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Recl. Trav. Chim. Pays-Bas **114**, 371-376 (1995) 0165-0513/95/08371-06$09.50
SSDI 0165-0513(95)00053-4

Synthesis and aggregation behavior of 2-(4-butyloctyl) malonic acid in aqueous solution. The formation of physically and colloidally stable vesicles by a branched-chain malonate

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(Received January 26, 1995)

Abstract. A new surfactant with a branched monoalkyl chain and a malonate headgroup has been synthesized: 2-(4-butyloctyl)malonic acid (BOMA). From the geometry of the surfactant, reflected in a packing parameter (*P*), it was anticipated that the surfactant would preferably aggregate in bilayers. This expectation was borne out in practice by the aggregation behavior, as studied by transmission electron microscopy (TEM) with negatively stained samples and freeze-fracture samples of aqueous vesicle dispersions. The structure of the surfactant headgroup has been varied by changes in pH of the vesicle dispersions. Over a wide pH range (pH 2.8-12.8) small unilamellar vesicles with a size of 20-50 nm were observed. Multilamellar vesicles were observed as well in the pH range 2.8-5.1. Phase-penetration experiments using an optical polarization microscope showed the birth and growth of myelins and multilamellar vesicles over a pH range 1.5-13.1. At pH < 2.8, a phase separation occured into a surfactant-rich, optically isotropic phase and a surfactant-lean phase. The physical and colloidal stability of the vesicular aggregates is high: up to one year as confirmed by TEM. The fusogenic properties of BOMA vesicles were examined. Upon the addition of Ca^{2+} ions, aggregates tended to become larger, but the formation of Ca^{2+} surfactant crystals was also observed.

Introduction

Eversince *Kunitake's* first report in 1977 on a completely synthetic surfactant capable of forming bilayers[1], many new bilayer-forming surfactants have been synthesized[2]. Most of these surfactants contain two hydrophobic tails. However, it has been shown that surfactants with a single, branched alkyl chain can also form bilayers[3,4,5]. In order to rationalize the preferred morphology of a surfactant aggregate, one can employ an approach couched in terms of a geometric packing parameter (*P*), introduced by *Tanford*[6] and *Israelachvili*[7]:

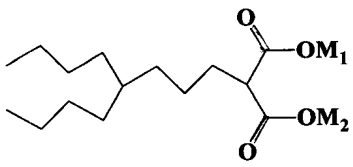
$$P = V / (a_0 \, l_c)$$

In this equation V corresponds to the volume of the hydrocarbon chain(s), a_0 is the optimal cross-sectional headgroup area in the aggregate and l_c is the critical alkyl chain length. According to this theory, P should be in the range of 0.5 to 1.0 for preferential bilayer formation. It is clear that a short chain and a large volume favor the formation of bilayers, depending on the size of the headgroup.

Herein we describe the synthesis and aggregation behavior of 2-(4-butyloctyl)malonic acid (BOMA), see Figure 1. In surfactant chemistry a malonate headgroup has seldom been investigated, only a few examples are known in the literature[8,9,10,11,12]. In the present study the dicarboxylate functionality has been used to influence the morphology of the surfactant by changing the pH. The pK_As of the two carboxylate groups in non-substituted malonic acid are 2.85 and 5.70, respectively[13]. The effect of alkylation at the 2-position of malonic acid on the pK_A is believed to be small. Changing the pH can change the charge of the headgroup and the size of the hydration layer. These factors are reflected in the magnitude of a_0. *Ravoo*[4] found for his branched monoalkyl phosphates that protonation of the phosphate headgroup tended to reduce the size of the hydration layer. Therefore, the bilayers are less curved and larger unilamellar vesicles are formed.

The aggregation behavior of BOMA has been studied using transmission electron-microscopy (TEM, negatively stained samples and freeze-fracture samples) and optical polarization microscopy in phase-penetration experiments.

