 by vol) were used and spots were visualized by spraying with 4.2 M sulfuric acid followed by charring on a hotplate (Type 2600; Thermolyne, Dubuque, IA) at 260 °C. Spot intensity on the charred plates was quantified using a digital scanner (Perfection 1200U Scanner; Epson, Long Beach, CA). Integrated intensities were estimated using the program FIT2D [12].

2.8. Monoolein and lipid additive sample preparation

The effect of oleic acid, diolein, and triolein on the phase behavior of hydrated monoolein was determined by X-ray diffraction. Samples were prepared by adding appropriate amounts of dry monoolein to molten oleic acid, diolein, and/or triolein in a microcentrifuge tube (0.5 ml). Incubation for 5 min at 40 °C brought about complete melting, and homogenization was effected by centrifugation and vortex mixing. Samples, with a final composition of ~60% (wt/wt) buffer, were prepared following the procedure outlined in Section 2.3 and were placed in X-ray capillaries, as described (Section 2.6).

2.9. Tryptophan alkyl ester stability

^1H and ^{13}C NMR were used to track the stability of the tryptophan alkyl esters in water and aqueous DMSO. For this purpose, the butyl tryptophan was dissolved in deuterated water, while the methyl tryptophan was dissolved in a 50/50 (by vol) mix of deuterated DMSO and deuterated water. All samples were prepared at ~102 mg/ml, and measured at 25 °C by NMR after 1, 2, 4, and 6 days at room temperature and compared to their initial spectra. The NMR results follow.

2.9.1. Methyl tryptophan (HCl salt)

^1H NMR [D_2O - $\text{C}_2\text{D}_6\text{SO}$ (1:1)]: δ 3.46 (dd, $J=14$, 8 Hz, 1H, ArCH₂), 3.54 (dd, $J=14$, 6 Hz, 1H, ArCH₂), 3.71 (s, 3H, OCH₃), 4.41 (t, $J=7$ Hz, 1H, CHN), 7.24 (t, $J=8$ Hz, 1H, ArH), 7.33 (t, $J=8$ Hz, 1H, ArH), 7.39 (s, 1H, =CHN), 7.60 (d, $J=8$ Hz, 1H, ArH), 7.65 (d, $J=8$ Hz, 1H, ArH); ^{13}C NMR [D_2O - $\text{C}_2\text{D}_6\text{SO}$ (1:1)]: δ 26.9 (CH₂), 53.9 (CH), 54.2 (CH₃), 107.1 (C), 112.9 (CH), 119.0 (CH), 120.4 (CH), 122.9 (CH), 125.9 (CH), 127.7 (CH), 137.2 (C), 170.7 (C=O).

2.9.2. Butyl tryptophan (HCl salt)

^1H NMR (D_2O): δ 0.7 (t, $J=7$ Hz, 3H, CH₃), 1.05 (sextet, $J=7$ Hz, 2H, CH₂CH₂CH₃), 1.36 (quintet, $J=7$ Hz, 2H, OCH₂CH₂), 3.35 (d, $J=7$ Hz, 2H, ArCH₂), 4.02 (t, $J=7$ Hz, 2H, OCH₂), 4.29 (t, $J=7$ Hz, 1H, CHN), 7.10 (t, $J=8$ Hz, 1H, ArH), 7.15 (t, $J=8$ Hz, 1H, ArH), 7.18 (s, 1H, =CHN), 7.40 (d, $J=8$ Hz, 1H, ArH), 7.49 (d, $J=8$ Hz, 1H, ArH).

2.10. The diffusion model

The experimental release profiles for tryptophan and its derivatives (collectively referred to here as additives) from the lipidic cubic phase presented below can be described by a Fickian diffusion model. The features of the model follow (refer to Fig. 5A).

Additives within the source cubic phase are identified as belonging to two populations. One is in the aqueous channels of the source and is free to diffuse into the sink. The other resides, or is associated with, the lipid bilayer component of the source. These are identified respectively, by fractions of the total additive population as F and B , where the sum of F

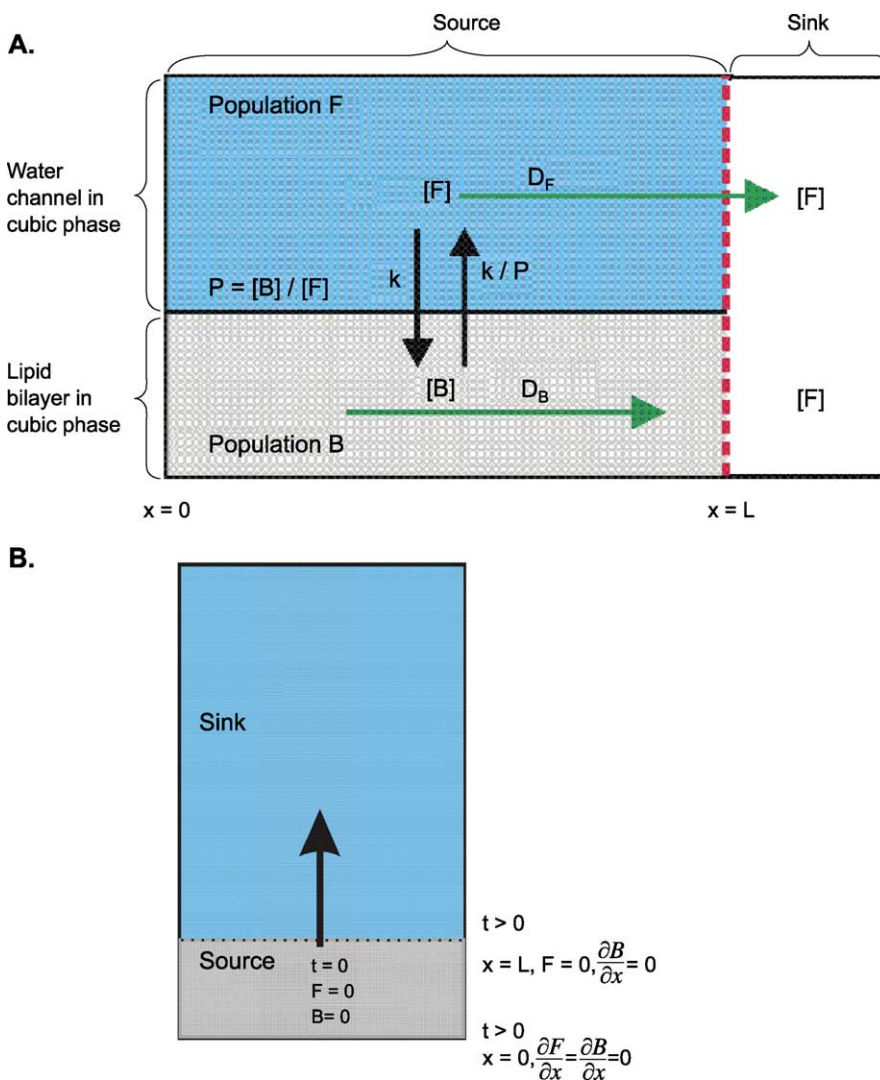


Fig. 5. Schematic representation of the diffusion model for release from the cubic phase and of the conditions imposed on the system for the water-soluble additives. (A) The Fickian diffusion model used to describe the release of tryptophan and its derivatives from the lipidic cubic phase. For clarity, the cubic phase is represented by two compartments: one aqueous and the other lipid bilayer. The parameters referred to in the text are included in the model. (B) Schematic representation of the conditions prevailing during release of the more water-soluble tryptophan derivatives. The initial ($t=0$) and boundary conditions ($t>0$) are indicated. The cubic phase source and the buffer sink are shown in light and dark grey, respectively. The direction in which additives move from source to sink is indicated by the arrow (see text for details).

