

See discussions, stats, and author profiles for this publication at: <https://www.researchgate.net/publication/5235595>

Comparison of the interaction of tomatine with mixed monolayers containing phospholipid, egg sphingomyelin, and sterols

ARTICLE in BIOCHIMICA ET BIOPHYSICA ACTA · JULY 2008

Impact Factor: 4.66 · DOI: 10.1016/j.bbamem.2008.06.004 · Source: PubMed

CITATIONS

15

READS

14

3 AUTHORS, INCLUDING:



Keith J. Stine

University of Missouri - St. Louis

70 PUBLICATIONS 1,752 CITATIONS

[SEE PROFILE](#)



Comparison of the interaction of tomatine with mixed monolayers containing phospholipid, egg sphingomyelin, and sterols

Barry W. Walker, Nathan Manhanke, Keith J. Stine *

Department of Chemistry and Biochemistry, University of Missouri – St. Louis, One University Boulevard, Saint Louis, MO 63121, USA
Center for Nanoscience, University of Missouri – St. Louis, One University Boulevard, Saint Louis, MO 63121, USA

ARTICLE INFO

Article history:

Received 16 February 2008

Received in revised form 19 May 2008

Accepted 3 June 2008

Available online 13 June 2008

Keywords:

Phospholipid

Sphingomyelin

Cholesterol

Sterol

Tomatine

Glycoalkaloid

Monolayer

ABSTRACT

The interaction of the glycoalkaloid tomatine with monolayers of a phospholipid (dimyristoylphosphatidylcholine, DMPC), and sphingolipid (egg sphingomyelin), and cholesterol is compared. Using measurements of the surface pressure response as a function of the subphase concentration of tomatine, interfacial binding constants are estimated for mixed monolayers of DMPC and cholesterol and for those of egg sphingomyelin and cholesterol of mole ratio 7:3. The binding constants obtained suggest a stronger interaction of tomatine with DMPC and cholesterol mixed monolayers, reflecting easier displacement of cholesterol from its interaction with DMPC than from its interaction with egg sphingomyelin. Mixtures of tomatine and cholesterol are found to spread directly at the water-air interface and form stable monolayers, suggesting that cholesterol holds tomatine at the interface despite the absence of observed monolayer behavior for tomatine alone. The interaction of tomatine with DMPC and cholesterol monolayers is found to exhibit a pH dependence in agreement with previously reported results for its interaction with liposomes; in particular, the interaction is much less at pH 5 than at pH 7 or pH 9. It is found that while tomatine interacts strongly with monolayers containing sitosterol, it does not interact with monolayers containing sitosterol glucoside. The response of monolayers of varying composition of DMPC and cholesterol to tomatine is also examined. Brewster angle microscopy (BAM) reveals further evidence for formation of suspected islands of tomatine+cholesterol complexes upon interaction with mixed monolayers of lipid and sterol.

© 2008 Elsevier B.V. All rights reserved.

1. Introduction

The glycoalkaloids are a family of compounds found in plants that consist of a steroid nitrogen-containing aglycone and an attached oligosaccharide (Fig. 1). A large literature exists concerning the agricultural and biological consequences of these compounds, and has been reviewed by Friedman [1,2]. These compounds are a part of plant defense mechanisms against pests and pathogens, although the relationships uncovered are complex and still under investigation [3–4]. The glycoalkaloids bind strongly to 3 β -hydroxy sterols and form complexes, resulting in membrane disruption [5–7] and thus are interesting compounds to study with respect to their interactions with monolayers and other model membrane systems. The presence of the intact oligosaccharide is required; removal of one or more sugar units drastically reduces the ability of these compounds to bind sterols [7]. A widely studied glycoalkaloid is tomatine, found in the tomato plant, whose properties have been extensively reviewed [1]. The level of tomatine in the tomato fruit decreases during ripening, while the levels in the rest of the plant remain significant.

The complex formed between tomatine and cholesterol has been found in precipitation experiments to be 1:1 in stoichiometry [7,8]. The activity of tomatine is maximal at pH 7.2 [5], and exposure to harshly acidic conditions at 100 °C is required to fully hydrolyze the oligosaccharide portion of tomatine. [9] The main direct biological effects of glycoalkaloids on mammals are membrane disruption by sterol binding and cholinesterase inhibition [10]. Glycoalkaloids have been explored for potentially useful applications due to their observed activity against certain cancer cell lines [11,12] and tomatine has been reported to have use as an immunological adjuvant [13,14]. The compound exhibits antifungal activity [15], inhibits feeding by snails [16], and can deter feeding by a variety of insects [17,18]. Fungi can resist these compounds by acidifying their environment or expressing enzymes that cleave sugar units off of the oligosaccharide [19].

The interaction of glycoalkaloids with liposomes was investigated by examining the promotion of the release of horseradish peroxidase encapsulated within liposomes by tomatine [5]. Tomatine promoted greater enzyme release from liposomes composed of egg yolk phosphatidylcholine and cholesterol than from those composed of bovine brain sphingomyelin and cholesterol. Tomatine promoted electrolyte leakage from plant tissues except from tomato and potato, which include low amounts of free sterol [15]. Glycoalkaloids promoted release of the self-quenching dye carboxyfluorescein from

* Corresponding author. Tel.: +1 314 516 5346.

E-mail address: kstine@umsl.edu (K.J. Stine).

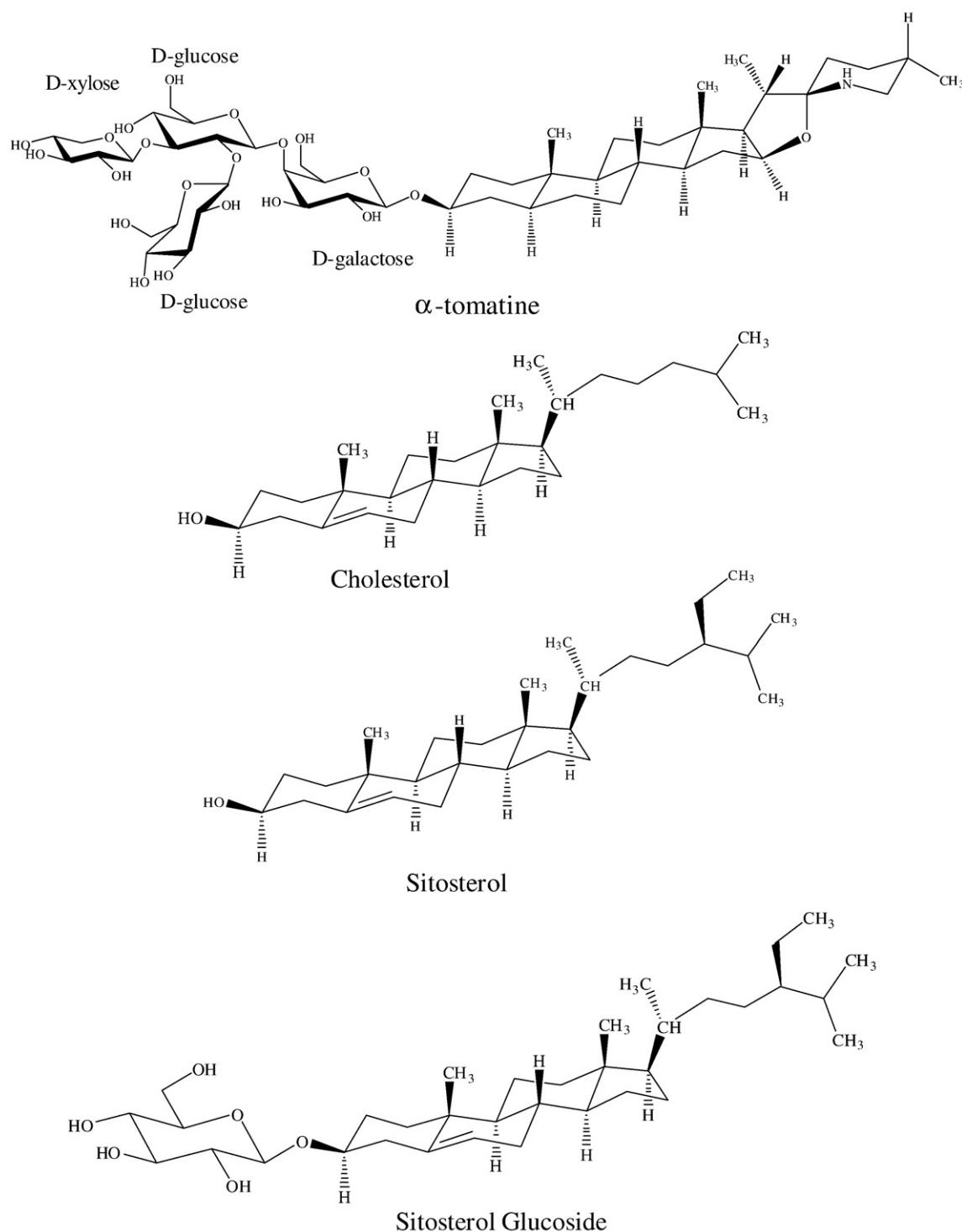


Fig. 1. Structures of α -tomatine, cholesterol, sitosterol, and sitosterol glucoside.

sterol-containing liposomes in a manner highly dependent on the sterol structure and on the presence of the intact oligosaccharide [7]. A model was proposed in which the glycoalkaloid binds to the sterols in the outer leaflet of the bilayer, these complexes undergo lateral aggregation, and the geometrical requirements of the glycoalkaloid oligosaccharide groups results in formation of tubular or globular structures rich in the 1:1 glycoalkaloid+sterol complexes that disrupt membrane integrity and result in the leakage of encapsulated molecules. Evidence for such structures was found in electron microscopy studies of liposomes and cells treated with tomatine. Tomatine has found application in adjuvant formulations, stimulating

the immune response to ovalbumin in mice [20–22]. The immune response was primarily the generation of antigen-specific cytotoxic T-lymphocytes. Use of the tomatine adjuvant in vaccination experiments on mice demonstrated enhanced immune responses to malaria [23]. The tomatine adjuvant was found to be non-toxic and preferable to certain saponin adjuvants. The mechanism by which the tomatine adjuvant helps to bring antigen into antigen-processing cells has not yet been elucidated but likely involves permeabilization of the membranes of antigen-presenting cells.

In our prior study, the interaction of tomatine with monolayers of phospholipid dimyristoylphosphatidylcholine (DMPC) and a range of

sterols was examined [24]. It was found that the strength of the interaction was highly sensitive to sterol structure and especially to the stereochemistry of the 3 β -hydroxy group since the morphology changes observed for cholesterol-containing monolayers were not seen for epicholesterol and the surface pressure response for epicholesterol was much smaller. The surface pressure response of a DMPC monolayer was small, while that of a cholesterol monolayer was very large. In mixed monolayers, visual evidence was found for bright island formation after interaction with tomatine which was proposed to insert into the monolayer and complex with cholesterol followed by separation away from the lipid for some of the sterols studied. For monolayers containing DMPC and coprostanol, evidence for the formation of chiral domains after the interaction was noted.

In the present study, we examine additional details of the interaction of tomatine with monolayers beyond the sensitivity to sterol structure previously studied. The response to subphase tomatine as a function of concentration is studied and analyzed in order to extract an effective interfacial binding constant for the overall interaction. The effect of changing the lipid from phospholipid DMPC to egg sphingomyelin is examined, and the response to tomatine as a function of concentration studied for this case as well. Sphingomyelin was chosen for comparison with DMPC in these experiments based on the significance of sphingolipids [25] in formation of 'raft' domains rich in cholesterol [26] believed to have significance for hosting proteins involved in cell signaling pathways [27] and their implication in cell signaling as ligands [28]. The choice of sphingomyelin also was motivated by the reported stronger interaction of cholesterol with sphingolipids than with phospholipids, attributed to the presence of a hydrogen-bond accepting amide group in the sphingolipid not present in the phospholipid [29], and how this difference might manifest itself in the interaction with tomatine. The behavior of directly spread binary mixtures of tomatine and cholesterol is examined to provide evidence for interfacial complexation and monolayer formation. Ternary spread mixtures of lipid, cholesterol, and tomatine are also examined with the goal of determining if similar morphologies can be seen using Brewster angle microscopy on spread monolayers containing tomatine as observed during the interaction of lipid and cholesterol monolayers with injected tomatine. Additional data on the response of the monolayers as a function of mole fraction of cholesterol is presented. The response of sitosterol and sitosterol glucoside containing monolayers is compared given the widespread interest in glycosylated plant sterols and how their presence effects the interaction of glycoalkaloids with membranes.