A remarkable property of branched-chain surfactants appears to be the stability of the corresponding bilayers. For example, di-*n*-alkyl phosphate vesicles are thermodynami-



M_1 = Na, H
M_2 = Na, H

Figure 1. Structure of 2-(4-butyloctyl)malonic acid (BOMA) and its mono- and disodium salt.

cally unstable and crystallization occurs within one day[14]. On the other hand, upon storage at room temperature, branched monoalkyl phosphate vesicles are stable for more than three days[4] and branched monoalkyl phosphorylcholine vesicles for more than one week[5]. The stability of vesicles can be of great importance for possible applications in drug targetting processes[15]. It has been shown previously that synthetic bilayer membranes can fuse asymmetrically with phospholipid membranes and biomembranes[16]. In this context the potential fusogenic properties of vesicles formed of BOMA were examined by adding the fusogenic cation Ca^{2+} to the vesicle dispersions.

Experimental

General procedures

PBr_3, diethyl malonate, $CDCl_3$, D_2O and CD_3OD were obtained from Merck. All solvents were distilled prior to use (diethyl ether and CH_2Cl_2 were distilled from P_2O_5).
^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Varian VXR 300 spectrometer or a Varian-200 spectrometer. Refractive indices were measured on a Carl Zeiss Refractometer equipped with a thermostat. Melting points were determined with a Reichert microscope equipped with a thermometer and heating plate. The elemental analyses were performed in the analytical department of our laboratory. The water used in the preparation of vesicle dispersions was demineralized and distilled twice in an all-quartz distillation unit.

Synthetic procedures

4-Butyloctyl bromide. 4-Butyloctyl bromide was obtained using the procedure described by *Nusselder*[3]. Under a nitrogen atmosphere, 28.0 g (0.15 mol) of 4-butyloctan-1-ol and 10 ml of CH_2Cl_2 were stirred and cooled in an icebath to 0°C. To this solution was added dropwise 14.8 ml (0.15 mol) of freshly distilled PBr_3. The reaction mixture was heated to 70°C in a waterbath and stirred for 2 ½ h. Subsequently the mixture was stirred at room temperature for one night. Then the mixture was cooled in an icebath and neutralized with 200 g of crushed ice and 75 ml of NaOH (4N). The organic product was extracted with 4 × 80 ml of ether. The combined ether layers were washed with saturated $NaHCO_3$(aq) and brine and dried with Na_2SO_4. The crude product was distilled *in vacuo* (84°C/0.08 mmHg) yielding 27.2 g (73%) of 4-butyloctyl bromide as a colorless oil (n_D^{20} = 1.4610). ^{1}H-NMR ($CDCl_3$): *d* 0.89 (6H,t), 1.24-1.40 (15H,m), 1.84 (2H,q), 3.39 (2H,t) ppm. ^{13}C-NMR ($CDCl_3$): *d* 14.0 (CH_3), 23.0 (CH_2), 28.8 (CH_2), 30.1 (CH_2), 32.1 (CH_2), 33.2 (CH_2), 34.3 (CH_2Br), 36.8 (CH) ppm. Anal. Calcd. for $C_{12}H_{25}Br$: C 57.83, H 10.11, Br, 32.06; found: C 57.60, H 10.00, Br 32.07%.

2-(4-Butyloctyl)malonic acid diethyl ester. This di-ester was prepared using a standard procedure[17]. After work-up the crude product was distilled *in vacuo* from a round-bottomed flask, while the product was stirred magnetically. Classical methods using a capillary or wooden stick in a Claisen flask failed because of foaming by the di-ester. The stirring method (135°C/0.08 mmHg) gave a pure, colorless oil in a yield of 65%. ^{1}H-NMR ($CDCl_3$): *d* 0.89 (6H,t), 1.21-1.31 (17H,m), 1.27 (6H,t), 1.87 (2H,q), 3.33 (1H,t), 4.20 (4H,q) ppm. ^{13}C-NMR($CDCl_3$): *d* 14.1 (CH_3), 23.1 (CH_2) 24.4 (CH_2), 28.8 (CH_2), 29.1 (CH_2), 33.2 (CH_2), 37.0 (CH), 52.0 (*C*H(COO)$_2$), 61.2 (CH_2O), 169.6 (COO) ppm. Anal. Calcd. for $C_{19}H_{36}O_4$: C 69.47, H 11.05; found: C 69.31, H 10.78%.