and B equals unity. The mathematical model describing the time-dependent change in additive concentration within the cubic phase takes the form of two partial differential equations, one for each population, as follows:

$$\frac{\partial F}{\partial t} = D_F \frac{\partial^2 F}{\partial x^2} - kF + \frac{k}{P}B \quad (2)$$

$$\frac{\partial B}{\partial t} = D_B \frac{\partial^2 B}{\partial x^2} + kF - \frac{k}{P}B \quad (3)$$

Here t is time (days); x is distance (cm) from the bottom of the sample holder measured in the direction of the source/sink interface; D_F and D_B are the diffusion coefficients (cm^2/s) of the additives in the water channel and in the lipid bilayer, respectively; k and k/P are rate constants (s^{-1}) describing the speed of additive partitioning into and out of the bilayer, respectively; and P is the additive partition coefficient as defined in Eq. (1). The maximum value of x is L , which corresponds to the thickness of the bolus of cubic phase used in the release study (Fig. 5).

The model assumes that the water-soluble additives (tryptophan, methyl tryptophan, ethyl tryptophan, and butyl tryptophan) are all initially in the water channel. This is based on how these samples were prepared. Additives were dissolved in buffer for cubic phase preparation and, within an hour, were being used in the release study.¹ The initial conditions that apply to the water-soluble additives are as follows:

$$t = 0, \quad 0 < x < L, \quad F = 1, \quad B = 0 \quad (4)$$

The lipid-soluble additives (octyl tryptophan, oleoyl tryptophan, and N -oleoyl-tryptophan ethyl ester) were all combined (individually) in a molten lipidic solution prior to cubic phase preparation. Thus, they started out, by and large, confined to the lipid bilayer.

¹ It is likely that the additives will begin to equilibrate as soon as the lipid and aqueous media are brought into contact. However, it will take a finite amount of time for them to reach equilibrium in partitioning between the two environments. The release experiments were set up as soon as was physically possible after mixing. Thus, the assumption that the additives are all initially in the aqueous channel is reasonable.

This third assumption gives rise to the following initial conditions for these additives:

$$t = 0, \quad 0 < x < L, \quad F = 0, \quad B = 1 \quad (4a)$$

Since the release chamber is sealed at its base where $x=0$, the additive cannot move in the negative x direction. Thus, the first boundary condition takes the form:

$$t > 0, \quad x = 0, \quad \frac{\partial F}{\partial x} = \frac{\partial B}{\partial x} = 0 \quad (5)$$

At the source/sink interface where $x=L$, the additive is free to diffuse from the cubic phase into the source buffer. Since the cell is constantly shaken and the sink solution is replaced frequently throughout the release study, additive is not assumed to accumulate at the interface. Thus, additive concentration at the interface can be set to zero, thus defining the second boundary condition:

$$t > 0, \quad x = L, \quad F = 0, \quad (6a)$$

$$t > 0, \quad x = L, \quad \frac{\partial B}{\partial x} = 0 \quad (6b)$$

Eq. (6a) means perfect sink conditions in that additive concentration in the sink is insignificant relative to that in the cubic phase. Eq. (6b) means that additive in or on the lipid bilayer does not directly enter the source from the source/sink interface in significant amounts. This makes sense when the relevant surface area (0.5 cm^2) is compared with the other surface, across which the additive passes on its way to the aqueous channels of the cubic phase. The latter is on the order of 15 m^2 . A schematic of the release cell with the initial and boundary conditions identified is shown in Fig. 5B.

With these initial and boundary conditions, the appropriate partition coefficient P and the time-dependent release data, it is possible to numerically solve Eqs. (2) and (3) and to obtain best estimates of D_F and k . To this end, a MatLab (v 6.0; The Math Works, Natick, MA) program was written. The latter finds the values of D_F and k for which the numerical solutions to Eqs. (2) and (3) give the best fit to the release profile data. An additional conclusion of our numerical simulations is that, if it is assumed that D_B is less than D_F , then the exact value of D_B is irrelevant to the results of the numerical simulations.

3. Results and discussion

The purpose of this study was to introduce a novel approach for extending and controlling the time for release of a water-soluble drug from the lipidic cubic phase. The approach taken involved using tryptophan to represent the drug and to modify it with alkyl chains of increasing lengths. The hypothesis under investigation is that the longer the chain, the more the drug will partition into the 'stationary' hydrophobic compartment of the cubic phase and the slower will be its release. It was tested with six alkyl derivatives having chains ranging from 1 to 18 carbon atoms long.

3.1. Initial phase conditions

To perform this study in a systematic way, it was critical that all of measurements be performed under the same initial conditions. Thus, the objective was to monitor additive release specifically from the cubic Pn3m phase (Fig. 1A) at 20 °C. The latter mesophase forms in the simple monoolein/water system under conditions of excess water at 20 °C (Fig. 2B). To proceed, therefore, it was important to resolve two issues. The first concerned knowing accurately the amount of additive in the cubic phase at the start of the release study. To realize this, it was necessary to work with a cubic phase sample that was at, or just below, full hydration. Should an excess of aqueous medium be present, its volume could not be determined reliably. This would lead to uncertainties in the additive loading level at the start of the experiment. The proper hydration level of the cubic phase was ascertained by preparing samples with buffer contents in the 30–50% (wt/wt) aqueous medium range. Visual inspection of the dispersion in the mixing apparatus revealed the presence of an excess aqueous medium at and above 40% (wt/wt) buffer. Thus, all samples used in the study were prepared with a buffer content of 39% (wt/wt) where no excess aqueous medium was seen. X-ray diffraction confirmed that the phase present under these conditions at 20 °C was of the cubic Pn3m type with a lattice parameter of ~105 Å. This is consistent with previous work on related systems [2].

The second concern was that the additives themselves might alter the phase character of the dispersion. At high loading levels, this indeed is likely to be the case, especially for the longer-chain derivatives

[13]. However, at loading levels actually used, the system remained in the cubic Pn3m phase with a lattice parameter of ~105 Å for all additives studied, as determined by X-ray diffraction.

3.2. Modulating release

A home-built, multicompartament dialysis cell was used to study the release of additives from the cubic phase. The cubic phase was loaded initially to the extent of 4–11 mmol of additive per mole of lipid (~6 mg additive/g monoolein). This corresponds to a payload of 5–13 additive molecules per unit cell of the cubic phase. A unit cell of the cubic Pn3m phase at 20 °C is made up of ~1200 molecules of monoolein and ~15,000 molecules of water.

