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Presence of lipids in urine, crystals and stones: Implications for the formation of kidney stones

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Presence of lipids in urine, crystals and stones: Implications for the formation of kidney stones.

Background. Cell membranes and their lipids play critical roles in calcification. Specific membrane phospholipids promote the formation of calcium phosphate and become a part of the organic matrix of growing calcification. We propose that membrane lipids also promote the formation of calcium oxalate (CaOx) and calcium phosphate (CaP) containing kidney stones, and become a part of their stone matrix.

Methods. Human urine, crystals of CaOx and CaP produced in the urine of healthy individuals, and urinary stones containing struvite, uric acid, CaOx and CaP crystals for the presence of membrane lipids were analyzed. Crystallization of CaOx monohydrate at Langmuir monolayers of dipalmitoylphosphatidylglycerol (DPPG), dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidylserine (DPPS), dioleoylphosphatidylglycerol (DOPG), palmitoyloleoylphosphatidylglycerol (POPG) and dimyristoylphosphatidylglycerol (DMPG) was investigated to directly demonstrate that phospholipid assemblies can catalyze CaOx nucleation.

Results. Urine as well as CaOx and CaP crystals made in the urine and various types of urinary stones investigated contained some lipids. Urine of both CaOx and uric acid stone formers contained significantly more cholesterol, cholesterol ester and triglycerides than urine of healthy subjects. However, urine of CaOx stone formers contained more acidic phospholipids. The organic matrix of calcific stones contained significantly more acidic and complexed phospholipids than uric acid and struvite stones. For each Langmuir monolayer precipitation was heterogeneous and selective with respect to the orientation and morphology of the CaOx crystals. Crystals were predominantly monohydrate, and most often grew singly with the calcium rich (10-1) face toward the monolayer. The number of crystals/mm^2 decreased in the order DPPG > DPPS > DPPC and was inversely proportional to surface pressure and mean molecular area/molecule.

Conclusions. Stone forming conditions in the kidneys greatly impact their epithelial cells producing significant differences in the urinary lipids between healthy and stone forming individuals. Altered membrane lipids promote face selective nucleation and retention of calcium oxalate crystals, and in the process become a part of the growing crystals and stones.

Key words: nephrolithiasis, calcium oxalate, phospholipids, phosphatidylserine, cell membrane.

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Lipids are integral to the organic matrices of mineralized tissues as well as pathologic calcifications [1–3]. Even though they account for a relatively small proportion of the organic matrix; 7 to 14% in bone, 2 to 6% in dentin, 12 to 22% in newly mineralized enamel [2], approximately 9.6% in submandibular salivary gland calculi and 10.2% in supragingival calculi [4–6], lipids are proposed to play a significant role in the calcification process. They promote crystal nucleation and become incorporated in the growing calcifications.

Our investigations of calcium oxalate (CaOx), struvite and uric acid stones showed that all of them contain some lipids [7] and that lipid matrix is a good nucleator of CaOx crystals from a metastable solution [8]. In addition, we have shown that membranes of renal epithelial cells are involved in crystallization of CaOx and CaP in vivo in kidneys of male and female rats, respectively [9, 10] and renal brush-border membrane vesicles isolated from rat kidneys can induce CaOx crystallization in vitro [11, 12]. Lipids that participate in crystallization of calcium phosphate form complexes with calcium and bind tightly to the crystals [13–15]. The current study was undertaken to elucidate further the role of lipids in CaOx nephrolithiasis and identify membrane lipids most likely involved in nucleation of CaOx crystals. We analyzed lipids of CaOx, CaP, struvite and uric acid stones and separated complexed and non-complexed lipids. We also isolated and analyzed urinary lipids from uric acid and calcium oxalate/phosphate stone formers and investigated the lipids associated with the CaOx and CaP crystals induced in the human urine. In addition, we used monolayers at the air/water interface for in vitro studies to directly demonstrate that phospholipid assemblies can nucleate CaOx. These monolayers, called Langmuir monolayers or films, have been used extensively to investigate the nucleation and growth of biominerals at organized interfaces [16]. The monolayer studies also were used to compare the CaOx nucleating potential of various phospholipids.

METHODS

Collection of human urine

Twenty-four–hour urine samples were collected from male and female uric acid and calcium oxalate stone formers (33 to 83 years) and healthy people with no evidence of kidney disease (31 to 54 years). Individuals were classified as uric acid or CaOx stone formers based on their history and composition of their stones. Individuals with hyperparathyroidism and primary or enteric hyperoxaluria were excluded. Urinary protein was determined using a protein-assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Absence of proteinuria, overt crystalluria and blood cells in the urine were considered symbolic of the absence of kidney disease. In the case of calcium oxalate stone formers, renal functions were further evaluated by determining the rate of creatinine clearance. During collection, the specimens were maintained at room temperature, approximately 24°C. Prior to collection, 1 mL of 20% sodium azide, an antibacterial agent, was placed in the collection bottles. The pH and total urinary volume were recorded. The total urinary protein was determined using dipsticks and/or a Bio-Rad protein assay kit. Urine was examined microscopically.

Induction of calcium oxalate crystals in the human urine

Previously reported procedures were utilized [17]. The urine samples were allowed to warm to 37°C in a shaking water bath (Fisher Scientific, Norcross, GA, USA) and divided into 250 mL aliquots. CaOx and CaP crystals were induced by the addition of 15 mL/L of 0.1 mol/L sodium oxalate or 0.1 mol/L sodium phosphate, followed by incubation for three hours. At the end of the incubation period, the urine specimens were centrifuged at $10{,}000 \times g$ for 25 minutes, and the supernatant was aspirated. The crystal-containing pellet was placed in a micro-centrifuge tube, washed three times and then dried for 24 hours in the Flexi-Dry lyophilizer.

Isolation, identification and quantification of lipids

The methods for isolation, identification and quantification of lipids from urine and stones are described in detail in earlier publications [7, 18]. Here we will provide only a brief account using urine as an example.