2-(4-Butyloctyl)malonic acid (H_2BOMA). 2-(4-Butyloctyl)malonic acid diethyl ester 13.5 g (41.1 mmol) was cooled in an icebath and 10 ml of an aqueous 2N NaOH solution was added dropwise in 10 min. This mixture was refluxed for 24 h. The alkaline solution was acidified to pH 1 with 2N HCl. The crude organic acid was removed by filtration under suction, dissolved in ether and washed with brine. Separation of the aqueous and organic layer took about 1 h. The ether layer was dried with Na_2SO_4. The ether was removed on a rotary evaporator and the crude product was allowed to crystallize overnight. White crystals (m.p. 81-82°C) were obtained by recrystallization twice from hexane and drying *in vacuo* (0.01 mmHg) in a yield of 8.37 g (75%). ^{1}H-NMR ($CDCl_3$): *d* 0.90 (6H,t), 1.23-1.30 (17H,m), 1.93 (2H,q), 3.46 (1H,t) ppm. ^{13}C-NMR($CDCl_3$): *d* 14.1 (CH_3), 23.1 (CH_2), 24.4 (CH_2), 28.8 (CH_2), 29.0 (CH_2), 33.1 (CH_2), 37.0 (CH), 51.7 (*C*H(COO)$_2$), *d* 175.5 (COOH) ppm. Anal. calcd. for $C_{15}H_{28}O_4$: C 66.14, H 10.36; found: C 65.81, H 10.31%.

Monosodium 2-(4-butyloctyl)malonate (NaHBOMA). Under a nitrogen atmosphere, 780 mg (2.86 mmol) H_2BOMA was dissolved in 20 ml of dry ethanol. To this solution, 19.2 g (24.0 ml) of sodium ethoxide in ethanol (0.149 M) was added dropwise and the mixture was stirred for 30 min. The ethanol was removed with a rotary evaporator. In order to remove traces of water, the salt was stripped twice with dry ethanol. The salt was dried *in vacuo* (0.005 mmHg) for two days in a drying pistol over P_2O_5 at a maximum temperature of 55°C. NaHBOMA was obtained as a slightly yellow, rather sticky solid (840 mg) in an almost quantitative yield (99.7%). ^{1}H-NMR (CD_3OD): *d* 0.90 (6H,t), 1.25 (17H,s), 1.88 (2H,q), 3.07 (1H,t) ppm. ^{13}C-NMR (CD_3OD): *d* 14.2 (CH_3), 23.8 (CH_2), 24.9 (CH_2), 29.7 (CH_2), 32.2 (CH_2), 34.1 (CH_2), 34.4 (CH_2), 38.2 (CH), 52.1 (*C*H(COO)$_2$, 177.9(COO) ppm. Anal. calcd. for $C_{15}H_{27}NaO_4$: C 61.20, H 9.25, Na 7.81; found: C 60.76, H 9.17, Na 7.72%.

Disodium 2-(4-butyloctyl)malonate (Na_2BOMA). The disodium salt was prepared using the same method as described for the monosodium salt but using two equivalents of sodium ethoxide instead of one. The disodium salt was obtained as an almost white amorphous powder. Attempts to recrystallize the salt from methanol/water or ethanol/water mixtures failed. ^{1}H- and ^{13}C-NMR and elemental analyses showed that there was no need for further purification. ^{1}H-NMR (D_2O): *d* 0.83 (6H,s), 1.20 (17H,s), 1.62 (2H,m), 3.01 (1H,t) ppm. Internal reference, CH_3OH: *d* 3.30 ppm. ^{13}C-NMR (D_2O): *d* 13.8 (CH_3), 22.9 (CH_2), 25.0 (CH_2), 28.5 (CH_2), 31.0 (CH_2), 32.9 (CH_2), 33.4 (CH_2), 36.8 (CH), 59.0 (*C*H(COO)$_2$, 180.2 (COO) ppm. Internal reference, CH_3OH: *d* 49.0 ppm. Anal. calcd. for $C_{15}H_{26}Na_2O_4$: C 56.95, H 8.28, Na, 14.54; found: C 56.61, H 8.12, Na 14.37%.

Preparation of vesicle dispersions

The vesicle dispersions were prepared by stirring a weighed amount of surfactant (5-20 mM) in 1 or 2 ml of water or buffer (5 mM NaAc/5 mM HEPES [a], pH 7.4) vigorously for 1 h. The temperature was kept at 25 or 30°C with a waterbath. The stirring speed was 900 turns/min on an Ikameg Ret stirring plate. In some cases, the stirring was followed by sonication. Some vesicle dispersions were prepared using the ethanol injection method[18]. In some cases, small aliquots of aqueous HCl or NaOH solutions were added to the water, prior to the preparation of the vesicle dispersion. No differences were observed between the various preparation methods. After the preparation of the vesicle dispersion, the pH of the solution was measured with an Orion SA 720 pH electrode.