2. Materials and methods

Experiments were conducted in a home-built Teflon trough positioned beneath a BAM-1plus Brewster angle microscope (Nano-film Technologies) at 21±1 °C. This configuration was used for the studies of directly spread monolayers. For the injection experiments, the main trough (24 cm×7 cm water surface) was augmented by the addition of a smaller Teflon insert trough on the side of the trough where the surface pressure transducer is located and the laser beam of the BAM is incident on the water surface. The BAM-1plus does not have a synchronized mechanical mirror scanner to correct for focus across the entire image as do the more recent BAM instruments; therefore, there is some loss of focus in the left and right sides of the images due to the fact that the monolayer is being imaged at an angle. The images therefore have been cropped to remove a 100 μm strip on the left and right edges of the image where the focus is poor. The subphase in the insert (volume=14 mL) is separate from the main subphase (volume~150 mL) with the monolayer allowed to flow across a 4.0 mm wide and 2.5 mm deep canal in the Teflon wall of the insert. The lower stage of the insert encloses a miniature cuvette stirrer (Spinette Magnetic Cell Stirrer, Starna Cells) beneath the upper stage of the insert containing the subphase. The cuvette stirrer drives a

miniature stir bar used to gently stir the subphase in the insert. The insert is placed in the trough underneath the illumination of the BAM and the surface pressure transducer (Nima ST9000) from which a piece of filter paper serves as the Wilhelmy plate. Surface pressure data are acquired by a multifunction board in a PC via a program written in GW-Basic. With this configuration, it is possible to carry out experiments in which the monolayer is compressed to an initial surface pressure prior to injection of the tomatine solution. In order to isolate the monolayer in the insert just prior to injection, a small Teflon 'gate' in the form of a 8 mm×14 mm piece of 0.5 mm thick Teflon that fits neatly into a slot is placed across the canal to prevent flow of the monolayer back into the main trough due to surface pressure gradients and leakage of the injected glycoalkaloid back into the main subphase volume.

Dimyristoylphosphatidylcholine (DMPC) and egg sphingomyelin (egg SM) were obtained from Avanti Polar Lipids (Alabaster, AL) and used as received. Cholesterol was obtained from Steraloids (Newport, RI) and used as received. β -Sitosterol and β -sitosterol glucoside were obtained from Chromadex (Irvine, CA) and used as received. The subphase was buffered to pH 7 using a 0.05 M phosphate buffer prepared in Millipore water, which was also 0.10 M in NaCl. Potassium dihydrogen phosphate (99.99%), potassium hydrogen phthalate (99.5%–100.5%), and sodium hydroxide (99.99%) were obtained from Sigma-Aldrich (St. Louis, MO), sodium chloride (metals-basis grade, 99.99%) was obtained from Alfa-Aesar (Ward Hill, MA). Sodium acetate (Biochemika grade) was obtained from Sigma and used to prepare pH 5.0 acetate buffer, also of 0.10 M NaCl concentration. Sodium tetraborate (99.98%) was obtained from Aldrich and used to prepare pH 9.0 borate buffer, also of 0.10 M NaCl concentration. α -Tomatine was obtained from Sigma (St. Louis, MO) and used as received. Solutions of tomatine of concentration 280–300 μ M were prepared in 0.010 M pH 5.0 phthalate buffer that was also 0.60 M in NaCl. The solutions were prepared fresh for each experiment. The solutions were injected through an angled needle hole in the side of the insert trough using either a 25.0 μ L, 100 μ L, or 250 μ L microsyringe, depending upon the desired final subphase concentration, into the subphase beneath the monolayer. The addition of NaCl to the buffer was found to be important as it results in the injected solution having a greater density than the density of the solution in the trough, which prevents the injected drops from rising after injection and promotes better mixing prior to diffusion and prevents artificially rapid increases in surface pressure after injection. The injected volumes had a small effect on the water level and the immersion of the Wilhelmy plate, resulting in a small shift in surface tension of ~0.10 mN m⁻¹ for injection of 50 μ L.

Monolayers were spread from solutions in chloroform (Fisher Scientific, Optima grade). After spreading and allowing 15 min for solvent evaporation, the monolayers were compressed at 4.0 Å² molecule⁻¹ min⁻¹ to the target surface pressure during injection experiments. Compression isotherms used a rate of 2.0 Å² molecule⁻¹ min⁻¹ for monolayers of sterols or of spread mixtures with tomatine, and of 4.0 Å² molecule⁻¹ min⁻¹ for mixed monolayers of lipid and sterol. The slower rate used for the sterol and sterol+tomatine mixtures was chosen as these isotherms are steeper and it was deemed best not to traverse the steep portion of the isotherm too quickly. Upon reaching the target surface pressure, the Teflon 'gate' was inserted across the canal to isolate the monolayer in the smaller insert trough, and then after 5 min gentle stirring was initiated at ~10–20 rpm. Glycoalkaloid dissolved in buffer was then injected into the subphase after waiting an additional 5 min for monolayer relaxation and approach to an initial equilibrium surface pressure. Prior to the injection, the monolayer surface pressure would typically relax by 0.5–1.0 mN m⁻¹ for the mixed monolayers of lipid and sterol. Subsequently, the surface pressure versus time was followed while the subphase was stirred. It has long been known that coverage of a water surface by a monolayer reduces the evaporation rate of water, with the

extent of the retardation of evaporation dependent on the amphiphile and on the surface pressure [30]. When the canal is closed by insertion of the Teflon ‘gate’, the monolayer and the subphase in the insert trough segment are separated from the remaining bulk of the water in the trough, including the uncovered water behind the main barrier. Monolayers of 7:3 DMPC and cholesterol (for example) compressed to 10 mN m^{-1} and then followed for 120 min showed small change in surface pressure of typically around $+0.5 \text{ mN m}^{-1}$, possibly due to the slow accumulation of airborne impurities. Evidence for an evaporation effect of significance was not observed in these experiments.

The fitting of the $\Delta\Pi$ versus subphase concentration of glycoalkaloid was carried out using the hyperbolic function fit available in SigmaPlot 10.0 (Systat Software, San Jose, CA).

3. Results

The response of mixed monolayers of DMPC and cholesterol of 7:3 mole ratio, chosen as a simple model membrane system, to injection of tomatine to varying subphase concentrations was studied by measurements of $\Delta\Pi$ versus concentration. The data for the maximum $\Delta\Pi$ as a function of concentration was analyzed following the model applied by Ruyssen and Joos [31] to the penetration of cholesterol monolayers by digitonin. Penetration of monolayers by

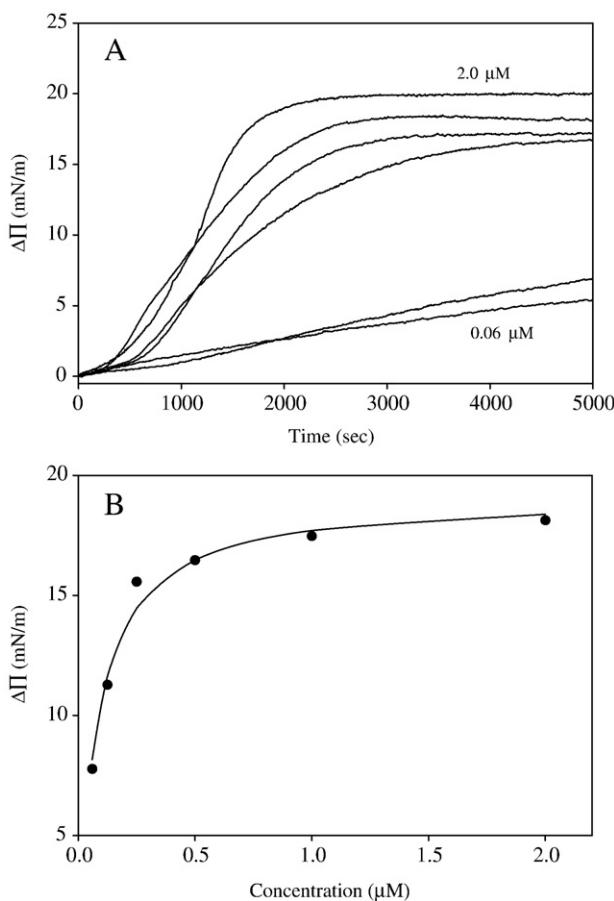


Fig. 2. (A) The response of mixed monolayers of 7:3 mole ratio DMPC/cholesterol to injection of varying concentrations of tomatine. The monolayers were compressed to 10 mN m^{-1} prior to injection. The concentrations studied were $2.0 \mu\text{M}$, $1.0 \mu\text{M}$, $0.5 \mu\text{M}$, $0.25 \mu\text{M}$, $0.125 \mu\text{M}$, and $0.06 \mu\text{M}$. At the later times, these curves fall in order of concentration on the graph from lowest to highest. The subphase is $0.05 \text{ M pH } 7.0$ phosphate buffer, 0.10 M NaCl . (B) Binding curve analysis of the measured plateau values of $\Delta\Pi$ at long time plotted versus subphase concentration of tomatine, the curve represents a fit to Eq. (2). The $\Delta\Pi$ values for the two lowest tomatine concentrations are determined at much longer times near 12,000–14,000 s. The $\Delta\Pi$ values are an average for two complete data sets.

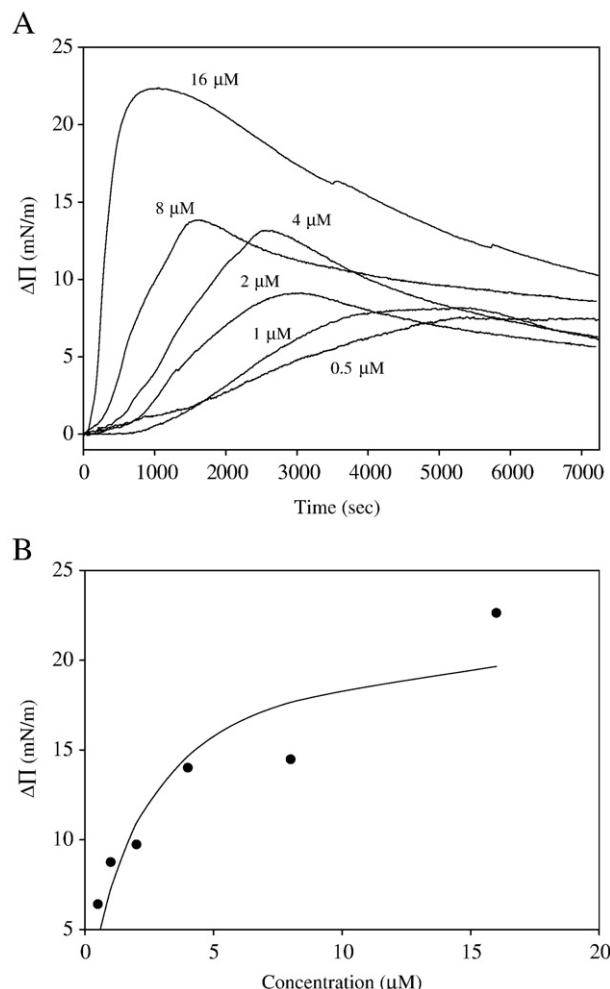


Fig. 3. The response of mixed monolayers of 7:3 mole ratio egg sphingomyelin/cholesterol to injection of varying concentrations of tomatine. (A) The monolayers were compressed to 10 mN m^{-1} prior to injection. The concentrations studied were $16 \mu\text{M}$, $8.0 \mu\text{M}$, $4.0 \mu\text{M}$, $2.0 \mu\text{M}$, $1.0 \mu\text{M}$, and $0.5 \mu\text{M}$. The subphase is $0.05 \text{ M pH } 7.0$ phosphate buffer, 0.10 M NaCl . (B) Binding curve analysis of the measured values of maximal $\Delta\Pi$ plotted versus subphase concentration of tomatine, the curve represents a fit to Eq. (2). The $\Delta\Pi$ values are an average for two complete data sets and represent the maximal peak points of each curve.