While the release measurement was carried out in a dialysis cell, the study itself was performed under infinite, or close to infinite, sink conditions. Thus, the sink solution, after accumulating a small amount of additive from the cubic phase on the source side of the membrane, was removed and analyzed for additive content. It was immediately replaced with fresh buffer devoid of additive, thereby imposing infinite sink conditions.

The results of the release study are presented as the fraction of additive lost from the cubic phase as a function of time (Fig. 6). The data show clearly that tryptophan is released rapidly from the cubic phase. Under conditions of measurement, essentially all the tryptophan escapes in a day. However, adding just a single methyl group to tryptophan slows release such that the same level of unloading requires almost a week. Lengthening the chain further significantly slows release. With 18 carbons in the chain, release has slowed to the point where less than 10% is lost over a 2-week period. Included in the study is *N*-oleoyl-tryptophan ethyl ester (Fig. 3G), which incorporates an 18-carbon amide and a two carbon ester. Unlike the other tryptophan additives examined, *N*-oleoyl-tryptophan ethyl ester is a neutral molecule. In this case, the long-chain additive becomes locked in the cubic phase with no release detectable on the time scale of the experiment. Some metrics characterizing the release of tryptophan and its alkylated derivatives are included in Table 1.

The interpretation of these data is that lengthening the alkyl chain attached to the water-soluble trypto-

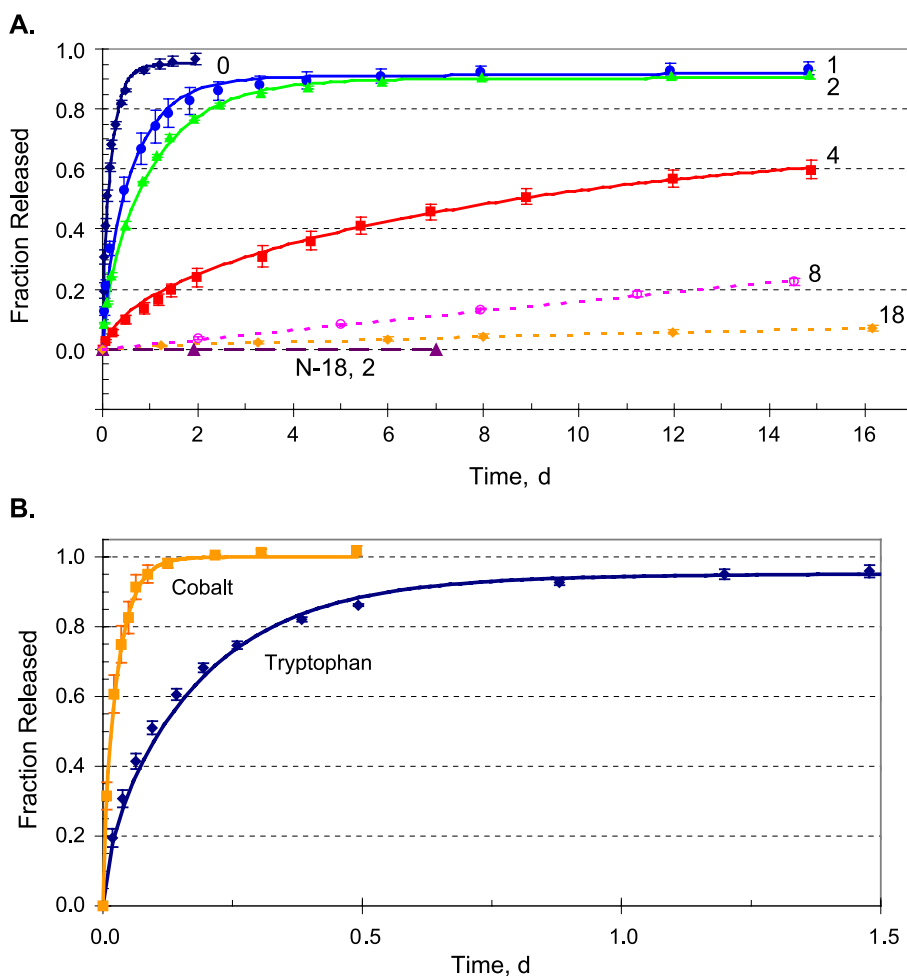


Fig. 6. Release profiles of additives from the cubic phase of hydrated monoolein as a function of time at 20 °C. (■) Cobalt ion (Co^{2+}); (◆) tryptophan; (●) tryptophan methyl ester; (▲) tryptophan ethyl ester; (■) tryptophan butyl ester; (○) tryptophan octyl ester; (◆) tryptophan oleoyl ester; and (▲) *N*-oleoyl-tryptophan ethyl ester. The number of carbon atoms in the alkyl chains of the tryptophan derivatives is indicated. The *N*-oleoyl-tryptophan ethyl ester release profile is labeled N-18, 2. The solid lines in (A) represent fits to the release data based on average D_F and k values obtained from the diffusion model (Table 2). Dashed straight lines were drawn to guide the eye. (B) Release profiles for cobalt and tryptophan are shown on an expanded time scale for clarity. Error bars are included for all data points and are based on triplicate measurements.

phan serves to anchor it more securely and to increase its residence time in the apolar compartment of the cubic phase. Thus, the concentration of additive in the aqueous channel is reduced and the driving force for release toward the (infinite) sink on the other side of the retaining membrane is lowered. A direct conclusion drawn from these data is that release of water-soluble drugs (as mimicked by tryptophan in this study) from the cubic phase can be manipulated by selective alkylation. The longer the chain, the slower is

the release from the hosting cubic phase. In this regard then, release rate can be rationally engineered by the appropriate choice of the modifying alkyl chain.

3.3. Partition coefficient

The release study above suggests that enhanced partitioning into the apolar part of the cubic meso-phase occurs as alkyl chain length increases. To test this hypothesis, the partition coefficient P for four of

Table 1
Effect of alkyl chain length on the characteristics of release from the cubic phase of tryptophan and its derivatives

Number of carbons in alkyl chain	Release in first 24 h (%)	Time for 20% release, $t_{20\%}$ (h)
0	94	0.5
1	75	1.3
2	61	3.3
4	14	35
8	2	300
18	0.01	≥ 300
(N-18) 2 ^a	0	–

^a Refers to *N*-oleoyl-DL-tryptophan ethyl ester.

the seven tryptophan derivatives used was measured with respect to the cubic phase. The study was limited to the more water-soluble compounds for reasons of sensitivity. The results in Fig. 7 (circles) show that P rose exponentially with chain length, as expected. Thus, P increased by a factor of 2–3 when chain length doubled from one to two carbons. In going from two to four carbons, the factor increase was 6. However, the line relating $\log P$ to the number of carbons in the alkyl chain N extrapolates to a value of P that is considerably in excess of that observed for free tryptophan at $N=0$. This may possibly reflect the fact that the underivatized amino acid is zwitterionic and more polar than the relevant alkylated derivatives that all bear a single positive charge.