Transmission electron microscopy (TEM)

Samples of the vesicle dispersions were prepared for TEM by bringing a small aliquot of a vesicle dispersion on a carbon-coated formvar grid (300 mesh). In most cases the sample was subsequently negatively stained with a 1% (w/v) aqueous solution of uranyl acetate. Freeze-fracture samples were obtained by the method described by *Koehler*[19] on a Balzer EVM 052 A with evaporation head EK 552. The samples were fractured at -165°C, shadowed with 20 Å of platinum/carbon under an angle of 45°, and coated with 200 Å of carbon. The replicas were cleaned in concentrated chromic acid and water. All samples were examined with a Philips EM 201 electron microscope operating at 60-kV accelerating voltage.

Optical polarization microscopy

Phase-penetration experiments were carried out at 25°C using a Nikon polarization microscope equipped with a monocular, binocular, lambda wave plate and crossed polarizers. A small quantity of NaHBOMA was molten between a microscope slide and a cover slip and subsequently squeezed in order to obtain a round uninterrupted rim of solid surfactant. With a Pasteur pipet, a little amount of water was brought into contact with the solid surfactant. The pH of the water was varied from 1.5 to 13.1 using small aliquots of aqueous HCl or NaOH solutions. Photographs were taken with a Minolta 7000 photocamera using a Kodak ektacolor pro (160 asa) film.

[a] HEPES = 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid.

Results and discussion

Synthesis

4-Butyloctyl bromide was prepared from the reaction of *g*-butyrolactone with two equivalents of *n*-butylmagnesium bromide, followed by dehydration of the tertiary alcohol function and protection of the primary alcohol function with an acetate moiety[3]. The carbon-carbon double bond was hydrogenated and the ester was reduced to yield 4-butyloctan-1-ol. Subsequently the hydroxyl group was converted into a bromide functionality using PBr_3[3,4].
In a next step the 4-butyloctyl bromide was used to alkylate malonic acid diethyl ester[17]. 2-(4-Butyloctyl) malonic acid (H_2BOMA) was obtained after hydrolysis of the diethyl ester in an aqueous 2N NaOH solution followed by acidification[11].
The conversion of H_2BOMA into the mono- and disodium salt was carried out by adding an appropriate amount of sodium ethoxide in ethanol to a solution of H_2BOMA in ethanol. The ethanol was evaporated and pure amorphous solids were obtained.

Calculation of the packing parameter for BOMA

As indicated in the introduction, the packing parameter P can be helpful in predicting the aggregation behavior of a surfactant. *Tanford*[6] introduced equations to calculate the volume of the hydrocarbon chain(s) (V) and the critical alkyl chain length (l_c).

$$V \approx (27.4 + 26.9n_c) \cdot 10^{-3}\ \text{nm}^3$$

$$l_c \approx (0.154 + 0.1265 \cdot n_c)\ \text{nm}$$

In these equations n_c represents the number of carbon atoms that determines the hydrocarbon chain volume (V) and the critical alkyl chain length (l_c). In the case of the 4-butyloctyl chain of BOMA, the chain volume is determined by all carbon atoms (n_c = 12) and V equals 0.35 nm^3. The critical alkyl chain length (l_c) is only determined by the octyl part of the chain (n_c = 8) and the value of l_c is taken as 1.17 nm.
Estimation of the optimal cross-sectional head group area (a_0) is less straightforward. For monoalkyl phosphates a value for a_0 was obtained using the aggregation numbers of micelles consisting of monoalkyl phosphates[20]. Unfor-