species from the subphase has been previously considered [32] and also recently reviewed [33]. The $\Delta\Pi$ values represent $\Pi(t) - \Pi(t=0)$ where $\Pi(t=0)$ is the surface pressure at the time of injection and is very near 10 mN m^{-1} in these experiments. This is the same initial surface pressure used in our prior study [24] and allows for a range of response in surface pressure increase after injection without pushing the monolayer into collapse. In addition, at this surface pressure the mixed monolayers studied are in a uniform one phase state according to BAM observation and thus any observed visual changes can be attributed to the interaction with the injected tomatine. An interfacial binding constant K can be defined as:

$$K = \Gamma_S C_G / \Gamma_{S,G}. \quad (1)$$

In Eq. (1), Γ_S is the surface concentration of free sterol, $\Gamma_{S,G}$ is the surface concentration of sterol-glycoalkaloid complex, and C_G is the concentration of glycoalkaloid in the subphase. The total surface concentration of sterol, Γ_S^* , is equal to the sum of the surface concentration of the free sterol and the surface concentration of complexed sterol, $\Gamma_S^* = \Gamma_S + \Gamma_{S,G}$. Using the approximation that the surface pressure increase upon complexation of sterol in the monolayer with glycoalkaloid is proportional to $\Gamma_{S,G}$, or $\Gamma_{S,G} = \alpha \Delta\Pi$,

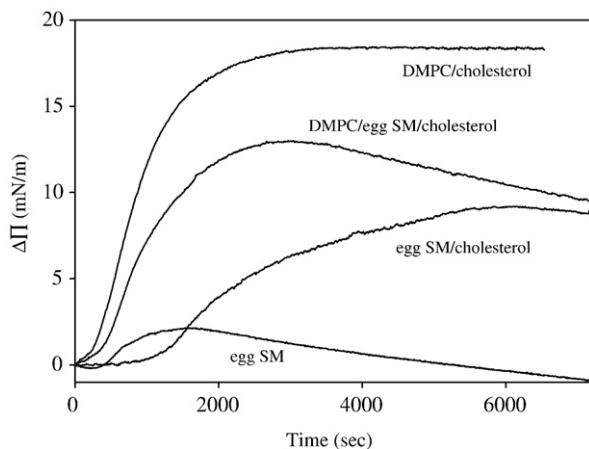


Fig. 4. Surface pressure response ($\Delta\Pi$) versus time for ternary mixture of DMPC, egg sphingomyelin, and cholesterol of molar proportion 7/1.5/1.5 compared with that for binary mixtures of 7:3 mole ratio DMPC and cholesterol, and 7:3 mole ratio egg sphingomyelin and cholesterol. The monolayers were compressed to 10 mN m^{-1} prior to injection. The responses for egg sphingomyelin and pure DMPC are also shown. The subphase is 0.05 M pH 7.0 phosphate buffer, 0.10 M NaCl.

the following equation can be derived by replacing $\Gamma_S = \Gamma_S^\circ - \Gamma_{S\cdot G}$, solving Eq. (1) for $\Gamma_{S\cdot G}$, and then substituting $\Gamma_{S\cdot G} = \alpha \Delta\Pi$ to obtain

$$\Delta\Pi = [C_G/\alpha(C_G + K)]\Gamma_S^\circ. \quad (2)$$

Data are fit using this equation in the form $\Delta\Pi(C_G) = \beta C_G/(C_G + K)$ with the two parameters K and β determined. The parameter β represents $\Delta\Pi_{\max}$, the surface pressure response in the limit of high glycoalkaloid concentration, and also equals Γ_S°/α . A fit to this equation of $\Delta\Pi$ as a function of C_G data can be used to determine the binding constant K , which represents the equilibrium

$$S \cdot G = S + G. \quad (3)$$

In Eq. (3), G represents glycoalkaloid (tomatine) in the subphase, S represents sterol in the monolayer and $S \cdot G$ represents a 1:1 complex in the monolayer. The parameter α is a proportionality constant relating the surface pressure increase to the surface concentration of complexes formed. The parameter α should be related to the compressibility of the monolayer and to the area occupied per complex, and it should be smaller for less compressible monolayers provided the same glycoalkaloid and sterol are studied and monolayer sterol composition is unchanged. In the limit of high glycoalkaloid concentration where complexation of all of the sterol in the monolayer is approached, α can be calculated from $\Gamma_S^\circ/\Delta\Pi_{\max}$.

K is a dissociation constant, and $K' = K^{-1}$ is the corresponding association constant. The values of K found for a mixed monolayer system of lipid and cholesterol or other sterol should be expected to vary with the nature of the lipid and with the composition of the monolayer, in addition to possible variation with the initial surface pressure which influences the fluidity. The fluidity of the mixed monolayer and the strength of the interaction of the lipid with cholesterol should influence the ease with which tomatine can penetrate the monolayer and complex with cholesterol and hence the value of K will incorporate effects of the intrinsic association of cholesterol with tomatine, the partitioning of tomatine between the subphase and the monolayer, and the physical properties of the environment surrounding the complexes in the monolayer such as dielectric constant. The dielectric constant near the interface in the region occupied by the molecular head-groups will differ from that of bulk water and can affect the strength of hydrogen-bonding involving the sterol hydroxyl group and hydroxyl groups on the sugars of the glycoalkaloid; estimates based on surface potential data suggest $\epsilon \sim 5-10$ in this region [34]. The complexes formed at the interface

also appear to aggregate and higher order associations between complexes may also influence the effective value of K .

The surface pressure response of mixed monolayers of DMPC and cholesterol of mole ratio 7:3 initially compressed to 10 mN m^{-1} to injected tomatine is shown as a function of concentration in Fig. 2A. As can be seen, the surface pressure response increases with concentration of injected tomatine. The surface pressure response of a monolayer of DMPC alone is small, near $\sim 1.5 \text{ mN m}^{-1}$, and was reported in our prior study [24]. Using the $\Delta\Pi$ values determined at long times for each concentration where $\Delta\Pi$ reaches equilibrium, the plot of $\Delta\Pi$ versus concentration shown in Fig. 2B is obtained. Fitting of the data to Eq. (2) for the DMPC and cholesterol yields a value of $K = 0.081 \pm 0.01 \mu\text{M}$, or $K' = 12 \times 10^6 \text{ M}^{-1}$. Fitting is done to a hyperbolic form $f(x) = \beta \cdot x / (K + x)$ where $x = C_G$ and $\beta = \Gamma_S^\circ/\alpha$, which is effectively the value for $\Delta\Pi_{\max}$ and is found to be $19.1 \pm 0.5 \text{ mN m}^{-1}$. In Fig. 2B, the $\Delta\Pi$ values used are an average for two entire sets of $\Delta\Pi$ versus concentration experiments.

A comparison of the response of sphingolipid and phospholipid mixed monolayers with cholesterol to injected tomatine was pursued to see if the expected stronger interaction of sphingolipid with cholesterol [29] would result in a lower value of K' . Experiments studying the response of mixed monolayers of egg sphingomyelin and cholesterol of mole ratio 7:3 initially compressed to 10 mN m^{-1} were pursued. These experiments were complicated by the consistently observed instability and surface pressure relaxation over time of egg sphingomyelin and cholesterol monolayers interacting with tomatine. This downward drift in surface pressure at longer times occurs even for monolayers of egg sphingomyelin alone, with which tomatine induces a small response in surface pressure. If the relaxation is a

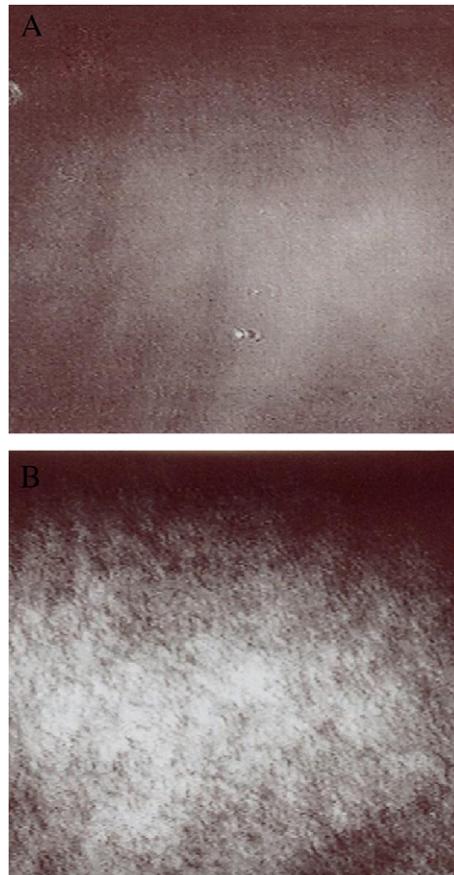


Fig. 5. BAM images of monolayers of mole ratio 7:3 DMPC and cholesterol interacting with $1.0 \mu\text{M}$ tomatine (A) just prior to injection, and (B) 41 min after injection of tomatine into the subphase. The monolayers were compressed to 10 mN m^{-1} prior to injection. The images are $700 \mu\text{m} \times 700 \mu\text{m}$.

structural instability effect not related to the kinetics of the interaction, then choosing a value at an earlier time before the structural relaxation effects become a dominant and variable factor seems advisable and thus we selected the maximum $\Delta\Pi$ at each concentration. The longer term values of $\Delta\Pi$ are not well correlated with subphase tomatine concentration compared to the relation that is seen when using the maximal $\Delta\Pi$ values. Clearly larger concentrations of injected tomatine were required to obtain surface pressure increases similar in magnitude to those observed with DMPC and cholesterol mixed monolayers. The maximal surface pressure responses as a function of injected concentration of tomatine are shown in Fig. 3A. The peak $\Delta\Pi$ values observed at each concentration are plotted versus concentration in Fig. 3B and subjected to analysis using Eq. (2). The resulting value of $K=2.1\pm 1.0 \mu\text{M}$, or $K'=0.48\times 10^6 \text{ M}^{-1}$ which is about 25 times smaller than the value for tomatine interacting with the 7:3 DMPC and cholesterol monolayer. The value of the parameter $\beta=\Delta\Pi_{\max}=22.2\pm 3.2 \text{ mN m}^{-1}$ is similar to the value of $\beta=19.1\pm 0.5 \text{ mN m}^{-1}$ found when the lipid is DMPC. Given that the mole fraction of cholesterol is 0.30 in each case, and only the lipid is changed from DMPC to egg sphingomyelin, the values of β are not expected to be very different between the two systems. The calculated value of $\alpha=I_s^\circ/\beta$ is the same for the two systems, with $\alpha=4.53\times 10^{-8} (\text{mol m}^{-2})/(\text{mN m}^{-1})$ for both the 7/3 DMPC+cholesterol and 7/3 egg sphingomyelin+cholesterol monolayers (with error bars of $\pm 0.11\times 10^{-8}$ and $\pm 0.65\times 10^{-8}$, respectively in the given units). The complexes of cholesterol and tomatine formed in each monolayer are expected to occupy similar surface area. The effect of lower compressibility for the egg sphingomyelin+cholesterol monolayer than for the DMPC+cholesterol monolayer appears to be compensated for by a higher value of I_s° for the egg sphingomyelin+

cholesterol monolayer of $3.35\times 10^{-6} \text{ mol m}^{-2}$ as compared to the value of $2.89\times 10^{-6} \text{ mol m}^{-2}$ for the DMPC+cholesterol monolayer. While the K' value is an approximation, it is consistent with the idea that the partitioning of tomatine into the egg sphingomyelin+cholesterol monolayer is less favorable than it is for the DMPC+cholesterol mixed monolayer.

The interfacial binding constants that we present indicate that injected tomatine interacts more strongly with the mixed monolayer of DMPC and cholesterol than it does with the mixed monolayer of egg sphingomyelin and cholesterol. In experiments evaluating the extraction of cholesterol from monolayers by cyclodextrin, it was found that extraction from a sphingomyelin/cholesterol mixed monolayer was much more difficult than extraction from a phospholipid/cholesterol mixed monolayer [29]. This was attributed to the stronger interaction between sphingolipid and cholesterol than between phospholipid and cholesterol, which could be assigned to hydrogen-bonding of the cholesterol hydroxyl group to the amide group unique to sphingomyelin. This constitutes a competing interaction decreasing the favorability of cholesterol complexing with tomatine. There may also be a contribution from a difference in the partitioning of tomatine from the subphase into the sphingomyelin containing monolayer due to its different fluidity. The strength of interaction with injected tomatine could possibly serve as a tool for assessing the strength of lipid–sterol interactions in monolayers serving as model membranes.