As noted, for reasons of sensitivity, it was not realistic to extend the measurement of P to the longer-chained derivatives. However, an estimate of the tendency of organic molecules to partition between polar (water) and apolar (octanol) environments can be obtained using the KOWWIN program, as described under Experimental. The result is a $P_{o/w}$ value. All that is needed to calculate $P_{o/w}$ is a molecular structure. Obviously, therefore, the exercise is not limited by chain length, as in the experimental study above. Thus, $P_{o/w}$ values were calculated for all tryptophan derivatives used and the results are presented in Fig. 7 (squares).

As expected, $P_{o/w}$ rises exponentially with alkyl chain length from $N=1$ to $N=18$. This makes good sense since, within the series, the only major change is chain length. A $P_{o/w}$ value for the *N*-oleoyl-tryptophan ethyl ester was calculated also and is included in Fig. 7 with an assigned value for N of 20. Its $P_{o/w}$ lies above the simple alkyl ester line. This would appear reasonable given that the derivative is uncharged and thus less polar. What does not make immediate intuitive sense is the finding that $P_{o/w}$ for tryptophan, with $N=0$, is above the extrapolated line, suggesting that the free amino acid is less polar. Perhaps this can be explained by the fact that, being zwitterionic, it is electrically neutral and thus the KOWWIN program considers it more likely to partition into the apolar octanol than its cationic counterparts.

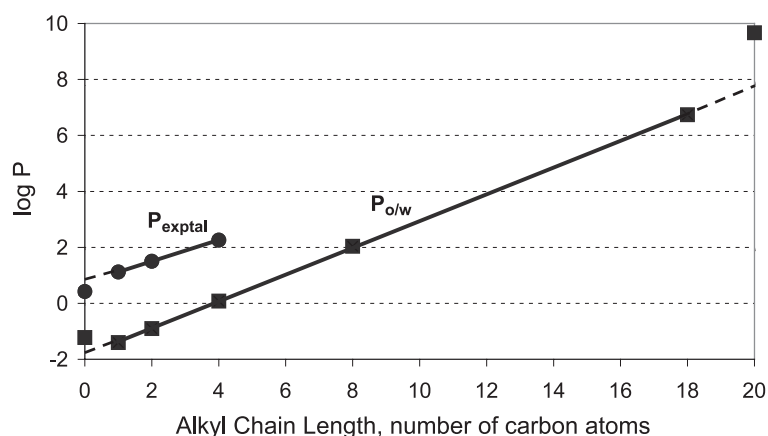


Fig. 7. Dependence of the partition coefficient for tryptophan and its derivatives on alkyl chain length. The experimental values (P_{exptal} , circles) measured with respect to the cubic phase of monoolein and the calculated $P_{o/w}$ (squares) values are included in the plot. Solid lines are best fits to the data in the range from $N=1$ to $N=18$. The corresponding lines of best fit are $\log P_{\text{exptal}}=0.38N+0.73$ ($R^2=1.00$) and $\log P_{o/w}=0.48N+1.85$ ($R^2=1.00$), where N is the chain length in number of carbon atoms.

Of particular note in Fig. 7 is fact that the experimental P (P_{exptal}) and calculated $P_{o/w}$ lines are displaced vertically, but that they have a similar slope. The common slope reflects a shared preference for an apolar environment that increases with alkyl chain length regardless of whether that environment is the interior of a lipid bilayer, as in the case of P_{exptal} , or bulk solvent, as in the case of $P_{o/w}$. The vertical displacement, however, is consistent with the view that the indole part of tryptophan has a preference for polar/apolar interfaces, which are present in the cubic phase but not (to the same degree at least) in the case of a water/octanol partitioning experiment.

The $P_{o/w}$ values for the longer-chained derivatives indicate that they extensively partition into the apolar phase with the highly apolar derivative, *N*-oleoyl-tryptophan ethyl ester, partitioning the most, as expected. This behavior correlates nicely with the release study results, where retention in the cubic phase is more pronounced for the longer-chained derivatives that partition preferentially into the lipid bilayer.

3.4. Diffusion model

As noted under Experimental, a Fickian model can be used to describe the experimental release profiles of tryptophan and its shorter-chain alkyl derivatives from the lipidic cubic phase shown in Fig. 6. The experimental P values in Table 2 were used to solve for D_F and k (Table 2), which in turn were used to generate the release profiles shown as solid lines in Fig. 6. The match between the model-generated profiles and the data is remarkably good, considering that the fits are based on just two adjustable parameters. It is interesting to note that the estimated $P_{o/w}$ value for tryptophan did not give a satisfactory

solution to its experimental release profile. This attests to the reliability of the P_{exptal} value for tryptophan.

The release data for the octyl and oleoyl tryptophan derivatives (Fig. 6A) could not be described satisfactorily with the current model. In both cases, release was extremely slow and essentially linear with time in the range measured. This latter feature makes the analysis difficult because the program needs some finite degree of curvature in the dependence of release on time to find reasonable values of D_F and k . In the case of the *N*-oleoyl-tryptophan ethyl ester, no release was observed (Fig. 6A) and so values for D_F and k are irrelevant.

A perusal of the data in Table 2 shows that the magnitude of D_F for tryptophan and its alkyl derivatives falls dramatically as chain length increases. The semilog plot in Fig. 8 indicates an exponential dependence. Thus, adding a single carbon, in going from tryptophan to the methyl tryptophan, causes D_F to decrease by a factor of 3.5. It decreases by 69-fold when four carbon atoms (butyl tryptophan) are added.

The rate constants describing partitioning into (k) and out of the bilayer (k/P) are tabulated in Table 2. These are referred to here as the ‘binding’ and ‘release’ rate constants. As with D_F , the values of k and k/P decrease exponentially as alkyl chain length increases. The observation that the rate constants for release are reciprocally related to alkyl chain length makes intuitive sense. The longer the chain, the more hydrophobic is the additive and the frequency with which it exits the apolar bilayer interior for the polar aqueous channels is expected to drop. Why the rate constant for binding should fall with chain length is not immediately obvious. It may have something to do with the declathrating of the alkyl chain of its water to suitably prepare that part of the additive for haven in the apolar recesses of the bilayer. It may also reflect the barrier nature of the polar/apolar interface, which must be breached as the alkyl tail bores through it.

A hypothesis to emerge from this latter discussion is that rate constants for binding and release should have magnitude zero for an additive that truly does not interact with the lipid bilayer. It was originally thought that this would be the case with the zwitterionic amino acid, tryptophan. However, the results indicate otherwise and tryptophan does appear to associate with the bilayer to a certain degree under

Table 2

Experimental partitioning and transport properties for tryptophan and its alkyl derivatives with respect to the cubic phase

Number of alkyl chain	P_{exptal}	D_F ($\times 10^8 \text{ cm}^2/\text{s}^{-1}$)	k ($\times 10^6 \text{ s}^{-1}$)	k/P_{exptal} ($\times 10^7 \text{ s}^{-1}$)
0	2.66	10.3 (0.9)	4.4 (2.1)	16.5 (8.0)
1	13.2	3.0 (0.8)	2.3 (0.9)	1.7 (0.7)
2	32.1	1.7 (0.1)	1.3 (0.2)	0.40 (0.06)
4	183	0.15 (0.02)	0.43 (0.12)	0.023 (0.007)

Numbers in parenthesis refer to the standard deviation ($n=3$).