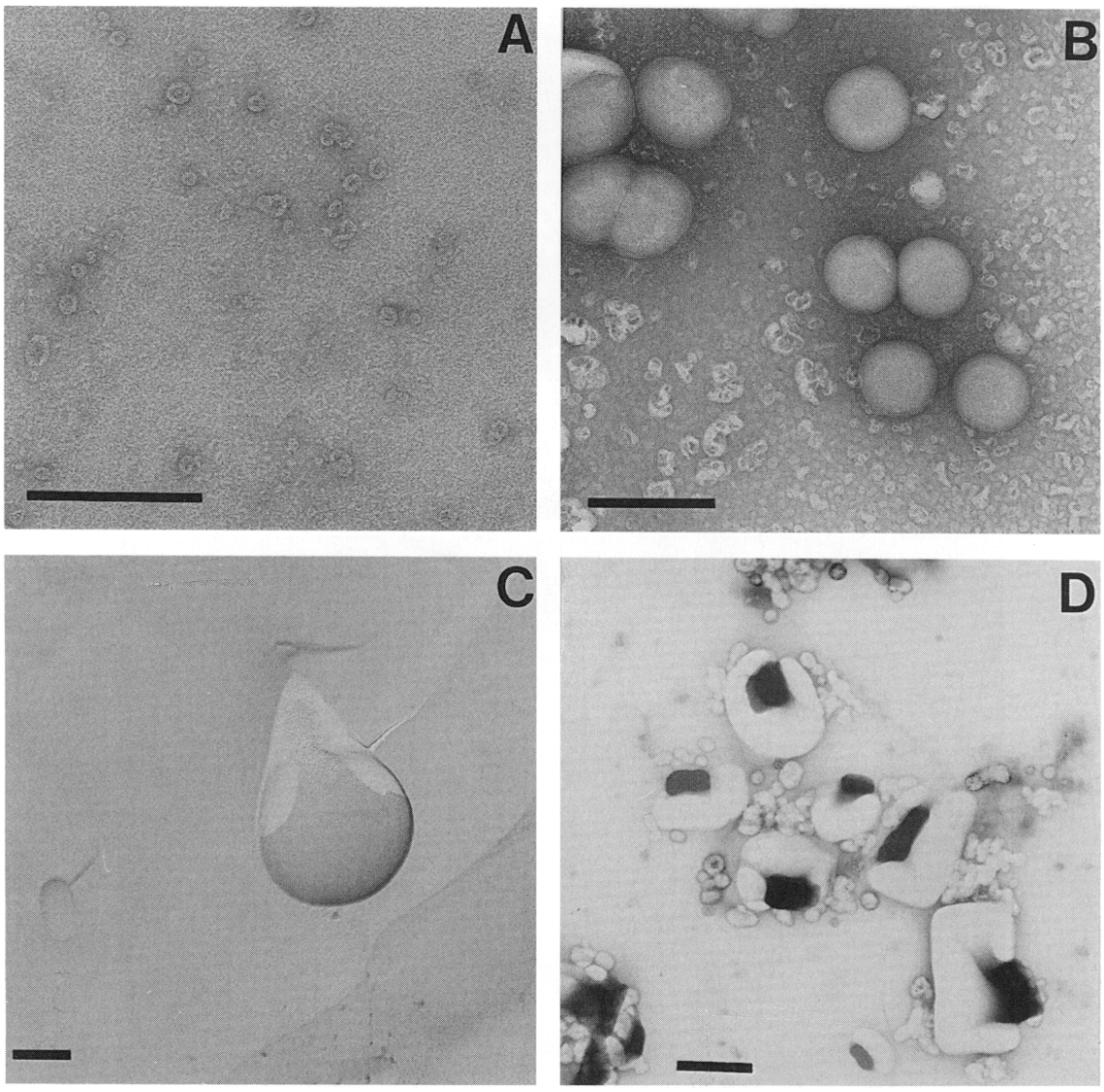


Figure 2. Transmission electron microscopy (TEM). (a) H_2BOMA with 2eq. NaOH(aq), pH 8.8, 18 mM, prepared by stirring at room temperature and sonication, stained with 1.0% UAc, after one day; (b) H_2BOMA in buffer, pH 5.1, 15 mM, prepared by stirring at room temperature, stained with 1.0% UAc, after one year; (c) H_2BOMA in buffer, pH 5.1, 15 mM, prepared with the ethanol injection method at 30°C, freeze-fracture replica, after 40 days; (d) H_2BOMA in buffer, pH 5.3, 3.4 mM, prepared by stirring at 25°C and sonication, $[Ca^{2+}]$ = 3 mM, unstained sample. Bars represent 250 nm.