Urinary lipids. To isolate the lipids, 400 mL of urine was mixed with 1.2 L of 2:1 chloroform:methanol. The mixture was shaken and placed on an end-over-end mixer for 24 hours at 4°C and then centrifuged at 7000 rpm to achieve phase separation. The top portion was removed and set aside as the aqueous layer, the middle layer was recovered as the interface, and the lower phase was collected as the organic layer. After evaporation to a smaller volume, the organic sample was Folch-washed twice, pooling the respective phases with the previous ones. The pooled organic phase consisting of the total lipids was then lyophilized, weighed and reconstituted with 2:1 chloroform:methanol to a final volume of 1 mL with 0.1% butyl hydroxytoluene (BHT) as a preservative, blanketed with dry nitrogen and stored in the dark at −70°C until further analysis. Lyophilized organic phase provided the dry weight for total lipid.

The organic extract was separated into various lipid classes using Bio Sil A silicic acid column. The sample was applied to a 1.5×15 cm column equilibrated with chloroform. Neutral lipids were eluted with 230 mL of chloroform, glycolipids with 900 mL of acetone and phospholipids with 230 mL of methanol. Each lipid class was concentrated with a rotary evaporator, dried under nitrogen gas, lyophilized, weighed, and re-suspended in a known volume of chloroform:methanol (2:1) with 0.1% BHT and stored at −70°C.

Phospholipids were further quantified using ammonium ferrothiocyanate (AMF) and Victorian Blue R (VBR) methods. The AMF method is suitable primarily for the determination of phosphatidylcholine (PC), phosphatidylethanolamine (PE) and sphingomylein (SM) while VBR method is appropriate for detecting phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylglycerol (PG), phosphatidic acid (PA) and cardiolipin. Neutral lipids were analyzed for total and free cholesterol, cholesterol esters and triglycerides. The amount of glycolipids was determined by analyzing for glucose. The lipid classes were further analyzed for identification of individual lipids by one-dimensional thin layer chromatography (TLC). Neutral lipid standards were purchased from Nu Chek Prep (Elysian, MN, USA); glycolipid standards from Sigma Chemical Ltd. (St. Louis, MO, USA), and phospholipid standards from Avanti Polar Lipids (Birmingham, AL, USA). Individual lipid spots were visualized by exposure to iodine vapor for 30 minutes and identified by comparison to known standards. Individual phospholipids were quantified by scraping the spots from the iodine-stained plates and analyzing them for phosphate using Bartlett's method.

Lipids of crystal matrix. The crystals were weighed and then demineralized by treatment with 5 mL of 0.25 mol/L ethylenediaminetetraacetic acid (EDTA) at pH 8.0 and 4°C for three days with continuous stirring. The extract was centrifuged at $10{,}000 \times g$ for five minutes. The supernatant was dialyzed against water for 24 hours at 4°C using dialysis tubing with a 6 to 8 kD cut-off (Spectrum Medical Industries, Gardena, CA, USA). Protein concentration in the crystal matrix was determined by the Lowry method using bovine serum albumin as the standard. Lipids were extracted by treating the crystal matrix with chloroform/methanol as described above.

Lipids of urinary stones. The specific purpose of this study was to further investigate the differences between stone types with respect to various lipids and determine

the amounts of complexed and non-complexed lipids in stone matrices. Calcium lipid complexes have been suggested to play a critical role in the nucleation of calcium phosphate [13–15]. To isolate complexed and non-complexed lipids from the same stones, we followed the procedures developed by Boyan and Boskey [14].

Urinary struvite, CaOx and uric acid stones were obtained from our departmental surgical pathology practice and stored at −80°C. Stone fragments were analyzed using x-ray diffraction and were classified according to their composition. For example, stones with 70% or more of CaOx were considered CaOx. Approximately 4 g of each stone were thoroughly washed and sonicated to remove blood and surface debris. After drying, stones were ground to a fine powder. Lowry's method was used to determine the protein contents of the stone matrix. The lipids were extracted from the stone powder with ice-cold chloroform:methanol:0.05 mol/L Tris-HCl, pH 7.4 (2:1:1); 30 mL of extraction solvent to 1 g of stone using sonication at 4°C. Sonication was carried out for 10 minutes, after which the sample was centrifuged to phase separate and pellet the residue. The upper and lower phases were removed separately and pooled as aqueous and organic substances, respectively. Fresh extraction solvent was added to the pellet and the process repeated several times pooling the respective phases. Pooled organic phases were extracted with ethanol:ether (3:1) and then centrifuged. Non-complexed lipids were isolated from the supernatant. Complexed lipids were recovered from the pellet.

Langmuir monolayers

Details of the experimental procedures are given in previous articles [19–21] and, therefore, only a brief description of the experimental methods is given here. The effects of changing head group, mean molecular area and surface pressure were investigated. Six different phospholipid interfaces, with different head groups and alkyl tails, were investigated. Monolayers of dipalmitoylphosphatidylglycerol (DPPG), dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidylserine (DPPS), dioleoylphosphatidylglycerol (DOPG), palmitoyl-oleoylphosphatidylglycerol (POPG) and dimyristoylphosphatidylglycerol (DMPG) were prepared at the air/water interface by spreading the lipid from a chloroform/methanol solution and compressing the films to the targeted surface pressure using opposing moveable barriers. The subphase was a metastable calcium oxalate solution of relative supersaturation (RS) 5. The RS values, specifically for the COM "whewellite" crystals, were computed using Finlayson's EQUIL v1.3 [22]. The Langmuir monolayers were held at the desired pressure at the solution/air interface at 25°C for a specified period of time (typically 4 h). Monolayers were then analyzed, either in situ, using Brewster angle microscopy (BAM), or ex situ, using SEM, TEM or optical microscopy. The BAM sees only what is taking place at the monolayer/water interface by measuring differences in refractive indices at the surface [21]. For ex situ analyses, Langmuir monolayers were deposited onto a solid support by carefully draining the trough to lower the monolayer onto a substrate that had been placed in the subphase before the monolayer was applied. To be sure that nucleation occurred only in the presence of the Langmuir monolayer, a control was performed for each experiment by placing a solid support outside the barriers where there was no monolayer. For each of the experiments described here, no COM crystals were observed on the control substrates.