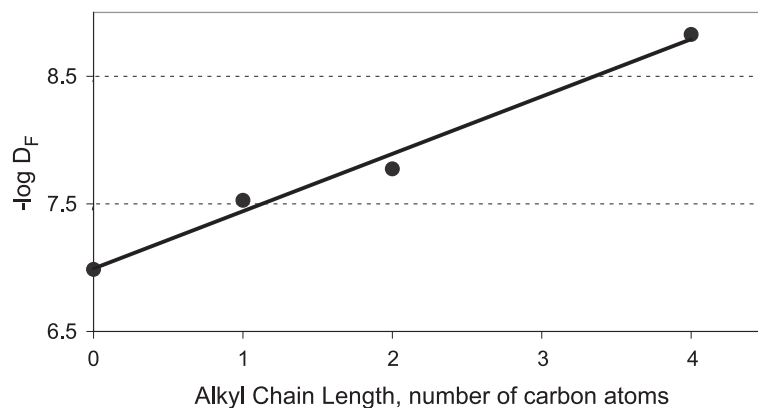


Fig. 8. Dependence of the diffusion coefficient (D_F) of tryptophan and its alkyl derivatives in the cubic phase on alkyl chain length. The line of best fit to the data is $\log D_F = 0.47N + 6.94$ ($R^2 = 0.99$), where D_F is in square centimeters per second (Table 2) and N is the chain length in number of carbon atoms.

present circumstances. To test the proposal, release measurements with cobalt chloride, a highly soluble salt, were repeated. It has a water solubility of 3.2 M at 20 °C [14], and the cobalt ion, which is a small (ionic diameter, 1.44 Å) [15], divalent cation, is easily quantified spectrophotometrically (see Experimental). As expected, the release of cobalt from the cubic phase was fast and complete (Fig. 6B). After 8 h under standard conditions, all of the cobalt was released. For comparison, ‘complete’ release of tryptophan under identical conditions required more than a day (Fig. 6B). The cobalt release data yielded a D_F value of 7.2×10^{-7} cm²/s, some seven times larger than that for tryptophan. The corresponding value of k was zero. This result supports the view that when an additive does not interact with the bilayer, the expected transport parameters are observed. It also lends credence to our findings that tryptophan associates to some finite degree with the lipid bilayer. However, it is important to point out that the latter interacts with the bilayer, but weakly in comparison to its longer-chain derivatives, that, when long enough, eventually ends up anchored there. Fig. 1B shows the probable locations of cobalt, tryptophan, and octyl tryptophan within the cubic phase.

3.5. Final phase state and transesterification

The release studies just described were set up under conditions where the initial hosting phase was of the cubic Pn3m type. Since the release experiments

were performed over protracted time periods in excess water and at 20 °C, the possibility existed for the lipid to undergo chemical change and for the initial phase to change also. This was investigated by taking samples of the lipidic material from the source chamber at the end of the release experiment. The samples were used in mesophase characterization by X-ray diffraction and in water content analysis by drying, followed by gravimetric analysis. Additional samples were used for chemical composition analysis by TLC.

It was found that neither the phase state nor the microstructure changed significantly when the release studies were of 1 week duration or less. Thus, for the cobalt, tryptophan, and *N*-oleoyl-tryptophan ethyl ester samples, the phase observed at the end of the investigation was of the cubic Pn3m type, with a lattice parameter of ~ 107 Å and a final hydration value of $\sim 42\%$ (wt/wt). However, in the case of the other tryptophan derivatives where the release study continued for 2 weeks, the final phase, while still cubic, was of the Im3m type with a lattice parameter of 152 ± 4 Å and a hydration level of $49 \pm 2\%$ (wt/wt).

Thus, prolonged incubation presumably facilitated a change in composition that triggered an alteration in cubic phase type. To determine the origins of this instability, the sample lipid profile was monitored during the course of the release experiment. The TLC results for one of the four solvent systems used (Fig. 9) clearly show that the sample after 7 days of incubation

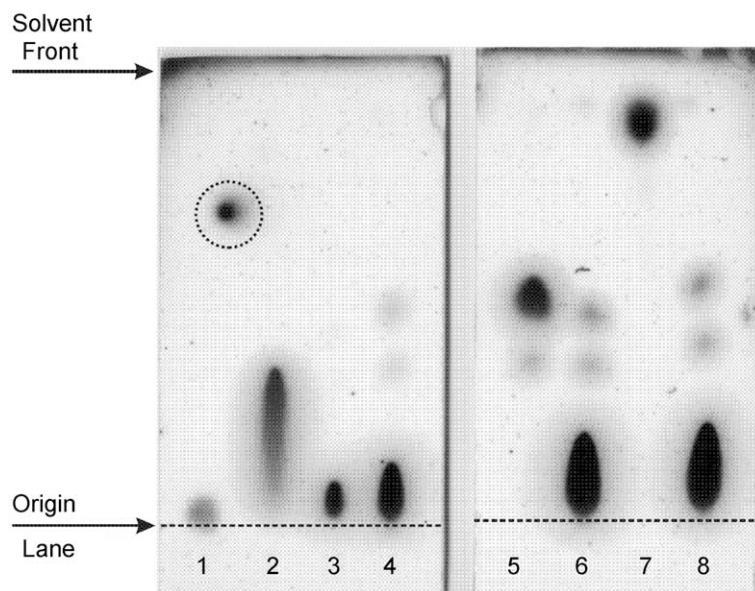


Fig. 9. Thin-layer chromatogram of vacuum-dried cubic phase 7 days after the start of the release experiment and of lipid standards. Sample identity (quantity used is reported in brackets) by lane is as follows: (1) glycerol (50 μg); (2) oleic acid (50 μg); (3) monoolein (50 μg); (4) vacuum-dried cubic phase (100 μg); (5) diolein (50 μg); (6) vacuum-dried cubic phase (300 μg); (7) triolein (50 μg); and (8) vacuum-dried cubic phase (300 μg). The development solvent used was chloroform/acetone (96/4 by vol). The positions of the origin and the solvent front are identified. Note that the diolein standard contains both 1,2-diolein and 1,3-diolein, which are clearly resolved in this system. The 1,3-diolein isomer has the highest mobility (front-running spot). The spot between lanes (1) and (2) identified by a dashed circle is an artifact and should be ignored. Quantitative analysis of spot density indicates that $\sim 0.8\%$ of the monoolein has converted to diolein and triolein during the course of the release experiment. The oleic acid and the 1,2-diolein standards comigrate in this solvent system. However, when the three other systems identified under Experimental were used, the oleic acid and diolein spots were clearly resolved (data not shown).

has small amounts of other lipids present in addition to the original lipid, monoolein. Comigration of these additional spots in the chromatogram with standard 1,2-diolein, 1,3-diolein, and triolein suggests that transesterification of the fatty acid between adjacent monoacylglycerols in the cubic phase takes place. The chromatograms were quantified as described under Experimental. These yield best estimates for the composition of the sample at the end of a 3-week incubation period at 99% monoolein, 1% diolein, and a trace of triolein. Glycerol and oleic acid standards were included in the chromatogram but no comigration was observed, suggesting that little, if any, of these breakdown products appears in the system. It is important to note, however, that both of these products, glycerol in particular, are water-soluble and may have exited the sample into the sink buffer during the course of the release experiment. Thus, not finding them by TLC is no guarantee that hydrolysis did not occur.