Table I Features of aqueous BOMA dispersions.

pH (solution)	Human eye	TEM	Phase penetration
pH < 2.8	clouding	-	Myelins/MLVs
2.8 < pH < 5.79	bluish	SUVs&MLVs	Myelins/MLVs
pH > 5.7	clear	SUVs	Myelins/MLVs

tunately, similar data are not available for surfactants with a malonate headgroup. Therefore, a value for a_0 was estimated. *Vikingstad*[21] measured group partial molar volumes for micellization of sodium alkylcarboxylates: $V_m(CH_2COO^-Na^+) = 28.3\ cm^3 \cdot mol^{-1}$ and $V_m(CH_2$,polar) = 16.6 $cm^3 \cdot mol^{-1}$. From these data, one can calculate the approximate value of V_m for a disodium malonate headgroup: $V_m(CH_2(COO^-Na^+)_2) \approx 2 \cdot 28.3 - 16.6 \approx 40\ cm^3 \cdot mol^{-1}$. In that case, the value for a_0 amounts to 0.43 nm^2, leading to $P = 0.70$, which is clearly within the range for which a surfactant is expected to form bilayers. On the other hand, one should be aware of the fact that the value of a_0 will depend on the pH of the surfactant solution.

Vesicle dispersions observed with the human eye

All vesicle dispersions prepared from H_2BOMA, NaHBOMA and Na_2BOMA, exhibited characteristic features depending on the pH of the solution, see Table I. Solutions with a pH > 5.7 (= pK_{A2}) containing dianionic $BOMA^{2-}$, were clear. Solutions with 5.7 (= pK_{A2}) > pH > 2.8 (= pK_{A1}) containing monoanionic $HBOMA^-$ were bluish. Finally, solutions with pH < 2.8 (= pK_{A1}) showed a phase separation into a surfactant-rich and a surfactant-lean phase, often referred to as clouding. The pK_{A1} and pK_{A2} values are estimates for the acid dissociation constants for the carboxyl moieties of the surfactants in the aggregates. All typical features are unchanged after a period of at least one month.

Transmission electron microscopy (TEM)

The formation of vesicles or other bilayer structures was investigated using TEM. Negatively stained samples of vesicle dispersions of H_2BOMA, NaHBOMA and