Table 1. Comparison of urinary excretion of proteins and lipids between healthy, calcium oxalate and uric acid stone formers

	Uric acid stone formers *mg/24 hr* ($N = 3$)	Normal *mg/24 h* ($N = 8$)	Calcium oxalate stone formers *mg/24 h* ($N = 12$)
Total proteins	71.35 ± 15.29^a	28.09 ± 2.47	42.43 ± 5.18^b
Glycolipids	8.57 ± 1.64^a	3.93 ± 0.85	5.35 ± 1.01
Cholesterol	3.86 ± 0.34^a	1.98 ± 0.28	3.39 ± 0.40^b
Cholesterol ester	2.43 ± 0.35^a	1.18 ± 0.20	2.23 ± 0.25^b
Triglycerides	34.70 ± 2.22^a	8.99 ± 2.24	18.08 ± 3.59^b
Phospholipid (VBR)	0.402 ± 0.14	0.351 ± 0.05	0.718 ± 0.13^b
Phospholipid (NH_4)	1.60 ± 0.59	1.10 ± 0.17	2.08 ± 0.56

N is the number of stone patients.
[a] $P < 0.05$ between normal and uric acid stone formers
[b] $P < 0.05$ between normal and calcium oxalate stone formers

Statistical analysis

Statistical analysis of the data was performed using Microsoft EXCEL's version of the Student t test. Results are presented as ± SD.

RESULTS

Urinary lipids

The mean creatinine clearance rate for calcific stone formers was 84 ± 22.19 mg/mL/min. The urine from stone formers contained higher amounts of total and individual phospholipids than urine from healthy subjects. Quantitative determination of phospholipids using the VBR method showed significantly more phospholipids in the calcium oxalate stone formers' urine than in the urine from healthy individuals or uric acid stone formers (Table 1). Thin-layer chromatography detected SM, PC, PE, as well as PS in all urinary samples investigated. In addition some urine samples from both stone formers and healthy subjects contained PI, cardiolipin (CL), and PA. Occasionally lyso (L)-PC, -PE, and -PA were detected also. More calcium oxalate stone formers' urine contained cardiolipin, and PA than urine from healthy individuals or uric acid stone formers. Densitometric quantification of individual phospholipids showed that urine from cal-

Table 2. Average protein and lipid contents of kidney stone as percent of the matrix

Stone type	Protein	Lipids
	%	
Struvite ($N = 5$)	74 ± 33.9	26 ± 3.9
Calcium oxalate ($N = 5$)	20 ± 6.3	80 ± 7.5
Calcium phosphate ($N = 3$)	33 ± 11.1	67 ± 5.6
Uric acid ($N = 5$)	75 ± 3.9	25 ± 6.6

cium oxalate stone formers contained higher amounts (mg/24 h) of SM (1.57 ± 0.76 vs. 0.98 ± 0.6), PC (1.66 ± 1.6 vs. 0.56 ± 0.34), and PS (0.72 ± 0.63 vs. 0.45 ± 0.16) than urine from the healthy subjects.

The urine of both uric acid and calcium oxalate stone formers contained significantly higher amounts of cholesterol, cholesterol ester and triglycerides than did the urine obtained from healthy subjects. Urine also contained glycolipids consisting of sulfatides, gangliosides, sphingosine, as well as glucocerebrosides. Even though urine from calcium oxalate stone formers contained more glycolipids, differences in the total or individual glycolipids between urine from healthy subjects and calcium oxalate stone formers were not significant.

Lipids of the matrix of urinary stones

As expected, the matrix of all stones investigated including struvite, uric acid, calcium oxalate and calcium phosphate contained both proteins and lipids (Table 2). The protein-to-lipid ratio, however, appeared higher in the matrix of struvite and uric acid stones than the matrix of calcium oxalate and calcium phosphate stones. Even though there were no significant differences in various types of lipids encountered in the stones (Table 3), there were some clear dissimilarities between struvite stones associated with infection and other non-infectious stones on the one hand and between calcium containing and uric acid stones on the other. Matrix of struvite stones contained more cholesterol and triglycerides than others. Calcific stones contained more phospholipids than uric acid stones. One-dimensional thin layer chromatography was used to separate and identify various phospholipids and glycolipids. SM, PC, PE, CL and trace amounts of PS were detected in matrices of all stones (Table 4). Matrix of struvite stones also contained quantifiable PS and PA. Occasionally the organic matrix of various stones showed the presence of PI, lyso-PC, lyso-PA and lyso-PE. Glycolipids identified in all stones included gangliosides, D-sphingnosine, and glucocerebrosides (Table 5). Struvite stone matrix also contained sulfatides and digalacto-diglycerides, while matrices of calcium oxalate and calcium phosphate stones contained cerebrosides 1 and 2 and digalacto-diglycerides.

The matrices of all stones contained both complexed and non-complexed lipids (Table 6). The amount of complexed lipids was highest in calcium phosphate stones and lowest in the uric acid stones. Both complexed and non-complexed lipids contained cholesterol, triglycerides, phospholipids and gangliosides.

Lipids of the matrix of crystals induced in healthy human urine

Both calcium oxalate and calcium phosphate crystals induced in the urine contained lipids (Table 7). There were no significant differences in either the nature of lipid constituents or the lipid amounts/g of crystals between two types of crystals. Glucocerebrosides were the most common glycolipids, while SM was the most common phospholipid. Gangliosides were the second most common glycolipids and PC and PE the most common phospholipids.

Crystallization at Langmuir monolayers

Observation of nucleation at phospholipid interfaces. Crystal growth was monitored under Langmuir monolayers of each lipid held at 20 mN/m (Fig. 1A). In all experiments the majority of the crystals nucleated with the (10-1) face toward the phospholipid monolayer (Figs. 2 and 3). While the overall selectivity in crystal habit remained the same for each of the lipids, the number of crystals observed under the monolayers changed, decreasing in the order: DPPG > DPPS ≥ DPPC (Fig. 1A). Even under lower surface pressure (Fig. 1B), the DPPG subphase produced more crystals than either the DPPS or DPPC.