The response of a ternary mixture of DMPC with egg sphingomyelin and cholesterol of mole ratio 7:1.5:1.5 to injected tomatine was also examined. The surface pressure response of the ternary mixture falls between that of the two mixtures, as seen in Fig. 4. BAM images are shown in Figs. 5–7 for the interaction of tomatine with these three systems. At the initial surface pressure of 10 mN m^{-1} , the mixed

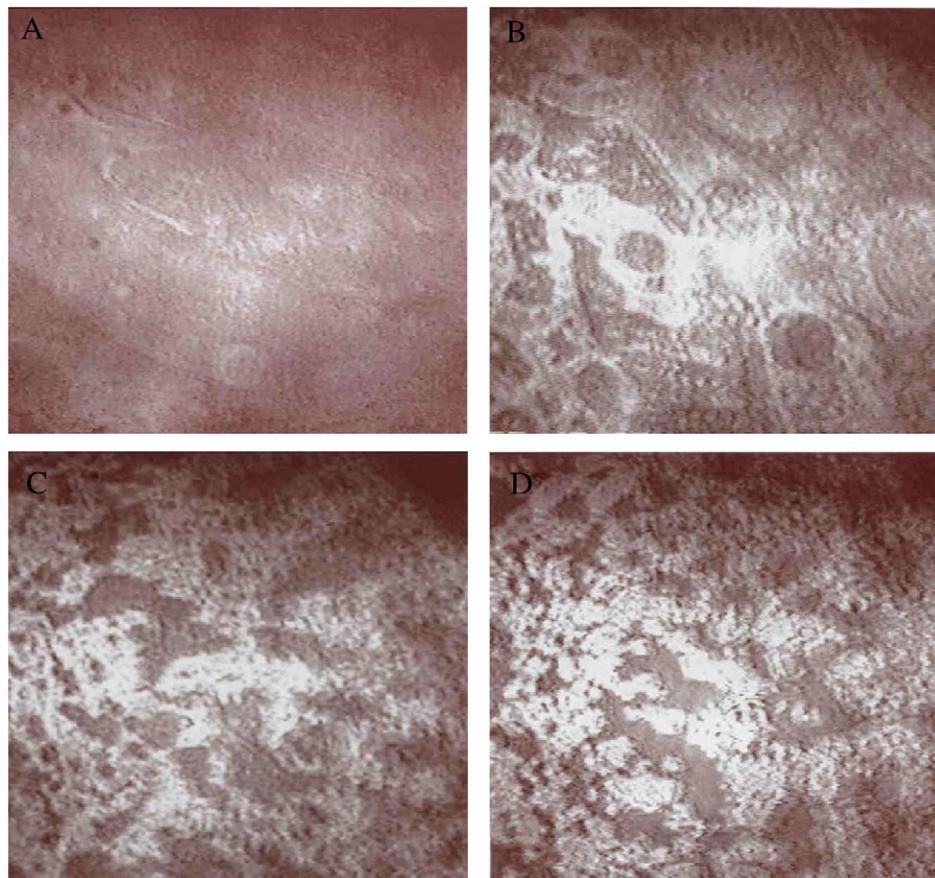


Fig. 6. BAM images of monolayers of mole ratio 7:3 egg sphingomyelin and cholesterol interacting with $1.0 \mu\text{M}$ tomatine (A) just prior to injection, (B) 15 min after injection, (C) 20 min after injection, and (D) 45 min after injection of tomatine into the subphase. The monolayers were compressed to 10 mN m^{-1} prior to injection. The images are $700 \mu\text{m} \times 700 \mu\text{m}$.

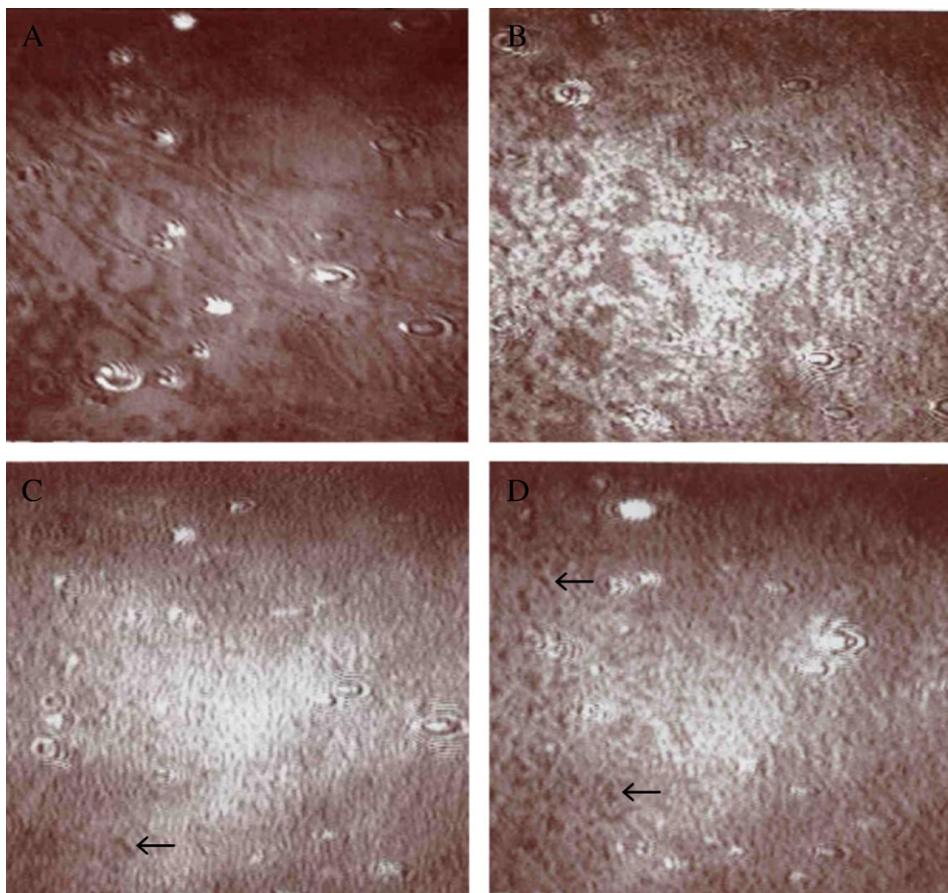


Fig. 7. BAM images of monolayers of a ternary mixture of DMPC, egg sphingomyelin, and cholesterol of molar proportions 7:1.5:1.5 interacting with 1.0 μM tomatine (A) just prior to injection, (B) 20 min after injection, (C) 80 min after injection, and (D) 120 min after injection of tomatine into the subphase. The monolayers were compressed to 10 mN m^{-1} prior to injection. The images are 700 $\mu\text{m} \times 700 \mu\text{m}$. Some of the many small dark holes that can be distinguished in the images are marked by arrows.

monolayers of DMPC and cholesterol are initially in a featureless one phase state (Fig. 5A). For the mixed monolayers with egg sphingomyelin and cholesterol there is evidence for some small islands of low contrast just before injection (Fig. 6A). The ternary mixture clearly shows visual evidence of phase separation prior to injection (Fig. 7A). After injection, the monolayers of DMPC and cholesterol evolve towards an interwoven matted texture (Fig. 5B) as previously reported [24] and shown here only for comparison. Monolayers of egg sphingomyelin and cholesterol evolve towards what appears as a more distinct phase separation (Fig. 6B) and then towards a pattern of irregularly shaped aggregated islands (Fig. 6C, D). The monolayers of the ternary mixture change from showing smooth fluid boundaries between phase-separated regions prior to injection (Fig. 7A) to showing small irregular islands (Fig. 7B) that gradually increase in number to yield a finely textured pattern with small dark holes (Fig. 7C, and especially D). In the 7:3 DMPC+cholesterol monolayer, the matted texture represents brighter and darker regions that are on a length scale too small to clearly resolve with the BAM (resolution ~4 μm). The 7:3 egg sphingomyelin and cholesterol monolayer clearly displays formation of bright islands that are larger and more resolvable. It is our conclusion from our prior work and this work that the bright islands that form are dominantly composed of 1:1 cholesterol+tomatine complexes that have phase separated from the lipid, either entirely or partially.

In order to characterize the behavior of tomatine at the water-air interface, spread monolayers of tomatine and cholesterol of mole ratios cholesterol:tomatine of 1:3, 1:1, and 3:1 were examined. The compound tomatine alone, when spread and compressed at the water-air interface, exhibits minimal surface pressure upon compression. It does not reveal any even transitory features under BAM

observation upon spreading. The Π - A isotherms for the spread mixtures of tomatine and cholesterol are shown in Fig. 8. The isotherm for the 1:3 mixture shows the buildup of some surface pressure upon compression. The 1:1 mixture gives an isotherm indicating formation of a stable monolayer with a high collapse pressure above 45 mN m^{-1} and a limiting molecular area near 40 $\text{\AA}^2 \text{ molecule}^{-1}$. The isotherm for the 1:1 mixture is consistent with significant retention of tomatine and spread cholesterol at the interface in the form of 1:1 complexes and most likely along with

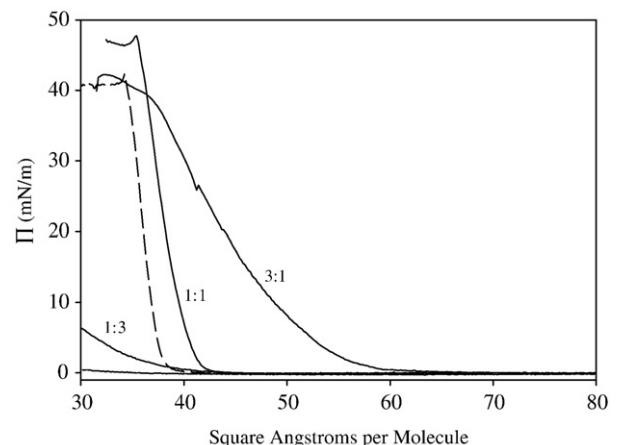


Fig. 8. Surface pressure (Π) versus mean molecular area (A) isotherms for mixtures of tomatine spread with cholesterol in mole ratio cholesterol:tomatine of 1:3, 1:1, and 3:1 as labeled, the dashed curve is an isotherm for cholesterol. The subphase is 0.05 M pH 7.0 phosphate buffer, 0.10 M NaCl.

some fraction of unbound cholesterol. The possibility that some fraction of tomatine, which is not complexed to cholesterol, is lost into the subphase seems probable given that the molecular area is not much greater than that for a cholesterol monolayer alone. The isotherm for the 3:1 mixture is significantly expanded relative to that of the 1:1 mixture; this is likely a consequence of two factors. First, the large tetrasaccharide on tomatine is proposed to be functioning as a head-group. Secondly, with an excess of cholesterol present, it is likely that less of the tomatine would be possibly lost into the subphase due to the 3 \times greater availability of a cholesterol partner for a tomatine molecule to pair up with during spreading. It seems unlikely that tomatine would be complexed to cholesterol in chloroform, and it is likely that complexes that form must do so during the spreading process. In testing the effect of varying the time interval between spreading and compression using the 1:1 tomatine + cholesterol mixture, it was observed that waiting 30 min instead of

15 min yielded an isotherm that was shifted to lower area by 3.0 \AA^2 molecule $^{-1}$, indicating that some small amount of tomatine may be lost upon allowing a longer evaporation time. The tomatine–cholesterol interaction is clearly very effective at retaining tomatine at the interface.