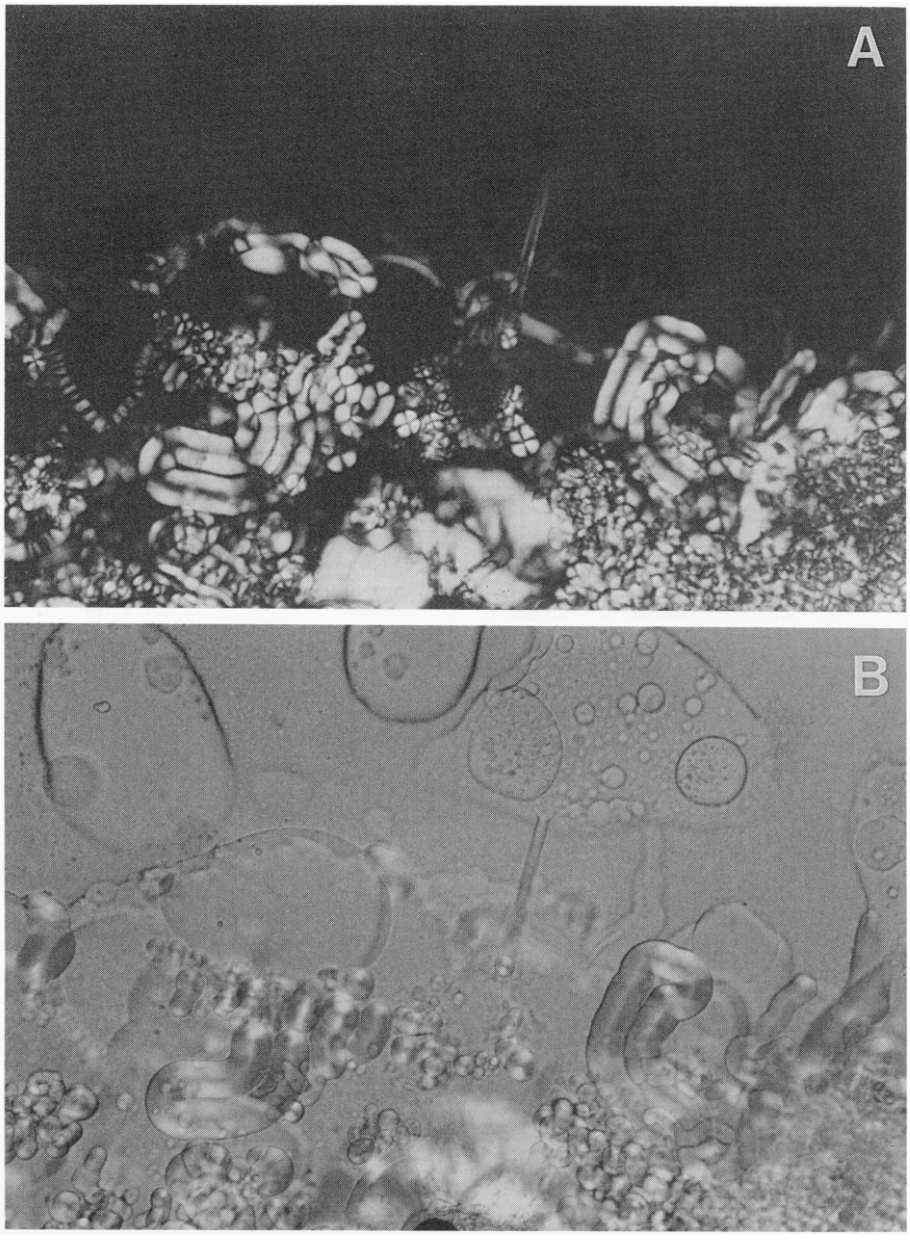


Figure 3. Phase-penetration experiment, using NaHBOMA, in the presence of aqueous HCl, pH 1.5; (a) crossed-polarizers mode; (b) lambda wave plate mode. The lower sides of the micrographs show myelins and MLVs, the upper sides of the micrographs show the optically isotropic surfactant-rich phase.

Na_2BOMA were studied by TEM over a wide pH range (pH 2.8-12.8). Small unilamellar vesicles (SUVs) were observed over the whole pH range for all vesicle preparations. In general, the size of the vesicles varied from 20 to 50 nm, with occasional sizes up to 200 nm (Figure 2a). The influence of the pH on the size of the SUVs was small. Only a slight tendency for formation of larger SUVs at lower pH could be confirmed with a TEM study. In some cases in the pH range from 2.8 to 5.1, multilamellar vesicles (MLVs) were observed as well, with a size varying from 90 to 180 nm (Figure 2b). The bilayer thickness (composed of surfactant and hydration layer) of the MLVs was approximately 3.5 nm. The MLVs were only observed when the solutions were not sonicated. Freeze-fracture replicas from a surfactant solution that had been stored for 40 days at room temperature, showed large MLVs varying in size from 140 to 1100 nm (Figure 2c). The difference in sizes observed for the MLVs is probably due to the electron-microscopic technique used. When the negative-staining technique is employed, large MLVs are likely to fall apart on the carbon-coated formvar grid at the moment that the water is blotted off. With the freeze-fracture technique, very large MLVs are caught in their rapidly vitrified aqueous surroundings, leaving the structure of the MLVs intact. After fracturing some MLVs can be broken through, revealing their multilamellar inner structure.

Stability of BOMA-vesicles

The vesicle dispersions of BOMA prepared by stirring at room temperature show a remarkably high physical and colloidal stability. After storage at room temperature for more than a year, no crystallization, flocculation or precipitation of the surfactant was observed. MLVs and SUVs were still observed using TEM. Previous work from our group indicated that vesicular structures of surfactants with branched tails can show remarkable physical and colloidal stability[4,5]. The high stability of branched monoalkyl phosphate vesicles is rationalized on the basis of the dianionic character of the phosphate headgroup at pH 11. The high negative charge of the surface of the vesicles gives rise to a large electrical double layer repulsion as well as a large hydration layer repulsion[22]. This prevents vesicles approaching each other and flocculating. It is likely that vesicles consisting of mono- or dianionic BOMA exhibit the same features in an even more drastic way. Because of the expected hardness of the two headgroup carboxylate functionalities, the headgroup will be strongly hydrated and the counter-ion binding of the vesicles will be low. Observations described for BOMA vesicles confirm this hypothesis.