Effects of monolayer fluidity on nucleation. In the Langmuir monolayer experiments, changing the surface pressure applied by the moveable barriers altered the fluidity and the local order within the model membranes. Increased pressure brought the molecules close together and decreased phospholipid fluidity. Lowering the surface pressure increased the number of crystals/mm^2 (Figs. 1A and B) without having a significant effect upon crystal orientation. Crystals with the (10-1) face oriented toward the monolayer still predominated except for a somewhat higher incidence of agglomerates under the DPPG monolayer. The effect of packing density of the headgroup on crystal formation was probed by employing DPPG, DMPG, POPG and DOPG monolayers. At surface pressure of 20 mN/m, mean molecular area increases in the order of DPPG < DMPG < POPG < DOPG ranging from 40 to 90 Å^2/molecule because different lipophilic tails had different sizes even though the headgroup remained the same. Clearly more crystals formed at lower mean molecular area (Fig. 1A). Still, most crystals formed with their (10-1) face toward the monolayer. When DPPG, DMPG, POPG and DOPG monolayers were held at the same mean molecular area

Table 3. Lipid constituents of the organic matrix

Stone type	Total cholesterol	Cholesterol ester	Triglycerides	Glycolipids	Phospholipids (AMF)	Phospholipids (VBR)
	mg/g stone					
Struvite	1.53 ± 0.72	0.21 ± 0.25	10.71 ± 9.17	0.13 ± 0.05	0.57 ± 0.5	0.06 ± 0.04
Calcium oxalate	0.64 ± 0.27	0.37 ± 0.10	1.64 ± 0.6	0.16 ± 0.06	0.18 ± 0.1	0.05 ± 0.03
Calcium phosphate	0.76 ± 0.5	0.37 ± 0.26	1.45 ± 0.13	0.17 ± 0.1	0.31 ± 0.23	0.05 ± 0.02
Uric acid	0.2 ± 0.07	0.11 ± 0.04	1.6 ± 0.34	0.09 ± 0.03	0.08 ± 0.03	0.03 ± 0.01

Number of stones is listed in Table 2.

Table 4. Major phosphilipids of stone matrix quantified by the Bartlett method

Stone type	SM	PC	PE	CL	PS	PA
	% of total phospholipids					
Struvite	30.4 ± 12.9	11.4 ± 10.3	8.7 ± 6.8	19.6 ± 16.6	7.1 ± 2.4	21.9 ± 29.0
Calcium oxalate	32.6 ± 6.6	16.8 ± 4.9	34.7 ± 7.6	11.2 ± 3.0		
Calcium phosphate	32.2 ± 18.0	15.0 ± 12.4	22.4 ± 8.6	12.7 ± 5.7		
Uric acid	20.5 ± 11.2	28.3 ± 5.5	29.8 ± 1.7	19.6 ± 11.6		

Abbreviations are: SM, sphingomyelin; PC, phosphatidylcholine: PE, phosphatidylethanolamine; CL, cardiolipin; PS, phosphatidylserine; PA, phosphatidic acid.

Table 5. Major glycolipids of stone matrix quantified by densitometric analysis of the spots after chromatography

Stone type	Gangliosides	D-sphingonosine	Sulfatides	Digalactodiglycerides	Cerebrosides	Glucocerebrosides
	% of total glycolipids					
Struvite	14.7 ± 3.9	3.0 ± 0.6	9.6 ± 4.4	13.8 ± 1.6		58.73 ± 3.7
Calcium oxalate	18 ± 7.6	1.9 ± 0.6		2.6 ± 2.3	36.7 ± 11.7	48.2 ± 23.1
Calcium phosphate	3.0 ± 2.3				40.8 ± 6.2	50.0 ± 0.5
Uric acid	52.0 ± 33.4					47.0 ± 36.2

Table 6. Complexed and non-complexed lipids of the stone matrix

Stone type	Non-complexed lipids	Complexed lipids
	mg/g stone	
Struvite	3.5 ± 2.53	0.7 ± 0.50
Calcium oxalate	9.93 ± 3	2.34 ± 0.82
Calcium phosphate	5.06 ± 1.2	2.76 ± 2.8
Uric acid	4.19 ± 1.45	0.76 ± 0.18

N is the same as in Table 2.

of 95 Å^2/molecule (Fig. 1B), again most crystals formed with their 10-1 face toward the monolayer.

DISCUSSION

Human urine usually contains only very small amounts of lipids. However, under certain nephrotic syndromes the urinary excretion of cholesterol, cholesterol esters, triglycerides, free fatty acids and phospholipids is considerably increased [23]. Many of these lipids originate from the plasma. In some diseases urinary excretion of specific lipids is increased. Patients with mitochondrial encephalomyopathy, for example, excrete PE, PS and cardiolipin derived from mitochondria and sulfatides specific to renal epithelial cells [24]. Increased lipiduria is caused also by intake of a variety of drugs and some common chemicals. Aminoglycoside antibiotics like gentamicin injure the proximal tubular epithelial cells inducing myeloid bodies in their lysosomes [25]. These bodies are later extruded out of the cells and cause an increase in urinary levels of phospholipids such as PE, PC, PS and PI. Nephrotoxins such as mercuric chloride [25] or ethylene glycol [9, 10] induce shedding of the microvillous brush border of the renal epithelium reflected in increased urinary excretion of phospholipids such as sphingomyelin. Thus, phospholipiduria is often an indication of membranuria.

Animal model and tissue culture studies have shown that an exposure to high levels of oxalate and/or CaOx crystals challenges the renal epithelial cells. Production of proteins such as osteopontin [26, 27], bikunin [28, 29] and Tamm-Horsfall protein [30] is increased. Cholesterol contents of proximal tubular cells are known to increase also when they are injured [31]. Prolonged exposure to oxalate and CaOx crystals results in cellular damage, resulting in shedding of microvillous brush border and ultimately in sloughing of the cells into the urine [9, 10]. These changes in membrane lipids must manifest as increases in their urinary contents. Thus, our results show-

Table 7. Lipids present in the matrix of calcium oxalate and calcium phosphate crystals produced in normal human urine

Crystal type	Total cholesterol	Cholesterol ester	Triglycerides	Glycolipids	Phospholipids (AMF)	Phospholipids (VBR)
	mg/total crystal/24 h					
Calcium oxalate	0.37 ± 0.09	0.20 ± 0.04	3.11 ± 2.9	1.15 ± 1.2	0.06 ± 0.04	0.09 ± 0.04
Calcium phosphate	0.27 ± 0.1	0.21 ± 0.03	2.51 ± 2.4	1.35 ± 1.04	0.07 ± 0.03	0.02 ± 0.01