Based on these findings, a hypothesis was put forward that the lipids appearing late in the release study are responsible for the cubic Pn3m-to-cubic Im3m phase change. This was tested by quantifying the effect that diolein and triolein have on the phase behavior of hydrated monoolein by X-ray diffraction. The results (Fig. 10) show convincingly that neither lipid, individually nor together, is responsible for the phase change seen. In all cases, the neutral lipid additives trigger a contraction of the cubic Pn3m phase lattice and, at relatively high concentrations (4–8 mol%), induce inverted hexagonal (H_{II}) phase formation. The latter effect has been reported previously for diolein [16].

Despite the fact that oleic acid could not be seen in samples that had formed the cubic Im3m phase, as noted, it could not discount the possibility that it was responsible for the phase change. Accordingly, its effect on the phase properties of hydrated monoolein was investigated. Interestingly, the fatty acid triggers

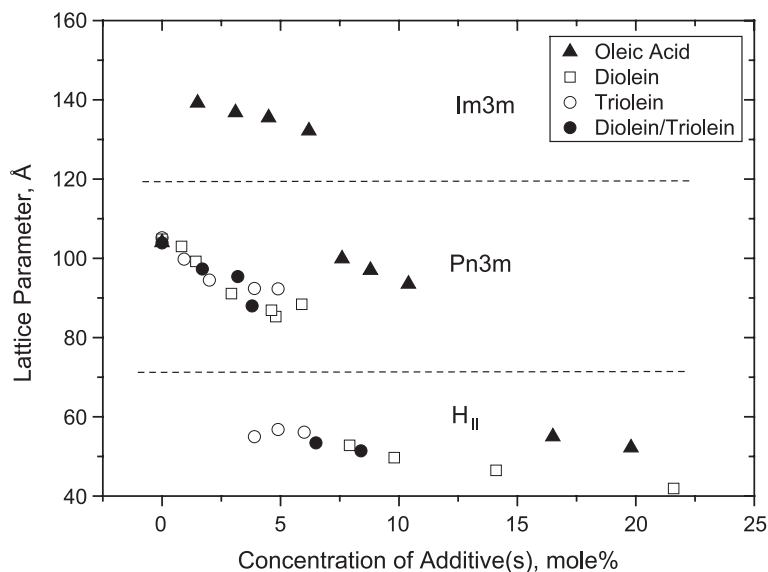


Fig. 10. Dependence of the lattice parameters of the phases formed by mixtures of monoolein and oleic acid, and diolein and/or triolein at 60% (wt/wt) buffer as determined by small-angle X-ray diffraction. Measurements were made at 20 °C. Additive identity: (▲) oleic acid, (□) diolein, (○) triolein, and (●) diolein/triolein mixture. Lipid concentration is expressed as mole percent and is calculated as $[100 \text{ (mol additive(s))} / (\text{mol additive(s)} + \text{mol monoolein})]$.

formation of the Im3m cubic phase variant and it does so at very low concentrations (Fig. 10). At higher concentrations, there is a reversion to the cubic Pn3m phase, which, with further additions of the acid, goes on to form the H_{II} phase. The disappearance and reappearance of the cubic phase in an additive-dependent way are not unusual [13].

Thus, it would appear to a first approximation that released oleic acid is responsible for the phase instability observed after lengthy incubation of hydrated monoolein. The oleic acid was not detected in such material, but this can be rationalized given the solubility of the acid and the likelihood that it simply diffused from the source compartment to the sink. The latter reasoning suggests that the cubic Im3m phase would revert to the Pn3m upon loss of the fatty acid. However, it is known that the liquid crystalline phases of lipids are notorious in their ability to exhibit phase metastability [2] and a failure to recover the cubic Pn3m phase may reflect this type of behavior.

The oleic acid formed presumably arises as a result of monoacylglycerol hydrolysis with glycerol as the second product. Diolein and triolein also form with time. These arise as a result of transesterification. It is known that such a reaction must occur

intramolecularly to produce an equilibrium mixture of 1-monoacylglycerol and 2-monoacylglycerol [17]. Presumably, it can also happen between molecules in a way that is facilitated by the proximity of the glycerol headgroups at the polar/apolar interface of the bilayer within the cubic phase. Since transfer is to a degree random, there is much less likelihood of forming triolein compared to diolein. This is borne out in the TLC analysis where the relative amounts of the two diolein isomers far exceed that of the triolein (Fig. 9).

3.6. Alkyl tryptophan stability

All but one of the alkyl tryptophan derivatives used in this study are simple esters. While chemically stable, hydrolysis might occur during the course of the release study, thus compromising the data. The issue was examined using butyl tryptophan as a representative and ¹H NMR to track changes during the course of the incubation. The results (see Experimental) show that the spectrum did not change during the 6-day trial supporting the view that the derivatives are indeed stable. A separate stability study was performed using methyl tryptophan in a 50/50 mix of deuterated

DMSO and deuterated water. Again, no degradation was found. While the stability measurements were performed in solution, it was noted that, for technical reasons, they were not carried out with the alkyl derivatives incorporated into the cubic phase.

3.7. Implications

This study demonstrates that it is possible to tailor over wide limits the rate at which a small, water-soluble organic molecule is released from the cubic phase. Tryptophan was used here as a drug surrogate because of its spectroscopic properties, which allow it to be easily quantified. By suitably alkylating the target molecule, rates can be adjusted to the point where release takes place over a period that can vary from a day to many weeks. The latter is advantageous in situations where repeat dosages are simply not practical for logistical, economic, and other reasons.