BAM images of spread monolayers of tomatine and cholesterol are shown in Fig. 9. Pure tomatine shows no features under BAM upon spreading, even just within the first minute after depositing drops on the surface, and is assumed on its own to be lost into the subphase. While it is likely that tomatine solutions of sufficient concentration should show adsorption of the molecule at the air–water interface, the amount spread (~ 25 nmol), if all dissolved, results in a bulk concentration of $\sim 0.2 \mu\text{M}$ that is not sufficient to result in an observable adsorbed monolayer. Studies of the formation of adsorbed monolayers of tomatine from bulk solutions of varying and much higher concentrations remain for future investigation. For the

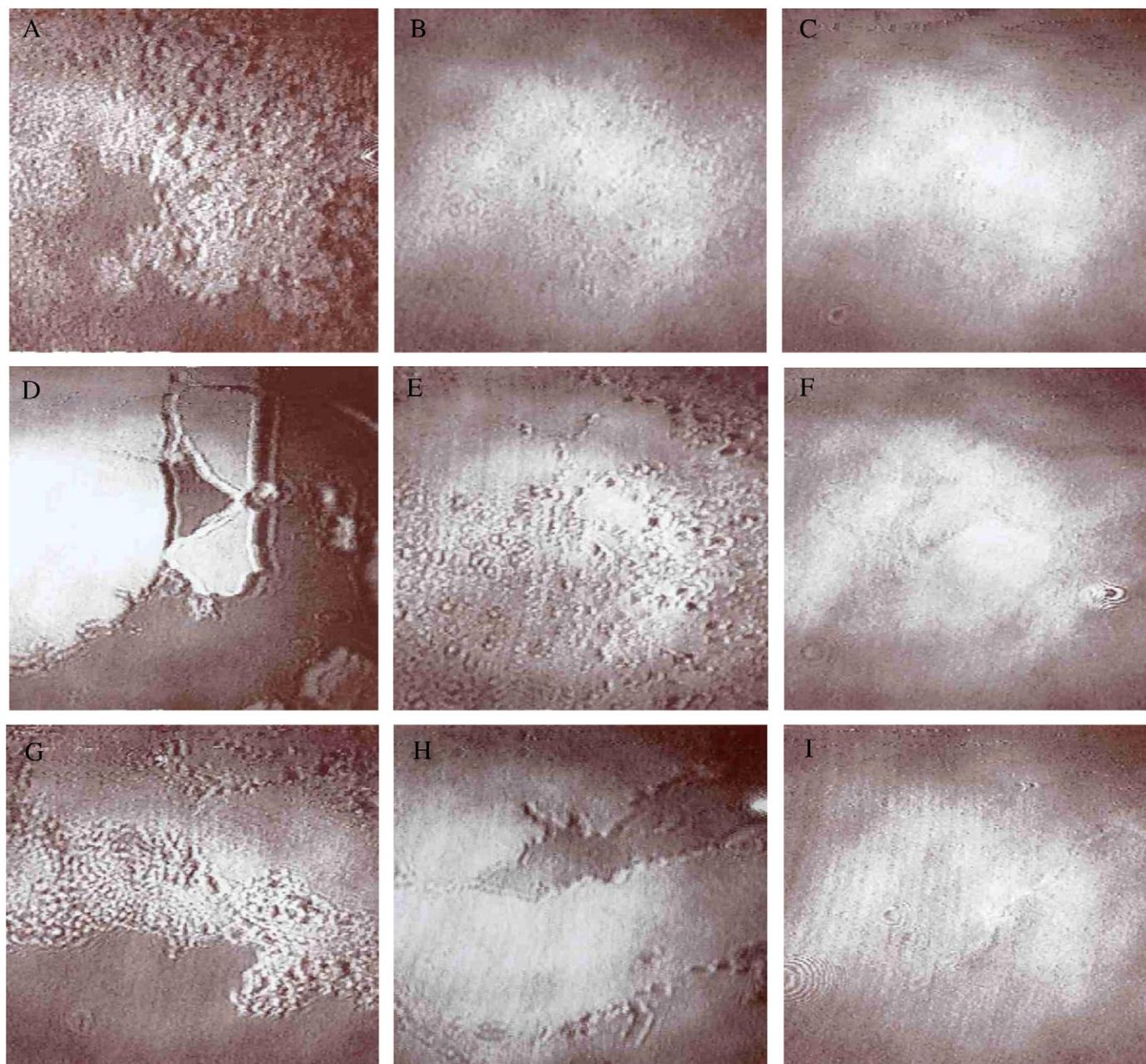


Fig. 9. BAM images of mixtures of tomatine spread with cholesterol of mole ratio cholesterol:tomatine of 1:3, 1:1, and 3:1. (A) 1:3 mixture at $82 \text{\AA}^2 \text{molecule}^{-1}$, (B) 1:3 mixture at $50 \text{\AA}^2 \text{molecule}^{-1}$, (C) 1:3 mixture at $34 \text{\AA}^2 \text{molecule}^{-1}$, (D) 1:1 mixture at $66 \text{\AA}^2 \text{molecule}^{-1}$, (E) 1:1 mixture at $50 \text{\AA}^2 \text{molecule}^{-1}$, (F) 1:1 mixture at $44 \text{\AA}^2 \text{molecule}^{-1}$, (G) 3:1 mixture at $78 \text{\AA}^2 \text{molecule}^{-1}$, (H) 3:1 mixture at $46 \text{\AA}^2 \text{molecule}^{-1}$, and (I) 3:1 mixture at $38 \text{\AA}^2 \text{molecule}^{-1}$. The images are $700 \mu\text{m} \times 700 \mu\text{m}$.

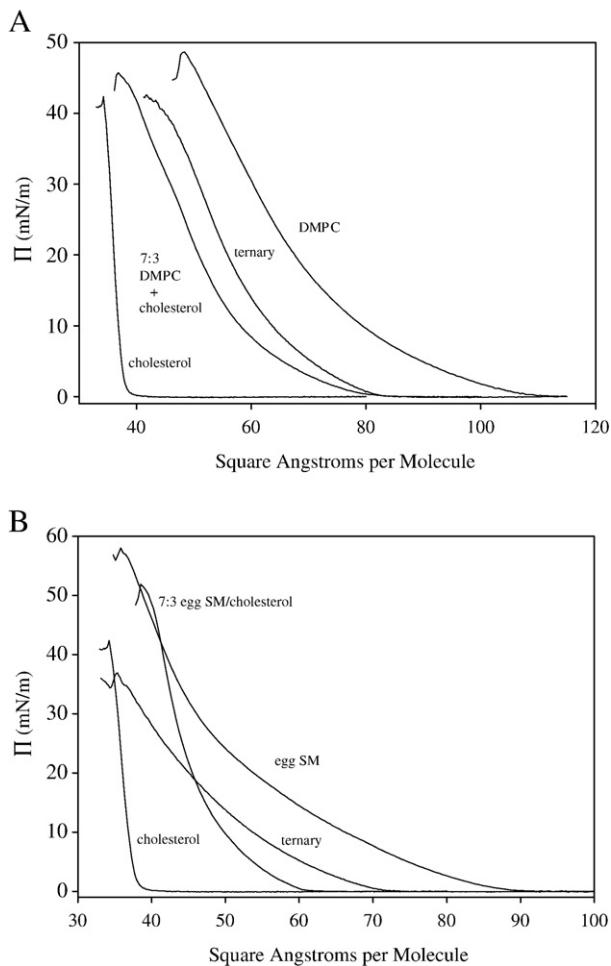


Fig. 10. (A) Surface pressure (Π) versus mean molecular area (A) isotherms for DMPC, a 7:3 mole ratio mixture of DMPC and cholesterol, cholesterol, and a ternary mixture of DMPC, cholesterol, and tomatine of molar proportions 7:1.5:1.5. (B) Surface pressure (Π) versus mean molecular area (A) isotherms for egg sphingomyelin, a 7:3 mole ratio mixture of egg sphingomyelin and cholesterol, cholesterol, and a ternary mixture of egg sphingomyelin, cholesterol, and tomatine of molar proportions 7:1.5:1.5. The subphase is 0.05 M pH 7.0 phosphate buffer, 0.10 M NaCl.

cholesterol:tomatine mixture of mole ratio 1:3, the images shown in Fig. 9A–C show that at $82 \text{ Å}^2 \text{ molecule}^{-1}$ (Fig. 9A) the monolayer has a grainy pattern suggesting the presence of many small islands aggregated together with significant coverage of the surface by darker regions. Compression brings these small aggregates closer together (Fig. 9B, $50 \text{ Å}^2 \text{ molecule}^{-1}$) and then ultimately the surface is filled in with relatively uniform brightness (Fig. 9C, $34 \text{ Å}^2 \text{ molecule}^{-1}$) in the range of the isotherm where the surface pressure is beginning to rise. It is possible that the monolayer of the 1:3 mixture consists of tomatine that has been retained at the surface and has complexed with cholesterol and some free cholesterol molecules. However, due to the low surface pressure buildup, it is clear that many molecules of tomatine have been lost into the subphase. For the mixture of mole ratio 1:1, the images shown in Fig. 9D–F show that at higher area per molecule, there are large islands with irregular geometry (Fig. 9D, $66 \text{ Å}^2 \text{ molecule}^{-1}$) suggesting a more solid-like arrangement. This structure differs from the more fluid view of elongated stripes, circular or smooth oval shaped domains, and dark holes seen for monolayers of pure cholesterol [35]. It is possible that the tomatine and cholesterol complexes link together through oligosaccharide interactions and are responsible for a more solid-like phase structure than that of cholesterol alone. Compression of the 1:1 mixture results in the surface filling in with the exception of some small holes (Fig. 9E, $50 \text{ Å}^2 \text{ molecule}^{-1}$) which ultimately disappear (Fig. 9F, $44 \text{ Å}^2 \text{ molecule}^{-1}$).

Further compression to $30 \text{ Å}^2 \text{ molecule}^{-1}$ results in a slightly grainy pattern for the 1:1 mixture, but no visually distinctive collapse structures are visible at the resolution of the BAM. For the mixture of mole ratio 3:1, the images shown in Fig. 9G–I show that at higher areas there are islands surrounded or bordered by groups of smaller aggregates (Fig. 9G, $78 \text{ Å}^2 \text{ molecule}^{-1}$). Given the excess of cholesterol present, this image is suggestive of regions of free cholesterol bordered by smaller aggregates that are hypothesized to consist of tomatine+cholesterol complexes since they resemble the kind of structures that appear when tomatine is injected beneath cholesterol containing monolayers. Compression decreases the fraction of open darker areas (Fig. 9H, $46 \text{ Å}^2 \text{ molecule}^{-1}$) and ultimately results in a fairly uniform filled-in appearance (Fig. 9I, $38 \text{ Å}^2 \text{ molecule}^{-1}$).

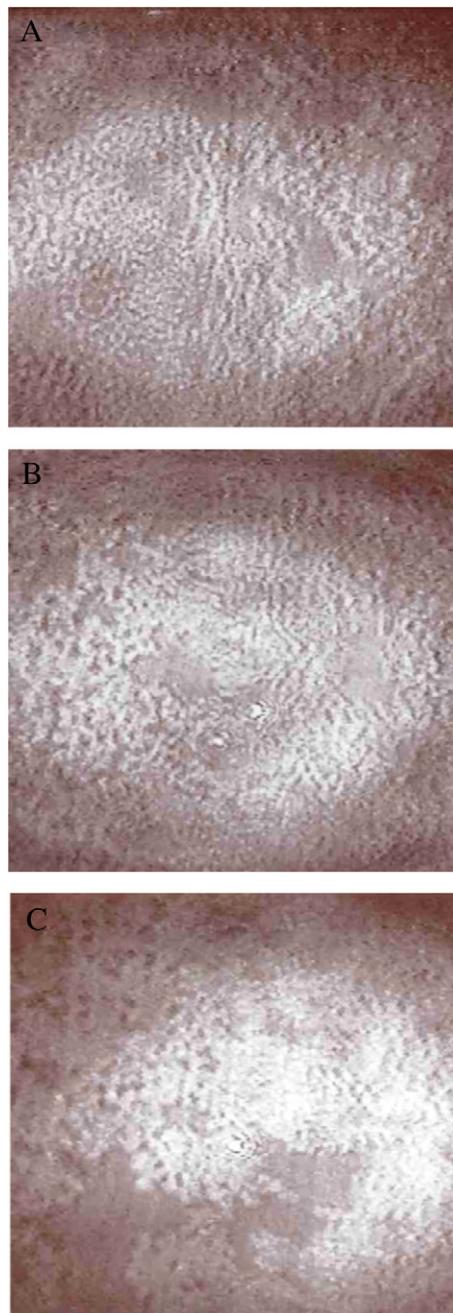


Fig. 11. BAM images of a monolayer of a ternary mixture of DMPC, cholesterol, and tomatine of molar proportions 7:1.5:1.5. (A) $86 \text{ Å}^2 \text{ molecule}^{-1}$, (B) $58 \text{ Å}^2 \text{ molecule}^{-1}$, and (C) $38 \text{ Å}^2 \text{ molecule}^{-1}$. The subphase is 0.05 M pH 7.0 phosphate buffer, 0.10 M NaCl. The images are $700 \mu\text{m} \times 700 \mu\text{m}$.