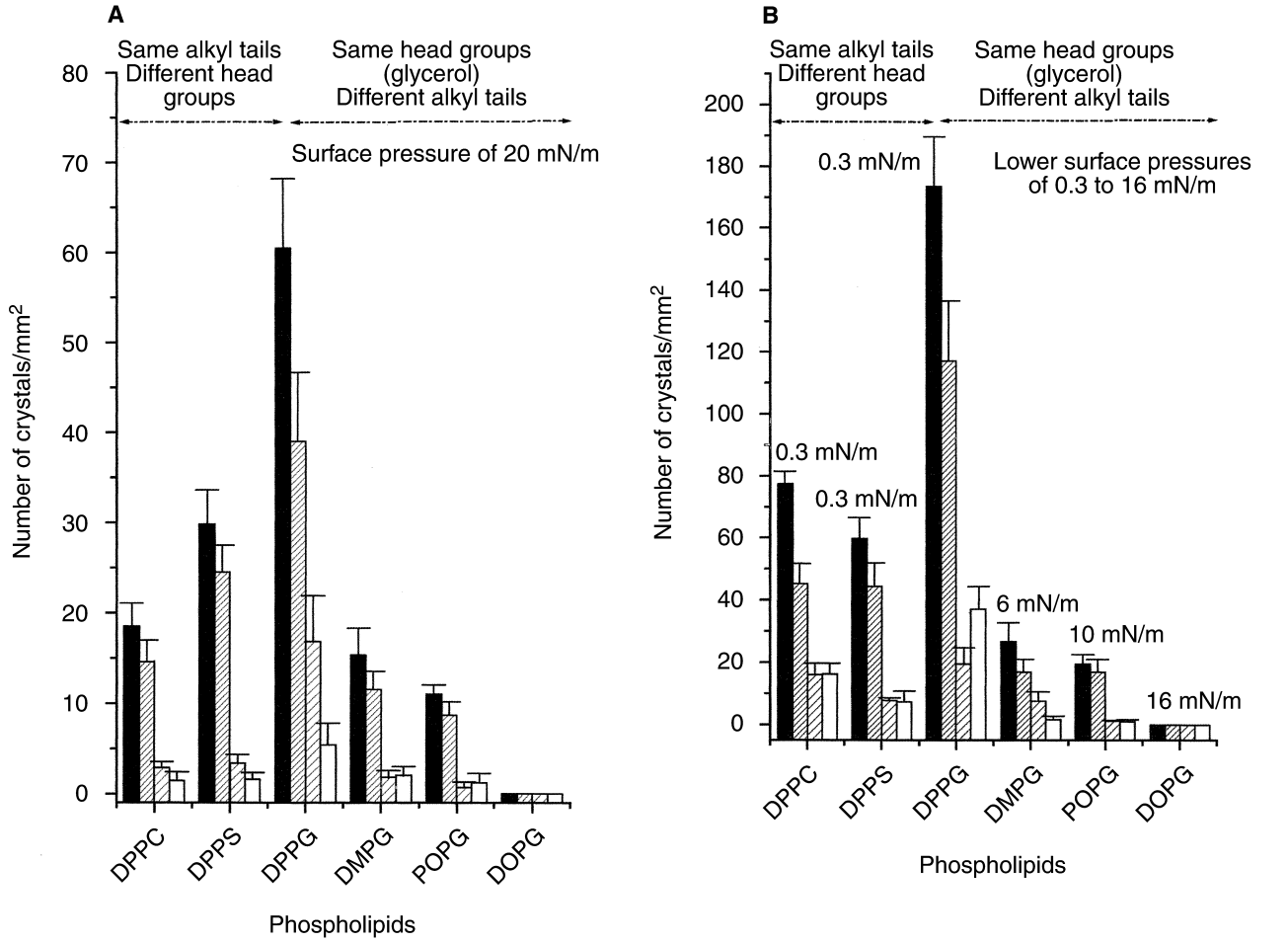

Fig. 1. Histograms showing the distribution of the observed COM crystal orientations under Langmuir monolayers of six different phospholipid interfaces from a calcium oxalate subphase of relative supersaturation of 5. Data are shown for high (*A*) and low (*B*) surface pressure. The number of densities reported, are on average over at least 40 mm^2. Note that DPPC, DPPS, DPPG, have different polar headgroups, while the series DPPG, DMPG, POPG, and DOPG has the same headgroup but different lipophilic tails. Symbols are: (■) total crystals: (▧) faces 10-1; (▨) faces 010; (□) agglomerates.

ing significantly increased urinary excretion of proteins and lipids by stone formers may be indicative of a renal response to prolonged exposure to oxalate and/or deposition of CaOx/CaP/uric acid crystals in the tubules. Lipiduria alone may be a non-specific reaction of the kidneys to various challenges, but in the case of stone formers, it is perhaps a surrogate marker for crystallization in the kidneys.

According to the current concepts on calcification, initial deposition of calcium phosphate (CaP) occurs on cellular membranes that are present at the calcification site either as a limiting membrane of the so-called matrix vesicles or as cellular degradation products [2, 32]. Even biomaterial associated calcification, such as that of bioprosthetic heart valves fabricated from porcine aortic valves or bovine pericardium, is associated with cellular membrane fragments derived from the pig cusp cells in the case of porcine valves and from connective tissue cells in the case of bovine pericardium [33]. Calcification of intrauterine devices also appears to be initiated by cellu-