The release profiles for tryptophan and its derivatives have been quantified experimentally. In addition, a mathematical model has been presented and used to describe the transport properties of the shorter-chained derivatives where release is relatively fast. In

conjunction with the modeling effort, the coefficients describing partitioning between the apolar and polar compartments of the cubic phase were measured. As expected, the longer-chained alkyl tryptophans partition in favor of the former in a way that correlates nicely with measured transport. The investigation was expanded to include inorganic ions that should not partition at all into the apolar compartment of the cubic phase. The experimental and theoretical results were entirely consistent with expectations, lending credence to the model. The parameters that describe transport emerging from the modeling exercise can now be used to predict behaviors such as release and uptake in clinically relevant applications. In the examples that follow, these metrics (Table 2) are used to predict the release profile from a bolus of cubic phase having different source geometries and sizes, and different transport properties (Fig. 11). The geometries chosen for investigation include a sphere, a cylinder, and a slab. To facilitate comparisons, a fixed diffusion path of 0.68 mm was used, which corresponds to the thickness of the ‘slab’ used experimentally (see Experimental and Fig. 6). While the slab simulates release from a patch, as in a

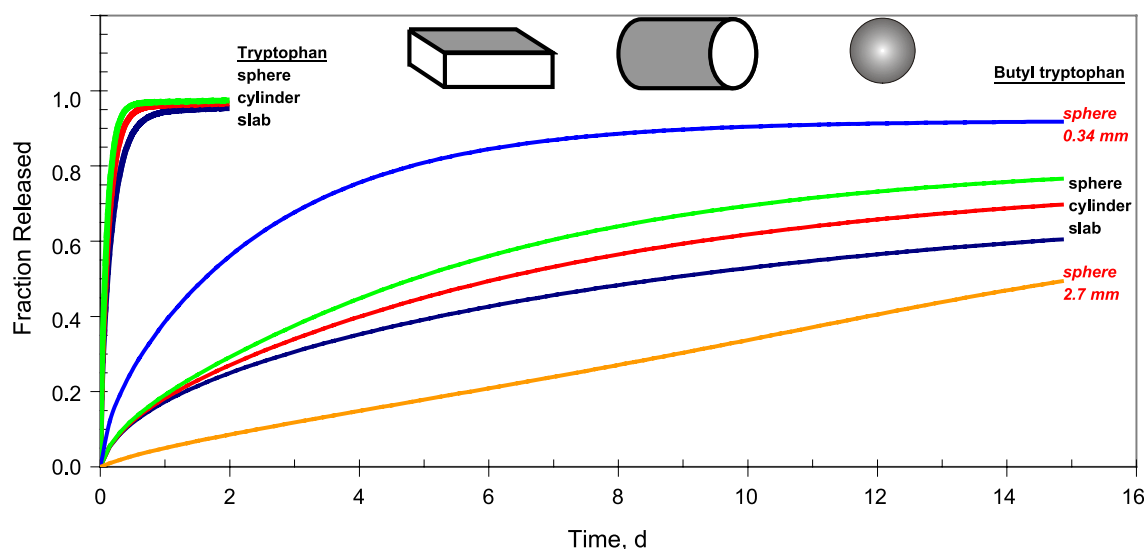


Fig. 11. Predicted release profiles for tryptophan and butyl tryptophan from the cubic phase under infinite sink conditions as influenced by source geometry and volume. Profiles were generated using MatLab and the partitioning and transport properties in Table 2. A radius of 0.68 mm was used for the cylinder and sphere geometries, unless otherwise noted. The slab (0.68 mm thickness) geometry corresponds to the fit to the experimental release data in Fig. 6. Release from the sphere was from its entire surface area. Release from the cylinder was limited to its curved surface with no release from either end. Release only occurred from one of the flat surfaces of the slab as prevailed in the actual experimental device (Fig. 4).

transdermal delivery application, the sphere, and possibly the cylinder, may be taken as representing geometries encountered with cubosome delivery. For a given diffusion path, the simulations show that release is fastest from the sphere, followed in turn by the cylinder and the slab. As expected, the release profile lengthens with source volume and with transport properties that reflect enhanced association with the lipid bilayer of the cubic phase. This type of analysis should prove useful in designing delivery systems for specific applications.

The transport studies with tryptophan provided a surprising result in that release was unexpectedly tardy. This is in contrast to cobalt where release was complete in several hours (Fig. 6B). The disparate behavior was explained, in part, by the measured partition coefficient, which shows that the amino acid, despite being zwitterionic, preferentially associates with the lipid component of the cubic phase. This is an interesting result that supports the idea that the indole of tryptophan has an affinity for the polar/apolar interface of membranes and may contribute to orienting proteins in and on membranes [18]. What sets the current finding apart is the fact that the lipid used here has a simple headgroup (viz., glycerol with a single ester linkage). Further, the interface is one of extreme curvature since it forms the lipidic fabric of the cubic mesophase (Fig. 1).

The integrity of the lipidic cubic phase was monitored throughout this study. The results show that the lipid is quite stable, which, in turn, translates to a mesophase that remained cubic and functional for the duration of the investigation. However, over protracted periods, a small change (<1%) in the lipid fraction does occur. This is attributed to transesterification and hydrolysis reactions that give rise to trace amounts of diolein, triolein, oleic acid, and glycerol. The former was identified tentatively as the culprit responsible for the alteration in cubic phase state that occurred at long incubation times. Such change is not unexpected given the prevailing experimental conditions that include excess water, mild temperatures, and long times. This is not unlike what might obtain *in vivo*, and the results point to adjustments likely to take place under conditions of use.

The modifications used in this study involve alkylation where chains of varying lengths were

combined with the α -carboxyl group of tryptophan by way of an ester linkage. In this case, the tryptophan that starts out as a zwitterion ends up bearing a single positive charge. Within this ammonium ion series, the release rate decreased exponentially as alkyl chain length increased. But even with a chain 18 carbon atoms long, there was some measurable release (Fig. 6A). However, when the tryptophan was rendered neutral by linking the 18-carbon chain to the amino group and by ethylating the carboxyl group, release was halted completely. This illustrates how sensitive release from the cubic phase is to the type of modification(s) imposed.

The focus of this study was primarily on modifications of the ester type where chain length controls release rate. Extending the work into the realm of drug administration may be direct in the case where having an alkyl chain in ester linkage with the pharmaceutical is inconsequential to its activity. It is possible too that chain removal will be required for activation. In this case, the hydrolysis reaction may occur spontaneously upon release of the derivative from the cubic phase bolus. It may also be accelerated by esterases that are ubiquitous in tissues. In these situations, the availability of the active drug would be modulated not only by release from the cubic phase but also by the rate of hydrolysis.

As demonstrated in this work, hydrophobicity and the tendency to partition into apolar environments rise with alkylation. For drugs that can be delivered transdermally, alkylation has been shown to enhance penetration [19]. Thus, alkylation as introduced in this study may serve double duty. On one hand, it will increase delivery across the dermal barrier. On the other hand, it will serve to anchor the drug in the apolar fraction of the skin, which can then act as a second reservoir. Release from the latter will also be controlled by the length of the alkyl chain.

A new theory for how the body responds immunogenically has been presented [20]. It explains the immune response to self and nonself molecules on the basis of a recognition of hydrophobic features shared by both. The theory is supported by convincing experimental evidence showing that antigen-presenting cells are activated by a host of molecular types, all of which have a hydrophobic moiety of varying size and complexity. The alkylation introduced in the

current study does just that: it adds a hydrophobic entity to a water-soluble molecule. If the latter is an antigen, then alkylation is likely to improve immunogenicity. This proposal for making a more effective vaccine is under investigation (Seong et al., in preparation).