The stability of the aggregates as expressed by their reluctance to crystallize from the solution can be explained in terms of the lower Krafft temperature of branched surfactants. For example, *Varadaraj* et al.[23] found a much lower Krafft temperature upon increasing the branching of the alkyl chains. We find that the Krafft temperatures for Na_2BOMA and NaHBOMA are below room temperature, as can be concluded from their high solubility. The presence of the branched chain implies that it is more difficult for the surfactant to pack efficiently, in a lattice compared to the linear chain analogue. An illustration of this phenomenon is provided by the comparison of the melting points of 2-*n*-dodecylmalonic acid: 121-121.5°C[8] *versus* 2-(4-butyloctyl)malonic acid: 81-82°C (*vide supra*).

Phase-penetration experiments

The formation of bilayers from 2-(4-butyloctyl)malonic acid was also investigated using the phase-penetration method (also known as contact-preparation method)[14,24,25]. Under an optical polarization microscope the lyotropic behavior of surfactants can be examined when water is brought into contact with solid surfactant. After initial hydration at the solid-water interface, the surfactants can aggregate into myelin structures, depending on their geometrical features and the state of hydration[6,7]. In the outermost region, closest to the bulk aqueous phase, the surfactant monomers are maximally hydrated. The presence of multilamellar aggregates in that region reflects the ability to form vesicles in an aqueous solution.

In the present experiments the monosodium salt (NaHBOMA) was used, because this solid could be molten to a liquid-crystalline state in which it could be sqeezed to a round shape, which is convenient for observing myelin birth and growth. Aqueous solvent of varying pH (pH 1.5, 3.0, 4.5, 9.5, 10.5, 13.1) was added to NaHBOMA at room temperature. Over this total pH range the birth and growth of myelin structures and MLVs could easily be observed.

Interestingly, the phase separation that occurred at pH < 2.8 could be studied under the microscope using both the lambda wave plate and the crossed polarizers. At the solid-water interface, myelins and MLVs were born upon addition of water of pH 1.5. After one minute some of these lamellar structures dissolved in an optical isotropic phase, observed with the crossed-polarizers mode: no birefringence (Figure 3a). With the lambda wave plate mode the phase separation was observed as small drops of surfactant-rich phase in the bulk aqueous phase (Figure 3b). It was found that the acid-induced phase separation was reversible. Rapid heating of the sample and evaporation of the HCl(g) from the water, gave rise to the rebirth of bilayer structures.

Addition of a $CaCl_2$ solution to vesicle dispersions

Vesicles composed of di-*n*-alkylphosphates undergo rapid fusion in the presence of a sufficiently high concentration of Ca^{2+} ions[16]. We have briefly examined whether vesicles prepared from H_2BOMA also exhibit fusogenic activity under these conditions. Thus, to a 3.5 mM vesicle dispersion of NaHBOMA in water (pH = 5.3) was added a small aliquot of a concentrated aqueous $CaCl_2$ solution, giving a 3 mM $CaCl_2$ solution. Immediately the formation of small white crystals could be observed. Samples stained with uranyl acetate and unstained samples were prepared for TEM. With TEM big rectangular shapes (300-1300 nm) were observed next to smaller spherical vesicles (50-120 nm). The rectangular shapes are most probably Ca^{2+} surfactant crystals (Figure 2d). The average size of the vesicles after addition of a Ca^{2+} solution is somewhat higher than before addition, indicating that some fusion might have taken place.

When small aliquots of a Ca^{2+} solution were titrated into a 1 mM vesicle dispersion, small crystals could be observed even at $[Ca^{2+}] = 0.05$ mM, illustrating the high affinity of the malonate head group for Ca^{2+} cations. This rapid formation of crystals strongly hampered experiments aimed at monitoring aggregation and fusion of the vesicles.

Acknowledgment

We are much indepted to Mr. *J.F.L. van Breemen* (Department of Biophysical Chemistry, University of Groningen) for assistance in TEM experiments and to Dr. *H.A. van Doren* (Netherlands Institute for Carbohydrate Research) for assistance with the phase-penetration experiments.

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