In Fig. 10, compression isotherms are shown for spread ternary mixtures of lipid, cholesterol, and tomatine. The compression isotherms shown in Fig. 10A compare DMPC, cholesterol, a 7:3 mole ratio mixture of DMPC and cholesterol, and a ternary mixture of DMPC, cholesterol and tomatine of molar proportions 7:1.5:1.5. The analogous compression isotherms using egg sphingomyelin as the lipid are shown in Fig. 10B. In Fig. 10A, the isotherm for the ternary mixture is shifted to slightly higher areas than that of the 7:3 mixture of DMPC and cholesterol. The isotherms shown for the 1:1 tomatine + cholesterol mixtures shown in Fig. 8 indicate that the mean molecular area occupied by spread 1:1 tomatine + cholesterol is also greater than that for cholesterol alone. In Fig. 10B, the ternary mixture of egg sphingomyelin, cholesterol, and tomatine is seen to be less stable than the 7:3 mixture of egg sphingomyelin with cholesterol and to collapse

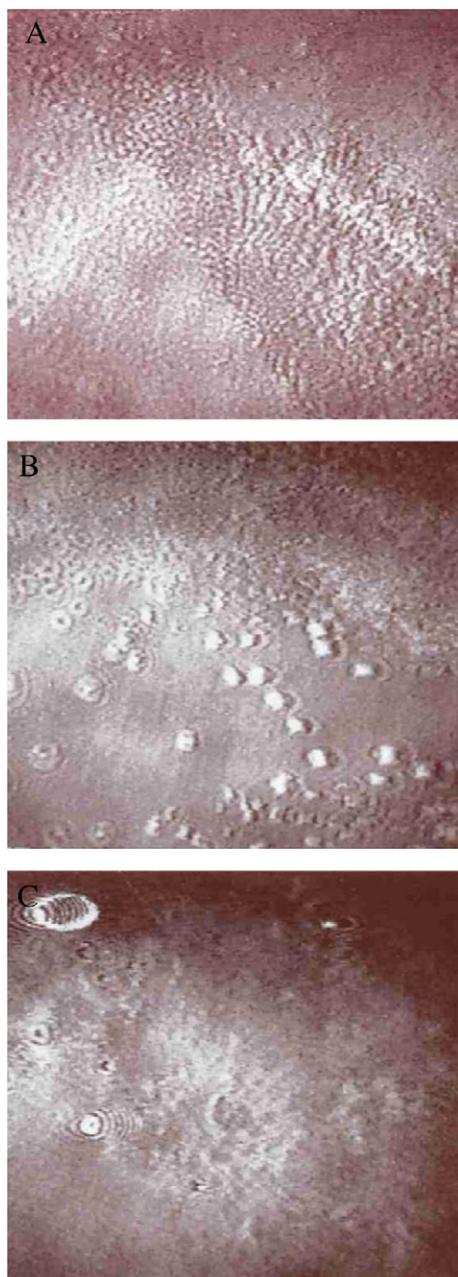


Fig. 12. BAM images of a monolayer of a ternary mixture of egg sphingomyelin, cholesterol, and tomatine of molar proportions 7:1.5:1.5. (A) $90 \text{ \AA}^2 \text{ molecule}^{-1}$, (B) $70 \text{ \AA}^2 \text{ molecule}^{-1}$, and (C) $38 \text{ \AA}^2 \text{ molecule}^{-1}$. The subphase is $0.05 \text{ M pH 7.0 phosphate buffer}$, 0.10 M NaCl . The images are $700 \mu\text{m} \times 700 \mu\text{m}$.

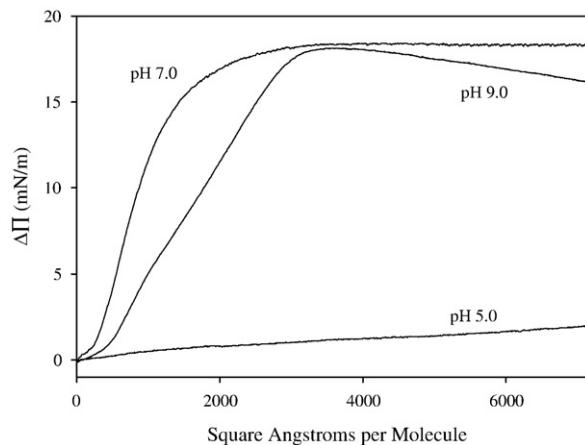


Fig. 13. Surface pressure response ($\Delta\Pi$) vs. time for mixed monolayers of DMPC and cholesterol of 7:3 mole ratio interacting with $1 \mu\text{M}$ tomatine compared on 0.05 M subphases of pH 5.0 (acetate buffer), pH 7.0 (phosphate buffer), and pH 9.0 (borate buffer). The monolayers were compressed to 10 mN m^{-1} prior to injection. Each subphase contains 0.10 M NaCl .

at a lower pressure and molecular area. The ternary mixture isotherm starts out at a higher area than the 7:3 mixture of egg sphingomyelin and cholesterol but crosses over to lower areas during the compression. The shift to lower area upon compression suggests some instability of the ternary mixture which is most likely due to some form of aggregation phenomenon involving three-dimensional structures. The lower stability of the spread ternary mixture is most likely related to the surface pressure relaxation that occurs when tomatine is injected beneath egg sphingomyelin containing monolayers.

BAM images of spread monolayers of DMPC mixed with tomatine and cholesterol in a 7:1.5:1.5 ratio are shown in Fig. 11A–C. At $86 \text{ \AA}^2 \text{ molecule}^{-1}$, the monolayer clearly shows the presence of a texture of many small aggregates. This texture is also seen in Fig. 11B at $58 \text{ \AA}^2 \text{ molecule}^{-1}$ as compression brings these aggregates closer together, and then at lower areas (Fig. 11C, $38 \text{ \AA}^2 \text{ molecule}^{-1}$) where the isotherm indicates collapse has occurred, the monolayer exhibits a pattern of many small domains that have not fully merged. The appearance of the monolayer resembles that found for DMPC and cholesterol mixed monolayers interacting with tomatine in general although the features are larger in the spread monolayer. BAM images of the spread monolayers of egg sphingomyelin with cholesterol and tomatine of mole ratio 7:1.5:1.5 are shown in Fig. 12A–C. The general observations are similar to those seen for the mixtures with DMPC. At a high area where the surface pressure is near zero, the presence of many small islands is observed (Fig. 12A, $90 \text{ \AA}^2 \text{ molecule}^{-1}$) against a background that appears alternately dark and grey. Given the near zero surface pressure, the darker background regions are likely the gas phase with a very low surface density of molecules. The regions of greyer background are likely composed primarily of sphingomyelin since the spread cholesterol is likely associated with tomatine in islands of higher surface density that appear as the bright spots in the image. Compression results in a grainy pattern of small domains (Fig. 12B, $70 \text{ \AA}^2 \text{ molecule}^{-1}$) and some larger bright islands that becomes more closely packed as the compression proceeds (Fig. 12C, $38 \text{ \AA}^2 \text{ molecule}^{-1}$). The images seen for these spread monolayers with lipid, cholesterol, and tomatine differ in a very important way from spread monolayers of lipid and cholesterol. For spread monolayers of DMPC and cholesterol, the monolayer becomes smooth and featureless on compression, and is so at 10 mN m^{-1} ; in contrast, the spread mixtures of DMPC, cholesterol, and tomatine show grainy domains that strongly resemble what is seen after tomatine is injected beneath a DMPC+ cholesterol mixed monolayer. The same observations apply to egg sphingomyelin and cholesterol spread monolayers compared to the ternary mixture including tomatine. The observation of small islands

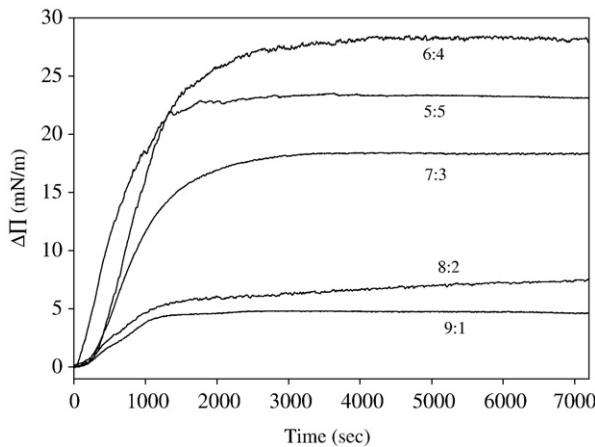


Fig. 14. Surface pressure response ($\Delta\Pi$) vs. time for mixed monolayers of DMPC and cholesterol of mole ratios DMPC:cholesterol of 9:1, 8:2, 7:3, 6:4, and 5:5 interacting with 1.0 μM tomatine. The monolayers were compressed to 10 mN m^{-1} prior to injection. The subphase is 0.05 M pH 7.0 phosphate buffer, 0.10 M NaCl.

and grainy patterns in the ternary mixtures provides support for the overall hypothesis of tomatine insertion, complexation, and domain formation by complexes.

The activity of tomatine against sterol containing liposomes is known to be pH dependent with the maximal activity near pH 7, and with the activity falling off significantly at lower pH but only decreasing modestly at higher pH. The surface pressure response

data shown in Fig. 13 is consistent with the reported trend for activity against liposomes [5]. In Fig. 13, the surface pressure response ($\Delta\Pi$) is shown for monolayers of DMPC and cholesterol of 7:3 mole ratio on buffered subphases of pH 5.0, pH 7.0, and pH 9.0 to injection of tomatine to a subphase concentration of 1.0 μM . The surface pressure response on pH 5.0 subphase is drastically reduced compared to that on pH 7.0, while that on pH 9.0 is just slightly lower. The reduced response at pH 5.0 can be attributed to protonation of nitrogen in the aglycone. The pK_b of the nitrogen on the aglycone has been reported as near 6.0 [1] and thus at pH 5.0, most of the tomatine is in the protonated form for which insertion into the hydrophobic membrane and interaction with cholesterol is very much less favorable. The properties of the monolayer of phospholipid DMPC are not expected to vary within the pH range studied [36], and neither should those for a mixed monolayer of DMPC and cholesterol.

The surface pressure response due to the interaction of tomatine with mixed monolayers of DMPC and cholesterol of mole ratios from 9:1 to 5:5 is shown in Fig. 14. The response to 1 μM tomatine increases with mole fraction of cholesterol up to 6:4 and then is somewhat lower for the 5:5 mole ratio. While the surface pressure response is higher for tomatine interacting with a pure cholesterol monolayer [24], the decrease in response between the 6:4 and 5:5 mole ratios could possibly be related to the less compressible nature of the 5:5 mole ratio monolayers. In our previous study, we hypothesized that tomatine inserts into the monolayer and complexes with cholesterol or other sterol present followed by phase separation of these complexes away from the lipid. In Fig. 15A–B, BAM images of tomatine interacting with monolayers of mole ratio 9:1 are shown. In these images, the formation of islands as a consequence of the interaction is