The current application focuses on the lipidic cubic phase as a drug delivery system. There are others in use, such as polymers [21,22] and vesicles [23,24], that, by analogy to the cubic phase, have polar and apolar compartments. Thus, controlling release rate by selective and judicious alkylation, as demonstrated here with the cubic phase system, should apply to these other systems, too. In the case of multilamellar vesicles, drug movement would have to occur from the inner to outer bilayered lipid leaflets and eventually into the surrounding medium. With polymer delivery, the route taken by the alkylated drug would depend on the microstructure of the formulation, which could, in certain circumstances [25], be similar to that of the bicontinuous cubic phase system described here. Thus, the transport properties quantified in the current study should provide an indication of how alkylated drugs will move in these other related systems.

4. Conclusions

Exquisite control over the rate at which water-soluble molecules diffuse from the lipidic cubic phase has been demonstrated. This has far-reaching implications in drug delivery where regulated release is critical to performance. The means by which control was effected exploited the dual hydrophobic and hydrophilic properties of the cubic mesophase. Under the standard conditions implemented here (infinite sink with a 30-mg cubic phase source at pH 7 and 20 °C), release of tryptophan, which served as a surrogate drug, was essentially complete in a day. By alkylating it with chains of increasing length, transport rates plummeted to the point where release extended to months with chains eight carbon atoms long and above. Release was also affected by changing simultaneously the alkyl linkage type and by rendering the target molecule uncharged. In the latter case, the modifications caused the molecule to remain anchored in the cubic phase and no release was

detected. Thus, by a judicious choice of chain length and type of linkage, release rates can be engineered over very wide limits.

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References

- [1] J.C. Shah, Y. Sadhale, D.M. Chilukuri, Cubic phase gels as drug delivery systems, *Adv. Drug Deliv. Rev.* 47 (2001) 229–250.
- [2] H. Qiu, M. Caffrey, The phase diagram of the monoolein/water system: metastability and equilibrium aspects, *Biomaterials* 21 (2000) 223–234.
- [3] M. Caffrey, Membrane protein crystallization, *J. Struct. Biol.* 142 (2003) 108–132.
- [4] K. Lindell, J. Engblom, M. Jonstroemer, A. Carlsson, S. Engstroem, Influence of a charged phospholipid on the release pattern of timolol maleate from cubic liquid crystalline phases, *Prog. Colloid & Polym. Sci.* 108 (1998) 111–118.
- [5] M.L. Lynch, A. Ofori-Boateng, A. Hippe, K. Kochvar, P.T. Spicer, Enhanced loading of water-soluble actives into bicontinuous cubic phase liquid crystals using cationic surfactants, *J. Colloid Interface Sci.* 260 (2003) 404–413.
- [6] A. Cheng, B. Hummel, H. Qiu, M. Caffrey, A simple mechanical mixer for small viscous samples, *Chem. Phys. Lipids* 95 (1998) 11–21.
- [7] K.A. McCall, C.A. Fierke, Colorimetric and fluorimetric assays to quantitate micromolar concentrations of transition metals, *Anal. Biochem.* 284 (2000) 307–315.
- [8] S. Engstrom, T.P. Norden, H. Nyquist, Cubic phases for studies of drug partition into lipid bilayers, *J. Pharm. Sci.* 84 (1999) 243–254.
- [9] W.M. Meylan, P.H. Howard, Atom/fragment contribution method for estimating octanol–water partition coefficients, *J. Pharm. Sci.* 84 (1995) 83–92.
- [10] V. Cherezov, H. Qiu, V. Pector, M. Vandenbranden, J.M. Ruyschaert, M. Caffrey, Biophysical and transfection studies of the diC14-amidine/DNA complex, *Biophys. J.* 82 (2002) 3105–3117.
- [11] T.N. Blanton, T.C. Huang, H. Toraya, C.R. Hubbard, S.B. Robie, D. Louer, H.E. Gobel, G. Will, R. Gilles, T. Raftery, JCPDS—International centre for diffraction data round robin study of silver behenate. A possible low-angle X-ray

- diffraction calibration standard, Powder Diffr. 10 (1995) 91–95.
- [12] A.P. Hammersley, S.O. Svensson, M. Hanfland, A.N. Fitch, D. Hausermann, Two-dimensional detector software: from real detector to idealized image or two-theta scan, High Press. Res. 14 (1996) 235–248.
- [13] V. Cherezov, J. Clogston, Y. Misquitta, W. Abdel-Gawad, M. Caffrey, Membrane protein crystallization in *meso*: lipid type-tailoring of the cubic phase, Biophys. J. 83 (2002) 3393–3407.
- [14] D. Linde, CRC Handbook of Chemistry and Physics, 84th ed., CRC Press, Boca Raton, FL, 2004.
- [15] R. Chang, Chemistry, McGraw-Hill, New York, NY, 2002.
- [16] J. Borne, T. Nylander, A. Khan, Microscopy, SAXD, and NMR studies of phase behavior of the monoolein–diolein–water system, Langmuir 16 (2000) 10044–10054.
- [17] R.A. Coleman, D.P. Lee, Enzymes of triacylglycerol synthesis and their regulation, Prog. Lipid Res. 43 (2004) 134–176.
- [18] J. Deisenhofer, H. Michel, The photosynthetic reaction centre from the purple bacterium *Rhodospseudomonas viridis*, EMBO J. 8 (1989) 2149–2170.
- [19] T. Akimoto, Y. Nagase, Novel transdermal drug penetration enhancer: synthesis and enhancing effect of alkylsiloxane compounds containing glucopyranosyl group, J. Control. Release 88 (2003) 243–252.
- [20] S.Y. Seong, P. Matzinger, Hydrophobicity, an ancient damage-associated molecular pattern that initiates innate immune responses, Nat. Rev., Immunol. 4 (2004) 469–478.
- [21] O. Pillai, R. Panchagnula, Polymers in drug delivery, Curr. Opin. Chem. Biol. 5 (4) (2001) 447–451.
- [22] P. Gupta, K. Vermani, S. Garg, Hydrogels: from controlled release to pH-responsive drug delivery, Drug Discov. Today 10 (2002) 569–579.
- [23] W. Mehnert, K. Mader, Solid lipid nanoparticles: production, characterization and applications, Adv. Drug Deliv. Rev. 47 (2001) 165–196.
- [24] M.A. Al-Meshal, S.H. Khidr, M.A. Bayomi, A.A. Al-Angary, Oral administration of liposomes containing cyclosporine: a pharmacokinetic study, Int. J. Pharm. 168 (1998) 163–168.
- [25] H. Matsuyama, M. Teramoto, H. Urano, Analysis of solute diffusion in poly (vinyl alcohol) hydrogel membrane, J. Membr. Sci. 126 (1997) 151–160.
- [26] B. de Foresta, J. Gallay, J. Sopkova, P. Champeil, M. Vincent, Tryptophan octyl ester in detergent micelles of dodecylmalto-side: fluorescence properties and quenching by brominated detergent analogs, Biophys. J. 77 (1999) 3071–3084.