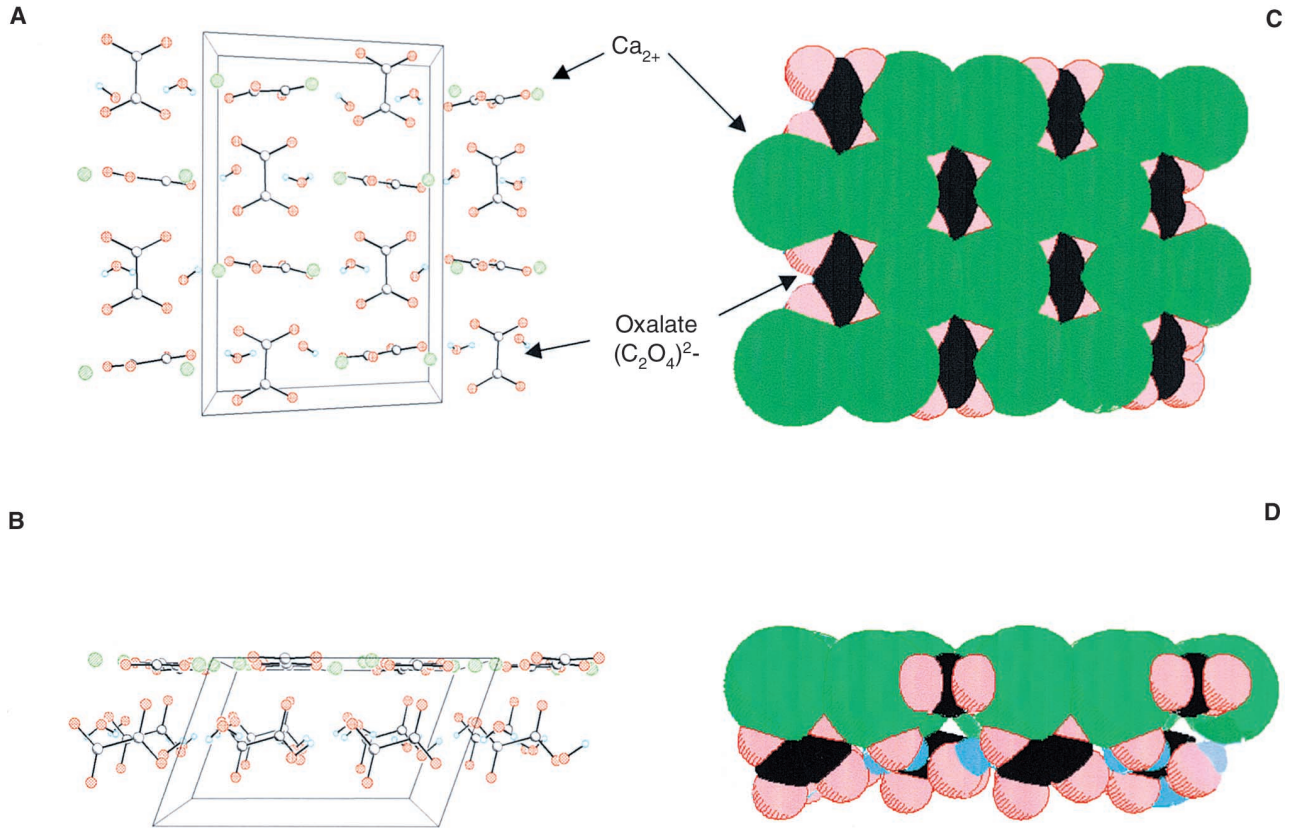

Fig. 2. Views of the calcium oxalate monohydrate (COM) crystal structure perpendicular (*A* and *C*) and parallel (*B* and *D*) to the (10-1) face. Views C and D are space-filling representations, showing the different sizes of the ions. Calcium ions and oxalate ions are indicated. The structure consists of sheets parallel to the (10-1) face that alternate between calcium-rich and oxalate-rich. Anionic additives are expected to stabilize the calcium-rich layers, giving the surface shown in C. The large ionic radius of Ca^{2+} causes it to project well beyond the oxalate ion (view D) and dominate the surface. Key to atom coloring: calcium is green; oxygen, red; carbon, black; hydrogen, blue.

lar membranous material that is deposited on the devices during their exposure to the uterine fluid [34]. Dental plaque and calculus formation is yet another example of calcification initiated by cellular membranes [4–6]. Membranes of microorganisms present in the dental plaque nucleate calcium phosphate and thus initiate calculus formation. One of the main reasons for cellular membranes to act as specific nucleators of calcium phosphate is proposed to be the presence of lipids and particularly the acidic phospholipids therein. We have proposed that membranes and their phospholipids are similarly involved in nucleation of calcium oxalate crystals and formation of kidney stones [11, 12, 35].

Our results show that lipids are present in stone matrices of all stones irrespective of the inorganic nature of their major crystalline components, be they calcium oxalate, calcium phosphate, struvite or uric acid. However, calcium oxalate and calcium phosphate stones contained 2 to 4 times more lipids than proteins (Table 2), while uric acid and struvite stones contained fewer lipids than proteins. Furthermore, calcium oxalate and calcium phosphate stones contained more complexed lipids than struvite and uric acid stones (Table 6). Formation of a complex between calcium and acidic phospholipids is considered the initial step in calcification [13–15]. Phosphatidylserine is one of the key membrane lipids involved in the formation of such complexes and, as reported here and earlier [7], was detected in matrices of all stones examined. However, PS amounts were so small that we were unable to quantify it in all samples and thus it was not included in Table 4. Lipid matrix of struvite stones contained comparatively higher amounts of triglycerides, cholesterol (Table 3) and sulfatides (Table 5), which may be a result of bacterial infection associated with struvite stone formation. In addition, cardiolipin constituted a higher percentage of phospholipids in the matrix of struvite stones. Cardiolipin is a significant part of bacterial membranes. The presence of cardiolipin in other stones is most likely a result of mitochondrial inclusion in the matrix. Mitochondrial membranes also contain significant amounts of cardiolipin. Ultrastructural examination of calcium oxalate stones has shown their matrix to contain cellular degradation products including mitochondria [35].