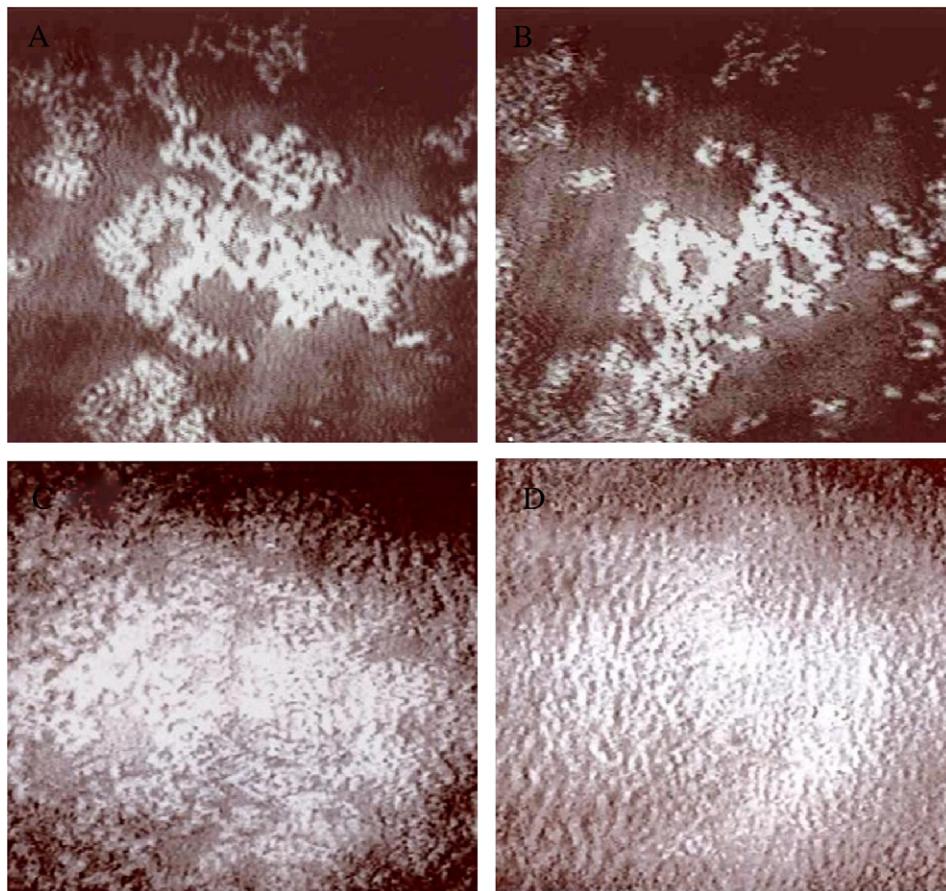


Fig. 15. BAM images of DMPC and cholesterol mixed monolayers interacting with 1.0 μM tomatine. (A) 9:1 mole ratio at 15 min after injection, (B) 9:1 mole ratio at 50 min after injection, (C) 8:2 mole ratio at 120 min after injection, (D) 5:5 mole ratio at 120 min after injection. The monolayers were compressed to 10 mN m^{-1} prior to injection. The images are $700 \mu\text{m} \times 700 \mu\text{m}$.

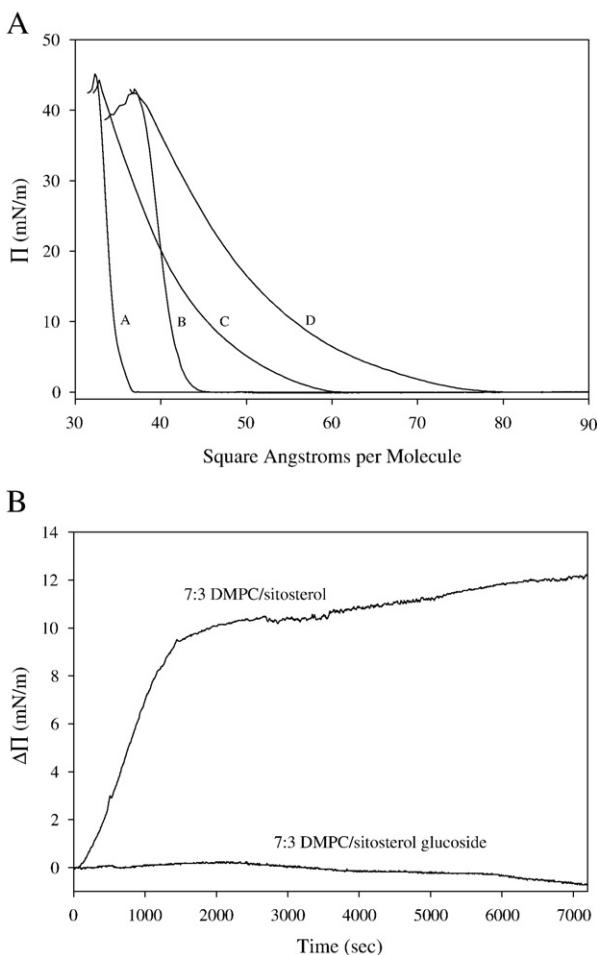


Fig. 16. (A) Surface pressure (Π) versus molecular area (A) isotherms for sitosterol (curve A), sitosterol glucoside (curve B), a 7: mole ratio mixture of DMPC and sitosterol (curve C), and a 7:3 mole ratio mixture of DMPC and sitosterol glucoside (curve D). (B) Surface pressure response ($\Delta\Pi$) vs. time for mixed monolayers of DMPC and sitosterol and of DMPC and sitosterol glucoside of 7:3 mole ratio interacting with 1 μM tomatine. The monolayers were compressed to 10 mN m^{-1} prior to injection. The subphase is 0.05 M pH 7.0 phosphate buffer, 0.10 M NaCl.

clearly evident. The lower fraction of cholesterol results in the formation of what we propose to be domains of 1:1 tomatine + cholesterol complexes being more visually evident than for the higher mole fractions of cholesterol due to the lower surface coverage and better separation of the bright domains. At higher mole fractions, what is observed resembles the emergence of an interwoven texture of regions of different brightness that we also believe represents separation of tomatine + cholesterol complexes away from lipid. In Fig. 15C, a BAM image of a monolayer of mole ratio 8:2 interacting with tomatine is shown. In Fig. 15D, a BAM image of a monolayer of mole ratio 5:5 interacting with tomatine is shown. In Fig. 15C, a greater fraction of the surface is covered by bright islands than for the 9:1 mixture. In Fig. 15D, a matted texture of more closely arranged darker and brighter regions is seen.

β -Sitosterol is a major plant sterol also found in tomato plants [37]. It has been stated that one reason why the presence of glycoalkaloids does not damage the cell membranes of the plant itself is due to the presence of glycosylated sterols such as β -sitosterol glucoside [15,38]. It was thus deemed of interest to compare the interaction of tomatine with monolayers containing β -sitosterol with those containing β -sitosterol glucoside with the expectation that tomatine would interact more weakly with the glucoside. In Fig. 16A, Π - A compression isotherms are shown for β -sitosterol and for β -sitosterol glucoside. The isotherm for the glucoside (curve B) is expanded relative to the

parent sterol (curve A), an observation consistent with the large sugar head-group. In Fig. 16A, the Π - A isotherms for monolayers of DMPC and β -sitosterol of mole ratio 7:3 (curve C) and for DMPC and β -sitosterol glucoside of mole ratio 7:3 (curve D) are shown. The isotherm for the 7:3 mixture with β -sitosterol glucoside is significantly expanded relative to that for the 7:3 mixture with β -sitosterol, also consistent with the larger glucose head-group. The response of these mixed monolayers to 1 μM tomatine is shown in Fig. 16B, and it can be seen that the mixed monolayer containing β -sitosterol glucoside does not interact with tomatine while the monolayer containing β -sitosterol interacts strongly with tomatine although not quite as strongly as with the analogous cholesterol containing monolayer. This observation indicates that the especially important interaction with the 3 β -hydroxy group proposed to drive the complexation of tomatine with sterols at membrane interfaces cannot be replaced by interaction with the hydroxyls on the glucose moiety. The larger glucose group also likely interferes with the possibility for favorable alignment and interaction of the ring systems of tomatine and the sterol. BAM images of the interaction of tomatine with mixed monolayers of DMPC and β -sitosterol are shown in Fig. 17A–B. The monolayer is initially featureless and a matted texture similar to that seen for DMPC and cholesterol mixed monolayers is observed after the interaction has proceeded. For the mixed monolayers of DMPC and β -sitosterol glucoside which are also featureless prior to injection, no observed features are seen as a result of the interaction with tomatine and they remain uniform in appearance. The lack of morphology change correlates well with the lack of an observed surface pressure increase as a result of tomatine injection.

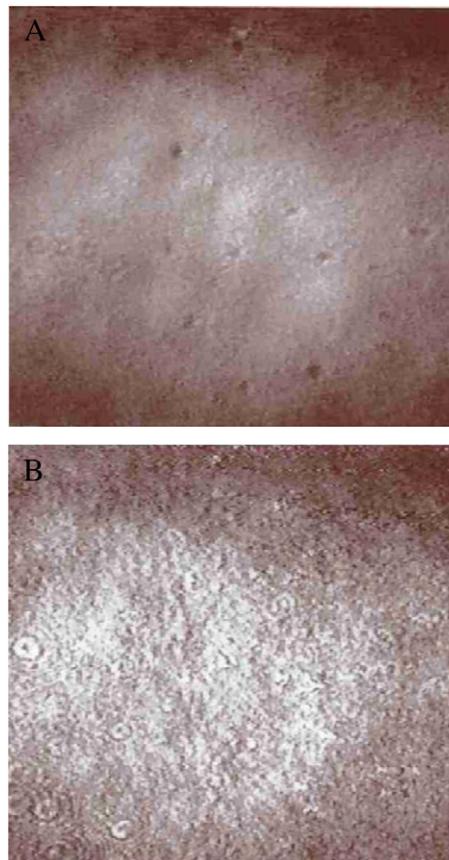


Fig. 17. BAM images of a DMPC and sitosterol mixed monolayer of 7:3 mole ratio interacting with 1.0 μM tomatine. (A) Just prior to injection, and (B) 60 min after injection. The monolayers were compressed to 10 mN m^{-1} prior to injection. The subphase is 0.05 M pH 7.0 phosphate buffer, 0.10 M NaCl. The images are 700 $\mu\text{m} \times 700 \mu\text{m}$.

4. Discussion

The interaction of tomatine with monolayers of lipids and sterols complements studies performed using liposomes. The phenomena of insertion, complexation, and aggregation present many variables for study as a function of monolayer composition and physical state. The value of the binding constant K' that can be obtained from studies of surface pressure response is not solely representative of a simple equilibrium between tomatine and cholesterol. The value obtained for the 7:3 monolayer of DMPC and cholesterol of $K' = 1.2 \times 10^7$ would correspond to a ΔG value of -40 kJ mol^{-1} at 298 K. The value obtained for the 7:3 monolayer of egg sphingomyelin and cholesterol corresponds to a value of -32 kJ mol^{-1} at 298 K. The two important contributions to the Gibbs free energy of formation of a 1:1 complex between tomatine and cholesterol in which the similar ring systems of the two are assumed to be aligned next to each other and there is a hydrogen-bond between the 3β -OH on cholesterol and some, as yet unspecified, hydroxyl group on one of the sugars of tomatine, would be from the hydrogen-bond and the attractive van der Waal's forces between the ring systems. The values estimated for ΔG in both cases are clearly larger than the typical value for a hydrogen-bond in aqueous medium of near -5 kJ mol^{-1} and are also larger than that for a hydrogen-bond in a hydrophobic medium of approximately -15 kJ mol^{-1} [39]. The nature of the medium near the monolayer interface is somewhere between these two limits. The contribution of attractive forces between ring systems must be an additional major factor. Contributions from competing interactions between cholesterol and lipid may also need to be considered, as well as that from the hydrophobic effect of removing tomatine from the subphase.

The surface pressure increases observed in the limit of high concentration of tomatine for the 7:3 mixtures appear to be consistent with area changes expected for insertion of tomatine into the monolayer. The values of $\Delta\Gamma_\infty$ of 19.1 mN m^{-1} found for the 7:3 monolayer of DMPC and cholesterol and of $\Delta\Gamma_\infty = 22.2 \text{ mN m}^{-1}$ for the 7:3 monolayer of egg sphingomyelin and cholesterol are similar. Both of these $\Delta\Gamma$ values increase the surface pressure of the respective monolayers to values below the collapse pressures for either system (see Fig. 10). These values of $\Delta\Gamma_\infty$ are in the limit of high concentration of tomatine where one could make the assumption that all of the cholesterol in the monolayer has been complexed, as was done in the analysis of digitonin–cholesterol interactions [25]. In this limit, the surface coverage after binding of every cholesterol molecule in the monolayer to an inserted tomatine would be $\Gamma^* = (1 + \delta)\Gamma_S^\circ$ where Γ_S° is the surface coverage prior to binding and Γ^* is the surface coverage after full binding. The factor δ would equal the cholesterol mole fraction of 0.30 if the area occupied per tomatine was identical to that of cholesterol. Tomatine likely occupies a somewhat larger molecular area than cholesterol and thus δ can differ from 0.30 as $\delta' = \varepsilon \delta$ where ε represents the ratio of molecular area between tomatine and cholesterol at the interface. At the initial surface pressure of 10 mN m^{-1} , the mean molecular area of the 7:3 mixed monolayer of DMPC and cholesterol is $58 \text{ \AA}^2 \text{ molecule}^{-1}$ or $\Gamma_S^\circ = 0.017 \text{ molecule \AA}^{-2}$. If $\Gamma^* = 1.3\Gamma_S^\circ = 0.022 \text{ molecule \AA}^{-2}$, then the molecular area after complexation would be $45 \text{ \AA}^2 \text{ molecule}^{-1}$. This value would ideally correspond to a surface pressure on an isotherm of composition ratio 7:3:3 DMPC:cholesterol:tomatine in which all of the tomatine was retained at the interface as was also complexed in the same manner as observed after an injection experiment. Such an area is clearly a plausible outcome; incorporating an estimated size for tomatine ($\varepsilon > 1$) would result in a lower value. In addition, for a mole ratio at the interface of 7:3:3, the fraction of the surface covered by complexes, which we assume is the bright phase, will be around 50% which is consistent with the matted pattern of dark and bright that is observed by BAM. Image analysis would be required to provide greater details.