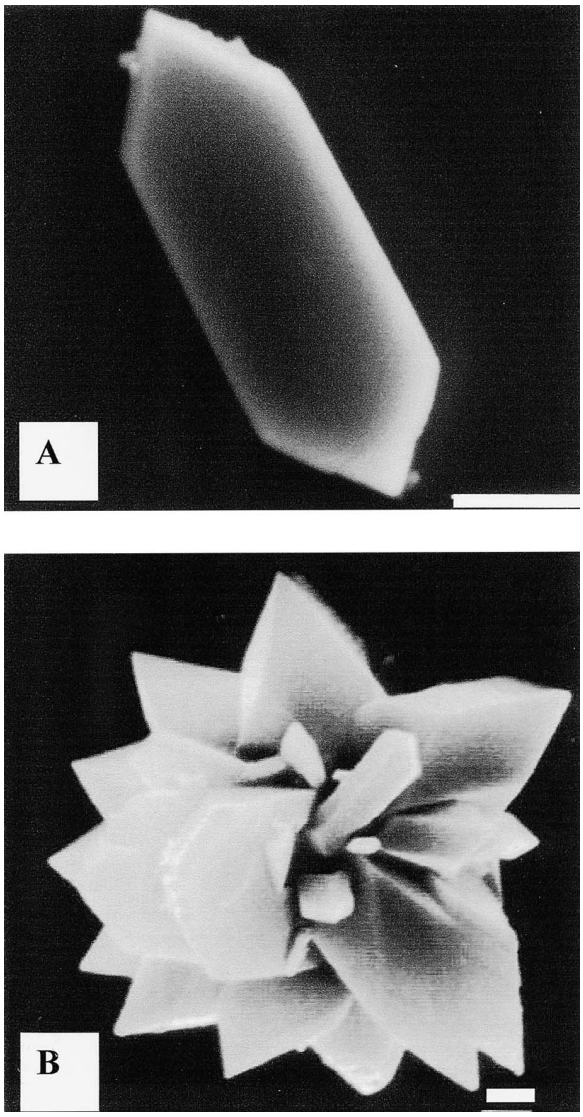

Fig. 3. Scanning electron micrograph of the CaOx crystals growing on a Langmuir monolayer. Bar = 1 μm. (*A*) A single CaOx monohydrate growing with 10-1 face toward the monolayer. (*B*) An aggregate of CaOx monohydrate crystals growing on the monolayer.

We propose that membrane lipids present in the urine promote crystallization of CaP and CaOx and then become a part of growing stones. It is possible, however, that cellular membranes and lipids found in stone matrix are accidentally incorporated by binding to surfaces of growing and aggregating crystals, since crystals and stones form in urine rich with these entities. Moreover, crystals and growing stones can physically damage the renal cells causing increased sloughing of cells into the urine. Similar arguments have been made about the accidental inclusion of urinary proteins. To deal with this issue, it is now customary to generate crystals in the healthy urine and study the macromolecules, which become associated with the induced crystals [17]. Our results demonstrate that both CaP and CaOx crystals induced in vitro in healthy human urine contained neutral lipids, phospholipids as well as glycolipids. Moreover, cholesterol, cholesterol esters, triglycerides, glucocerebrosides, gangliosides, sphingomyelin, phosphatidylcholine and phosphatidylethanolamine were identified, lipids that are the major constituents of renal cell membranes [36].

Langmuir monolayers of various membrane phospholipids were used to investigate their potential involvement in the crystallization of CaOx [19–21]. While the monolayer experiments are not physiological, they provide direct physical evidence that CaOx can be nucleated at phospholipid interfaces. Previous studies have shown that nucleation at a monolayer from a low supersaturation calcium oxalate solution is heterogeneous and selective with respect to the nature, orientation and morphology of the precipitated crystal [19–21, 37]. The rate of crystallization is higher at monolayers of acidic phospholipids. Crystals are mostly CaOx monohydrate and the majority nucleate as single cuboidal structures with their (10-1) face oriented toward the monolayer (Figs. 2 and 3). As seen in Figure 2, layers of the cations alternate with layers of anions parallel to the (10-1) direction so that this crystal surface either can be rich in calcium ions or rich in oxalate ions. In the presence of anionic phospholipids, it is reasonable to envision the layer of calcium ions at the interface as the point of interaction and crystal nucleation. Once the crystal forms, the anionic phospholipid will remain adsorbed to the surface, stabilizing the calcium layer and making the (10-1) face prominent in the observed crystal habit.

Monolayer studies can be extended to explore further the variables that can influence nucleation. For example, the effect of the fluidity of the monolayer was studied by maintaining the same head group with different alkyl tails and under different applied surface pressures. When comparing the same headgroups under different conditions, CaOx monohydrate crystals were observed to form at both high and low pressures (Fig. 1). Despite the significantly different mean molecular area/headgroup represented in the experiments, the selectivity for the (10-1) face relative to the other faces remained nearly constant. The monolayers held at 20 milliNewton/m cannot all present the same template because the lipophitic tails have different sizes, yet they show similar selectivity (Figs. 1A). Even though more crystals were observed under DPPG, the selectivity for the (10-1) and (010) COM faces was similar for DMPG and POPG, which cannot achieve the same packing geometry as DPPG. It appears that the highest density of crystals resulted under the phospholipids that have the potential to achieve the smallest mma in association with the higher anionic density.

The monolayer does not have to be held in a compressed state to nucleate CaOx, but if it is capable of organizing in a smaller area, the crystal precipitation in-