To recap and summarize the major conclusions of the studies presented here, we note:

- Interfacial binding constants can be estimated for the insertion of tomatine into lipid+sterol monolayers. The binding of tomatine into the monolayer appears to be more favorable when phospholipid DMPC is present versus egg sphingomyelin, possibly due to required displacement of a stronger competing interaction with cholesterol in the latter case. The binding constants indicate a free energy of interaction stronger than what can be attributed to only a hydrogen-bond between the 3β -hydroxy group of cholesterol and hydroxyl(s) on the sugar(s) of tomatine.
- BAM provides further evidence for a process of insertion, complexation, and aggregation when tomatine interacts with cholesterol containing monolayers or those of similar 3β -hydroxy sterols. The extent and coverage of formation of bright islands is correlated with the fraction of cholesterol in the monolayer.
- It is possible to directly spread complexes of tomatine and cholesterol at the water-air interface and, for sufficient composition of cholesterol, stable isotherms with high collapse pressures are observed. Tomatine itself has no observable ability to form a spread monolayer, but adsorbed monolayers may be expected at the surfaces of more concentrated solutions.
- The similarity in appearance under BAM of the aggregates seen for spread ternary mixtures of lipid+cholesterol+tomatine and those formed upon interaction of injected tomatine with lipid+sterol monolayers provides support that the domains formed during the interaction of the spread monolayers with tomatine injected into the subphase are dominantly tomatine+cholesterol aggregates.
- The pH dependence of the activity of tomatine against DMPC+cholesterol monolayers is in agreement with the reported pH dependence of the action of tomatine against cholesterol containing liposomes.
- While tomatine interacts strongly with the plant sterol sitosterol in a mixed monolayer with DMPC, it shows no interaction when sitosterol is replaced by sitosterol glucoside.
- In the case of egg sphingomyelin+cholesterol monolayers, interaction with tomatine induces some longer term structural relaxation effect that reduces the surface pressure.

The problem of tomatine and related glycoalkaloids interacting with lipid and sterol monolayers warrants further analysis using a range of surface chemistry techniques. Tomatine or related glycoalkaloids may be useful as tools for assessing the strength of cholesterol-lipid interactions in membranes. A more detailed study will be required to determine how to quantitatively model the kinetics of the surface pressure response and the dynamics of the growth of the observed domains. Image analysis and studies of transferred films using techniques such as AFM are also of interest. The details of these studies may spur further consideration of the application of these compounds in drug delivery and vaccine development.

Acknowledgement

The support of a UM-Saint Louis Research Award is gratefully acknowledged.

References

- M. Friedman, Tomato glycoalkaloids: role in the plant and in the diet, *J. Agric. Food Chem.* 50 (2002) 5751–5780.
- M. Friedman, Potato glycoalkaloids and metabolites: roles in the plant and in the diet, *J. Agric. Food Chem.* 54 (2006) 8655–8681.
- A. Osbourn, Saponins and plant defence – a soap story, *Trends Plant Sci.* 1 (1996) 4–9.
- Z. Chen, A.R. Miller, Steroidal alkaloids in solanaceous vegetable crops, *Hortic. Rev.* 25 (2001) 171–196.

- [5] J.G. Roddick, R.B. Drysdale, Destabilization of liposome membranes by the steroidal glycoalkaloid α -tomatine, *Phytochem.* 23 (1984) 543–547.
- [6] E.A.J. Keukens, T. de Vrije, C.H.J.P. Fabrie, R.A. Demel, W.M.F. Jongen, B. de Kruijff, Dual specificity of sterol-mediated glycoalkaloid induced membrane disruption, *Biochim. Biophys. Acta* 1110 (1992) 127–136.
- [7] E.A.J. Keukens, T. de Vrije, C. van den Boom, P. de Waard, H.H. Plasman, F. Thiel, V. Chupin, W.M.F. Jongen, B. de Kruijff, Molecular basis of glycoalkaloid induced membrane disruption, *Biochim. Biophys. Acta* 1240 (1995) 216–228.
- [8] J.G. Roddick, Complex formation between solanaceous steroidal glycoalkaloids and free sterols in vitro, *Phytochem.* 18 (1979) 1467–1470.
- [9] M. Friedman, N. Kozukue, L.A. Harden, Preparation and characterization of acid hydrolysis products of the tomato glycoalkaloid α -tomatine, *J. Agric. Food Chem.* 46 (1998) 2096–2101.
- [10] J.G. Roddick, The acetylcholinesterase-inhibitory activity of steroidal glycoalkaloids and their aglycones, *Phytochem.* 28 (1989) 2631–2634.
- [11] T. Nakamura, C. Komori, Y. Lee, F. Hashimoto, S. Yahara, T. Nohara, A. Ejima, Cytotoxic activities of *solanum* steroid glycosides, *Biol. Pharm. Bull.* 19 (1996) 564–566.
- [12] K.R. Lee, N. Kozukue, J.S. Han, J.H. Park, E.Y. Chang, E.J. Baek, J.S. Chang, M. Friedman, Glycoalkaloids and metabolites inhibit the growth of human colon (HT29) and liver (HepG2) cancer cells, *J. Agric. Food Chem.* 52 (2004) 2832–2839.
- [13] Y.-W. Yang, N.A. Sheikh, W.J.W. Morrow, The ultrastructure of the tomatine adjuvant, *Biomat.* 23 (2002) 4677–4686.
- [14] W.J.W. Morrow, Y.-W. Yang, N.A. Sheikh, Immunobiology of the tomatine adjuvant, *Vaccine* 22 (2004) 2380–2384.
- [15] C.C. Steel, R.B. Drysdale, Electrolyte leakage from plant and fungal tissues and disruption of liposome membranes by α -tomatine, *Phytochem.* 27 (1988) 1025–1030.
- [16] A. Alzerreca, G. Hart, Molluscicidal steroid glycoalkaloids possessing stereoisomeric spirosolane structures, *Tox. Lett.* 12 (1982) 151–155.
- [17] W.M.J. Van Gelder, O.M.B. De Ponti, α -Tomatine and other steroidal glycoalkaloids in fruits of tomato lines resistant to the glasshouse whitefly, *Euphytica* 36 (1987) 555–561.
- [18] J.A. Juvik, M.A. Stevens, Physiological mechanisms of host-plant resistance in the genus *Lycopersicon* to *Heliothis Zea* and *Spodoptera exigua*, two insect pests of the cultivated tomato, *J. Am. Soc. Hortic. Sci.* 107 (1982) 1065–1069.
- [19] M. Ruiz-Rubio, A. Perez-Espinosa, K. Lairni, T. Roldan-Arjona, A. Dipietro, N. Anaya, Metabolism of the tomato saponin α -tomatine by phytopathogenic fungi, *Stud. Nat. Prod. Chem.* 25 (2001) 293–326.
- [20] N.A. Sheikh, P. Rajananthan, G.S. Attard, W.J.W. Morrow, Generation of antigen specific CD8+ cytotoxic T cells following immunization with soluble proteins formulated with novel glycoside adjuvants, *Vaccine* 17 (1999) 2974–2982.
- [21] P. Rajananthan, G.S. Attard, N.A. Sheikh, W.J.W. Morrow, Evaluation of novel aggregate structures as adjuvants: composition, toxicity studies and humoral responses, *Vaccine* 17 (1999) 715–730.
- [22] P. Rajananthan, G.S. Attard, N.A. Sheikh, W.J.W. Morrow, Novel aggregate structure adjuvants modulate lymphocyte proliferation and Th1 and Th2 cytokine profiles in ovalbumin immunized mice, *Vaccine* 18 (1999) 140–152.
- [23] K.G. Heal, N.A. Sheikh, M.R. Hollingdale, W.J.W. Morrow, A.W. Taylor-Robinson, Potentiation by a novel alkaloid glycoside adjuvant of a protective cytotoxic T cell immune response specific for a preerythrocytic malaria vaccine candidate antigen, *Vaccine* 19 (2001) 4153–4161.
- [24] K.J. Stine, R.K. Hercules, J.D. Duff, B.W. Walker, Interaction of the glycoalkaloid tomatine with DMPC and sterol monolayers studied by surface pressure measurements and Brewster angle microscopy, *J. Phys. Chem. B* 110 (2006) 22220–22229.
- [25] J.C.M. Holtwijk, T. Pomorski, R.J. Raggers, H. Sprong, G. Van Meer, The organizing potential of sphingolipids in intracellular membrane transport, *Physiol. Rev.* 81 (2001) 1689–1723.
- [26] J.C. Lawrence, D.E. Saslowsky, J.M. Edwardson, R.M. Henderson, Real-time analysis of the effects of cholesterol on lipid raft behavior using atomic force microscopy, *Biophys. J.* 84 (2003) 1827–1832.
- [27] A. Meister, C. Nicolini, H. Waldmann, J. Kuhlmann, A. Kerth, R. Winter, A. Blume, Insertion of lipidated Ras proteins into lipid monolayers studied by infrared reflection absorption spectroscopy (IRRAS), *Biophys. J.* 91 (2006) 1388–1401.
- [28] M.L. Fanani, S. Hartel, R.G. Oliveira, B. Maggio, Bidirectional control of sphingomyelinase activity and surface topography in lipid monolayers, *Biophys. J.* 83 (2002) 3416–3424.
- [29] B. Ramstedt, J.P. Slotte, Interaction of cholesterol with sphingomyelins and acyl-chain-matched phosphatidylcholines: a comparative study of the effect of the chain length, *Biophys. J.* 76 (1999) 908–915.
- [30] O. Fiedler, New method for the demonstration of phase transitions in lipid films, *Biochim. Biophys. Acta* 345 (1974) 321–325.
- [31] R. Ruyssen, P. Joos, Penetration of gaseous cholesterol monolayers by saponins, *Mededel. Koninkl. Vlaam. Acad. Wetenschap., Belg., Kl. Wetenschap.* 27 (1965) 3–20.
- [32] R. Matalon, On monolayer penetration, *J. Colloid. Sci.* 8 (1953) 53–63.
- [33] D. Vollhardt, V.B. Fainerman, Penetration of dissolved amphiphiles into two-dimensional aggregating lipid monolayers, *Adv. Coll. Interface Sci.* 86 (2000) 103–151.
- [34] O.N. Oliviera Jr., D.M. Taylor, T.J. Lewis, S. Salvagno, C.J.M. Stirling, Estimation of group dipole moments from surface potential measurements on Langmuir monolayers, *J. Chem. Soc., Faraday Trans.* 85 (1989) 1009–1018.
- [35] R. Seoane, J. Miñones, O. Conde, J. Miñones Jr., M. Cass, E. Iribarne-Garay, Thermodynamic and Brewster angle microscopy studies of fatty acid/cholesterol mixtures at the air/water interface, *J. Phys. Chem. B* 104 (2000) 7735–7744.
- [36] D. Gorwyn, G.T. Barnes, Interactions of large ions with phospholipid monolayers, *Langmuir* 6 (1990) 222–230.
- [37] E.T.S. Chow, J.J. Jen, Phytosterol biosynthesis in ripening tomatoes, *J. Food Sci.* 43 (1978) 1424–1426.
- [38] D.S. Catz, J.S. Tandecarz, C.E. Cardini, Steryl glucoside and acyl sterol glucoside formation in the amyloplast membrane during the development of potato tuber, *Plant Sci.* 38 (1985) 179–184.
- [39] G.C. Pimentel, A.L. McClellan, *The Hydrogen-Bond*, San Francisco: Freeman, 1960.