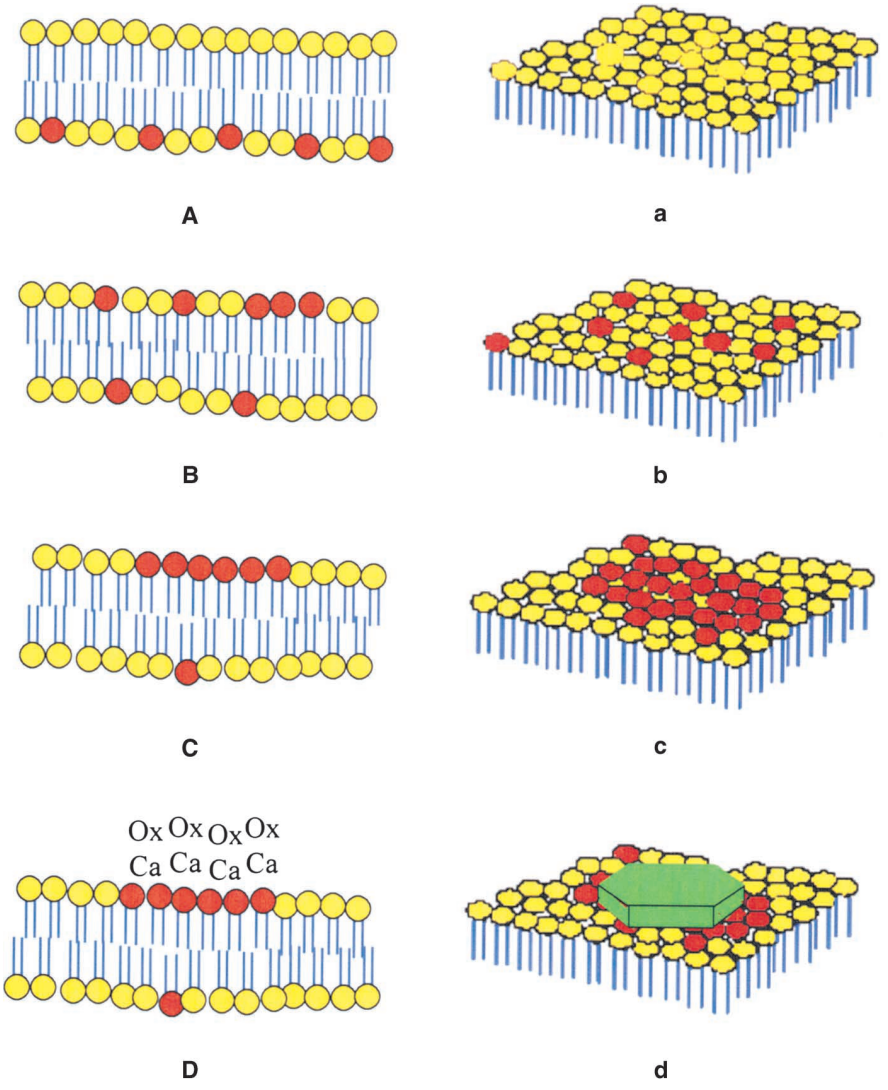

Fig. 4. Schematic presentation of changes in cell membrane phospholipids and nucleation of calcium oxalate crystals based on Wiessner et al [49] and Khan [50]. Two-dimensional (*A–D*) and three-dimensional (a–d) views. (A, a) Normal membrane with only the neutral phospholipids (yellow circles) on the outer surface. (B, b) Movement of acidic phospholipids (red circles) from inside to the outside. (C, c) Lateral movement of acidic phospholipids into specific domains and concentration of calcium ions. (D, d) Interaction between calcium and oxalate ions and nucleation of CaOx crystal.

creases. It is clear from Figure 1 that crystallization was enhanced at lower applied surface pressures. For each of the monolayers, crystal numbers increased as the monolayer expanded. Also, when the DPPG, DMPG, DOPG and POPG monolayers were compared at the same area (Fig. 1B), those at the lower corresponding surface pressure generated more crystals. These observations are consistent with the concept that crystal formation is enhanced if the interface is more fluid, or has the ability to rearrange to accommodate the nucleating or growing crystal.

Crystal attachment to the inner medullary collecting duct cells has previously been correlated with membrane fluidity [38]. Results of other tissue culture studies also indicate that dynamic regions at the lipid interface lead to increased chances of crystal nucleation and or crystal attachment [39–41]. Membrane damage, which is so prevalent after exposure to oxalate and CaOx crystals, may lead to exceptionally fluid sites that can catalyze crystal nucleation and adhesion.

Templating by organic interfaces is often invoked in biomineralization. However, there does not appear to be a direct lattice match between closely packed phospholipid monolayers and CaOx monohydrate [19–21]. More likely, there is a more complex process involving concentration of Ca^{2+} ions at the phospholipid interface followed by reorganization of the lipids to stabilize the nascent crystal face. The (10-1) face of CaOx monohydrate can be calcium-rich (Fig. 2), and the tendency of the phospholipids to bind Ca^{2+} may provide a mechanism to sufficiently concentrate calcium ions, mimicking the calcium-rich face, to lead to specific nucleation. Since the structure is layered in the (10-1) direction, the phospholipid anions do not need to be displaced to bind oxalate beneath the calcium layer (Fig. 2B). The phospholipids stabilize this face, making it the predominant

facet in the observed crystals. The calcium rich (10-1) crystal face has been implicated also in adhesion of CaOx monohydrate crystals to membranes in lipid enriched cell culture studies [38]. The calcium-rich face of CaOx dihydrate crystals has similarly been implicated in their attachment to renal epithelial cells in culture [39].

The influence of the lipid headgroups on precipitation of CaOx monohydrate correlates with the anionic character of the head group (glycerol > serine ≥ choline). Most crystals were observed at the DPPG interface, which has an anionic headgroup, while fewer crystals were observed at the negatively charged zwitterionic DPPS interface and the neutral zwitterionic DPPC interface. The observations further suggest that calcium binding at the interface, either electrostatic or specific, plays a role in CaOx monohydrate nucleation. All three lipids are known to bind Ca^{2+} from aqueous solution and concentrate ions at lipid interfaces. However, calcium binding is greater at the negatively charged lipids.

Increased urinary excretion of oxalate and calcium are the major risk factors for stone formation. Exposure to both high calcium and oxalate induces several changes in the renal epithelial cells [42–49]. Catastrophic changes can induce lateral and *trans*membrane migration of phospholipids, sequestering them in specific domains (Fig. 4). Oxalate exposure induces a redistribution of phosphatidylserine from the inner leaflet of the plasma membrane to the outside [43, 45, 47] and promotes adhesion of CaOx crystals to the epithelium. Membranous vesicles derived from such cells with phosphatidylserine on the surface can promote crystallization of calcific crystals. Partitioning of acidic lipids in specific domains available to calcium and oxalate ions will attract them and nucleate CaOx crystals.

This study demonstrates the presence of lipids in human urine, CaOx crystals generated therein, and in both, the calcium as well as and non-calcium containing urinary stones. Since millions of tubular epithelial cells are discarded daily into the urine, it is highly likely that membranes of some of these cells accidentally become incorporated into the growing stones. We also provide evidence that membrane phospholipids promote heterogeneous nucleation of CaOx crystals, and suggest the possibility that their aggregation may be promoted also by an interaction with the cell membranes. Negatively charged phospholipids may be involved also in crystal attachment to the cell membranes. Thus, cell membranes and their lipids, by virtue of their involvement in crystal nucleation, aggregation and retention, and accidental incorporation become a part of the organic matrix of growing stones.

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APPENDIX

Abbreviations used in this article are: AMF, ammonium ferrothiocyanate; BAM, Brewster angle microscopy; CaOx, calcium oxalate; CaP, calcium phosphate; CL, cardiolipin; COM, calcium oxalate monohydrate; DMPG, dimyristoylphosphatidylglycerol; DOPG, dileophosphatidylglyerol; DPPC, dipalmitoylphosphatidycholine; DPPG, dipalmitoylphosphatidylglycerol; DPPS, dipalmitoylphosphatidylserine; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; POPG, palmitoyloleoylphosphatidylglycerol; PS, phosphatidylserine; RS, relative supersaturation; SEM, scanning electron microscopy; SM, sphingomylein; TEM, transmission electron microscopy; TLC, thin layer chromatography; VBR, Victorian Blue R.

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